UNIVERSITAT DE BARCELONA

Evaluation of biotoxins in marine ecosystems by new analytical and biological strategies

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PhD Thesis

2020

EVALUATION OF BIOTOXINS IN MARINE ECOSYSTEMS BY NEW ANALYTICAL AND BIOLOGICAL STRATEGIES

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CSIC
Consejo Superior de Investigaciones Científicas

Universitat de Barcelona

Programa de Doctorado
"Química Analítica i Medi Ambient"

Evaluation of biotoxins in marine ecosystems by new analytical and biological strategies

Memoria de Tesis presentada para optar al grado de
Doctor por la Universidad de Barcelona

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Barcelona, noviembre 2020

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Certifica:

Que la presente memoria presentada para optar al título de Doctor, titulada "Evaluation of biotoxins in marine ecosystems by new analytical and biological strategies" ha sido realizada bajo mi dirección por la Sra. Cristina Bosch Orea en el Instituto de Diagnóstico Ambiental y Estudios del Agua (IDAEA), perteneciente al Consejo Superior de Investigaciones Científicas (CSIC), y que todos los resultados presentados son fruto del trabajo experimental realizado por la mencionada doctoranda.

Barcelona, 21 de noviembre de 2020

Dra. Marinella Farré Urgell

Este trabajo de tesis ha contado con el apoyo financiero del Ministerio de Economía y Competitividad a través de los proyectos INTEGRA-COAST (CGL2014-56530-C4-1-R) y PLAS-MED (CTM2017-89701-C3-1-R), de la Unión europea a través de los proyectos Sea-on-a-Chip (FP7- OCEAN2013.1 no. 614168) y BRAAVOO (FP7-OCEAN2013.1 no. 614010) y del Banco Santander para la realización de estancias en el extranjero mediante la beca Iberoamérica- Santander Investigación.

“Each dream you leave behind
is a part of your future
that will no longer exist”
Steve Jobs

“There is a dream that I have had
since lunch
and I’m not giving up on it now”
Michael Scott

“O drama e a comédia,
as perdas e os ganhos,
o deserto e o oásis,
o relaxamento e o estresse
são privilégios dos vivos”
Augusto Cury

“El pollo a l’ast
huele mejor de lo que sabe”
Ana Almena

A Schwarzenegger

Agradecimientos

Más complejo que entender la transformada de Fourier, será resumir aquí a todos a quienes quiero agradecer. Y es que ha sido un placer descomunal, compartir con tantos y tantas estos años de tesis doctoral. Durante este camino, un sinfín de gente he conocido y de todos siempre algo he aprendido. Las ha habido de todos los colores, desde risas tochas a días muy llorones, y me siento muy privilegiada de haber estado siempre tan bien rodeada. En esta etapa con intensos sentimientos, a todos los que me han acompañado quisiera expresarles mis más sinceros agradecimientos.

Así que, sin más dilación, primero quisiera agradecer a todo mi grupo de investigación. Aprecio muchísimo todas vuestras aportaciones, cada ayuda, cada enseñanza y todas vuestras explicaciones. A Marinella, con quien he tenido trambolikas vivencias, gracias por compartir conmigo toda tu experiencia y a Damià, quien siempre tiene un momento para conversar y miles de contactos que proporcionar, gracias a ambos por hacer posible que al final me pueda doctorar (aunque alguno durante el depósito se haya tenido que sacrificar). También a Òscar Núñez por ayudarme con tanta documentación, sin tus pautas habría sido todo aún más follón.

Gracias al Marinella's team por todo por ser tan buen equipo. Voy a echar de menos los muestreos en el delta del Ebro siempre tan variados, se sabe a qué hora empiezan, pero no en qué playa quedarás varado. Tampoco se sabe cuántas toneladas de agua recolectaremos, ni si alguna rueda pincharemos, pero nos espera siempre la caseta de fusta cuando el hambre ataca y como no, todo siempre al ritmo de la cobra taca taca. Gracias Josep y Marta por toda la peceaciencia, vuestro saber estar y el aguantar en la ciencia, a Mar y a Diana por vuestro salero y encanto del que nunca me harto.

A Dori y a la Rusi, por ese trato siempre tan cercano, menos cuando tienen el látigo en la mano (o un capilar obturado, hipotéticamente, no es que eso haya pasado, muy frecuentemente).

Han sido muchísimas las personas y animalicos de granja que por el CSIC hemos pasado, pero quisiera agradecer especialmente a aquellos que se han quedado (en mi coração, XD). Desde que os conozco que sé, que ya no existen dolores más grandes que la reina del martes santo, que los cabarets de drag queens tienen mucho más encanto, que el pan que pacha, también despacha y que las celdas del ASE se vuelven de color dorado, si alguna vez por error las has muflado, y es que anécdotas dentro y fuera del laboratorio existen ya un buen repertorio. Hay momentos que pasaron a la historia y ya nunca sacaremos de nuestra memoria, como la conmovedora despedida y pica-pica de nuestros queridos Jens y Annika, tampoco a nuestro saxofonista predilecto el cual sin saberlo inventó un dialecto, eeeh claro claro, como no, l'essenza più pura de la Italia y también de Brasil, con quien hemos compartido fiesta y poyata hasta las mil, los findes de casas rurales y los viajes conociendo capitales, a Simón y sus troleos de campeonato con los que siempre nos hace pasar un buen rato, el perfeccionismo de Marianne en estado puro, siempre dispuesta a ayudarte a afrontar hasta el día más duro, al más canalla de los que he conocido, no sé cuántas veces nos habremos reído, aunque tenga la sensibilidad de un zapato y no quiera reconocer que tiene cara de gato, a las compis de bodybalance, jam y abdominales, aunque sufridos han sido siempre ratos geniales, en can Vila nos hemos puesto como gorrinos a base de calçotadas, y hemos hecho en la playa la mayoría de las escapadas, gracias a Gemma y todo su encanto, Mas Aulina nos queda pendiente para cuando vuelvas del sentimiento blanco. A la professoressa napoletana, y a la más divina Rayana. A Carmín y su bondad, su alegría y su amistad. También gracias al santo padre de la astrología, por compartir conmigo toda tu sabiduría, también tu perversión que sin duda ha sido pura diversión, como la compartida con Alba aquí en o en cualquier rincón, de la terra alta lo més valuós, no és la garnatxa sino el seu bateà més ros, de Albacete no es la carne sino las tortillas y obviamente también es Esterilla, i de l'Esquirol arriba la Berta amb tota la força, qui en cuidar els detalls sempre s'esforça. A otra a quien siempre adoro, es a Mavi, que es un tesoro, a Massimo, il raggio di sole que alla donde entra aporta luz y colores. Y aunque Albert sea un coñazo, al final nos

mola mazo, su pelo tan voluminoso nos parece muy hermoso, Mari Pau que habla solo con sus caretos y la novia de Margareto. Al consejo de sabios de la tercera planta, que entre cafés y sonrisas siempre me aguanta, Marianne, Nico y Cris Postigo en particular. A los recién llegados, gracias por el aire fresco que habéis aportado, y a todos los de cuando empecé, que ya queda bien lejos, gracias por todo vuestro apoyo y los sabios consejos. A alguno, pero, me quiero dejar, por ser tan puto y quererme vacilar, aunque estoy deseando que nos lie sin dejarnos cenar.
A mi Cabesuelo por aguantar mis tremendas chapas y darme consuelo, y sobretodo por ser mi ornitólogo favorito, al cual con mis encantos lo tengo siempre loquito ¿o es más bien al revés? También a quien siempre ha estado a mi vera, espero haber sido para ti tan buena parcera, como tú lo has sido conmigo pero en sentido inverso, gracias al rolo más querido de todo el universo. Y obviamente a mi sorella italiana, para quien me sobran las palabras, pero nunca los ratos a tu lado, y aunque mucho hayamos madurado, y compartido frustraciones, siempre sabemos pasarlo bien al ritmo de buenas canciones. Espero que así siga siendo y mil ratos más sigamos compartiendo.

Y fuera del CSIC también hay vida, gente estupenda a la que le estoy también muy agradecida. A todita la family, en especial a mi papichulo el rey del tritio, él sí que es un hidrogenio, a mi incondicional Troli que, aunque ya no me pone cuentos para dormir, me sigue cuidado desde Madrid, junto a Karen la traviesa, pero sobretodo a mi mamasita Omá, que sin su apoyo y ternura no habría acabado con esta amargura, y aunque no le gustan las mequendeces, siempre sabe cuándo un abrazo te mereces. A las tertulianas de Can Vidalet por los ratos de ocio y por ser tan geniales, nada se soluciona mejor que con unos buenos carnavales, un pepi-brie en la furguiro de buena mañana o ahora que me gustan las infusiones, con una buena valeriana.
Como no, también quería agradecer, a mi familia tetudil, la cual no para de crecer. Y aunque no por falta de talento, lo mejor que he sacado de la facultad, no han sido los muchos premios y reconocimientos, sino sin duda alguna vuestra valiosa amistad, que sigue creciendo, bioaccumulating and biomagnifying along the food chain y más allá. A los Poznianos y los encuentros en lugares inesperados, siempre siguiendo la tradición de estar bien alimentados. A los vintage climbers y can oliveres, por los raticos de alta calidad y montaña, siempre dispuestos a nuevas aventuras y hazañas. Y a los sirénidos, que siempre alimentan mi sed de bióloga marina, y al Hospi team que me tienen como vecina, mi sed de orujo, con los que me lo paso de lujo.

Ólho, lhó! Tásh tola Cristina tásh? Qué cagáçu, por pouquinho esqueço de expressar minha mais profunda gratidão à equipa do MAPA, que mazânza! Gente, que bom compartilhar com vocês meus dias no Brasil, ainda que forem complicados pelo COVID, adorei o tempo tudo que passei no seu lado. Queria agradecer de tudo coração a todos, porém mais especialmente à deliciosa Sol e a gostosona Michelle, quem sempre estão espalhando sua alegria pelo laboratório. Ao chuchuzinho Luan e o mônshtru Mauricio quem me ensinaram a ser uma ótima manezinha das formas mais engraçadas. Ao explorador Lucas com quem adorei conhecer a natureza selvagem da ilha. Ao cabuloso Heitor por seu afeto e detalhes. Também não vou esquecer as conversações com o Marcelão e a Joana, nem a doçura das Andris. E claaaaro, a equipa das biotoxinas marinhas por sofrer juntos a síndrome não conhecida que as toxinas provocam, a depressão profunda quando elas não são recuperadas, mas também pelas conversas, as aulas para abrir mexilhões na velocidade da luz, as dicas e as risadas, agora tenho um novo olhar da pesquisa, waaaw. Muito obrigada Carol pelos momentos dentro e fora do laboratório, ainda com uma perna quebrada você é imparável, também ao futuro papai Cris o melhor churrasqueiro que conheci e obviamente, ao falso impostor Rodrigo, quem fiz possível tudo, cuidou-me desde o início e me fiz voltar a motivação na ciência com sua inteligência e humor. Obrigadão!

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Abbreviations

Ab	Antibody
ACN	Acetonitrile
Ag	Antigen
AOAC	Association Of Analytical Chemistry
ASE	Accelerated Solvent Extraction
ASMAT	Analytical System for Marine Algal Toxins
ASP	Amnesic Shellfish Poisoning
AZAs	Azaspiracids
AZP	Azaspiracids Shellfish Poisoning
BE (2)-M17	Human neuroblastoma cells
BSA	Bovine Serum Albumin
C_{18}	Octadecyl Column
CAC	Codex Alimentarius Commission
CBAs	Cell-Based Assays
CCFFP	Committee on Fish and Fishery Products
CCβ	Detection Capability
CE	Capillary Electrophoresis
CE	Cellulose Ester
CFP	Ciguatera Fish Poisoning
CI	Cyclic Imines
CSIC	Spanish Council for Scientific Research
CTD	Conductimetry Temperature Depth
CTXs	Ciguatoxins
CY5	Cyanine
DA	Domoic Acid
DAD	Diode Array Detection
DNA	DeoxyriboNucleic Acid
DSP	Diarrheic Shellfish Poisoning
DTXs	Dinophysistoxins
EC	European Commission
EC_{50}	Half maximal effective concentration
ECL	ElectroChemiLuminescence
EDABs	Ecosystems Sisruptive Algal Blooms
EFSA	European Food Safety Authority
ELIMC	Enzyme-Linked Immune-Magnetic Colorimetric
ELISA	Enzyme-Linked ImmunoSorbent Assay
ESI	Electro Spray Ionization
FAO	Food and Agriculture Organization
FCM	Flow Cytometry
FD	Fluorescence Detection
FDA	Food and Drugs Administration
FISH	Fluorescence In-Situ Hybridization

FITC	Fluorescein IsoThioCyanate
FWHM	Fifty percent of the maximum peak height
GC	Gas Chromatography
GMOs	Genetically Modified Organisms
GTXs	Gonyautoxins
HABs	Harmful Algal Blooms
HAc	Acetic acid
HAEDAT	Harmful Algae Event DATabase
HGG	Human Gamma Globulin
HILIC	Hydrophilic Interaction LIquid Chromatography
HLB	Hydrophilic-Lipophilic Balance
HPLC	High-Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
hYTX	Homoyessotoxin
IC_{50}	Half maximal inhibitory concentration
IDAEA	Institute of Environmental Assessment and Water Research
iLOD	Intrumental Limit Of Detection
iLOQ	Intrumental Limit Of Quantification
IQAC	Institute for Advanced Chemistry of Catalonia
IT	Ion Trap
LC	Liquid Chromatography
LDH	Lactate DeHydrogenase
LIT	Linear Ion Trap
LLE	Liquid-Liquid Extraction
LOD	Limit Of Detection
LOQ	Limit Of Quantification
MALDI	Matrix-Assisted Laser Desorption/Ionization
MBA	Mouse BioAssay
MBTs	Marine Biotoxins
MCF-7	Michigan Cancer Foundation-7
MeOH	Methanol
mLOD	Method Limit Of Detection
mLOQ	Method Limit Of Quantification
MRLs	Maximum Regulated Limits
MS	Mass Spectrometry
MWCO	Molecular Weight Cut-Off
N2a	Neuroblastoma 2a
Na^+/K^+-ATPase	Sodium-potassium Adenosine TriphosPhatase
NADH	Nicotinamide adenine dinucleotide
Nb4D	Nanobiotechnology for Diagnostics
NOAA	National Oceanic Atmospheric Administration
NSP	Neurotoxic Shellfish Poisoning
OA	Okadaic Acid

OEG-ATs	Oligo-Ethylene Glycol AlkaneThiols
OMA	Official Methods of Analysis
OVA	OVAlbumin
PbTxs	Brevetoxins
PCR	Polymerase Chain Reaction
PLE	Pressurized Liquid Extraction
PLTXs	Palytoxins
PMS	Phenazine MethoSulphate
PRM	Parallel Reaction Monitoring
PSP	Paralytic Shellfish Poisoning
PTXs	Pectenotoxins
qPCR	Quantitative Polymerase Chain Reaction
QqLIT	Quadrupole coupled to Linear Ion Trap
QqQ	Triple quadrupole
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RBAs	Receptor-Based Assays
RL-EU	Reference Laboratories of the European Union
RNA	RiboNucleic Acid
RPLC	Reverse Phase Liquid Chromatography
RSD	Relative Standard Deviation
RT-PCR	Real Time Polymerase Chain Reaction
SAM	Self-Assembled Monolayer
SAX	Strong Anion eXchange
SCP	Solid Phase Cytometry
SEM	Scanning Electron Microscope
SHA	Sandwich Hybridization Assay
SLE	Solid Liquid Extraction
SOP	Standard Operation Procedure
SPATT	Solid Phase Adsorption Toxin Tacking
SPE	Solid Phase Extraction
SPR	Surface Plasmon Resonance
STXs	Saxitoxins
TEFs	Toxicity Equivalency Factors
TOF	Time of flight
TTXs	Tetrodotoxins
UAE	Ultrasounds Assisted Extraction
US	United States
UV	UltraViolet
VGSCs	Voltage-Gated Sodium Channels
WCX	Weak Cationic eXchange
WFD	Water Framework Directive
WHO	World Health Organization
XICs	eXtracted Ion Chromatograms
YTXs	Yessotoxins

Abstract

In recent years, the frequency of harmful algal blooms (HABs) has increased in worldwide coastal areas. Even though these phenomena occur naturally, anthropogenic activities seem to have significant implications regarding to HABs. The eutrophication of coastal waters, by way of the large discharge of organic matter from industries, agriculture and run-off from farms, and the fact of global warming, are believed to be the main triggers of these disproportionate phytoplankton proliferations.

Ecosystems are highly impacted during and after the formation of the events. However, one of the main concerns is related to the production of marine biotoxins (MBTs) by a subset of algae. These substances have toxic properties and are capable of bio-accumulating along the food chain. Several intoxication episodes have been reported in marine fauna and humans by the consumption of MBT-contaminated food. Most of the intoxications are resolved by supportive treatment but in some cases the effects can be lethal. The syndromes caused by the biotoxins are well described and depend on the nature and mode of action. Main classification of MBTs is defined by the syndromes that are produced when the intoxication is via shellfish or fish: diarrheic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), azaspiracids shellfish poisoning (AZP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP) and ciguatera fish poisoning (CFP).

Consequently, the occurrence of MBTs supposes a risk for the ecosystem and the public health. Based on this classification, monitoring programs for the surveillance of these toxins have been established as mitigation strategies. However, in this classification all existing biotoxins with potential toxicity are not included. Since 1980, exhaustive monitoring programs of the toxigenic-producer algae has been carried out with the aim of managing the appearance of HABs formations and palliating the unfavourable effects that they can give rise to. The identification and quantification of the algae up to genus level has been based on the observation of the morphological characteristics of cells using microscopy. From the early 1960s, molecular techniques were implemented to facilitate the quantification of the cells using flow cytometry or polymerase chain reaction in combination with fluorescence. Further, the detection of high biomass on the water surface has been carried out using remote sensing. Even though these techniques are suitable for monitoring the toxigenic species and estimating the possibility of MBTs release, they are not sufficiently reliable to confirm the presence of MBTs or to determine the toxicity of the algae.

Thus, the monitoring of MBTs was established by the public authorities with a particular on analysing commercial marine products. Maximum regulated limits of MBTs in shellfish were determined for the different groups. Limits for each group were determined by toxicological assays in mouse. In fact, mouse bioassay was the first method used as reference to determine the MBTs occurrence. To date, mouse bioassay is still the methodology of reference in some countries, however, due to the little specificity and also the ethical controversy, new methodologies have been developed for the analysis of the compounds. It is noteworthy that immunoassays are the most employed biochemical assays for the determination of MBTs. There are also cell-based and receptor-binding assays which provide information about the toxin activity. These techniques have been implemented as biosensors providing high sensitivity and rapid analysis. However, the most employed analytical methodologies are those based on liquid chromatography coupled to mass spectrometry (LC-MS). The combination of these instrumental techniques provides high selectivity and sensitivity to the analysis of MBTs, and that is why they have been established as methods of reference for the analysis of specific biotoxins. Moreover,

with the improvement of accuracy in MS analysers, LC coupled to high resolution MS (HRMS) have permitted the unequivocal identification of the MBTs groups and the characterization of new analogues.

Furthemore, the surveillance of MBTs is mainly focused on the commercial seafood harvest, particularly bivalve molluscs, while ignoring other organisms that could potentially be affected by the presence of the biotoxins or be transport vectors for other species. Moreover, to the best of our knowledge, there is scant information related to the occurrence of these toxins in the media where they are produced, i.e. in seawater. The characterisation of toxins and the environmental parameters could provide information regarding the production, distribution and transport of biotoxins.

For these reasons, the objectives of this thesis were the development of highly sensitive and selective methods for the unequivocal detection and quantification of the different MBTs groups in seawater. The application of these sensitive/selective methods could contribute to the study of the dynamics of MBTs by the detection of very small concentrations. In addition, the analysis of the biotoxins directly in the medium where they are produced, could be employed as early warning detection strategy. For instance, high performance liquid chromatography (HPLC) coupled to HRMS techniques has been employed. The selected toxins were Okadaic acid (OA) and related dinophysistoxin-1 (DTX-1), pectenotoxins-2 (PTX-2), azaspiracids-1,2,3,4 and 5 (AZA-1,2,3,4 and 5), yessotoxin (YTX) and homoyessotoxin (hYTX), domoic acid, saxitoxin (STX) and analogues decarbamoylsaxitoxin (dcSTX), neosaxitoxin (Neo), gonyautoxin-2,3 (GTX-2,3), and tetrodotoxin (TTX) that have been frequently reported in the Mediterranean Sea. Seawater was necessarily pre-treated to eliminate any interferences, which would mainly be organic matter and salts, and to isolate and concentrate the target MBTs. The water was filtered and the particulate and filtrate fractions were separated and MBTs were extracted from separately by ultrasound assisted extraction (UAE) and solid phase extraction (SPE), respectively. Due to the wide differences in polarity of these biotoxins, different modes of chromatography were employed as well as different sample pre-treatments.

The most lipophilic MBTs of the group, OA, DTX-1, AZAs, PTX-2 and YTXs were separated by reverse phase LC (RPLC). The extraction from seawater was carried out via UAE with methanol and SPE using hydrophilic-lipophilic balance (HLB) cartridges. The performance of the method was satisfactory and high sensitivity was achieved with limits of detection (LODs) at pg/L levels. The method was successfully applied to samples from the Catalan littoral (nostheast Spain) which were collected from inside marinas and from beaches. Only OA was detected at concentrations ranging from 2 ng/L to 9.5 µg/L.

Most hydrophilic MBTs, Neo, dcSTX, GTX-2,3, TTX and STX were separated by using hydrophilic interaction LC. Pre-treatment of the particulate and filtrate was by UAE with methanol and SPE with Silica cartridges, respectively. The recoveries for these toxins were low, due to the low retention in the cartridges, as a result of the high polarity character, but the results are reproducible. The method was applied to samples from Mar Menor (a coastal saltwater lagoon) off the coast of the Spanish province of Murcia (located in the southeast of the Iberian Peninsula, Spain), but no toxins were detected.

Further, DA was separated by using RPLC and HILIC, but obtaining better resolution with HILIC. DA was extracted from seawater via SPE using HLB cartridges and the method was applied to real samples from Ebro delta wetland (Catalan coast, northeast Spain), which were collected during different seasons. Concentrations ranged from 0.9 to 69.6 ng/L, with the highest being concentrations determined during the summer months. The

environmental parameters were measured during the sampling campaigns and compared with the occurrence of toxins. Nevertheless, there was no relation or tendency to be found. The production mechanism of MBTs is still unknown and is influenced by several biotic and abiotic factors.

Finally, one methodology was applied in the validation of a multiplexed immunoassay type of enzyme-linked immunosorbent assay (ELISA) which is able to detect and quantify five different groups of pollutants in seawater, one of them being DA. The addition of the SPE pre-treatment prior to the ELISA analysis, allowed the concentration of DA in the sample to reach a lower LOD equal to 1.39 ng/L. The immunoassay proved to be highly sensitive and specific for the analysis of DA in seawater with no cross-reactivity and no matrix effects.

Resumen

Las floraciones de algas nocivas se han incrementado en las zonas costeras de todo el mundo a lo largo de los últimos años. Se cree que estos fenómenos estas promovidos por el aumento de las temperaturas y la eutrofización de las aguas debido a la gran descarga de materia orgánica procedente en su mayoría de actividades antropogénicas. Dichas condiciones favorecen el crecimiento de especies fitoplanctónicas que en ocasiones pueden liberar altas concentraciones de compuestos con propiedades toxicas para la fauna y los humanos, conocidas como biotoxinas marinas. Estas substancias tienen la capacidad de bioacumularse y biomagnificarse a lo largo de la cadena trófica, causando episodios de intoxicación especialmente cuando son consumidas a través de pescados y mariscos que las contienen. Dependiendo del tipo de biotoxina, su modo de acción y la cantidad, éstas pueden causar efectos leves o letales. Los principales grupos de biotoxinas se clasifican según el síndrome que producen y son: biotoxinas diarreicas, amnésicas, paralizantes, azaspiracidas, neurotóxicas y ciaguatera.

En consecuencia, la presencia de biotoxinas marinas supone un riesgo para el ecosistema y la salud pública. Basados en la clasificación de biotoxinas previamente expuesta, diversos programas de vigilancia se han establecido como estrategia de mitigación. Sin embargo, en esta clasificación no están incluidas todas las toxinas que se conocen hasta día de hoy y algunas de ellas con alto potencial toxico. Numerosos esfuerzos se están llevando a cabo para controlar y mitigar la presencia de estas substancias tanto en el medio ambiente como en productos marinos. En 1980, se implantó el monitoreo de especies productoras de toxinas con el objetivo de gestionar la aparición de biotoxinas y aminorar los efectos desfavorables que estas pueden provocar. La identificación de estas algas es mediante su observación al microscopio, y a día de hoy aún se utiliza esta metodología. Posteriormente, la aplicación de técnicas moleculares como la PCR o la citometría de flujo han facilitado la detección de dichas algas. También, los últimos avances en telemetría han proporcionado muchas ventajas para la detección de grandes cantidades de biomasa, típicas de floraciones. Sin embargo, estas técnicas no proveen información específica de la toxicidad debido a las biotoxinas. Por normalidad, la mayor concentración de toxinas se produce en el inicio de la floración y hasta que ésta lleva a su punto máximo. Sin embargo, esto no es siempre tan predecible. Diversos estudios han demostrado que a grandes concentraciones de algas productoras de biotoxinas, no ha habido presencia de dichas toxinas. Por lo contrario, la presencia de biotoxinas ha sido detectada en ocasiones donde no había presencia de algas productoras o éstas estaban a muy baja concentración. Además, la producción de toxinas no es igual incluso para individuos de la misma especie de alga ya que está muy influenciada por factores biológicos y físico-químicos.

Todavía se desconoce el mecanismo de producción de estos compuestos y existe mucha diversidad de condiciones en las que ciertas algas. Por ello, la implementación de los programas de vigilancia, han determinado el análisis directo de las biotoxinas, principalmente en productos comercializados como bivalvos. Límites máximos regulatorios en mariscos han sido establecidos para cada grupo de toxinas mediante unos valores de toxicidad determinados por ensayos toxicológicos en ratones. De hecho, el ensayo toxicológico de raton era el método de referencia para determinar la presencia de biotoxinas. Hasta la fecha, este ensayo se sigue utilizando como referencia en algunos países, aunque dado lo poco específico y éticamente cuestionable que es, otras metodologías han sido desarrolladas. Los inmunoensayos son los métodos bioquímicos más utilizados para la determinación de toxinas, así como los ensayos funcionales. La incorporación de estas técnicas a biosensores han aportado una gran sensibilidad y rapidez

en los análisis de biotoxinas. Por otro lado, las técnicas analíticas más utilizadas son la cromatografía liquida acoplada a espectrometría de masas (LC-MS). La combinación de estas técnicas instrumentales ha proporcionado una mayor selectividad y sensibilidad en el análisis de biotoxinas y por ello has sido establecidas como técnicas de referencia para algunos grupos específicos de toxinas. Además, la alta resolución en MS ha supuesto un gran avance en la caracterización de nuevos compuestos a la vez que permite la identificación y cuantificación de los compuestos dirigidos.

Por otro lado, la vigilancia de biotoxinas marinas está básicamente enfocada para productos de comercialización, concretamente mejillones, dejando de lado, muchas otras especies que a su vez podrían sufrir intoxicaciones por la presencia de estos compuestos o que podrían hacer de vectores de transporte. También, poca información existe en relación a la presencia de toxinas en su medio de vida y de producción, el agua. La caracterización de toxinas en su ambiente y sus diferentes parámetros podrían proporcionar información acerca de la producción, distribución y transporte.

Por estas razones, los objetivos principales de esta tesis fueron los de desarrollar métodos de análisis muy selectivos y sensibles para la detección inequívoca de las toxinas y su cuantificación en agua de mar. La aplicación de estos métodos podría ser una aportación clave en el estudio de la dinámica de las biotoxinas, incluso a muy bajas concentraciones. Adicionalmente, este método podría ser utilizado también como alarma temprana de la presencia de toxinas.

Para ello, se ha utilizado LC de alta eficacia acoplada a MS de alta resolución. Las biotoxinas seleccionadas están muy presentes en las aguas del Mediterraneo y son: ácido okadaic (OA), dinophysistoxina-1 (DTX-1), pectenotoxina-2 (PTX-2), azaspiracidos-1.2.3.4.5 (AZA-1,2,3,4,5), yessotoxina (YTX), homoyessotoxin (hYTX), ácido domoico acid, saxitoxina (STX), decarbamoylsaxitoxina (dcSTX), neosaxitoxina (Neo), gonayutoxina-2,3 (GTX-2,3) y tetrodotoxina (TTX).

El agua de mar fue necesariamente tratada previamente al análisis, para retirar el máximo de materia orgánica y el alto contenido en sales y además extraer y concentrar los analitos. El agua fue filtrada y la porción del particulado fue separada de la del filtrado. Ambas partes se trataros por separado, mediante extracción asistida con ultrasonidos (UAE) y extracción en fase sólida (SPE), respectivamente. Debido a la gran diferencia de polaridad entre las biotoxinas marinas, diferentes tipos de LC se llevaron a cabo, así como diferentes condiciones de extracción para las muestras.

Los compuestos más apolares, OA, DTX-1, AZAs, PTX-2 y YTXs se separaron mediante LC en fase reversa (RPLC). La extracción de las toxinas del agua de mar se hizo por UAE con metanol y SPE utilizando cartuchos de balance hidrofilico-lipofílico (HLB). El desarrollo del método fue satisfactorio y una alta sensibilidad fue conseguida con límites de detección que alcanzan los pg/L. El método se aplicó exitosamente para analizar muestras recolectadas del litoral catalán, procedentes de puertos y playas. Solo OA fue detectado, en concentraciones de 2 ng/L a 9.5 µg/L.

Por otro lado, los compuestos más polares Neo, dcSTX, GTX-2,3, TTX y STX, se separaron por LC de interacción hidrofílica. El tratamiento de muestra se hizo por UAE con metanol y SPE con cartuchos de silica para el particulado y la porción de filtrado, respectivamente. Para estos compuestos se obtuvieron recuperaciones bajas, debido a lo poco que se quedan retenido al cartucho dada su gran polaridad, sin embargo, la reproducibilidad es buena. El método se aplicó a muestras reales del Mar Menor en Murcia, pero ninguna de las toxinas fue detectada.

Finalmente, el DA se separó por ambos modos de LC, RPCL y HILIC, aunque se obtuvo una mejor resolución mediante HILIC. DA fue extraído del agua mediante SPE don cartuchos HLB. El método fue aplicado a muestras real del delta del Ebro, recolectadas en diferentes estaciones del año. Las concentraciones oscilaban entre 0.9 y 69.6 ng/L, siendo las más altas las pertenecientes a los meses de verano.

Algunos parámetros ambientales se midieron durante las campañas de muestreo y se compararon con las concentraciones de biotoxinas. No obstante, ninguna relación ni tendencia fueron determinadas.

Finalmente, uno de los métodos se utilizó en la validación de un inmunoensayo tipo ELISA (del inglés *enzyme-linked immunosorbent assay*) capaz de detectar y cuantificar cinco familias de contaminantes en agua de mar, siendo el DA uno de ellos. El hecho de añadir un tratamiento de muestra previo al análisis por ELISA, del tipo SPE, permitió la concentración de DA aumentando así su detectabilidad. El inmunoensayo manifestó una gran sensibilidad con un LOD de 1.39 ng/L y una alta especificidad para DA, sin reactividad cruzada con otros compuestos de estructura similar.

1. INTRODUCTION

1.1 Harmful algal blooms and marine biotoxins

Algal blooms are natural phenomena of rapid phytoplankton proliferation that can take place in all types of water bodies. Phytoplankton plays an important role in the environment; it is not only the basis of the food chain but also the major O_2 producer by the fixation of the atmospheric CO_2. However, when the magnitude of phytoplankton proliferation is critically affecting the environment, these phenomena are considered as harmful algal blooms (HABs). HABs are also colloquially referred to as "red tides" due to the reddish-brown colour of the blooms that are observed when species with red or brown pigments are predominant and covers the water surface. However, manifestations of HABs are varied depending on the species that are producing it, and with a variety of colours and textures. Normally, one-species is predominant during the bloom, but the co-occurrence of diverse species can take place simultaneously. Some examples of these events are shown in **Figure 1**.

Figure 1. Examples of algal blooms in coastal areas.
Credits: Cristina Bosch-Orea and Álvaro López Valiñas.

HABs can produce a variety of effects on the ecosystem. The riskiest events are toxic-HABs because of the presence of toxins that are produced by a subset of phytoplankton species. Nowadays, at least 60 species of marine phytoplankton are known to produce toxins, which are commonly known as marine biotoxins (MBTs) [1]. More than 200 MBTs and their analogues have been characterised, to date [2], by their occurrence worldwide, and most of them are associated with intoxication incidents. These substances can produce poisoning syndromes when they are ingested or by direct contact with the skin, and in the worst cases they can cause death [3-5]. Moreover, MBTs can bio-accumulate along the

food chain [6] causing intoxication problems in a wide variety of marine organisms even reaching birds, mammals, and also humans [7-9].

Marine ecosystems are seriously altered during and after the development of a HAB phenomenon, even if it does not turn into a toxic episode. These incidents can contribute to eutrophication processes, with the loss of oxygen/nutrients equilibrium, thus causing negative consequences for the environment and the activities which are conducted in coastal areas.

Environmental impacts The exponential growth of phytoplankton and the presence of MBTs are potential stressors for the living organisms of an ecosystem. In some cases, the disequilibrium is overcome, but in some other cases, affected party will have problems that become serious and consequently there are high mortalities. These kinds of episodes are also called ecosystems disruptive algal blooms (EDABs) [10].

During the bloom formation, high amounts of phytoplankton stay on the water surface, and even reaching the millions of cells/L [11, 12]. Some species which have colourful organelles into their cells, such as *Gonyaulax polyhedra* and *Prorocentrum micans* [13] can colour the water. Others species such as *Noctiluca scintillans* can create bioluminescence effects [14] while some other species produce a dense long-lasting foam on the water surface during the bloom, as is the case with *Phaeocystis* spp. such as *P. pouchetii*, *P. globulosa* and *P. antarctica* [15, 16]. Any of these perturbations at the water surface will alter the penetration of the solar radiation into the photic zone, thus affecting then the photosynthetic organisms living there. Also, when a bloom reaches its maximum growth and the phytoplankton starts to die due to the lack of nutrients, the decomposition of the cells rapidly takes place. These processes consume the dissolved oxygen, leading the water into a state of hypoxia. If the accumulation of cells is considerably great enough, the hypoxia can turn into anoxia and cause the commonly known “dead zones” [17] where the organisms that require oxygen in order to live cannot survive.

Notwithstanding, some phytoplankton species have cell walls that contain a lot of silica, which is a glass-like substance, and in direct contact with other organisms they can physically damage them. For example, they can cut fish gills, thus causing damage or suffocation [18]. But there are far greater potential problems, due to the presence of MBTs, because some organisms are intoxicated by the ingestion of water containing the substances or by consuming other organisms that have accumulated the toxins in their tissues. Toxicity magnitude depends on the mode of action of each toxin and the organism affected. However, some organisms seem to not be potentially affected by the presence of MBTs and are able to accumulate them without suffering complications, but some other organisms experience adverse effects, which can even cause their death. Furthermore, MBTs can affect the behaviour of some species during their larvae stage, consequently leading to developmental toxicity and low survival rates [19]. Also, fatal intoxications can occur in fishes, birds and mammals resulting in high mortality episodes. Some examples of these events are the high mortality of fish species, such as the sea bream *Sparus aurata* along the Spanish coast by the presence of *Karlodinium* spp. [20], the death of brown pelicans *Pelecanus occidentalis* in Mexico by the ingestion of mackerel *Scomber japonicas* contaminated by domoic acid (DA)-producing *Pseudo nitzschia* spp. (a marine planktonic diatom genus) [7], and the unusual mortality of sea lions *Zalophus californianus* from California, USA by eating anchovies *Engraulis mordax* containing the same neurotoxin, DA [21].

Intoxications by marine toxins and their effects on human health Phytoplankton proliferation and the posterior release of toxic metabolites are responsible for seafood poisoning events in which filter-feeding organisms, such as mussels, oysters and clams, among other, can accumulate these toxins throughout the food chain [6] and, consequently, present a threat for the health of consumers. Summarised in **Table 1** are the most common groups of MBTs according to the syndromes and effects that are produced with respect to human health. All of these effects are caused via ingested seafood containing MBTs. In the case of ciguatera fish poisoning (CFP), vectors of contamination are coral reef fishes. In addition to ingestion via seafood, the neurotoxins shellfish poisoning (NSP) is also caused by inhalation or dermal contact of seaspray aerosol containing brevetoxins (PbTxs).

Table 1. MBTs and the associated syndromes and effects on humans produced when are ingested, inhaled or in dermal contact.

Syndrome	Producer toxins	Effects
DSP: Diarrheic Shellfish poisoning	Okadaic acid and dinophysistoxins	Gastrointestinal distress
PSP: Paralytic shellfish poisoning	Saxitoxins	Gastrointestinal and central nervous system
AZP: Azaspiracids shellfish poisoning	Azaspiracids	Gastrointestinal distress and neurotoxic symptoms
ASP: Amnesic shellfish poisoning	Domoic acid	Gastrointestinal and central nervous system
CFP: Ciguatera fish poisoning	Ciguatoxins	Sensory and gastrointestinal dysfunction
NSP: Neurotoxic shellfish poisoning	Brevetoxins	Gastrointestinal, sensory effects and respiratory effects

During the last two decades, most outbreaks of intoxications by marine toxins were reported in Europe, North America and South America. For example, one of the largest outbreaks occurred in Belgium in 2002, whereby 403 persons suffered intoxication by diarrhetic shellfish poisoning (DSP) because of the ingestion of blue mussels that were imported from Denmark [22]. In that outbreak, okadaic acid (OA), dinophysistoxins (DTXs), yessotoxins (YTXs), pectenotoxins (PTXs) and azaspiracids (AZAs) were confirmed with a level of 529 µg OA-eq/kg. Recently, six cases of DSP following consumption of mussels that were harvested in the United Kingdom were reported. *Dinophysis spp*. in the water column was found to have increased rapidly at the production site resulting in high levels of OA in the flesh of consumed mussels [23]. It is noteworthy that thanks to the new detection approaches and monitoring programmes in most of the cases the detection of MBTs avoids the commercialisation of contaminated seafood. For example, a bloom of *Dinophysis spp*. dominated by *D. sacculus* and its related DSP outbreak in Alfacs Bay (Catalonia, northeast Spain and northwest Mediterranean Sea) was carried in 2016 [24]. In addition, recurrent algal blooms are produced in some areas. For example, in Portugal, the presence of both DSP and PSP toxins leads to recurrent seasonal bans on bivalve harvesting [25]. In the USA a DSP outbreak involving three people after the consumption of contaminated mussels was reported in Washington State [26]. At the same time, 62 DSP illnesses occurred in British Columbia due to the ingestion of Pacific coast mussels [27]. In South America, an important outbreak occurred in 2002 on Chiloé Island due to mussels contaminated with both DSPs and paralytic shellfish poisoning (PSP) resulting in 50 persons being intoxicated [28]. In 2005, 35 people were hospitalised after the consumption of mussels contaminated with DSPs reported by the Puerto Montt Hospital

(Lo Lagos, Chile) [29]. In **Figure 2** the main routes of accumulation and transfer of MBTs are summarised.

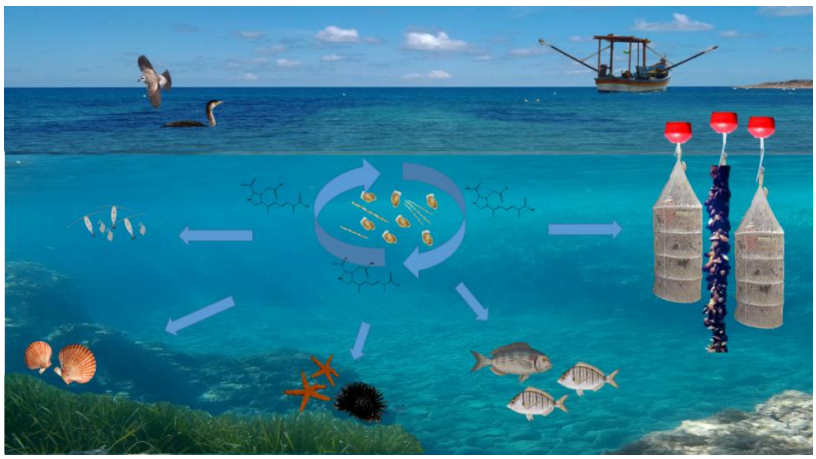


Figure 2. Scheme of the main routes of accumulation and transfer of MBTs.

The effects and magnitudes of these events vary, depending on the mode of action of each toxin but also depending on the type of exposure and the concentrations ingested, going from a simple dermal annoyance up until mortality. It is noteworthy that in the same conditions and concentrations of exposure, certain organisms could accumulate more than others. For example, it has been reported that mussels accumulate higher concentrations of saxitoxins (STXs) than oysters in the same conditions [30]. In addition, MBTs are frost-stable and heat-stable and are not affected by cooking processes (until 150 ºC), then, their toxicity will remain intact in the food [31]. Despite the fact that less than 0.02% of the existing species of phytoplankton are capable of producing marine biotoxins, due to global warming and therefore due to changes in weather conditions and current patterns, specific HAB taxa tend to occur more often and in unexpected places [32]. As a result, marine biotoxins are considered a growing concern for public health.

Impacts on coastal activities Whilst HABs are a natural event, in addition to the impacts of these episodes in human health through inhalation, direct contact or ingestion, they also have adverse effects on the environment and generates significant losses in the coastal economy (fisheries, aquaculture, and tourism) [33]. For example, the clean-up activities to remove biomass which has accumulated after HABs from the littoral suppose considerable losses [15]. However, the most damaged sectors are those related to the food industry, such as fisheries and aquaculture. Bivalve molluscs culture is a relevant commercial activity in Europe, with a production of ~ 625k tonnes and value of EUR 1.24 billion in 2017 [34]. Also, shellfish farming is predominantly carried out by small producers, being an essential industry in many rural areas. It is noteworthy that, in contrast to the increasing aquaculture production of mussels worldwide, in the EU there has been a decreasing trend in aquaculture production over the last two decades. The mussel aquaculture production in the EU reached more than 600 000 tonnes in the late 1990s, but by 2016, the production volume had dropped by 20%. Considering that mussel production represents more than one third of the total EU aquaculture production, this decrease is an important contributor to the stagnation of the sector. This decrease can be attributed to different factors such as diseases, lack of mussel seed (spat), and low profitability, but the primary cause is related to HABs. For example, in Spain, Galician protected areas (e.g. bays or rias) represented 73% of the total Spanish aquaculture production in terms of quantities, with the mussel production being the most important product. Years 2015 and

2016 show how controlled by the HABs this sector is, being that they are the cause of the closing of production areas for long periods of time. In 2016, the industry suffered a decrease of 4.3% with respect to 2015, by up to 215,000 tonnes. Notwithstanding, the mussel production in Galicia was also severely affected in years 2010, 2013 and 2014 by red tides. Moreover, the number and intensity of these events have been multiplied by four during the decade (from 2000 days of closure in 2007 to 8000 days of closure in 2016), and this tendency seems to have consolidated because of the global warming and the contamination of coastal areas. These facts make this industry more vulnerable to climate change impacts and place their capacity to supply the seafood markets at risk [35]. Another example is the case of the diarrhoeic shellfish toxins produced by the genera *Dinophysis*. These toxins, on average, reduced shellfish production by 0.66% in Scottish (northern UK) shellfish farms, with an average yearly negative variation of 15% (1,080 tonnes) [33]. Another example is the shellfish industry in Ireland (European Union), where AZAs intoxications involved the closure of aquaculture facilities up to more than 12 months after certain events [36]. High penalties have been applied to the industries when products have exceeded the limits of concentrations that are permitted by the regulatory agencies [37].

Outside of Europe, for example, in Japan, the fish mortality by the HABs is calculated to be in the order of tens of millions of dollars per year [38].

Also, other economic divisions, such as the tertiary sector, are affected by the formations of blooms. As previously commented, tourist and recreational activities are in danger with the presence of HABs and such activities can be interrupted to ensure the safety of the swimmers, who could be affected simply by direct contact or even because of the toxic aerosols formed by waves when neurotoxins such as PbTxs are present [30]. Public health agencies have to properly manage the detection of MBTs and to proceed with the most protective of measures.

Economic losses are difficult to calculate since many sectors are involved in and they are affected at different magnitudes. Annual estimations of losses in Europe due to the presence of HABs have been estimated at 862 million Euros [39]. In the USA, annual costs corresponded to 75 million dollars in the period from 1987 to 2000 [38]. In Korea, the aquaculture industry estimated a total of 121 million dollars in solely economic losses due to the presence of PSP toxins [40]. However, these estimations integrate the economic costs derived from the direct losses generated by a hazardous episode, but also the costs that suppose the mitigation actions that are required to face up the event.

For these reasons, aquaculture activities require surveillance programms in order to ensure the quality and safety of the consumable products and this value is elevated.

1.2 Marine biotoxins: Main classes and properties

MBTs are secondary metabolites which are produced by a subset of marine diatoms, dinoflagellates and bacteria, and have potential bio-activity. Their mechanism of production has not already been elucidated, to the best of our knowledge, but is believed to synthesised as a defence mechanism against other predators or competitors. In fact, MBTs are considered to be allelochemicals, which are able to influence other organisms, causing beneficial or pernicious effects on them. MBTs are beneficial for some organisms that can accumulate these chemicals in their bodies and use it as a defence against their predators. Examples of this benefit are from the case of heterotrophic dinoflagellates that accumulate MBTs which are produced by other algae and use them as intoxication vectors against their

consumers [41], as in the case of pufferfish which uses the potent neurotoxin TTX as a defence against predators since the larvae stage and also as a male-attracting pheromone during spawning [42]. On the contrary, harmful consequences are suffered by some other organisms, as commented on the previous sections. In birds and mammals, intoxications are produced by the ingestion of MBT-contaminated seafood or by its direct contact with the skin. Moreover, other imperceptible alterations are produced when ingested by molluscs. It is thought that mussels were not affected by the accumulation of MBTs in their tissues, but a recent study has proved that their metabolism could be altered by this accretion [43].

Another important property is the capability to bioaccumulate and biomagnify along the food chain [6]. Nevertheless, the toxicity of each MBT widely varies depending on the nature of the toxin, its concentration, the co-occurrence of diverse toxins or the affected organism.

MBTs encompass a wide variety of different chemical structures, showing diverse chemical behaviour and mechanisms of action [44]. Several classifications have been considered. In **Table 2** are diagrams of the MBTs which are classified by their bio-synthetic origin, chemical structure, molecular size, intoxication syndrome and polarity.

1.2.1 Structure of marine biotoxins

MBTs can be grouped by the similarities in their main chemical structures in three main classes: polyketides, excitatory amino-acids and alkaloids [45]. According to this classification the main toxins in the Mediterranean Sea are summarised below.

1.2.1.1 Polyketides

Okadaic acid and is a high molecular lipophilic MBT that was first identified in sponges of the genus *Halichondria* in 1981 [46]. Later on, other analogues were characterised, such as **dinophysistoxins** congeners [47]. Nowadays, there are more than 80 acyl-esters and diol-esters OA-congeners which have been identified [2]. The chemical structure of this group is presented in **Figure 3**. As it can be seen, these structures are composed by a long carbon chain with two spiro-fused tetrahydropyrans, a spiro tetrahydrofuran-dioxadecalin unit and terminal carboxylic group [45].

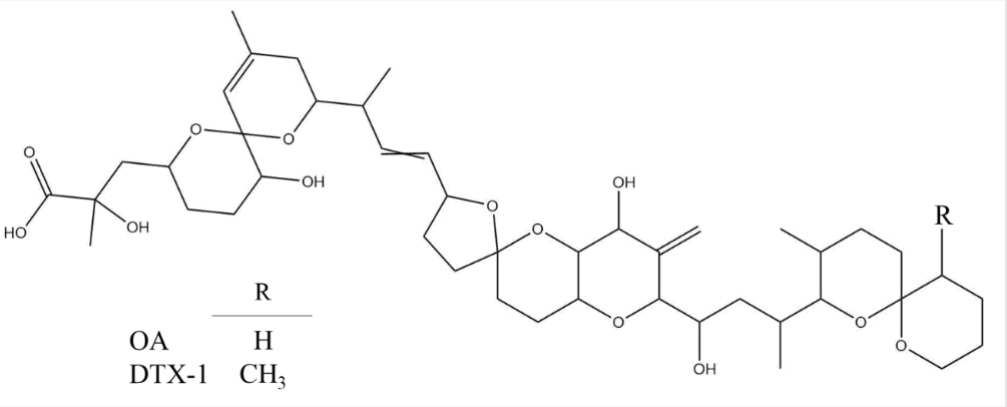


Figure 3. Chemical structure of OA and DTX-1.

Table 2. Classification of all MBTs by their chemical structure, synthetic origin, syndrome and polarity.

Bio-synthetic origin	Chemical structure	Toxins	Molecular size	Syndrome	Polarity
Polyketides	Linear and macrocycled polyethers	**Okadaic acid and dinophysistoxins**	Medium	DSP	Lipophilic
		Pectenotoxins	Medium	-	
		Azaspiracids	Medium	ASP	
		Goniodomins		-	
	Polyethers ladders	**Yessotoxins***	Large	-	
		Brevetoxins	Large	NSP	
		Brevisucenals	Large	-	
		Ciguatoxins and maitotoxins*	Large	CFP	
		Gamberdiscus	Large	-	
		Prymnesins	Large	-	
	Cyclic imines	Spirolides	Medium	-	
		Gymnodimines	Medium	-	
		Pinnatoxins-pteriatoxins	Medium	-	
		Prorocentrolides, spiroprorocentroimine, portimine and symbioimines	Medium	-	
	Long carbon-chain polyols	Amphidiniols, karlotoxins, karmitoxins	Large	-	
		Palytoxins*	Large		Hydrophilic
Excitatory amino-acids	Amino-acids	**Domoic acid**	Small	ASP	
		β-methylamino-L-alanine	Small	-	
Alkaloids	Tetrahydro-purines	**Saxitoxins**	Small	PSP	
		Tetrodotoxins	Small	-	

*These substances are also considered to have an amphiphilic behaviour

Pectenotoxins are macrocyclic lactones with heat-stability unless they are in alkaline conditions [48]. These lipophilic compounds were isolated for the first time in 1984 from scallop *Patinopecten yessoensis* in Japan [49]. More than 25 methyl-, hydroxymethyl-, aldehyde-, carboxylic acid- and epimerization analogues have been described to date [2, 45]. The chemical structure of PTXs is presented in **Figure 4**. The structure contains a spiroketal, a bicyclic ketal, cyclic hemiketals, and oxolanes [50]**.**

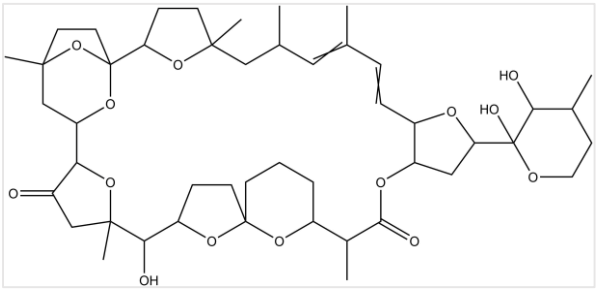
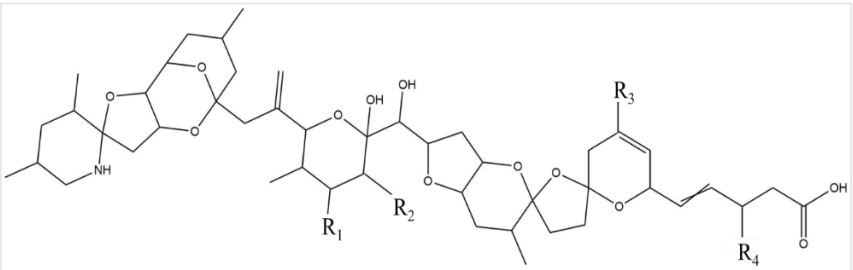


Figure 4. Chemical structure of PTX-2.

Azaspiracids (AZAs) form a group which was firstly identified in 1995 in The Netherlands during a toxic episode by the consumption of shellfish that were produced in the Killary Harbour on the west coast of Ireland [51]. These substances have been widely studied and almost 40 congeners have been identified [2] containing dihydroxy-, carboxy-, carboxy-hydroxy-, dehydro-, methyl-esters structures [36]. AZAs are structurally related to OA, and the molecules contain a heterocyclic amine, a unique tri-spiro-assembly and an aliphatic carboxylic acid [50]. The main structure is represented in **Figure 5**.



	R_1	R_2	R_3	R_4
AZA-1	H	CH_3	H	H
AZA-2	H	CH_3	CH_3	H
AZA-3	H	H	H	H
AZA-4	H	H	H	OH
AZA-5	OH	H	H	H

Figure 5. Chemical structure of AZA-1,2,3,4 and 5.

Yessotoxins were also identified for the first time in scallops *Patinopecten yessoensis* in Japan in 1986 [52]. The structures of YTXs have eleven transfused ether rings, two sulphate esters and an unsaturated side chain. The structures of these polyether ladders are shown in **Figure 6**. To date, more than 100 congeners have been described, with different length in the carbon chain, hydroxylations, carboxylations, methylations, oxidations, amidations, and glycosyl derivates [45].

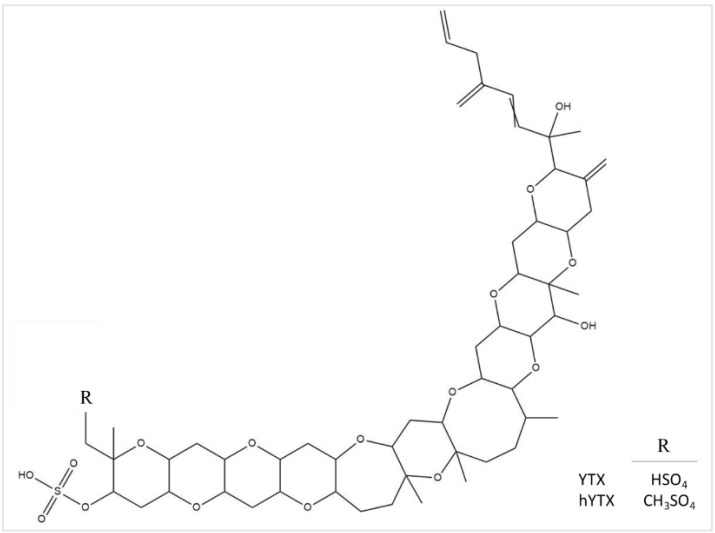


Figure 6. Chemical structure of YTX and the analogue homoyessotoxin (hYTX).

1.2.1.2 Excitatory amino acids

Domoic acid (DA) is a compound that is derived from glutamic acid. Since its first identification in 1987 on Prince Edward Island, Canada, this compound and its 9 conformational isomers have been deeply investigated. The main structure of DA is represented in **Figure 7**. The molecule possesses a proline ring, three carboxylic acids, a secondary amine group and one unsaturation, and it is highly water soluble [53].

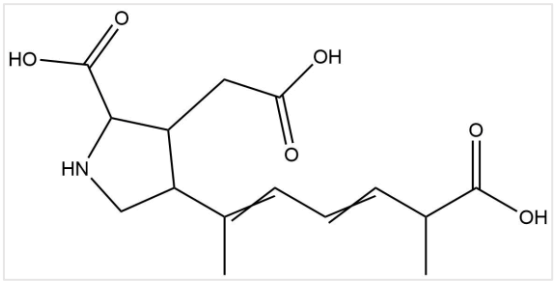
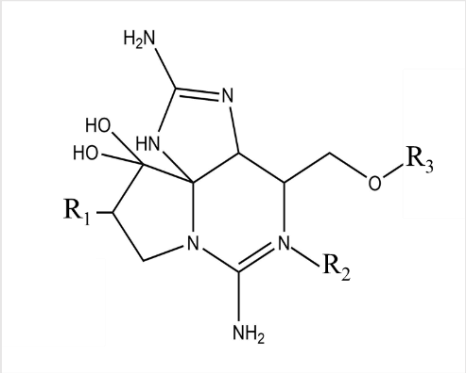


Figure 7. Chemical structure of DA.

1.2.1.3 Alkaloids

Saxitoxin (STX) structure was firstly characterised in 1975, being isolated from seafood and plankton [54]. Several analogues were identified and can be grouped into four categories; carbamate, N-sulfocarbamoyl, decarbamoyl and deoxydecarbamoyl-STXs, and less frequent groups; mono-hydroxy-benzoate, di-hydrobenzoate and sulphate benzoate analogues [55]. These substances are guanidinium alkaloids possessing a tricyclic 3.4-propinoperhydropurine system with two guanidinium moieties formed by the NH_2- groups and a reduced purine [45]. The general structure is presented in **Figure 8**. More than 38 congeners have been described to date [56], being characterised for high water solubility and pH dependent heat stability [50].



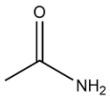
	R_1	R_2	R_3
dcSTX	H	-	H
STX	H	-	
GTX-2.3	HSO4	-	
NeoSTX	H	O^-	

Figure 8. Chemical structure of STX and analogues decarbamoylsaxitoxin (dcSTX), neosaxitoxin (NeoSTX), gonyautoxin 2 and 3(GTX-2,3).

Tetrodotoxins TTX was isolated during the period of 1910-1913 [57], and the structure was confirmed in 1964 [58, 59]. Nowadays, more than 30 congeners have been characterised [60]. TTX is constituted by a guanidine structure with a hydroxylated dioxaadamantane carbon backbone. The structure of TTX and its analogues are shown in **Figure 9**.

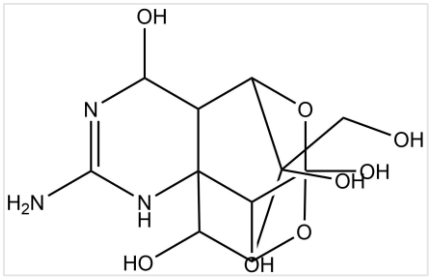


Figure 9. Chemical structure of TTX.

1.2.2 Mode of action and effects on humans

To date, several poisoning episodes have been described and associated with MBTs. Pieces of evidences of intoxication by the ingestion of pufferfish date from the time period of the Egyptian Pharaohs (3100-343 BC), when some species such as *Tetraodon lineatus* were recognised as poisonous fishes and were frequently appearing in the illustration within Egyptians tombs [61, 62]. Nowadays, it is known that the cause of intoxication is related to the TTX that is contained in the organs, skin and flesh of some fishes of the Tetraodontidae family [63]. Also, the foodborne illness ciguatera was reported in the West Indies during year 1555, being associated with the consumption of poisonous fishes [64]. This illness is currently connected to the toxin ciguatoxin (CTX) that is contained in some coral reef fishes. This toxin is produced by benthic dinoflagellates of the genus Gamberdiscus, which live in association with corals in tropical waters [65] and are easily consumed by the fishes. In other cases, the description of the effects has made possible the identification of MBTs as causative agents. For example, the symptoms described by an intoxication episode in the aboriginal population of Patagonia in southern South America in the year 1886, are those caused by the intoxication with STXs [66].

The seriousness of the intoxication will be led by the type of toxins and dose. The specific mode of action of each toxin determines the main effects and symptoms produced in humans that are summarised in **Table 3**.

Table 3. Mode of action of the main toxins and the effects on humans.

Toxins	Mode of action	Effects	Symptoms	Supportive treatment
OA and DTXs	Inhibition of the serine/threonine protein phosphatase PP2A, PP1 and 2B	Gastrointestinal distress	Diarrhoea, nausea, vomiting, abdominal pain, sometimes headache, chills and fever	Replacement of fluid loss and electrolytes
PTXs	Unknown	Not determined in humans		
AZAs	Partially inhibition of the voltage-gated sodium channels	Gastrointestinal distress and neurotoxic symptoms	Nausea, vomiting, severe diarrhoea, stomach cramps and in some cases headaches	Replacement of fluid loss and electrolytes
YTXs	Unknown	Not determined in humans		
DA	Inhibition of the voltage-gated sodium and calcium channels	Gastrointestinal and neurological distress	Nausea, vomiting, diarrhoea or abdominal cramps, confusion, disorientation, headaches, memory loss, seizures, coma as well as hemodynamic instability, cardiac arrhythmias and even the death	Correction of cardio-respiratory failures
STXs	Inhibition of the voltage-gated sodium channels	Gastrointestinal and central nervous system distress	Paraesthesia, headache, dizziness, nausea, ataxia, incoherence of speech, incoordination, weakness, myalgia, respiratory difficulty and muscular paralysis	Correction of cardio-respiratory failures
TTXs	Inhibition of the voltage-gated sodium channels	Nervous system distress with or without gastrointestinal symptoms	Paraesthesia, perioral numbness, lingual numbness, early motor paralysis, incoordination, aphonia, severe respiratory failure, hypoxia, hypotension, cardiac dysrhythmias and unconsciousness	Removal of unabsorbed toxin

OA and DTXs are classified as diarrhoeic shellfish poisoning (DSP) toxins. These substances inhibit the serine/threonine protein phosphatase 2A (PP2A), 1 (PP1) and 2B (PP2B) [67-69] that is translated to gastrointestinal disorders when these substances are ingested via seafood: diarrhoea, nausea, vomiting, abdominal pain and sometimes headache, chills and fever [70]. Additionally, it is thought that these substances inhibit the protection action against diarrhoea by the neuropeptide Y due to other toxins that inhibit protein phosphatases, which are not responsible for diarrhoeic symptoms [55, 71]. Other studies also confirmed that they are tumour promoters in mice [72], which would suggest that this group of toxins could have similar effects on humans. However, to date, no fatal effects have been reported [73] and the patients recovered totally after the intoxication by a treatment based on the replacement of the loss of fluids and electrolytes [55].

PTXs were firstly classified as DSP because of their notorious co-occurrence with OA and DTXs. PTXs have been demonstrated to cause cell death and apoptosis [50, 74] and toxicity in mice by intraperitoneal injection [75]. However, it has been proved that this does not cause diarrhoea [76], and consequently, these substances have been excluded from the DSP classification. No toxic symptoms in humans have been reported but due to their noticed worldwide occurrence and the possibility of being an intoxication trigger, PTXs are included in the monitoring programmes.

AZAs are classified as a known syndrome, azaspiracid shellfish poisoning (AZP) which is known to cause neurotoxic symptoms and gastrointestinal disorders [77] and recent studies have demonstrated that they are partially inhibiting the voltage-gated sodium channel [78]. Main syndromes are nausea, vomiting, severe diarrhoea, stomach cramps and in some cases headache [30, 55]. Also, metabolic activities decreased in human cells after long-term exposure to the toxin group. However, the total recovery of azaspiracid poisoning is completed after 2-5 days by supportive therapy that is similar to the OA treatment [55].

YTXs have not been involved in any human intoxication episode by the ingestion of contaminated seafood and the mechanisms of action still remains unknown. The first studies demonstrated that as with PTXs, they are not causing diarrhoea [79], and that is why are not further considered as DSP toxins. Cardio-toxic effects have been reported only when the group of toxins has been injected, but not because of oral ingestion [80]. Later studies indicated that YTXs can produce apoptosis and human neuroblastoma [81] but further investigation will be required to clarify the mode of action.

DA causes the amnesic shellfish poisoning (ASP) syndrome when ingested via seafood, leading to a series of gastrointestinal and neurologic effects: nausea, vomiting, diarrhoea or abdominal cramps, confusion, disorientation, headache, memory loss, seizures, coma as well as hemodynamic instability, cardiac arrhythmias and even the death in the worst cases. Again, the treatment is based on supported therapy giving correction to the cardio-respiratory failures, and the recovery is fulfilled in days or even weeks when the intoxication is acute [55]. The mechanism of action is based on the inhibition of the central nervous system receptors, leading the sodium and calcium channels to be free and causing neuron depolarisation [82].

STXs have been classified as PSP toxins due to the intoxication that is produced when ingested via seafood. These substances act in the sodium channel, inhibiting the neuromuscular transmission [83]. Neurological symptoms can even become fatal cases of cardio-respiratory paralysis as early as 3-4 hours after the ingestion [55]. The main symptoms are paraesthesia, headache, dizziness, nausea, ataxia, the incoherence of speech, non-coordination, weakness, myalgia, respiratory difficulty and muscular paralysis. Palliative treatments are employed until the toxins are removed via respiratory support and

the replacement of fluids. Recovery is accomplished after some days when poisoning is not severe [55].

TTXs present similar symptoms as STXs, even though they have not been included in the PSP group to date. TTXs bind to the voltage-gated sodium channel, also affecting the neuromuscular transmission too, but with a different affinity to STXs [84]. The main symptoms are paraesthesia, perioral numbness, lingual numbness, early motor paralysis, incoordination, aphonia, severe respiratory failure, hypoxia, hypotension, cardiac dysrhythmias and unconsciousness. In addition, the toxicity of both TTXs and STXs has been proved to be additive [85]. This toxin can produce death caused by respiratory failure and cardiac collapse [86], but in the case of acute intoxication, recovery is achieved in a few days by supportive treatment.

1.3 Analytical methods

A wide variety of analytical methods have been developed to assess MBTs in both the environment and seafood.

In the environment, indirect approaches based on the determination of algae that are potentially linked to the production of MBTs, have been used, including molecular probes for phytoplankton cell detection, and even satellite sensing and imaging technologies for remote acquirement of information on the occurrence of blooms. In addition, many laboratory experiments that were carried out under controlled conditions have been used combined with detection methods that are based on microscopy and/or molecular techniques in order to identify phytoplankton which are responsible for the production of MBTs. However, the link between algal blooms and MBTs depends on many different environmental parameters, and correlations between the concentration levels of toxins and the levels of biomass cannot be established.

A great effort has been made to assess both MBTs concentrations and their toxicity in seafood. These analytical methods have included from mouse bioassays to a variety of alternatives, to avoid the ethical and technical problems associated with bioassays using vertebrates. These alternatives can be classified into three major groups including functional assays, immunochemical methods and chemical analysis. However, much less attention has been paid to developing quantitative analytical methods for the quantification of MBTs in environmental samples, such as seawater. Notwithstanding, information can be used as an early warning approach to assess the potential contamination of aquaculture molluscs and also an approach is needed to distinguish whether an episode of algae growth, beyond its impact in terms of eutrophication may have adverse effects that are associated to the presence of MBTs.

In the following sections the different steps on the analysis and the different analytical approaches of MBTs in environmental samples and food are presented but with the special focus on environmental samples.

1.3.1 Sample collection

Different sampling procedures must be carried out, depending on the type of the matrix. The collection of phytoplankton samples takes place via plankton nets (**Figure 10**) in vertical or horizontal position, depending on the purpose of the study, and in the case of the identification of producers which species will be monitored. Depending on the purpose of the analysis, if only the analysis of MBTs will be carried out or if phytoplankton species

will be also identified, immediately after collection, the samples shall be treated in a different manner: for the analysis of MBTs and producer species, identification by in vivo assays will only be preserved in cool conditions. In the case of the analysis of producer species using non-in vivo approaches, a sub-sample will be fixed and precipitated. The fixation of the samples can be undertaken using a buffered solution of Lugol's iodine, aldehydes, saline ethanol or simply by freezing. Further, precipitation of the cells for the further analysis, can be carried out using sedimentation, centrifugation or filtering [87].

For the analysis of biota, in general samples are manually collected in the aquaculture facilities or after fishing and they are then preserved in cold or frozen conditions.

The collection of sediments is carried out by using Van Veen grabs (**Figure 10**) or box core grabs. But, in general, the most common method is to use the Van Veen grabs because greater amounts of sediments can be sampled. The sample preservation is achieved in cold or frozen conditions and away from light sources.

The seawater sampling is normally carried out by the using Niskin bottles (**Figure 10**). These bottles have two taps and are connected to a pressure sensor that is activated to make the closure of the bottle at the desired depth. Niskin bottles can be used in combination with CTD-probes using a carrousel or Rosette (**Figure 10**) to measure the conductivity, temperature and pressure at the sampling site. The sample preservation is achieved in cold or frozen conditions and away from light resources.

During the last decade the use of passive samplers increased in popularity. This system is based on the free attachment of the toxins into a resin that is held on the water during a defined period of time. There is a wide variety of formats, but the most common is solid phase adsorption toxin tacking (SPATT), that uses a porous synthetic resin to retain the target toxins [88].

Marine aerosols are also collected to monitor the presence of some MBTs such as PbTxs. In general, high-content air samplers are used, being equipped with specific filters for target compounds or the impact cascade samplers, in which the aerosol passes through filters of different mesh size and material and then the particles are distributed. These devices are left near the water during a specific period of time.

Figure 10. Environmental samplers: 1) Phytoplankton net, 2) and 3) Van veen grabs, 4) Niskin bottle, 5) Rossete sampler and 6) High volume air sampler.
Credits: Cristina Bosch-Orea.

1.3.2 Detection of harmful algal blooms and identification of species

As previously noted, in the last two decades HABs have increased in both frequency and intensity, driven by the global warming and activities that introduce growth-stimulating nutrients into the aquatic environments. The impact of HABs is not only the potential production of toxins; because other consequences should be properly considered, such as to conduction of anoxic conditions that can kill aquatic life, unpleasant odours and economic damages. Therefore, the rapid detection of these events is of primary importance in order to employ prevention strategies. The rapid detection is based on monitoring metrological and water quality parameters that will lead to results from HABs (**Figure 11**) and the detection of unique pigments of algae that allow their monitoring using fluorescence-based tools, ranging from handheld meters to satellites.

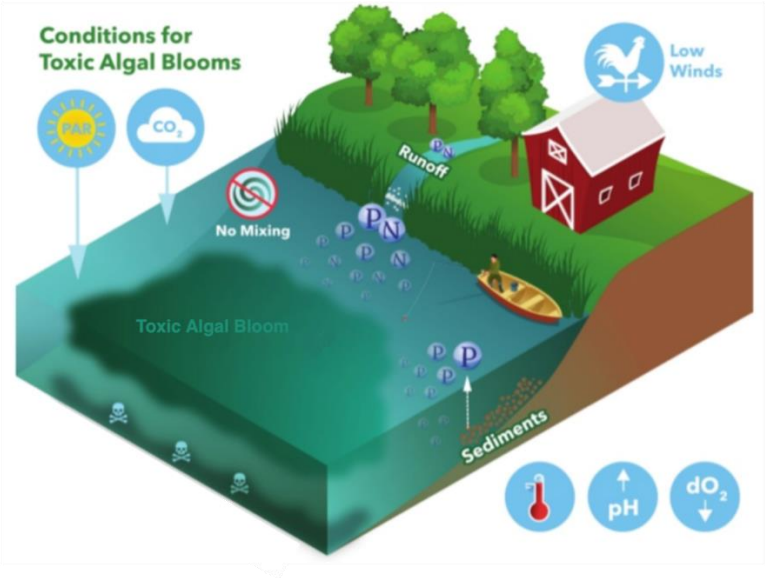


Figure 11. Main water quality and environmental conditions favouring a HAB.

The algal pigments make their macroscopic display easy, for instance, *Noctiluca scilliants* has orange colour due to the carotenoids which are present in its cells [89]. However, chlorophyll is the most studied pigment of the phytoplankton and it has been employed for this type of investigations [90]. Remote-sensing instruments to be used into satellite or aircrafts together with powerful elucidation software tools have been developed for the remote identification of algal blooms [91]. More robust options are able to distinguish algae shape between different superclasses of phytoplankton, such as between diatoms and dinoflagellates [92], or the blooms of specific species such as *Karenia brevis* [93]. A summary of HAB monitoring systems based on fluorescence detection is presented in **Figure 12**. However, the monitoring systems are, not able to analyse the composition of the algae that are able to produce toxins and in this case, laboratory analysis is required.

For the taxonomic investigation of the potential occurrence of toxin-producing algae, traditional techniques are based on the microscopic counting of cultures. The official method of Utermölh technique is based on the use of inverted microscopes to perform the taxonomic identification of algae species [94, 95]. In **Figure 13** there is an example of two algae of the genus Pseudo-Nitzschia and Dinophysis that were identified by observation under light microscopy. Another powerful microscopy system that is employed in the characterisation of toxic species of algae is the scanning electron microscopes (SEM) that provides information about morphology and even chemical composition [96, 97].

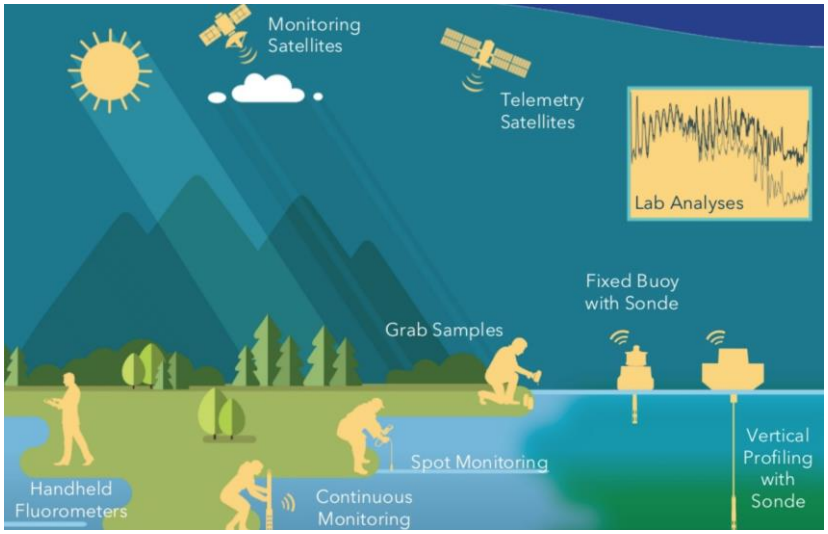


Figure 12. HAB monitoring systems based on fluorescence detection.

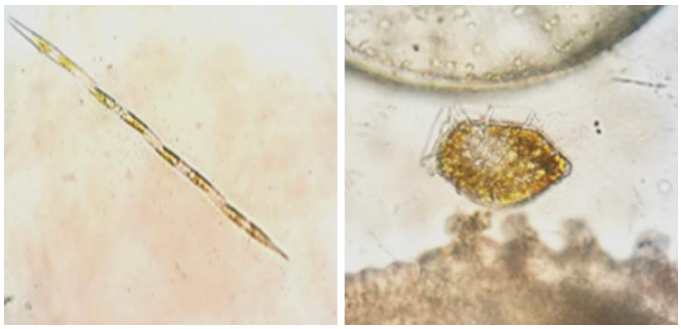


Figure 13. *Pseudo-nitszchia* spp. (left) and *Dinophysis* spp. (right) imaged by a light microscopy. Credits: Cristina Bosch-Orea.

Molecular detection techniques have also been also very much employed because they offer easier, faster, and more accurate means of monitoring species, in comparison with traditional techniques [98]. Moreover, molecular techniques offer additional advantages when multiple species need to be detected and/or are in very low abundance [99]. Molecular techniques can be used in combination with a range of techniques such as microscope-based enumeration and fluorescence in-situ hybridization (FISH) and molecular cell-free formats, such as the sandwich hybridisation assay (SHA), biosensors, microarrays, quantitative polymerase chain reaction (qPCR), and real time PCR (RT-PCR). Among the most advanced approaches are those that combine one or several laboratory functions into a single integrated system (lab-on-a-chip), which are techniques that generate a much higher throughput of data, and those that enhance the performance of molecular techniques such as the nano-bioengineered probes, the pre-concentration and magnetic separation systems.

Among these approaches, FISH is widely employed to identify HAB species [100, 101] using the labels of the fragments of RNA or DNA that can be fluorescently marked without destroying the cell. In combination with epifluorescence microscopy and flow cytometry, FISH is a rapid technique, even though it is not really all that sensitive and requires large amounts of cells [102]. However, the main limitation of FISH approaches is the number of species that can be detected within one experiment. In addition, only two different kinds of fluorochromes, FITC and CY5, are routinely used for detection.

In combination with fluorescence genetic markers or imaging using solid phase cytometry (SPC), these techniques have demonstrated an achievement of high sensitivity and rapid analysis [102]. For example, imaging in flow cytometry (FCM) algorithm has been employed in the monitoring of *Karenia Brevis*, the causative factor of PbTxs, during blooms in the Gulf of Mexico (Atlantic Ocean, North America) [103]. Also, *Heterosigma akashiwo*, one of the most important HAB species in China, was determined as being joined by five more HAB species, by using FCM combined with immunofluorescence [104].

If the total nucleic acids are extracted from samples then all cell morphology is lost. However, several methods have been developed that rely on DNA or RNA extracted from environmental samples and have been successfully used to detect organisms from many different water types. Among these approaches, the SHA should be highlighted, in which two specific oligonucleotides probes capture and immobilise a target sequence of nucleic acids (DNA or RNA). Any of the probes can be detectable by being marked and then they can easily be quantified. This technique is highly sensitive and has been employed in the determination of the HAB species *Prymnesium parvum, Pseudo-nitzschia australis* and *Gymnodinium catenatum,* which are responsible for the production of toxins Prymnesins, DA and STXs, respectively [105].

Some combinations of the SHA detection technique and biosensors have also been developed and showing excellent performance. A biosensor is an analytical platform consisting of a biological receptor that is immobilised onto a transducer surface. The combination of the SHA method with electrochemical detection of bound nucleic acid target molecules has proven to be the successful [106]. Moreover, Diercks et al. [107] adapted this system into a multiprobe for its use in a semi-automated device for the simultaneous detection of 14 target species of toxic algae.

The PCR, probably the most employed technique because it is able to distinguish between species which are morphologically similar by the differences in their genetics. Several types of quantitative PCR have been developed and applied in the study of toxigenic-species, but qPCR is probably the most used technique. In qPCR, quantitative information can be calculated because fluorescent markers are incorporated into each PCR product as amplification proceeds, and data can be collected over each PCR cycle. Then, the changes in fluorescence, measured as PCR labelled amplicons, are accumulated in each cycle which is directly proportional to the starting amount. This technique has been successfully used in complex environmental samples, for example, Eckford *et al.* determined *Alexandrium tamarense*, *Karenia mikimotoi*, *Karlodinium veneficum and Prymnesium parvum,* the causative species for the STXs, PbTXs, karlotoxins and pyrmnesin toxins by employing a multiplex real-time qPCR assay [108]. In another example, Perini *et al.* quantified the toxic-dinoflagellate resting cysts of *Lingulodinium polyedrum, Protoceratium reticulatum, Gonyaulax spinifera, Alexandrium minutum* and *A. pacificum*, the producers of the YTXs and STXs toxins, from marine sediments that were collected from commercial ports [109].

1.3.3 Analysis of marine biotoxins

In vitro and *in vivo* toxicology assays were the first methodologies which were employed to assess the intoxication cases that appeared in seafood commercial samples and to characterise the toxicity of the determined toxins. The most well-known test is the mouse bioassay (MBA) which was widely used for the analysis of ciguatoxins (CTXs) since the 1980s [110, 111]. The assay consists in the *in vivo* intraperitoneal injection of seafood extracts into mice to determine the presence or absence of MBTs [112]. Nevertheless, in

some countries MBA is still being used for the determination of PSP toxins and CTXs [113].

Currently, most of the analytical techniques for the analysis of MBTs can be classified into two main groups:

- Biological approaches
  - immunochemical techniques
  - cell-based assays
  - receptor-based assays
- Chemical analysis

In the following subsection these approaches are summarised and discussed.

1.3.3.1 Biological tools for marine biotoxins analysis

Biosensors, biochemical assays and cell-based assays are excellent analytical tools for the detection of MBTs because of their selectivity, sensitivity, ease-of-use and low cost. In this section, the primary biochemical assays are summarised. Immunoassays, cell-based assays (CBAs) and receptor-based assays (RBAs) which are sometimes based on the same mechanism of action as CBAs, but using receptors instead of whole cells will be discussed. Finally, since biosensors are based on any of these assays, they are described at the end of each subsection.

Immunochemical techniques Immunoassays are biochemical assays based on the recognition and affinity between an antibody (Ab) and its antigen (Ag). They have been used for the screening and precise quantification of some MBTs, because of their high selectivity [114]. However, Abs may have cross-reactivity to detect different toxin analogues or derivatives, if they share a structurally similar fragment, although it can be to a different extent. This ability is known as class recognition, and it can be advantageous (or not), depending on whether the purpose is to detect the whole family of toxins or just one specific toxin [115].

The most commonly used immunoassay format is the enzyme-linked immunosorbent assay (ELISA), which relies on the use of specific antibodies (Abs) against the target analyte, and enzymes as labels. This type of assay can be carried out according to direct, indirect and sandwich formats (**Figure 14**). The direct assays imply the labelling of the primary Ab while the indirect assays use a labelled secondary Ab against the primary Ab. The indirect assays require more steps and thus longer analysis times. ELISAs can also be competitive, in which the free and immobilised toxins compete for the capture of an Ab and, if required, a labelled secondary Ab is added to the system. In addition to the direct and indirect assays for large molecules, another format is the sandwich which can be useful for particular classes of MBT such as palytoxins (PLTXs) [116]. For instance, Boscolo et al. [116], developed an indirect sandwich ELISA for palytoxin and 42-hydroxy-palytoxin with a limit of detection (LOD) of 1.1 ng/mL and a limit of quantitation (LOQ) of 2.2 ng/mL. But, the most common formats that are employed for the detection of MBTs are competitive ELISAs under both direct and indirect formats. For example, Dubois et al. [117] developed three direct competitive ELISAs to detect OA, DA, and SXT. The method was proved for the analysis of shellfish extracts with detection capabilities (CCβ values) of 150 µg/kg for OA, 50 µg/kg for DA, and 5 µg/kg for SXT. In another work, Sato et al. [118], presented the development of a quantitative direct screening approach coupled a sample pre-treatment for the PSP group [118]. More recently, the same research group [119] developed a new polyclonal Ab against TTX, and developed a direct ELISA. However, this method does not

meet the current sensitivity requirements and needs further research to make future improvements.

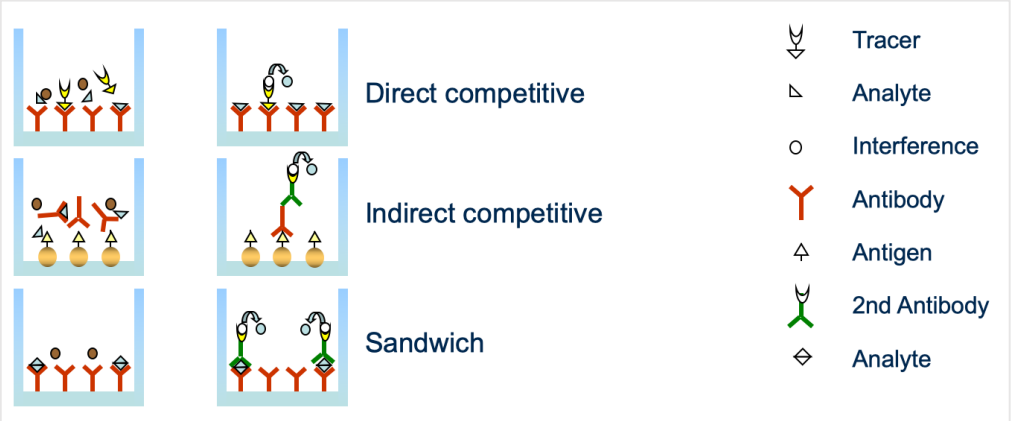


Figure 14 Scheme of ELISA formats.

Indirect assays have also been reported, for example and assay for the detection of PLTX was reported by Bignami et al. [120] using the classical PLTX-BSA conjugate as a coating agent and the obtained IC_{50} value was 6.2 ng/mL. Further, in Frolova et al. [121] for PLTX, those authors took advantage of the large size of this analyte and PLTX was directly immobilised on the microtiter plate surface with an IC_{50} of 20 ng/mL.

Other formats are based on strips or sticks that provide a visual sign when the toxin in present, making these assays a rapid screening tool, and the ELISA approach has been heavily employed in the detection and quantification of MBTs in seafood [122, 123] and water [124]. In general, these approaches are admitted for screening purposes, however, due to the cross-reactivity and sometimes the lack of sensitivity of some congeners their use can be avoided under certain regulatory criteria. A summary of ELISA assays for the analysis of MBTs is summarised in **Table 4**. ELISA permits fast screening and different commercially available kits are currently in the market place for different groups such as PSP toxins (R-Biopharm, AG, Darmstadt, Germany, RIDASCREEN® Fast PSP SC and Biosense Abraxis® Saxitoxin (PSP) kits), for DSP, AZP and other lipophilic toxins (UBE Eurofins Industries DSP-Check; Eurofins Abraxis® Okadaic Acid (DSP) and Rougier, Montreal, Canada Bio-Tech® tests) and for ASP toxins (Biosense® ASP ELISA). The latter case meets the requirements to be an official method (Association of official agricultural chemists, USA, AOAC, Official Method 2002.06). Also, other rapid immunoassays formats are commercially available such as the lateral flow tests (dip-stick style tests), such as the Jellett® Bioteck, Dartmouth, Canada, PSP Rapid Test for PSP and ASP.

Table 4. Examples of enzyme-linked immunosorbent assay (ELISA) for the detection of MBTs.

Toxins	ELISA type	Antibody	Detectability	Matrix	Ref.
CTXs	Sandwich	Monoclonal	1 pg/ml	Fish	[125]
DA	Indirect	Monoclonal	6 pg/ml	Shellfish	[126]
STXs	Direct	Polyclonal	0.02 ng/ml	Urine	[127]
PbTxs	Indirect	Monoclonal	14 ng/mL	Shellfish	[128]
OA	Indirect	Monoclonal	12 pg/mL	Shellfish	[129]
AZAs	Indirect	Polyclonal	57 µg/kg	Phytoplankton and shellfish	[130]

During the last decade, an important development effort has been undertaken to develop biosensors that detect MBTs. Some examples of immunosensors for the detection of MBTs are presented in **Table 5**. In spite of these developments that were mainly focussed on seafood analysis, but can also be applied to assess water samples and environmental extracts. Most of these biosensors are surface plasmon resonance (SPR) immunosensors, which employ an optical technique allowing the label-free detection of the toxin of interest in real time, but also some developments based on electrochemiluminescence (ECL) and electrochemical detection have also been exploited.

For instance, based on a direct immunoassay with monoclonal Abs, Yakes et al. [131], developed a SPR immunosensor for PLTX with LODs of 0.52, 2.8 and 1.4 ng/mL in buffer, grouper and clam samples, respectively. In another example, a sandwich immunoassay combined with ECL detection has been developed [48], thus providing a LOD of 0.07 ng/mL and a LOQ of 0.24 ng/mL.

Several immunosensors based on SPR transduction have been reported for TTX detection [132-136]. Nevertheless, the small molecular size of TTX make it difficult their detection using direct formats, and improved results were obtained by indirect approaches or approaches that involve the Ag immobilisation and detection of the Ab interaction. Functionalisation of the gold chip surfaces is in general obtained by a mixed self-assembled monolayer (SAM) of hydroxy- and amino-terminated oligo-ethylene glycol alkanethiols (OEG-ATs), because the ethylene glycol units minimise non-specific interactions. In general, the LOD of these immunosensors showed enough good sensitivity below 1 ng/ml in an assay buffer.

Table 5. Examples of immunosensors for the detection of MBTs.

Toxins	Transductor type	Antibody	Detectability	Matrix	Ref.
AZAs	Electrochemical	Polyclonal	63 µg/kg	Shellfish	[137]
TTX	Optical	Monoclonal	0,4 µg/g	Fish	[138]
DA	Electrochemical	Monoclonal	0.7 ng/ml	Shellfish	[139]
OA	Optical	Monoclonal	0.05 µg/L	Shellfish	[140]
CTXs	Electrochemical	Monoclonal	0.01 µg/kg	Fish	[141]
PbTxs	Electrochemical	Monoclonal	5.0 pg/ml	Shellfish	[142]

Cell-based assays (CBA) these assays are based on the cytotoxicity of MBTs, that produces changes in the morphology, the physiology or the cell viability, which can be measured [143].

Some examples of the application of CBAs for the analysis of MBTs are shown in **Table 6**. In most of the cases, the good performance of CBAs requires the presence of agonists or antagonist substances that act in counteracting or emphasising the actions of the toxins. Some examples of these substances are veratridine and ouabain. Veratridine is an activator of the voltage-gated sodium channels (VGSCs), and it binds to these channels and blocks them in an open position. However, ouabain attaches to the Na^+/K^+-ATPase pump and blocks it in a closed position. MBTs affect on these channels and pumps, in the presence or absence of ouabain and veratridine at appropriate concentrations, giving a specific response on the cells. Despite the fact that different toxins can share the same mechanism

of action, they may act on the cells to a different extent, therefore showing a different degree of toxicity.

Table 6. Application of CBAs in the analysis of MBTs in biota samples.

Toxins	Cell line	Detectability	Matrix	Ref.
CTXs	Neuroblastoma (N2a)	0,031 μg/kg	Fish	[144]
Palytoxin	Erythrocytes	0.375 ng/L	-	[145]
CTXs	Neuroblastoma (N2a)	0.4 ng/L	Blood	[146]
Palytoxin	Neuroblastoma (BE (2)-M17)	0.2 μg/L	Shellfish	[147]
CTXs	Neuroblastoma (N2a)	26 ng/kg 15 ng/kg	Fish Shellfish and sea urchins	[148]

These assays can be carried out using primary cultures or immortal cell lines. Primary cultures are obtained from tissues before the assay. They reflect the properties, to a larger extent, that the cells have in the organism and they could be more sensitive than immortal cell lines. In spite of that, they may involve the use of laboratory animals which present a higher variability related to the organism source and the cell isolation process, and for this reason they have not been extensively exploited.

Bellocci et al.,[149] developed the first CBA for PLTX using MCF-7 cells and ouabain as the antagonist substance. The reduction of cytosolic lactate dehydrogenase (LDH) after cytolysis was measured. The assay presents an EC_{50} of 530 pM of PLTX. Later studies [150, 151] measured the mitochondrial activity and quantified the cell viability because it does not involve transferring supernatants, thus increasing the reproducibility of the assay. In another example, an interesting method based on a dynamic assay was developed by Espiña et al. [152], using rat hepatocytes (clone 9) and human neuroblastoma BE (2)-M17 cells. Alamar Blue, a fluorescent dye, was used to continuously monitor of cell viability. The ouabain preventive effect on the decrease of cell viability caused by PLTX was more evident in human N2a cells than in hepatocytes.

In another example, Manger et al. [153] developed an innovative variant of the N2a CBA using FCM for the detection of CTXs. In this case, the assay was performed with cells in suspension, and veratridine was used as the agonist. Fluorescent voltage-sensitive dyes and a flow cytometer were employed to assess the membrane potential caused by sodium currents resulting from VGSC activation. This assay reduces the time of analysis to minutes and provides more direct mechanistic data.

An interesting approach is the combination of CBAs in biosensors. Some examples of CBA-sensors are summarised in **Table 7**. For instance, Volpe et al. [87] measured the haemolytic activity of PLTX with amperometry on 8-screen-printed electrode strips. Ouabain was employed to guarantee the assay specificity for PLTX. The optimal approach uses pyruvate and NADH as enzyme substrates and phenazine methosulphate (PMS). This latter compound reacts with the remaining NADH to produce PMSH, which subsequently reduces the hexacyanoferrate (III) to hexacyanoferrate (II), which is oxidised on the electrode surface. The LOD for 4 hours of haemolysis time, was 0.16 ng/mL.

In spite of the interest of CBA approaches for the detection of MBTs, their application to assess certain environmental samples presents a series of drawbacks such as lack of specificity, potential of matrix effects, and lack of sufficient sensitivity to be used in early warning systems, for example, measuring the ultra-trace presence of these MBTs in

seawater. Therefore, most of the applications of these approaches are focussed on food safety analysis.

Table 7. Application of CBA-biosensors in the analysis of MBTs in shellfish.

Toxins	Transductor type	Cell line	Detectability	Matrix	Ref.
STXs PbTxs	Electrochemical	Cardiomyocyte	0.35 µg/L STX 1.55 µg/L PbTX-2	-	[154]
STXs	Electrochemical	Neuroblastoma (N2a)	31 ng/L	Shellfish	[155]
OA	Optical	Liver cancer (HepG2)	33.95 µg /L	Shellfish	[156]
STXs TTXs	Electrochemical	Cardiomyocyte	87 ng/L STX 89 µg/L TTX	-	[157]
OA	Piezoelectrical	Liver cancer (HepG2)	10 µg/L	-	[158]
OA	Electrochemical	Cervical cancer (HeLa)	10.2 µg/L	Shellfish	[159]
		Liver cancer (HepG2)	3.3 µg/L	Shellfish	

Receptor-binding assays (RBAs) are assays based on the affinity of certain cellular receptors to bind to a specific analyte. In general, these competitive formats use a labelled toxin in competition with the toxin that is present in the sample for the receptor is usually carried out. Like happens in immunochemical assays, the main drawbacks are the cross-reactivity between structurally-related compounds. During the recent decade different RBAs have been developed for the detection of cyclic imines (CIs) [160, 161] and ciguatoxins [162].

1.3.3.2 Chemical analysis of marine biotoxins

Sample preparation The extraction of MBT analytes from solid samples is in general achieved by solid-liquid extraction (SLE), whereby a solvent is added to the sample and the mixture is agitated to extract MBTs into the solvent, with insoluble material being separated by centrifugation or vacuum filtration. The solubility of MBTs is relative depending on the group; however, all of the MBTs are soluble in MeOH [163]. The Standard Operation Procedure (SOP) developed by the Europena Union Reference Laboratories (EURLs) employs a solid-liquid extraction with 100% MeOH assisted by centrifugation, in the analysis if lipophilic MBTs in bivalve molluscs [164]. Ultrasonication can be used to aid the extraction process. Ultrasounds assisted extraction (UAE) is one the cheapest techniques with low instrumental requirements [165] and it can extract a simultaneously high number of samples. Consequently, it is widely used in environmental and industrial applications [166]. In the analysis of MBTs, Wang et al. used UAE to extract the DA contained in Pseudo-Nitzschia cultures by the lysis of the phytoplankton cells from sonication [167], and Barbaro et al. from mussel's tissue [168].

Some applications have been developed based on the use of accelerated solvent extraction (ASE). In this case, the sample and solvent are placed in a sealed container and slightly heated under pressure, increasing the speed of the SLE process. The technique provides

good recoveries for thermostable compounds such as CTX [169] [170, 171]. One of the main advantages of this technique is the automatisation of the process, which is attractive for routine analysis.

The extract can be used for direct analysis or it can be subjected to a clean-up. Sample extract clean-up is performed to remove unwanted matrix interference that may disturb the instrumental analysis, thus producing ion enhancement or ion suppression which can drive the process towards over- or under-estimation of the results [172]. A clean-up is typically carried out using solid-phase extraction (SPE), with one or more of a variety of stationary phases, which may be standard or reverse-phase, ion exchange resins, or immunoaffinity materials. SPE also contributes to the pre-concentration of the extracts or samples. The versatility of this technique yields an huge number of stationary phases which are available, that allows the extraction and clean-up of various compounds, and that is the reason why it is extensively used in SPE as a broad-based extraction technique [173]. Lipophilic MBTs have been extracted from seawater by SPE employing hydrophilic-lipophilic balance (HLB) with recoveries up to 53-70% [174]. From solid matrices such as shellfish, SPE with HLB cartridges are usually employed as the clean-up step [175]. In the case of there being more polar toxins, DA can be extracted by using HLB and ionic exchange cartridges. For example, Gagez et al. employed HLB cartridges to extract DA from seawater [176] and Gimenez-Papiol et al. used strong anion exchange (SAX) cartridges in the clean-up step of mussels analysis [177]. In the case of hydrophilic MBTs, the extraction or clean-up by employing SPE is more difficult due to the high polarity of these compounds, that are not always easy to retain it in the cartridges. In some cases, the stationary phase that is used for the clean-up of mussel extracts has implemented amorphous graphitised polymer carbon [178], while in some other cases have needed C_{18} cartridges [179] or HLB [180]. The volume and polarity of the sample play important roles in the extraction of MBTs.

The QuEChERS (quick, easy, cheap, effective, rugged, and safe) method is another sample extraction and clean-up technique that has been used in the extraction of lipophilic MBTs from bivalve shellfish extracts. The approach consists of the extraction with an aqueous miscible solvent in the presence of high amounts of salts or buffering agents to induce liquid-phase separation of MBTs. Upon shaking and centrifugation, an aliquot of the organic phase is subjected to further clean-up using SPE. QuEChERS has been employed in seafood matrices such as mussels, with recovery values ranging from 83 to 112% [181] and 74 to 102% of PSTs and lipophilic MBTs [163], respectively. This technique is suitable for eliminating the lipid and protein content without losing the MBTs. The main drawback of this approach is the poor extraction recoveries for some MBTs that can be overcome by the use of isotopically labelled internal standards.

Some of the most currently used extraction and clean-up methods for the analysis of MBTs are summarised in **Table 8**.

Analytical techniques Capillary electrophoresis (CE) is a separation technique in which charged molecules are dissolved in a solution within an applied electric field and the separation of the compounds takes place. The ionic mobility of each analyte will determine the separation that will be modulated by the changes in the conductivity and pH of the solution. CE with fluorescence detection has been used for the analysis of DA in mussels [182]. Later, CE-with diode array detection (DAD) has been successfully employed for the analysis of STXs, YTX and DA [183]. In terms of resolution, CE-DAD is highly convenient for the separation of the most polar MBTs. However, the low sensitivity and the lack of a sufficient number of confirmation points for the identification of the analytes are the major drawbacks of this approach. The most widely used separation approach for the analysis of

MBTs is Liquid Chromatography (LC), particularly in the reverse phase (RP-LC). Several methods of High-Performance LC (HPLC) have been developed for the analysis of MBTs using different stationary phases, but C_{18} is the most employed method. LC coupled to ultraviolet (UV) and diode array (DAD) detection [96, 184, 185] have been widely employed. Actually, some of the current official methods of analysis employed by the reference laboratories are based on HPLC-UV, as in the case of DA analysis in shellfish [186]. However, the analysis of hydrophilic MBTs by RP-LC is more complex than for the lipophilic MBTs. Lawrence et al. developed a method of HPLC-FD (fluorimetric detection) with previous oxidation of the analysed PSP group of toxins (PreCOX-LC-FD) [187, 188]. Oxidation of the PSTs in basic conditions with hydrogen peroxide or periodic acid was required in order to form fluorescence products which are able to be detected by FD. Even with the fact that the sensitivity for some of the toxins was good with LODs of 7-12 ng/g, there are have some other inconveniences such as the co-elution of the GTXs, the long time period for the analysis and the need of high amounts of toxins.

Table 8. Extraction and clean-up techniques used in the analysis of MBTs. Total recovery is expressed in percentage (%).

Toxins	**Matrix**	**Extraction and clean-up technique**	**Recovery (%)**	**Ref.**
Lipophilic MBTs	Seawater	SPE	52.25-70.18	[174]
		SPE	54.5–83.3	[171]
	Shellfish	Centrifugation	>90	[189]
		On line SPE	97–102	[190]
	Sediment	PLE	30-83	[171]
		PLE	78–109	[170]
	Phytoplankton	LLE	92-107	[191]
Hydrophilic MBTs	Shellfish	Centrifugation	90-110	[192]
		QuEChERS	79-112	[181]
		SPE	28-107	[178]
Domoic acid	Seawater	SPE	57-69	[193]
		SPE	96	[176]
	Shellfish	UAE	94	[168]
		Centrifugation	95	[192]
	Phytoplankton	UAE + SPE	98	[167]
	Fish	SPE	74	[194]

A significant number of analytical techniques developed during recent decades have been based on the use of hyphenated techniques with mass spectrometry (MS) to achieve higher levels of confidence in the identification of the compounds.

The coupling of CE and MS has made the analysis of MBTs suitable but in particular, regarding the most polar compounds, the alkaloids group. TTX, STX and their analogues have been separated with very high resolution when employing CE-MS. In addition, the coupling to MS generally avoids the matrix interferences of the samples [195].

Gas chromatography (GC) coupled to MS has been used to analyse some MBTs such as TTX in shells [196]. Also, the first studies of TTX isolation from bacteria were performed by HPLC and extra confirmation analysis was performed by using GC-MS [197]. However, the use of GC did not become highly popular in the analysis of MBTs because of the need to derivatise the compounds to make them suitable for the analysis using this technique.

LC-MS is, in general, the technique of choice for the analysis of MBTs [198], due to their high selectivity and sensitivity. **Table 9** summarises some of these combinations and applications, and also the limits of detection (LODs) of the different methods.

Table 9. LODs of different methods of CE-MS and LC-MS for the analysis of MBTs in different matrices.

Techniques	Matrix	Toxins and mLOD (µg/kg)	Ref
CE-MS	Mussels	TTXs: 5.2 DA: 160 STXs: 1.8 - 120	[195]
	Mussels	YTX: 10 PTX-6: 130	[199]
LC-MS	Mussels	STXs: 1.5 - 9	[200]
	Mussels, clams, oyster and scallops	OA: 0.007 DTX: 0.01 AZAs: 0.035 - 0.08 PTX: 0.06 YTX: 0.09	[175]
	Mussel, oysters and clams	DA: 100	[177]
	Phytoplankton	DA: 0.03	[167]
	Phytoplankton	OA: 0.04 DTX-1: 0.03 PTX-2: 0.07 AZA-1: 0.07 YTX: 0.05	[191]
	Sediments	OA: 0.006 DTX-1: 0.013 YTX: 0.013 AZAs: 0.013- 0.062 PTX-2: 0.003	[170]

The combination of these techniques is physically possible because of the ionisation sources, which are the interphase approaches that permit the ionisation of the molecules and the passing to a vapour phase from the liquid phase as a result of CE and LC. Due to the characteristics of MBTs, the most employed ionisation source in the analysis of these compounds by CE-MS or LC-MS is the electro spray ionisation (ESI) source [181, 195, 200, 201]. The ionisation takes place at atmospheric pressure by the application of an electric field that forms charged droplets which are then evaporated, leading to the charged analytes entering the mass spectrometer. During recent years, hydrophilic interaction LC (HILIC) that are used to separate the hydrophilic MBTs has become more popular because this technique avoids the need for previous derivatisation. The first study of HILIC for the analysis of PSPs toxins in mussels was developed by Dell'Aversano et al. which in combination with MS, was able to resolve the separation between the different STXs congeners [200].

The latest advances in MS include their operation with powerful analysers of high resolution, that are able to distinguish between narrow mass/charge ranges, such as time of flight (TOF) and orbitrap analysers. Known as high resolution mass spectrometry (HRMS), the MS operating in conjunction with these analysers was recently employed for the structural elucidation of MBTs [44] and the discovery of new analogues. The high accuracy in the mass/charge determination allows the discrimination between isobaric compounds, which are those compounds with the same nominal mass but different molecular formula.

1.4 Occurrence of marine biotoxins

The frequency and worldwide distribution of HABs have increased during recent decades [38]. Although these events are occurring naturally, the influence of global warming and anthropogenic activities is critical for this increment. These activities have contributed to the increase in the global temperature and in the eutrophication of the waters. In combination, these two factors favour phytoplankton growth and consequently, the occurrence of HABs.

- **Eutrophication** is a phenomenon that is derived from the high amounts of nutrients that reach the oceans and are driven by the rivers and run-offs from industry, agriculture and wastewater. The amplification of the tourism/travel industry, other industry and domestic run-off has contributed to the eutrophication of the waters, especially in coastal regions. These waters are rich in nutrients that provide suitable conditions for phytoplankton proliferation.

- **Global warming** is the increment in the global temperature during recent years which has become another important factor for the promotion of phytoplankton growth and the migration of species to areas that are more temperate in order to escape harsh environments.

The growth of algal bloom has increased in parallel with the increment of eutrophic regions and the waters warming. Further, the occurrence and distribution of these toxins stem from the habitat where the producer-algae live and blooms and they are naturally determined by environmental conditions or forced by stressful conditions.

The toxigenic phytoplankton species and the corresponding MBTs, are summarised in **Table 10**. This type of identifications is carried out under laboratory conditions, and only the species that have produced specific toxins in such studies are considered as producers, in order to avoid misleading information with regards to the co-occurrence of algae or other organisms. For example, TTX was thought to be produced by pufferfish because this toxin was determined at high concentrations in their bodies. Later, it was demonstrated that TTX are produced by bacteria, and is bioaccumulated by pufferfish and other species such as the blue-ringed octopus [202]. Even though marine bacteria are producing TTX [50], this information still remains contradictory in the body of literature. The number of producing bacteria could have been overestimated by the lack of specificity in the methods used when determining the production of TTX [203].

The adaptation of the species to the new habitat could be the key to explaining its presence in remote areas. Some species seem to have developed new strategies of survival. In the case of dinoflagellates, which constitute the majority of MBT producer species, these are able to move along the water column to look for the sunlight, nutrients or whatever they need to live. However, diatoms, are not motile, hence they developed a strategy of movement through the density of their cells, in which they are able to sink or “control” their buoyancy. Another example that is related to survival strategies for nutrition is the case of the genus Dinophysis. This group of dinoflagellates is formed by mixotrophous organisms with the capacity to use different sources of energy and carbon, from photosynthesis and from the uptake of other organisms [113], thus taking greater advantages of the environmental conditions. Another point to consider is the lack of previous information, to the best of our knowledge, about the presence of toxigenic species, and this fact creates uncertainty in the knowledge of autochthonous species and the cryptogenic species.

Table 10. Toxigenic phytoplankton species and corresponding MBTs.

Toxin	Producer	Genus	Species	Ref
OA, DTXs	Dinoflagellates	Dinophysis Prorocentrum	*Dinophysis acuminata, D. acuta D. tripos, D. caudata, D. norvegica, D. fortii, D. ovum, D. sacculus, D. tripos, D. mitra, D. rotundatum* *Prorocentrum lima, P. minimum P. rhathymum*	[204] [205] [206] [207]
PTXs		Dinophysis	*Dinophysis acuminata, D. acuta, D. caudata, D. infundibula, D. fortii, D. sacculus, D. tripos, D. norvegic, D. tripos, D. caudata, D. rotundatum*	[204] [207]
AZAs		Azadinium	*Protoperidinum crassipes, Azadinium spinosum, A. poporum, A. Spinosum, A. Trinitatum, A. cuneatum, A. concinnum, A. dalienense, A. poporum, A. obesum*	[15] [208] [209]
YTXs		Gonyaulaux	*Protoceratium reticulatum* *Lingulodinium polyhedral* *Gonyaulax polyhedral, G. spinifera*	[210] [211] [212]
DA	Diatoms	Pseudo-nitzschia	*Pseudo-nitzschia brasiliana, P. Fraudulenta, P. Multistriata, P. Pungens, P. Calliantha, P. Delicatissima, P. Galaxiae,P. australis*	[37] [213]
STXs	Dinoflagellates and freshwater bacteria	Alexandrium Gymnodinium Pyrodinium Cyanobacteria	*Alexandrium catenella, A. tamarense, A. ostenfeldii* Gymnodinium catenatum Pyrodinium bahamense Trichodesmium erythraeum	[214] [215] [216] [217]
TTXs	Bacteria	Serratia Bacillus, Shewanella, Roseobacter, Vibrio, Pseudomona, Alteromona, Aeromona, Nocardiopsis	*Serratia marcescens* Vibrio spp. V. Aeromonas spp. Microbacterium Arabinogalactanolyticum Pseudomonas spp. Shewanella putrefaciens Alteromonas spp. Pseudoalteromonas spp. *Nocardiopsis dassonvillei.*	[50]

The worldwide occurrence of MBTs during the last five years are represented in **Figure 15**. STXs and TTXs are the MBTs which are more widespread around the world, followed by OA and DTXs, PTXs and YTXs. Further, DA is extensively occurring in coastal areas. These groups of toxins have also been the most studied because of a surge in seafood consumption. AZAs have a distribution that is mainly in European waters and the North of Africa. The absence of toxins in regions like Africa, the north of Russia or the Antarctica cannot be fully corroborated because of the non-existence of monitoring programmes.

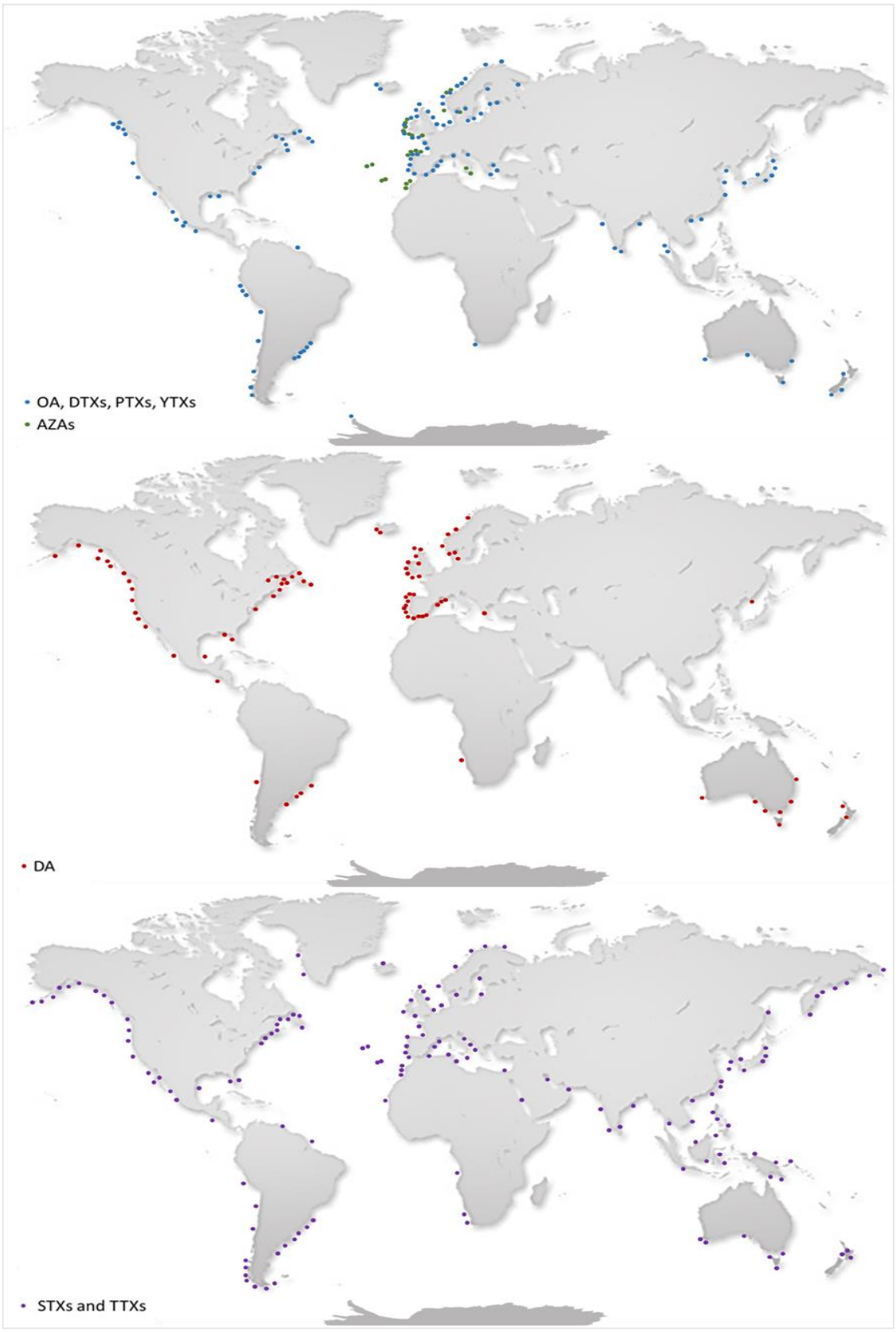


Figure 15. Worldwide occurrence of MBTs during the period comprised during the years 2015-2020. Data collected from database of National Oceanic Atmospheric Administration (NOAA), Harmful Algae Event Database (HAEDAT) and references in the manuscript.

Since 1980, the countries which are most affected by the occurrence of MBTs established exhaustive monitoring programmes to determine the toxigenic-producer species and the corresponding toxins. Thereafter, ample data have been gathered, and all the information provided by these programmes has been employed to deeply study the phytoplankton species, their habitat and the production of MBTs. Different matrices have been analysed with the aim of characterising the concentrations of the toxins. The most reported matrices have been the seafood species of commercial interest. However, to date, some other environmental matrices have been considered in order to characterise the distribution of the toxins and to identify the possible vectors of intoxication. The maximum concentrations of MBTs that have been reported in different matrices during the last five years are shown in **Table 11**.

Bivalve molluscs, and mussels in particular, are the organisms which have been studied more in relation to the contamination by MBTs. These feeder-filters have been employed as sentinels of worldwide coastal areas to determine the pollutants which are present in the water and consequently in the ecosystem. Mussels are benthic organisms that act as passive samplers, thus providing suitable characterisations of the specific areas where their habitats are located. Moreover, the commercial interest has promoted the monitoring of these species. The higher concentrations of MBTs which have been reported during the last five years correspond to mussels of the species *Mytilus chilensis* and *Mytilus galloprovincialis* which reached concentrations of 24,950 µg/kg of STX in Chile and 7,693 µg/kg of OA in Spain, respectively [218, 219]. These amounts of toxins are above the maximum residue limits (MRLs) that ensure the safety of this consumption, which are 800 µg STX equivalent/kg and 160 µg OA equivalent/kg, respectively. Also, clams and oysters have been widely reported due to the toxins content. DA was measured at concentrations of 346,000 µg/kg in clams (*Pecten maximus)* from Nova Scotia, eastern Canada [220] and STX at 32,300 µg/kg in Chilean clams (*Gari Solida)* [218]. Other molluscs, such as sea snails, have reported high concentrations of STXs. *Argobuccinum ranelliforme*, *Charonia lampas* and *Ceritium vulgatum* have reported high concentrations of STXs, 36,370 µg/kg, 1,423 µg/kg and 2,556 µg/kg in areas from Chile, Portugal and Morocco, respectively [218, 221]. *A. ranelliformes* and *C. lampas* are carnivorous gastropods while *C. vulgatum* is a grazer.

Moreover, other non-conventional vectors of MBTs propagation have been reported, with high concentrations of these substances. Also, non-edible animals such as starfishes have been affected. For example, starfishes from Portugal such as *Marthasterias glacialis* reported a maximum concentration of STXs equal to 7,744 µg/kg [221] and in the case of *Ophidiaster ophidianus*, 125 µg/kg of AZAs [222]. These animals accumulate high concentrations of toxins and are a vector of propagation to their predators.

The highest concentration that has been determined in these matrices has been 710,000 µg/kg of DA, corresponding to whale faces of *Eubalaena australis* from Argentina [223]. However, the lowest concentrations of toxins belong to sediment and seawater samples. Concentrations reported in these matrices are scarce compared to those accumulated in the seafood and they could be fundamental to the characterisation of the propagation into the ecosystem.

Table 11. Maximum levels of MBTs in environmental samples from different locations.

Toxins	Matrix		Maximum concentration (µg/kg)	Location	Ref.
OA and DTXs	Mussel	*Mytilus chilensis*	51.2	Chile	[218]
		Mytilus galloprovincialis	758	Italy	[224]
			7,693.4	Spain	[219]
		Perna perna	234.1	Brasil	[225]
	Clam	*Cerastoderma edule*	541	Spain	[226]
		Tagelus tombeii	51.3	Chile	[218]
	Sea snail	*Concholepas concholepas*	400.3	Chile	[218]
		Thais haemastoma	43.5	Brasil	[225]
	Shrimp	*Callichirus major*	1,476.2	Brasil	[225]
	Bird	*Sphenicus magellanicus* (liver)	4.1	Brasil	[225]
	Dolphin	*Sotalia guianensis*	5.2	Brasil	[225]
	Sediment		3.34	China	[171]
	Seawater		0.014 (µg/l)	China	[171]
PTXs	Mussel	*Mytilus galloprovincialis*	93.5	Italy	[224]
			2.9	Spain	[219]
	Clam	*Talegus tombaii*	25.3	Chile	[227]
	Sediment		2.23	China	[171]
	Seawater		0.008 (µg/l)	China	[171]
			14.7 (ng/SPATT)	Antarctica	[228]
AZAs	Mussel	*Mytilus galloprovincialis*	3.2	Spain	[219]
		-	518	United Kingdom	[229]
	Limpet	*Petella ordinaria*	1.23	Portugal	[222]
	Sea snail	*Haliotis tuberculata*	0.29	Portugal	[222]
		Charonia lampas	14.75	Portugal	[222]
			1.63	Morocco	[222]
	Starfish	*Ophidiaster ophidianus*	124.91	Portugal	[222]
		Marthasterias glacialis	2.78	Morocco	[222]
	Sea urchin	*Sphaerechinus granularis*	5.89	Portugal	[222]
YTXs	Mussel	*Mytilus* spp	29.93	Morocco	[222]
		Mytilus galloprovincialis	0.49	Italy	[224]
	Shellfish	-	1,800	United Kingdom	[229]
	Clam	*Cerastoderma edule*	57	Spain	[226]
	Sea snail	*Charonia lampas*	28.63	Portugal	[222]
		Penion lineatus	1.16	Morocco	[222]

Table 11. (Continued) Maximum levels of MBTs in environmental samples from different locations.

	Starfish	*Marthasterias glacialis*	3.51	Portugal	[222]
			9.15	Morocco	[222]
DA	Mussel	*Mytilus galloprovincialis*	800	Tunisia	[230]
		Perna viridis	454	Thailand	[231]
	Clam	*Spondylus spinosus*	4	Lebanon	[232]
		Acanthocardia tuberculata	770	Croatia	[233]
		Callista chione	280	Croatia	[233]
		Pecten maximus	346,000	United Kingdom	[220]
		Chlamys farreri	14,900	China	[234]
	Cuttlefish	*Sepia officinalis*	75,910	Portugal	[235]
	Whale	*Eubalaena australis (feces)*	710,000	Argentina	[223]
	Sediments		0.17	United States	[236]
STXs	Mussel	*Mytilus galloprovincialis*	10,851	Italy	[237]
		Mytilus chilensis	24,950	Chile	[218]
	Clam	*Acanthocardia tuberculata*	108.82	Croatia	[233]
		Callista chione	33.92	Croatia	[233]
		Gari solida	32,320	Chile	[218]
		Argopecten purpuratus	3,317	Spain	[238]
	Limpet	*Petella ordinaria*	1,123.3	Portugal	[221]
		Patella spp.	3,622.5	Morocco	[221]
	Sea snail	*Argobuccinum ranelliformes*	36,370	Chile	[218]
		Charonia lampas	1,423.4	Portugal	[221]
		Cerithium vulgatum	2,556	Morocco	[221]
	Starfish	*Marthasterias glacialis*	7,744.3	Portugal	[221]
			1,852.4	Morocco	[221]
	Sea urchin	*Diadema africanus*	276.3	Portugal	[221]
	Seawater		0.08 (µg/l)	Italy	[237]
TTXs	Mussel	*Perna canaliculus*	160	New Zealand	[203]
		Mytilus edulis	33.3	Netherlands	[239]
		Mytilus galloprovincialis	6.4	Italy	[237]
	Oyster	*Ostrea edulis*	124.1	Netherlands	[239]
		Saccostrea glomerata	3	New Zealand	[240]
		Crassostrea gigas	253	England	[241]
	Fish	*Lagocephalus sceleratus*	13,480	Cyprus	[242]
		Sphoeroides marmoratus	9,918	Portugal	[243]
		Takifugu oblondus (ovary)	34.5 µg*	India	[244]

1.5 Risk assessment and regulatory framework of marine biotoxins and harmful algal blooms

As aforementioned, the high bioactivity of MBTs and their occurrence in worldwide seafood supposes a threat to human health. Evaluation of the potential risk is necessary to determine the maximum dose of MBT that would guarantee no adverse effects in humans. Risk assessment involves the identification and description of the symptoms of intoxication induced by the toxin, the determination of the mode of action and the dose-response relationship of a specific organism which is exposed to a defined concentration of the toxin, and finally, the characterisation of the associated risk. Regulations concerning that maximum concentrations of MBTs which are present in seafood come from the necessity to ensure the safety of the commercialised marine products. Numerous benefits of including seafood in the diet are known due to the high protein content, unsaturated fatty acids, vitamins and the association to the low risk of suffering heart diseases [245]. However, foodborne diseases caused by the shellfish and fish consumption are causing outbreaks to impact human health, with special regard to the MBTs content. Regulatory authorities have determined fixed maximum levels of MBTs in edible molluscs and fishes to prevent unsafe products being placed in the international market. Moreover, methods of analysis have also been stipulated for the proper identification of MBTs in seafood and the estimation of possible toxicity.

1.5.1 Regulations on maximum limits of marine biotoxins

The Codex Alimentarius Commission (CAC), formed by the worldwide public safety agencies, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), have compiled in the Codex Alimentarius International Food Standard, guidelines, codes and standard programmes to protect consumers and to promote fair practices in food trade. In the case of bivalve mollusc commercialisation, the CODEX Committee on Fish and Fishery Products (CCFFP) has determined standard maximum levels of MBTs that are present in molluscs (CODEX STAN CXS 292-2008) [246]. The European Commission (EC) has regulated these levels of concentration for the main group of toxins through the regulation No. (EC) 853/2004 [247] and No. (EU) 786/2013 [248]. Due to the diversity of compounds within each family of toxins, the regulatory levels are grouped according to the total toxicity of the analogues, represented as equivalents for the most relevant biotoxin of the group. The values of the MRLs of the MBTs groups that are permitted in shellfish are summarised in T**able 12**. The establishment of these regulatory limits is based on the toxicity which is the focus of an experiment when employing the MBA for a known concentration of the toxins. Currently, the re-adaptation of the toxicity of the MBTs is proposed as being related to toxic units, which are expressed as toxicity equivalency factors (TEFs). TEFs are defined as the toxicity ratio of a compound from a chemical group that shares the same mode of action of a reference compound in the same group. Then, the toxicity of each analogue is expressed as a fraction of the toxicity of the reference compound [249]. The importance of these TEFs yields on the fact that the toxicity of each sample can be estimated once the concentration of the MBTs has been determined. Moreover, TEFs provide the most reliable data due to the absence of human intoxication information [250]. The TEF of MBT analogues that were ascertained by the European Food Safety Authority (EFSA) are presented in **Table 12** [251].

Table 12. MRLs and TEFs of the MBTs groups.

Group of MBTs	MRLs in shellfish	TEFs
OA and DTXs	160 µg OA equivalent/kg	OA: 1 DTX-1: 1 DTX-2: 0.6
PTXs	160 µg OA equivalent/kg	PTX-2: 1
AZAs	160 µg AZA equivalent/kg	AZA-1: 1 AZA-2: 1.8 AZA-3: 1.4 AZA-4: 0.4 AZA-5: 0.2
YTXs	3.75 mg YTX equivalent/kg	YTX: 1 hYTX: 1
DA	20 mg DA equivalent/kg	DA: 1
STXs	800 µg STX equivalent/kg	STX: 1 GTX-2: 0.4 GTX-3: 0.6 dcSTX: 1 dcGTX-2: 0.2 dcGTX-3: 0.4

Regarding TTX, no concentration limits have been regulated in seafood to date. The consumption of pufferfish, the potentially most associated TTX-vector, is not authorised in Europe as a preventive measure to avoid intoxications. The entry of derivative products from *Tetrodontidae* family is regulated by the No. (EC) 853/2004 [247]. A toxicity study by the EFSA in the EU determined that the consumption of large portions of fish containing 44 µg TTX equivalent/kg was not causing adverse effects [239]. In other countries, such as Japan, only specialised chefs are able to prepare this fish after years of training. Even though the limits are not defined, similitudes in the mode of action with STX have been fixed as guidance. However, studies in Japan, where the consumption of pufferfish is relevant, have proved that quantities of TTX ranging from 0.18 to 0.2 mg are causing severe symptoms and fatal intoxication when the consumption reaches 2 mg of the toxin [252].

A similar situation takes place with the related CFP via ciguatoxin fishes which are forbidden in the European and Australian markets, but not in Japan, Mexico or the USA [253]. Regulations that are related to fish present more discrepancies. While the Codex suggests to not consume any raw derivate of fish containing ciguatoxins in amounts with detrimental effects, no limits have been concluded. Further, the US Food and Drugs Administration (FDA) guidance recommends MRLs of ciguatoxins corresponding to 10 ng Pacific CTX-1 equivalent/kg and 100 ng Caribbean CTX-1 equivalent/kg [254].

In the case of other toxins, such as CI, for example, no recommendation has appeared since no toxic episode has occurred to date. In contrast, for NSPs such as PbTXs, the Codex Alimentarius has regulated the MRL of these toxins in 0.8 mg PbTX-2 equivalents/ kg or 200 mouse units/kg.

However, regulatory limits of MBTs in seawater, the medium where are produced, have still not been established. Methods for the analysis of MBTs in phytoplankton samples have also been developed by LC-MS/MS [167, 200]. Due to the different dynamics of production and excretion of MBTs from the toxigenic phytoplankton, the analysis of intracellular and extracellular content is recommended [204] and should be considered to have a deeper comprehension of the behaviour of MBTs. Methods for the detection and quantification of

MBTs in seawater are scarce compared to those that existent for their analysis in bivalves. The main differences between seawater and shellfish analyses would be the expected levels of concentration of MBTs. Shellfish accumulate the MBTs that are present in the water or contained in the phytoplankton cells by direct filtration [30]. The concentrations reached in the tissues of the bivalves are higher than those that could be expected in the seawater, when there are any bloom phenomena to which they can be attributed. This difference in concentration levels could be the most challenging aspect for the analysis of MBTs in seawater because of the need to achieve high sensitivity methods.

1.5.2 Official methods of marine biotoxins analysis

The Official Methods of Analysis℠ (OMA) for the analysis of the different MBTs groups are described by the Association of Analytical Chemistry (AOAC, Rockville, MD, USA). These methods are considered as the reference, although alternative analyses can be used if they provide equivalent levels of protection [253]. In Europe, the Commission Implementing Regulation (EU) 2019/627 [255] has established the following OMA for the analysis of the different MBTs groups, based also in the EU-RL criteria:

- **PSP group:**

The group of STXs has to be analysed by the OMA 2005.06 of the AOAC, known as the Lawrence method, within which is employed a LC method with pre-column oxidation and fluorescence detection [256]. Also, the MBA (AOAC OMA 959.08) [257] or any method of analysis can be employed that follows the criteria stipulated by the EU-RL, but in the case where there are discrepancies, the official method to be used must be the Lawrence method. Other official methods are widely employed, such as the OMA 2011.02, that is based on HPLC with post-column oxidation and fluorescence detection [258]. In fact, in the USA and Canada, the authorised method of analysis of PSP in shellfish is the RBA (AOAC OMA 2011.27) [259].

- **ASP group:**

The determination of DA or any of its isomers in the edible part of the bivalve molluscs has to be performed by HPLC with UV detection following the OMA 991.26 of AOAC [260]. Alternative methods can be employed but the official method is HPLC-UV. Another official method based on ELISA, the AOAC OMA 2006.02 [256], is also employed and is recommended as a screening method for a high throughput analysis.

- **Lipophilic group:**

The group of lipophilic MBTs that have been determined by the Regulation Commission encompasses the OA and relative DTXs, the AZAs, the YTXs and the PTXs. There is a SOP that has been developed by the EU-RL and validated in inter-laboratory tests of the EU state members. This method is based on LC coupled to MS in tandem, with a previous hydrolysis of the OA group, in order to have the OA-molecule and not the possible esters [164]. Again, alternative methods of analysis can be employed if they are following the EU-RL criteria, but the official method in the case where there are discrepancies is HPLC-MS/MS.

- **Emerging MBTs:**

In the case of new discoveries of MBTs, any method of analysis can be applied that is always following the basis of the national control programmes which have been elaborated by the EU state members.

1.5.3 Assessment of harmful algal blooms: Prevention, control and mitigation strategies

The assessment of HABs, and especially MBTs, has become necessary in order to avoid or to minimise their negative effects on the environment and to humans by the risk that supposes the consumption of contaminated seafood. Since HABs and MBTs are naturally occurring and their causes are highly difficult to control, the prediction of their appearances has become an arduous challenge. Moreover, preventive strategies do not ensure the reduction of HABs and, consequently, management actions have to be arranged to prepare for inconveniences. To date, no unique protocols or procedures have been established due to the diversity of the blooms and the associated risks of MBTs. However, different approaches are employed with the aim of preventing the occurrence of HABs, controlling the extension of the event, the magnitude of damage, or the extent of the consequences that they can produce into the environment or those that are a threat to human health. These strategies of prevention, control and mitigation are detailed below.

Prevention The unpredictable occurrence of these events, has made harder their prevention and methodologies for approaches the situation more difficult, due to the many factors that are involved in the way that an algal bloom takes place. One of the well-known triggers that could somehow be managed is the concentration of nutrients in the water. The reduction of the organic matter contribution from the human activities is presented as a proper prevention methodology. Control of the sewage from industry, agriculture and urban areas could make a decrease in the concentration of nitrates, phosphates and other nutrients that promote the proliferation of phytoplankton. Many countries have implemented the treatment of wastewater so as to not contribute to the rise of HABS and MBTs, as well as to reducing the eutrophic areas and contamination [261]. In Europe, some directives, such as the Water Framework Directive (WFD) [262], have been created to assure the better quality of the water and, in this way, reduce the eutrophication that is by anthropogenic activities.

Control The control of HABs is based on the suppression or destruction once it has been formed. According to Anderson et al. [38], there are different tactics with which to control the HABs which can be classified into five categories;

- **Mechanical**: An easy way to end bloom creation is by removing the high concentration of phytoplankton cells at the water surface. Some techniques would include, the catching of the cells using nets, or the addition of products that produce the precipitation of the cells. An example of this action is the addition of clay that forms algal-clay-aggregates with the cells and the sediment at the sea bed [263]. Despite of the fact that this strategy seems to be effective in high-production areas where the production is really affected by the presence of HABs and also an acceptable benefit/cost solution [264], it results in a controversial practice.

- **Biological:** The addition of biological agents to avoid the reproduction of the phytoplankton cells. This involves the introduction of pheromones that stop the growth or inhibit their reproduction or by the introduction of infertile specimens or even viruses [265]. Even though this practise is widely used in terrestrial agriculture, the difficulties involved in controlling the widespread nature of the new agent into the ocean and all the possible adverse effects that can be caused, make this a more controversial than beneficial practise.

- **Chemical:** Similarly to the addition of biological agents is the addition of chemicals, but in this way, these substances would be employed to directly destroy the

phytoplankton cells or the produced MBTs. A typical example is the addition of copper sulphate by aeroplane directly into a bloom [266] or the addition of algaecides [267]. Once again, this strategy is questionable in terms of its specificity to kill the target phytoplankton without affecting the rest of the organisms that are present in the ecosystem.

· **Genetic:** This strategy would be driven by the introduction of genetically modified species to stop the reproduction, or compete for the survival, of the toxin-producer algae. This strategy has a biological function and is more specific than the chemical control. However, yet again, it has the same problems which are associated to their difficult control in the sea and the negative effects in the ecosystem. Moreover, an additional controversial aspect derives from the ethical issues that the use of genetically modified organisms (GMOs) supposes.

· **Environmental:** These actions would be related to physical or chemical processes that would cause some variation in any environmental condition in order to diminish the phytoplankton proliferation. Some examples of these actions could be the aeration of the water to destroy the water stratification, the interruption of light irradiance [268] or the decrease in the residence time by the recirculation of the water through channels [269]. The disadvantage is that the low specificity of the exercises can interrupt other activities in the ecosystem.

Due to the many adverse effects that the use of controlling tactics involves, their application is not always easy. In other words, the situations have to be deeply studied before applying any of these measures because the cure must not become more threatening than the disease.

Mitigation Mitigation strategies take into account the actions that extenuate or minimise the impacts of the occurrence of HABs and MBTs once they are present in the environment. The monitoring of the aquatic areas is essential in order to command the appearance of blooms, more especially when they become toxic episodes.

Then, the direction of palliative activities is essential to maximising the avoidance of the inconveniences that are derived from these events. Some of these actions are based on the closure of the aquatic activities affected by the bloom. The cancellation of the leisure activities can ensure the safety of humans due to some algae produce irritant MBTs in contact with the skin, and more dangerous toxins could be ingested by accidentally swallowing water. Also, the closure of the aquaculture facilities when the presence of biotoxins is over the regulatory limits is another remediation action to consider in harvesting areas. Moreover, to guarantee the security in the marine resources that are consumed, many of them are treated using depuration processes prior to their commercialisation. However, this operation is not always effective in eliminating all of the groups of MBTs. Further, the rate of accumulation of toxins is highly variable depending on the type of the toxin and the shellfish and also on the given area where they are placed, as well as their elimination rate. For example, AZAs require an extended time of depuration, in some cases reaching periods of six months of toxicity, due to the accumulation of the toxins occurring in all of the shellfish tissue, and not only in the digestive gland as typically happens with polyether toxins, that would mean a slow rate of natural depuration [30]. In another study, DA was 90% depurated in 72 hours from a blue mussel collected on the southeast coast of Canada and only the 7.5% from razor clams during more than 3 months on the west coast of the USA, demonstrating the differences in the depuration rate with respect to the type of shellfish and the area [270]. Another action related to seafood would be the relocation of the harvesting production nets to areas that are far away from where the bloom is located, however, it is not a feasible practise.

Due to the difficulties in preventing the formation of an algal bloom, some countries have established the monitoring of the toxigenic species and the MBTs through official programmes of control, with the aim of preserving the public health, and minimising the effects.

2. OBJECTIVES

HABs are naturally occurring events, but global warming, the water contamination and eutrophication by anthropogenic activities have contributed to the increase in their frequency, intensity and worldwide distribution. Due to the consequences for the environment, human health, and, therefore, for the development of coastal industries, the early detection of MBTs is of primary importance.

The exhaustive monitoring of the toxigenic-producer algae has been carried out since 1980 with the aim of managing the appearance of HAB formations and to palliate the unfavourable effects that they can give rise to. However, these estimations do not provide information about the levels of MBTs nor do they identify the toxins which are produced by an algal bloom event. Moreover, gaps in information continue to exist concerning to the behaviour of MBTs into the marine ecosystem.

Under this context, the overall objectives of this doctoral thesis were:

1. The development of highly sensitive and selective analytical methods based on LC-MS for the reliable determination and quantification of MBTs in seawater for their use as early warning information. In addition, HRMS will be used with the aim of working on the analysis of a target group of MBTs and a possible retrospective analysis to determine the future for new emerging MBTs or other contaminants.
   The selection of the target MBTs has been defined by the frequency of their appearance in the Mediterranean Sea. These toxins are: Okadaic acid (OA) and related dinophysistoxin-1 (DTX-1), pectenotoxins-2 (PTX-2), azaspiracids-1.2.3.4 and 5 (AZA-1,2,3,4 and 5), yessotoxin (YTX) and homoyessotoxin (hYTX), domoic acid, saxitoxin (STX) and analogues decarbamoylsaxitoxin (dcSTX), neosaxitoxin (NeoSTX), gonayutoxin-2,3 (GTX-2,3) and tetrodotoxin (TTX).

2. The use of the developed analytical methods to study the occurrence of MBTs in seawater and the possible relations with the environmental parameters, such as the temperature, the salinity, the dissolved oxygen, the pH and the concentration of algal nutrients in the water.
   The areas of the study are located in coasts of the occidental Mediterranean Sea, in particular, along the Catalan coast and the Ebro Delta wetland and on the salted water lagoon of Mar Menor in Murcia.

3. The comparison between the analyses of MBTs in seawater by using HPLC-HRMS and the bioassay type ELISA, in order to stablish the main advantages and limitations for their employment as rapid and sensitive methods for the detection and quantification of MBTs in seawater.

3. DEVELOPMENT OF LC-MS METHODOLOGIES FOR THE ASSESSMENT OF MARINE BIOTOXINS IN SEAWATER

3.1 Development and validation of LC-MS methods

In order to fulfil the requirements for the selective and sensitive analysis of MBTs in seawater, the development and validation of analytical methodologies based on LC-MS will be the first step, and is the basis of this chapter.

For the proper development of the methods, the physico-chemical properties of MBTs will be the first parameter to consider, in order to select the optimal conditions of the LC-MS techniques. T**able 13** presents almost all of the target toxins and they are organised from the most hydrophilic, TTX, to the most lipophilic, DTX-1 by the corresponding value of the logP. Also, the values of the strongest acidic or basic pKas are expressed for the toxins.

Another important consideration is the matrix in which they are going to be analysed. Seawater is a complex matrix that is mainly composed of salts and dissolved organic matter. These interferences have to be removed or decreased in order to avoid any obstruction of the equipment during the analysis or any misleading results because of signal disturbances that would be produced by the matrix effect. The election of the pre-treatment techniques will also be conditioned too by the nature of the matrix and the MBTs. Not only is the removal of the matrix interferences a challenge when monitoring contamination in seawater, but there is also the low levels of contamination. The combination of the sample pre-treatment techniques should allow the concentration of the analytes in order to increase their detectability.

Table 13. Values of log P of the different toxins and the corresponding strongest acidic and basic pKas. All values are extracted from https://chemicalize.com.

	log P	Strongest acidic pKa	Strongest basic pKa
Tetrodotoxin	-4,59	10,30	9,20
Saxitoxin	-4,25	10,74	9,14
Gonyautoxin-2,3	-3,86	-1,66	9,98
Neosaxitoxin	-2,62	10,53	9,12
Domoic acid	-1,79	1,68	11,59
Yessotoxin	-0,44	-2,30	-
Homoyessotoxin	0,15	-2,27	-
Azaspiracid-3	4,24	3,95	9,32
Azaspiracid-1	4,95	3,95	9,32
Azaspiracid-2	4,97	4,03	9,32
Okadaic acid	5,13	3,76	-
Pectenotoxin-2	5,40	10,22	-
Dinophysistoxin-1	5,61	3,76	-

An additional drawback that is presented in this process is related to the purity of the standards. Available standards are scarce and expensive. The commercial standards are mussel extracts contaminated by MBTs that have been isolated and purified until leading

to 96-99% of purity. Moreover, certified reference material has only been prepared for mussel extract, thus maintaining a distance away from seawater matrix comparisons.

3.1.1 LC-MS

LC-MS are the most appropriated techniques for the unequivocal identification of MBTs. The medium-high polarity and the medium-low volatility are the properties of MBTs that makes a propitious analysis by using these techniques. Several methods have been developed for its determination and quantification in different matrices. However, most of the methods are focused on their application in shellfish. Some of the conditions for the LC-MS techniques at were employed in the analysis of the different groups of MBTs in shellfish and seawater are summarised in **Table 14.**

As commented, MBTs involve a wide range of biomolecules with different behaviours. The polarity is the property that more strongly determines the selection of the stationary and mobile phases for the separation in LC. As can be seen in **Table 14**, RPLC is employed for the analysis of the most lipophilic MBTs such as OA, DTXs, YTXs, PTXs and AZAs. Silica-based octadecyl columns (C_{18}) are the most commonly used chromatographic columns for the analysis of lipophilic MBTs.

In contrast, HILIC is the most applied technique in the analysis of the most polar toxins: STXs and TTXs. The chromatographic columns that are most employed in HILIC are those based on a silica structure and with amide groups. Even DA has a strong polar behaviour, and it is mainly analysed by using RPLC. Some other columns such as cyano, with cyanopropylsilane groups have been employed by Molognoni *et al.* to analyse a wider groups of toxins including lipophilic and hydrophilic toxins (**Table 14**).

Water, MeOH and ACN are the most employed solvents as mobile phases. MeOH or ACN are used in RPLC and HILIC, but ACN is more convenient as an organic modifier because of the eluotropic and the aprotic characteristics. Buffers can be used to control the pH, which is a factor that really interferes with the retention mechanisms of the analytes into the stationary phase, especially in HILIC. Ammonium formate buffers are usually employed because of their high solubility in acetonitrile. Moreover, these salts are volatile, hence they are not causing problems during the ionisation process.

The coupling of LC and MS techniques takes place through an interface of ionization, in which the neutral molecules from the liquid phase of LC are converted to charged molecules in the gaseous state. Electrospray ionization (ESI) is the method that is more employed for the ionisation of MBTs in LC-MS analysis. ESI is a soft ionisation mode that provides negative and positive multiple charged ions. All methods which are summarised in **Table 14** use ESI as an interface in the analysis of MBTs.

Table 14. Conditions of LC-MS methods for the detection and quantification of MBTs in shellfish and seawater. All methods use ESI as interface.

Toxins	Technique	LC conditions			MS	REF
		Chromatographic column	Mobile phase		Analyser	
			A	B		
SHELLFISH						
OA, DTXs, YTXs, AZAs, PTXs, SPXs	LC-HRMS/MS	BEH C18 (100 x 2.1 mm, 1.7 µm)	Water 6.7 mM NH_4OH	ACN	Orbitrap	[163]
OA, DTXs, YTXs, AZAs, PTXs, SPXs, GYM, PbTxs, DA	LC-HRMS/MS	Xselect HSS T3 (100 x 2.1 mm, 2.5 µm)	Water 2 mM ammonium formate, 50 mM FA	ACN/water (95:5) 2 mM ammonium formate, 50 mM FA	QTOF	[271]
OA, DTXs, YTXs, AZAs, PTXs, SPXs, GYM, PbTxs, DA, STXs	LC–MS/MS	Zorbax 300 Stand Boat-CN (150 × 4.6 mm, 5 µm)	Water 2.5 mM ammonium formate, 0.1% FA	ACN 2.5 mM ammonium formate, 0.1% FA	QqLIT	[192]
OA, DTXs, YTXs, AZAs, PTXs	LC-MS/MS	X-Bridge C18 (50 × 2.1 mm, 2.5 µm)	Water 2 mM ammonium formate, 50 mM FA	ACN/water (95:5) 2 mM ammonium formate, 50 mM FA	QqQ	[224]
STXs	LC-MS/MS	Amide-80 TSK-gel (250 x 2 mm, 5 µm)	Water 2 mM ammonium formate, 3.6 mM FA	ACN/water (95:5) 2 mM ammonium formate, 3.6 mM FA	QqQ	[200]
STXs	LC-MS/MS	Acquity UPLC BEH Amide (150 x 2.1 mm, 1.7 µm)	Water/FA/NH_4OH (500:0.075:0.3) ACN/water (95:5)	ACN/water/FA (700:300:0.1)	QqQ	[178]
STXs	LC-MS/MS	Amide-80 TSK-gel (150 × 2 mm, 3 µm)	Water 2 mM ammonium formate, 3.6 mM FA	ACN	QqQ	[129]

Table 14. (Continued) Conditions of LC-MS methods for the detection and quantification of MBTs in shellfish and seawater. All methods use ESI as interface.

STXs, TTXs	LC-MS/MS	Acquity UPLC BEH Amide (150 × 2.1 mm, 1.7 µm)	Water/FA/NH_4OH (500:0.075:0.3) ACN/water (95:5)	ACN/water/FA (700:300:0.1) ACN/water (95:5)	QqQ	[272]
STXs	LC-MS/MS	Amide-80 TSK-gel (250 x 2 mm, 5 µm)	Water 2 mM ammonium formate, 50 mM FA	ACN/water (95:5) 2 mM ammonium formate, 50 mM FA	QqQ	[273]
SEAWATER						
OA, DTXs, PTXs, AZAs, YTXs, GYM, SPXs	LC-MS/MS	Zorbax Extend-C18 (150 x 3 mm, 3.5 µm)	Water 3.3 mM NH_4OH	ACN/water (9:1) 3.3 mM NH_4OH	QqLIT	[274]
DA	LC-MS/MS	ZIC-HILIC (4.6 × 150 mm, 3.5 µm)	Water 0.01% FA	ACN 0.01% FA	QqQ	[193]
OA, DTXs, AZAs, PTXs, YTXs, GYM, PbTxs, SPXs, DA, OvTXs	LC-HRMS/MS	Kinetex XB-C18 (100 × 2.1 mm, 2.6 µm)	Water 2 mM ammonium formate, 50 mM FA	ACN/water (95:5) 2 mM ammonium formate, 50 mM FA	Orbitrap	[275]
DA	LC-HRMS/MS	UPLC BEH C18 (2.1 × 50 mm, 1.7 µm)	Water 0.01% FA	MeOH 0.01% FA	TOF	[176]
STXs, DA, SPX, OA, Palytoxin	LC-MS/MS	Gemini C18 (150 × 2.00 mm, 3 µm)	ACN/Water (95:5) 22 mM FA	Water 30 mM FA	QqQ	[276]

Multiple MS analysers are employed in the analysis of MBTs and triple quadrupole (QqQ) is the most frequently used, as can be seen in **Table 14**. This type of low resolution analyser provides high sensitivity and robustness in the analysis of MBTs. Hybrid combinations with other low-resolution analysers, such as the linear ion traps (LIT), have increased the versatility in the acquisition modes of the analysis of MBTs. Mass spectrometers with these analysers have a moderate cost compared to those of high resolution, and they are the most used in routine analyses. However, the surge of high resolution MS analysers has opened up new possibilities in the analysis of MBTs. TOF or orbitrap analysers, in combination with ion traps (ITs) or quadrupoles, provide excellent improvements in terms of mass accuracy and resolution, becoming effective tools for the analysis of target and non-target MBTs. In this thesis research, a hybrid Q-orbitrap has been used in the MS analysis. It is noteworthy that several characterisation studies of new analogues of MBTs are being carried out using these analysers [44, 176, 277].

3.1.2 Sample pre-treatment

The analysis of MBTs in seawater has become more popular during the last five years by the characterisation of the toxins in both the particulate and the filtrate fractions [278]. The protocol employed for the pre-treatment of seawater is based on a prior filtration through filters of micrometric mesh. The extraction of the toxins from the particulate and filtrate fractions is carried out by using UAE and SPE, respectively, as shown in **Figure 16**.

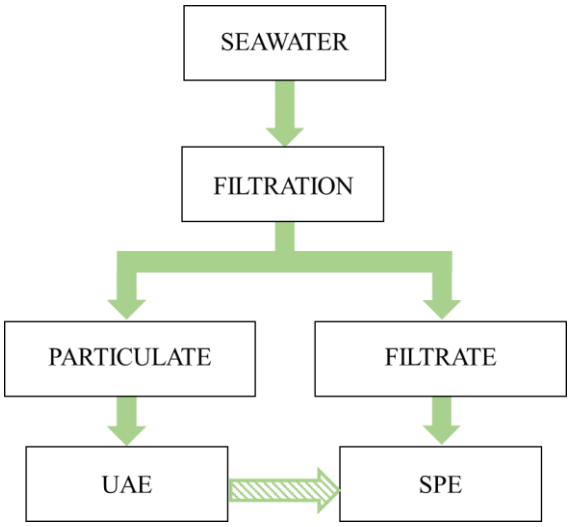


Figure 16. Scheme of the seawater pre-treatment for the extractions of the MBTs from the particulate and the filtrate fractions.

UAE has been used in the extraction of MBTs from solid samples such as sediments, seafood phytoplankton or biota matrices. However, SPE has been widely used as the clean-up step after the extraction of MBTs from solid samples by UAE or other extraction methods, and from seawater. Some examples of the UAE and SPE application in the extraction of the toxins from different matrices, and the recovery obtained after the process are shown in **Table 15**.

Table 15. Pre-treatment of sample for the extraction of MBTs from different matrices.

Toxins	**Matrix**	**Sample pre-treatment**		**Recovery (%)**	**REF**
	Shellfish	**Extraction**	**Clean-up**		
DA		**SLE** MeOH/Water (1:1)	**SPE** SAX AccuBOND II	-	[177]
OA, DTXs, YTXs, PTXs, AZAs, GYM, SPXs		**UAE** MeOH	**SPE** Strata X	83-126	[175]
STXs, TTXs		**SLE** Water 0,01% Acetic acid	**SPE** EnviCarb	34-74	[272]
STXs		**SLE** Water 0.1M Hydrochloric acid	**SPE** OASIS HLB	94-104	[273]
Oa, YTX, AZAs, PTX, SPX, GYM		**SLE** MeOH	**SPE** Strata X	75-103	[201]
	Sediment	**Extraction**	**Clean-up**		
OA, DTXs, PTXs, YTXs, AZAs, SPXs		**PLE** MeOH	**SPE** OASIS HLB	35-109	[170]
OA, DTXs, YTXs, PTXs, AZAs, GYM, SPXs		**UAE** MeOH	**SPE** Strata X	-	[175]
	Seawater	**Extraction of particulate**	**Extraction of filtrate**		
OA, YTX, PTX		-	**SPE** OASIS HLB	52-70	[174]
DA		**UAE** MeOH/Water (1:1)	**SPE** OASIS HLB	57-69	[193]
OA, DTXs, AZAs, YTXs, GYM, SPXs, PTXs		**UAE** MeOH	**SPE** OASIS HLB	55–83	[274]
STXs, DA, SPXs, OA, Palytoxin		-	**SPE** Bond elut LRC-C18 + Carbograph4	77-114	[276]

As can be observed, the extraction of MBTs from the different solid matrices is normally carried out using MeOH, for the different modalities of SLE. MeOH is the strongest eluotropic organic solvent that is employed in chromatography, and it is because of this

that it is widely used for extracting or eluting solvent in SPE. The most employed SPE modality is the hydrophilic-lipophilic balance (HLB) for the extraction of lipophilic and hydrophilic MBTs with good recovery values.

3.1.3 Validation of the analytical methods

Analytical quality/control parameters have been assessed for the validation of the different developed methods based on LC-MS. In order to estimate the accuracy of the method and the instrumental measures, different parameters have been tested. Since no certified reference material is available, in this research thesis, instrumental parameters were evaluated with the analysis of fortified instrumental blanks and fortified seawater blank samples were used during the validation experiments that were undertaken using the different methods.

Sensitivity and linearity Limits of detection and quantification (LODs and LOQs, respectively) were determined experimentally by the injection of MBTs standards in serial dilutions. Instrumental LODs (iLODs) were defined as the lowest concentration that each compound is detected in pure solvent. Then, iLOQs were estimated as being 10/3 of the iLODs. Following this criterion, LODs of the method (mLODs) for each of the MBTs were determined as the lowest concentration detected by a matrix-matched calibration curve. Because of the different fractions of seawater that were analysed, particulate and filtrate calibration curves were employed in each case. The method LOQs (mLOQs) were calculated as being 10/3 of the mLODs.

The slope of the calibration curves determined the sensitivity and the linearity at the linear ranges of concentrations, as measured by using the Spearman correlation (R^2).

Selectivity The identification of the compounds was fulfilled by the comparison of the retention time, the exact mass and the full scan signals of the analytes of the standards analysed in pure solvent and in seawater extracts, following the same instrumental conditions.

Precision and trueness Precision was determined by inter-day (n=6) and intra-day (n=3) analysis of the MBTs standards at the same experimental conditions.

The trueness was estimated by the recovery and the matrix effect of each biotoxin in the different methods**.** Recoveries were estimated by the comparison between fortified extracts of seawater before and after the extraction procedures using the formula:

$$R\ (\%) = ([Toxin\ Area]_{before\ extraction} / [Toxin\ Area]_{after\ extraction}) \times 100,$$

where $[Toxin\ Area]_{before\ extraction}$ is the integrated area of fortified blank extracts of seawater before the pre-treatment process and $[Toxin\ Area]_{after\ extraction}$ is the integrated area of fortified blank extracts of seawater after the pre-treatment process and prior to the analysis.

The matrix effect was calculated by the comparison of fortified solutions of extracts and pure solvent using the formula:

$$ME\ (\%) = ([Toxin\ Area]_{extract} / [Toxin\ Area]_{solvent}) \times 100,$$

Where $[Area]_{extract\ is}$ the integrated area of fortified blank extracts of seawater and $[Area]_{solvent}$ is the integrated area of fortified pure solvent.

3.2 Results

The experimental results of this chapter are presented in different scientific publications, divided into different groups of MBTs.

3.2.1 Polyketide MBTs: OA, DTXs, PTXs, AZAs and YTXs

In this publication has been developed an HPLC-HRMS method for the detection and quantification of polyketide MBTs in seawater and it has been applied to samples from all along the Catalan coast (Spain). The occurrence of the MBTs will be discussed in section 4.3.2.

Scientific publication 1: Analysis of lipophilic marine biotoxins by liquid chromatography coupled with high-resolution mass spectrometry in seawater from the Catalan Coast.Cristina Bosch-Orea, Josep Sanchís, Marinella Farré and Damià Barceló. Analytical and Bioanalytical Chemistry, 2017. 409(23): pp. 5451-5462.

3.2.2 Excitatory amino-acids MBTs: DA

In this publication has been developed a HILIC-HRMS method for the detection and quantification of DA in seawater. The method was successfully applied in the study of the occurrence of DA in the wetland of Ebro Delta, in the Catalan coast (Spain), and these results will be discussed in section 4.3.2.

Scientific publication 2: Ultra-trace determination of domoic acid in the Ebro delta estuary by SPE-HILIC-HRMS. Cristina Bosch-Orea, Josep Sanchís, Damià Barceló and Marinella Farré. Analytical methods, 2020, 12: p.1966-1974.

3.2.3 Alkaoid MBTs: STXs and TTX.

In this last publication a HILIC-HRMS method for the detection and quantification highly polar MBTs in seawater has been developed. These polar compounds are alkaloids of the family of STXs and TTX. Real seawater samples from Murcia (Spain) were analysed using this methodology and the results will be discussed in section 4.3.2.

Scientific publication 3: Analysis of highly polar marine biotoxins in seawater by hydrophilic interaction liquid chromatography coupled to high resolution mass spectrometry. Cristina Bosch-Orea, Josep Sanchís, Marinella Farré. (Submitted to the journal Methods X)

This work has been supported by the European Union through the projects Sea-on-a-chip (FP7-KBBE-OCEAN2013.1 grant no. 614168) and BRAAVOO (FP7-OCEAN2013.1 grant no. 614010) and the Spanish Ministry of Economy and competitiveness through the projects IntegraCoast (CGL-2014-56530-C4-1-R) and PLASMED (CTM-2017-89701-C3-1-R).

3.2.1 Polyketide MBTs: OA, DTXs, PTXs, AZAs and YTXs

Scientific publication 1: Analysis of lipophilic marine biotoxins by liquid chromatography coupled with high-resolution mass spectrometry in seawater from the Catalan Coast

DOI: https://doi.org/10.1007/s00216-017-0536-y

Anal Bioanal Chem (2017) 409:5451–5462
DOI 10.1007/s00216-017-0536-y

RESEARCH PAPER

Analysis of lipophilic marine biotoxins by liquid chromatography coupled with high-resolution mass spectrometry in seawater from the Catalan Coast

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Received: 25 April 2017 / Revised: 23 June 2017 / Accepted: 19 July 2017 / Published online: 16 August 2017

Abstract Marine biotoxins regularly occur along the coast, with several consequences for the environment as well as the food industry. Monitoring of these compounds in seawater is required to assure the safety of marine resources for human consumption, providing a means for forecasting shellfish contamination events. In this study, an analytical method was developed for the detection of ten lipophilic marine biotoxins in seawater: azaspiracids 1, 2, 3, 4 and 5, classified as azaspiracid shellfish poisoning toxins, and pectenotoxin 2, okadaic acid and the related dinophysistoxin 1, yessotoxin and homoyessotoxin, classified as diarrheic shellfish poisoning toxins. The method is based on the application of solid–liquid ultrasound-assisted extraction and solid-phase extraction, followed by high-performance liquid chromatography coupled with high-resolution mass spectrometry. The limits of detection of this method are in the range of nanograms per litre and picograms per litre for most of the compounds, and recoveries range from 20.5% to 97.2%. To validate the effectiveness of this method, 36 samples of surface water from open coastal areas and marinas located along the Catalan coast on the Mediterranean Sea were collected and analysed. Eighty-eight per cent of these samples exhibited okadaic acid in particulate and aqueous phases in concentrations ranging from 0.11 to 560 μg/g and from 2.1 to 1780 ng/L respectively. Samples from open coastal areas exhibited higher concentrations of okadaic acid in particulate material, whereas in samples collected in sportive ports, the particulate material exhibited lower levels than the aqueous phase.

Keywords Lipophilic marine biotoxins · Okadaic acid · Diarrhetic shellfish poisoning · Seawater · Solid phase extraction · High-performance liquid chromatography–high-resolution mass spectrometry

Published in the topical collection *Advances in LC-MS/MS Analysis* with guest editors Damià Barceló and Mira Petrovic.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-017-0536-y) contains supplementary material, which is available to authorized users.

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Introduction

Marine biotoxins are secondary metabolites that are produced by marine photosynthetic organisms such as dinoflagellates, diatoms and cyanobacteria. Depending on the water temperature and quantity of organic matter, rapid growth of these microorganisms can occur, with the production of high amounts of marine biotoxins. This phenomenon is known as harmful algal blooms and can cause damage in marine ecosystems, and, in addition, biotoxins can be accumulated in certain marine organisms, passing then to the human food web.

In recent decades, an increase in proliferation, frequency, and persistence of toxic algal blooms has occurred because of eutrophication from agricultural run-off and global climate change [1]. As a consequence, the risk of intoxication via recreational bathing in contaminated waters and consumption of contaminated food has increased.

Marine biotoxins can be classified by their toxic mode of action, such as paralytic toxins, amnesic toxins, diarrheic

toxins and neurotoxins, or by their physicochemical properties, as in lipophilic and hydrophilic biotoxins.

To protect public health, there is Europe-wide legislation to limit their levels in seafood for human consumption. The maximum permitted levels in molluscs for human consumption are compiled in Commission Regulation (EU) 786/2013 [2]. In addition, the methods used for determining the marine biotoxins in seafood are described in Commission Regulation (EU) 15/2011, amending Regulation (EC) 2074/2005 [3]. Mouse and rat bioassays were the regulatory approaches for the control of different marine biotoxins in previous regulations. However, for ethical reasons and the lack of specificity and sensitivity for the determination of some toxins, these methods have been replaced by others, based on liquid chromatography (LC) coupled with tandem mass spectrometry (LC–MS/MS).

For the particular case of lipophilic marine biotoxins, in 2001 Quilliam et al. [4] reported for the first time their chromatographic separation using acidic conditions. Since then, the method has been applied in different studies and, 10 years later, the European Union Reference Laboratory for Marine Biotoxins reported another method, also based on acidic conditions [5]. However, in 2009, Gerssen et al. [6] achieved the separation of 28 lipophilic marine biotoxins using water and acetonitrile at pH 11 with limits of detection (LODs) in mussels ranging from 1 to 22 ng/g. Since then, other authors have reported different analytical approaches using slightly alkaline conditions for the chromatographic separation [7]. The success of the methods based on LC–MS/MS has been proven in different interlaboratory exercises, and was reflected in the regulation. However, with use of selected reaction monitoring or single ion monitoring methods for target analysis, data on non-target analytes such as potential transformation products is lost. In this sense, application of full-scan acquisition schemes with high-resolution mass spectrometry (HRMS) instrumentation allows parallel non-target screening of the acquired data to be performed.

A limited number of studies have explored the advantages of HRMS to assess marine biotoxins [8–11]. The first method using LC–HRMS was developed for the determination of azaspiracids (AZAs) in shellfish with a limit of quantification (LOQ) of 0.010 μg/g [12]. More recently, Domènech et al. [8], presented a multitoxin method for quantification and confirmation of okadaic acid (OA), yessotoxin (YTX), AZA-1, gymnodimine, 13-desmethyl spirolide C, pectenotoxin 2 (PTX-2) and brevetoxin B in mussels. The LOQs ranged from 0.9 to 4.8 pg on column. More recently, the same group of researchers [9] presented a new method combining a quick, easy, inexpensive, efficient, rugged and safe (QuEChERS) extraction and LC–HRMS for the analysis of lipophilic marine biotoxins in fresh and canned bivalves. The method fulfilled all the requirements of current European legislation [9]. Some analytical methods based on immunoassays have also been reported, achieving LODs of 150 mg/kg in shellfish [13, 14]. However, early determination of marine biotoxins in seawater to prevent seafood contamination events has been almost unexplored. Zendong et al. [15] reported an efficient passive-sampling-based extraction method for the high-resolution profiling of marine biotoxins in water from the French coast.

In this article, we present the development and application of a method for the analysis of ten lipophilic marine biotoxins from different groups in seawater. The main objectives were as follows:

1. To develop a multiresidue method based on solid–liquid ultrasound-assisted extraction (UAE) and solid-phase extraction (SPE) followed by LC–HRMS for the detection and quantification of ten marine biotoxins in seawater, including five AZAs, OA and the related dinophysistoxin 1 (DTX-1), YTX, homoyessotoxin (hYTX) and PTX-2.
2. To apply the approach developed to assess the distribution of the selected marine biotoxins in seawater along the Catalan coast.

To the best of our knowledge, this is the first method which explores the use of HRMS for the analysis of marine biotoxins directly in seawater, and the method has improved the LOQ previously reported for marine biotoxins in seawater and in other matrices [16, 17]. Monitoring methods for the determination of marine biotoxins in seawater at trace levels can provide a means of forecasting shellfish contamination events in support of food safety.

Materials and methods

Chemicals and reagents

The structures and characteristics of the selected lipophilic marine biotoxins in this work are shown in Fig. 1.

Analytical standards of marine biotoxins were purchased from Cifga Laboratory (Lugo, Spain). The certified reference material were AZA-1 (1.36 ± 0.09 μg/g, purity 98% or greater; reference CRM-02-AZA1), AZA-2 (1.33 ± 0.09 μg/g, purity 97% or greater; reference CRM-02-AZA2), AZA-3 (1.30 ± 0.11 μg/g, purity 96% or greater; reference CRM-02-AZA3), OA (20.2 ± 1.4 μg/g, purity 99% or greater; reference CRM-00-OA), DTX-1 (8.08 ± 0.47 μg/g, purity 98% or greater; reference CRM-00-DTX1), YTX (7.42 ± 0.66 μg/g, purity 96% or greater; reference CRM-00-YTX) and hYTX (7.68 ± 0.49 μg/g, purity 99% or greater; reference CRM-00-hYTX). The quality control standards were AZA-4 (1.19 ± 0.07 μg/mL, purity 96% or greater; reference 02-AZA4), AZA-5 (1.20 ± 0.07 μg/mL, purity 97% or greater; reference 02-AZA5) and PTX-2 (7.16 ± 0.36 μg/g, purity 96% or greater; reference 00-PTX2). The auxiliary reagent ammonium hydroxide (99.99% purity) was

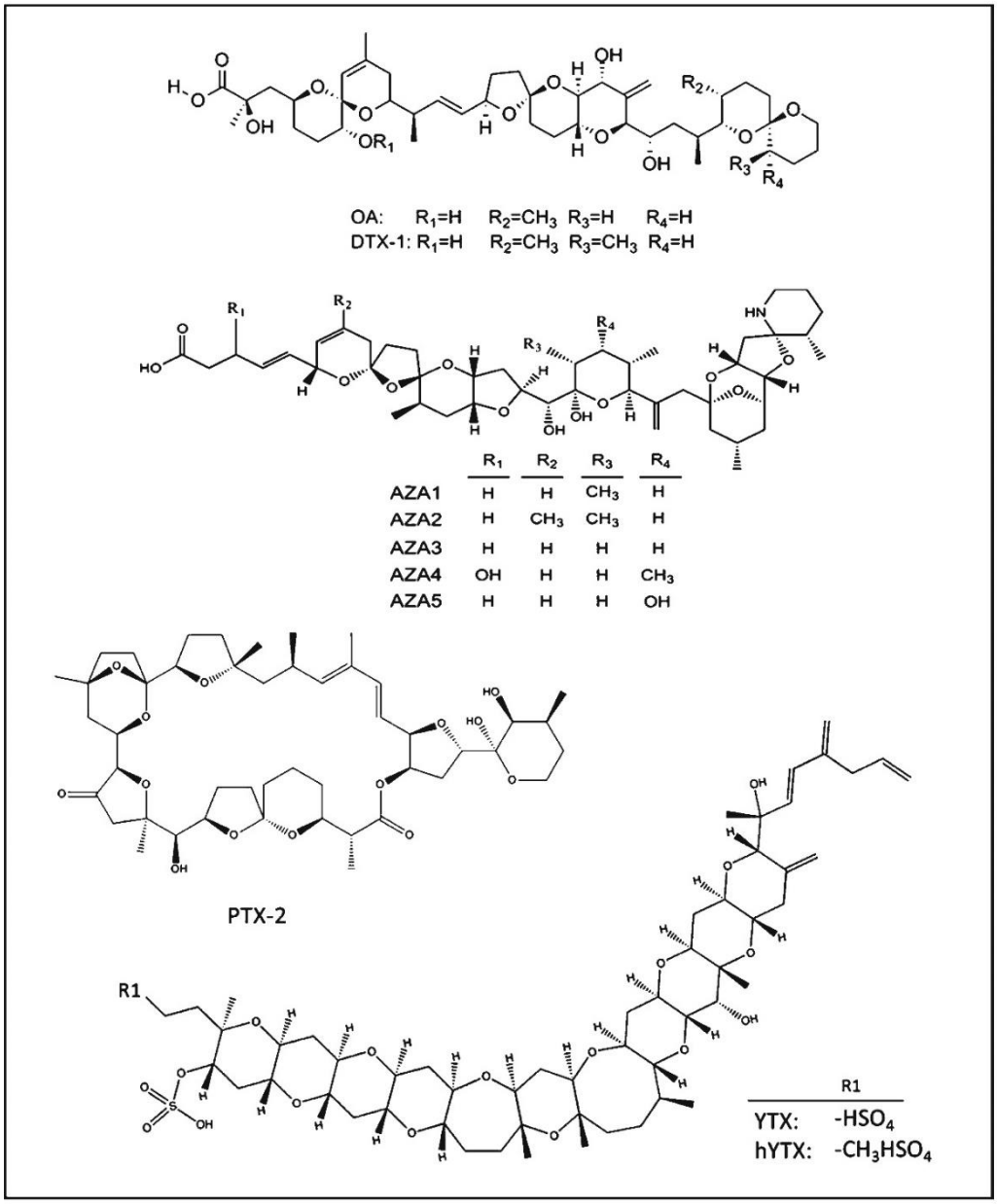

Fig. 1 Chemical structures of the selected lipophilic marine biotoxins in this study. *AZA* azaspiracid, *DTX-1* dinophysistoxin 1, *hYTX* homoyessotoxin, *OA* okadaic acid, *PTX-2* pectenotoxin 2, *YTX* yessotoxin

purchased from Sigma-Aldrich (Steinheim, Germany). High-performance LC grade methanol, acetonitrile, ultrapure water and formic acid were supplied by Merck (Darmstadt, Germany).

Sampling

In February and March 2015, seawater samples were collected from 18 different sampling sites along the Catalan coast (northeast Spain, Mediterranean Sea). Details of each location are summarised in Fig. S1. All of the selected locations have a marina and a public-access beach. Thirty-six samples of surface water (25-cm depth) from the shore of the marina and the beaches were collected in amber glass pots of 2.5 L, and they were immediately transported to the laboratory under cool conditions, and then frozen at −20 °C until their analysis.

Sample pretreatment

Each sample was analysed in triplicate. For each replicate, 500 mL of surface seawater was filtered through a nylon fibre filter of 0.45-μm mesh size (Whatman, Maidstone, UK). The seawater particulate and the dissolved phase were extracted separately. The particulate was extracted by UAE with 20 mL of methanol for 30 min. The filtrate phase was extracted and purified by SPE with 100-mg hydrophilic–lipophilic balance (HLB) cartridges (Waters, Cerdanyola del Vallès, Spain). Extraction consisted of preconditioning with 3 mL of methanol followed by 3 mL of water. Then, 500 mL of filtrate was loaded at 1 mL/min. Cartridges were washed with 3 mL of water to remove salts and interferences. Finally, the elution was completed with 6 mL of methanol. Extracts were concentrated to approximately 10 μL under a gentle N_2 stream and then reconstituted to 500 μL with the initial chromatographic mobile phase, 9:1 acetonitrile–water acidified with 0.1% formic acid.

Liquid chromatography coupled with high-resolution mass spectrometry

Chromatographic separation was performed with an Acquity ultra-high-performance LC system (Waters, Massachusetts, USA) using a reversed-phase column with C_{18} as the stationary phase (Synergy, 50 mm × 2 mm, 5 μm, 80 Å; from Phenomenex, Torrance, USA). The mobile phase was composed of acetonitrile (solvent A) and water (solvent B), both being acidified with 0.1% formic acid. The elution gradient was programmed as follows: 0 min (10% solvent A)—6 min (95% solvent A)—10 min (95% solvent A)—15 min (10% solvent A). The flow rate was maintained at 0.3 mL/min for the 15 min of the total chromatographic run. Samples were kept at 10 °C in the autosampler. The injection volume was 20 μL.

Mass spectrometry was performed with a Thermo Scientific Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ionisation (HESI) probe operating in positive and negative ion modes.

The optimal source parameters were as follows: spray voltage of 3.5 kV for positive mode and 2.5 kV for negative mode, sheath flow gas of 60 a.u., auxiliary gas of 20 a.u. and sweep gas of 2 a.u. The heater temperature was set at 350 °C, the capillary temperature was set at 300 °C, and the S-lens RF level was 60%.

Acquisition was performed in full-scan and data-dependant MS/MS modes, simultaneously obtaining the full-scan mass spectrum with high resolution of 70,000 full width at half maximum (measured at *m/z* 200) and the tandem mass spectrum at medium resolution of 35000 full width at half maximum for every compound. The normalised collision energy (NCE) was set at different percentages of intensity for each compound (see Table 1).

Table 1 Fragmentation pattern of the marine biotoxins studied at optimal normalised collision energy (*NCE*)[a] The relative standard deviation is given in *parentheses*

Compound	Chemical formula	Molecular ion	*m/z* calculated	Product ion 1	*m/z* calculated	Relative abundance	Fragment ion ratio[a]	Product ion 2	*m/z* calculated	Relative abundance	Fragment ion ratio[a]	NCE (%)
Okadaic acid	$C_{44}H_{68}O_{13}$	$[M + Na]^+$	827.4547	$[C_{40}H_{60}O_{10}Na]^+$	723.4086	82	1.11 (0.06)	$[C_{44}H_{66}O_{12}Na]^+$	809.4454	58	1.62 (0.12)	40
Dinophysistoxin 1	$C_{45}H_{70}O_{13}$	$[M + Na]^+$	841.4710	$[C_{41}H_{62}O_{10}Na]^+$	737.4234	68	0.99 (0.05)	$[C_{45}H_{68}O_{12}Na]^+$	823.4606	49	1.71 (0.14)	30
Pectenotoxin 2	$C_{47}H_{70}O_{14}$	$[M + Na]^+$	881.4655	$[C_{46}H_{70}O_{12}Na]^+$	837.4758	30	20.97 (0.89)	$[C_{47}H_{68}O_{13}Na]^+$	863.4526	20	5.45 (0.62)	30
Azaspiracid 1	$C_{47}H_{71}NO_{12}$	$[M + H]^+$	842.5049	$[C_{47}H_{70}O_{11}N]^+$	824.4941	100	0.07 (0.00)	$[C_{47}H_{68}O_{10}N]^+$	806.4834	49	2.09 (0.12)	30
Azaspiracid 2	$C_{48}H_{73}NO_{12}$	$[M + H]^+$	856.5218	$[C_{48}H_{72}O_{11}N]^+$	838.5102	100	0.06 (0.01)	$[C_{48}H_{70}O_{10}N]^+$	820.4998	50	1.86 (0.02)	35
Azaspiracid 3	$C_{46}H_{69}NO_{12}$	$[M + H]^+$	828.4894	$[C_{46}H_{68}O_{11}N]^+$	810.4778	100	0.94 (0.07)	$[C_{46}H_{66}O_{10}N]^+$	792.4672	45	2.21 (0.14)	35
Azaspiracid 4	$C_{46}H_{71}NO_{13}$	$[M + H]^+$	844.4856	$[C_{46}H_{68}O_{12}N]^+$	826.4733	100	0.03 (0.00)	$[C_{46}H_{66}O_{11}N]^+$	808.4631	43	2.20 (0.18)	35
Azaspiracid 5	$C_{46}H_{71}NO_{13}$	$[M+H]^+$	844.4856	$[C_{46}H_{68}O_{12}N]^+$	826.4733	100	0.06 (0.00)	$[C_{46}H_{66}O_{11}N]^+$	808.4631	100	1.00 (0.03)	35
Yessotoxin	$C_{55}H_{82}O_{21}S_2$	$[M - H]^-$	1141.4739	$[C_{55}H_{81}O_{18}S]^-$	1061.5157	100	0.43 (0.04)	$[C_{42}H_{63}O_{16}S]^-$	855.3845	61	1.62 (0.04)	35
Homoyessotoxin	$C_{56}H_{84}O_{21}S_2$	$[M - H]^-$	1155.4892	$[C_{56}H_{83}O_{18}S]^-$	1075.5300	100	0.40 (0.01)	$[C_{43}H_{65}O_{16}S]^-$	869.4004	58	1.71 (0.04)	35

Method validation and quality assurance/quality control

The proposed method was validated by our looking at the linearity range, intraday and interday precisions, method LODs and LOQs, and recovery rates. The validation experiments were performed by fortification of blank seawater samples with the selected marine biotoxins at three concentrations and by analysis of three replicates at each different spiking level following the method described in "Sample pretreatment" and "Liquid chromatography coupled with high-resolution mass spectrometry".

Instrumental blanks, extraction blanks and procedural blanks were analysed at the beginning and in between fortified extracts. The instrument blanks were marine-biotoxin-free solvent (methanol) blanks that were analysed at the beginning of the run. The extraction blanks were marine-biotoxin-free ultrapure water that was extracted and analysed together with the samples. The procedural blanks were blanks that had been subjected to the sampling, the extraction and the instrumental analysis. No interference, contamination or carryover was detected in the blanks.

Selectivity

Identification of the target compounds was accomplished by comparison of the relevant retention time, exact mass and full-scan signals of the analytes in the matrix with those obtained for standard solutions, being analysed under the same experimental conditions.

Limits of detection and quantification

Instrumental LODs, defined as the lowest concentration at which each compound could be detected (with a Gaussian peak shape, less than 2 ppm of exact mass error and molecular isotopic pattern accomplishing the standard ratio) were determined by progressive dilution. A standard solution containing the ten selected biotoxins was prepared at an initial concentration of 50 μg/L, and serial dilutions of 1:5 and 1:10 were injected from 50 μg/L to 1 ng/L. The instrumental LOQ was estimated as 10/3 times the instrumental LOD.

In the same way, the method LODs of each analyte were defined as the lowest concentration for which the peak area was detected in matrix-matched calibration points. Progressive dilutions of the ten compounds in extracts from the particulate (seawater-suspended material) and in filtered seawater were used. The method LOQ were established as 10/3 times the method LOD.

Linearity

Instrumental linearity and sensitivity were estimated as the Pearson correlation coefficient (R^2) and the slopes of the calibration curves respectively. Average retention times and relative abundances for each analyte are shown in Table 2. An acceptable chromatographic separation was achieved for most of the target analytes.

Intraday and interday precision

Instrumental reproducibility (interday precision) was determined with six replicates of standard solutions on three consecutive days. Method reproducibility was calculated on three different days, and these values are shown in Table 2.

Recoveries and matrix effects

Repeatability and recoveries were obtained in fortified blank samples of seawater particulate and filtrate fractions. Three replicates for each matrix were used at two concentrations. Also, the matrix effect, in terms of signal suppression/enhancement, was estimated for particulate and filtrate extracts. These parameters are shown in Table 3.

Safety conditions

Because of the toxic properties of these compounds, extreme caution was used during the manipulation of the standards and samples. All solutions were maintained and all extractions were prepared under a fume hood. Moreover, the instrumental analysis was performed with a covering curtain surrounding the equipment. Microsyringes and glass material in contact with standards and samples were carefully rinsed after their use and heated at 400 °C overnight.

Results and discussion

Optimisation of the analytical methods

Sample preparation

Because of interferences that are commonly present in complex matrices, such as seawater, and the low concentrations expected in seawater, a purification step is needed to increase the sensitivity and to reduce matrix interferences and the concentration of salts in the extract. Therefore, 500 mL of each seawater sample was filtered through a 0.45-μm nylon filter and submitted to SPE. Considering the versatility and the results that were previously reported with use of OASIS HLB cartridges [7], this type of stationary phase was preselected. After the preconditioning of the cartridges with 3 mL of methanol and 3 mL of ultrapure water, the optimal loading volume of the seawater sample was evaluated: 100-, 250-, 500- and 1000-mL samples were tested. The highest intensities were obtained for 500 and 1000 mL, but to avoid clogging of the

Table 2 Analytical parameters for the ten lipophilic marine biotoxins: average retention time, linearity and sensitivity expressed as Pearson correlation coefficients (R^2) of the calibration curves for the instrument and the analytical methods of particulate and the seawater, and intraday and interday instrumental and method precision represented as the relative standard deviation (*RSD*) of the standard solution

Compound	Instrumental parameters							Particulate				Filtrate seawater			
	Retention time (min)	Linearity (μg/L)	R^2	RSD (%)		ILOD (fg on column)	ILOQ (fg on column)	Linearity (ng/L)	R^2	MLOD (ng/L)	MLOQ (ng/L)	Linearity (ng/L)	R^2	MLOD (ng/L)	MLOQ (ng/L)
				Intraday (n = 6) (10 μg/L)	Interday (n = 3) (10 μg/L)										
Okadaic acid	4.97	0.25–50	0.99	3.39	7.92	5	15	0.5–50	0.99	0.4	1	0.5–50	1.00	0.3	1
Dinophysistoxin 1	5.86	0.25–50	0.99	7.89	6.14	5	15	0.8–50	0.98	0.3	1	0.2–20	0.99	0.3	1
Pectenotoxin 2	5.47	0.5–50	0.99	10.2	27.5	2	6	0.8–50	0.98	0.5	2	0.5–50	1.00	0.5	2
Azaspiracid 1	5.3	0.5–50	0.99	7.06	10.8	10	30	0.8–50	0.97	0.003	0.01	0.5–50	0.99	0.002	0.007
Azaspiracid 2	5.5	0.1–50	0.99	5.56	14.3	10	30	0.5–50	0.99	0.003	0.01	0.5–50	1.00	0.002	0.007
Azaspiracid 3	4.96	0.025–50	1.00	4.38	7.67	2	6	0.5–50	0.99	0.003	0.01	0.5–50	0.99	0.002	0.007
Azaspiracid 4	4.21	0.025–50	1.00	8.25	7	1	3	0.5–50	0.98	0.003	0.01	0.5–50	0.99	0.003	0.01
Azaspiracid 5	4.49	0.8–50	0.96	8.33	12.1	1	3	1–50	0.98	0.003	0.01	1–20	1.00	0.003	0.01
Yessotoxin	5.59	0.5–50	0.99	10.8	10.7	16	48	1–50	0.98	0.3	1	0.5–50	0.99	0.3	1
Homoyessotoxin	5.61	0.5–50	0.99	9.56	7.26	10	30	1–40	0.99	0.5	2	1–40	0.99	0.5	2

Limits of detection and quantification obtained for each compound in the instrumental approach and in both analytical methods are shown

ILOD instrumental limit of detection, *ILOQ* instrumental limit of quantitation, *MLOD* method limit of detection, *MLOQ* method limit of quantitation

Table 3 Recoveries of the analytical method for the ten lipophilic marine biotoxins at two concentrations for the particulate and the filtrate, and the corresponding matrix effect

Compound	Particulate								Filtrate				
	Recovery (%)							Matrix effect	Recovery (%)				Matrix effect
	30-min ultrasonication				3-min ultrasonication								
	10 ng/L[a]	RSD (n= 3)	1 ng/L[b]	RSD (n = 3)	10 ng/L[a]	RSD (n = 3)	1 ng/L[b]	RSD (n = 3)	25 ng/L	RSD (n = 3)	10 ng/L	RSD (n = 3)	
Okadaic acid	83.4	10.1	69.9	0.2	91.6	2.2	80.68	3.07	1.0	84.8	11.4	97.2	6.03	1.4
Dinophysistoxin 1	89.1	5.8	-	-	-	-	-	-	0.3	75.0	8.6	61.2	3.7	0.3
Pectenotoxin 2	75.3	3.2	-	-	64.2	21.5	55.1	2.0	1.3	84.7	19.7	81.5	9.6	1.5
Azaspiracid 1	33.9	5.1	69.5	39.6	106.8	4.0	64.6	19.9	0.8	40.1	11.9	33.8	11.1	1.8
Azaspiracid 2	20.5	0.3	61.3	51.2	66.2	8.8	48.0	13.4	1.8	41.0	13.5	24.0	0.8	2.0
Azaspiracid 3	57.1	8.0	29.4	3.4	104.4	21.5	49.2	8.9	1.0	83.3	7.8	44.4	11.2	1.3
Azaspiracid 4	74.4	13.8	72.4	4.9	121.2	34.0	63.8	14.0	0.9	66.4	2.7	47.4	2.4	1.1
Azaspiracid 5	76.2	22.2	51.2	1.6	101.8	38.7	58.4	21.2	1.0	50.9	9.5	38.8	7.8	2.0
Yessotoxin	43.0	2.1	76.1	4.2	47.6	8.4	29.5	0.5	0.6	53.2	14.9	51.5	20.1	1.5
Homoyessotoxin	46.1	1.6	79.2	5.8	35.6	8.1	45.6	4.3	0.6	47.6	12.5	48.8	24.0	1.5

RSD relative standard deviation

[a] Considering 17 mg of particulate per litre of seawater (diameter greater than 0.45 μm), this concentration is equivalent to 588 μg of okadaic acid per kilogram of particulate.

[b] Considering 17 mg of particulate per litre of seawater (diameter greater than 0.45 μm), this concentration is equivalent to 58.8 μg of okadaic acid per kilogram of particulate.

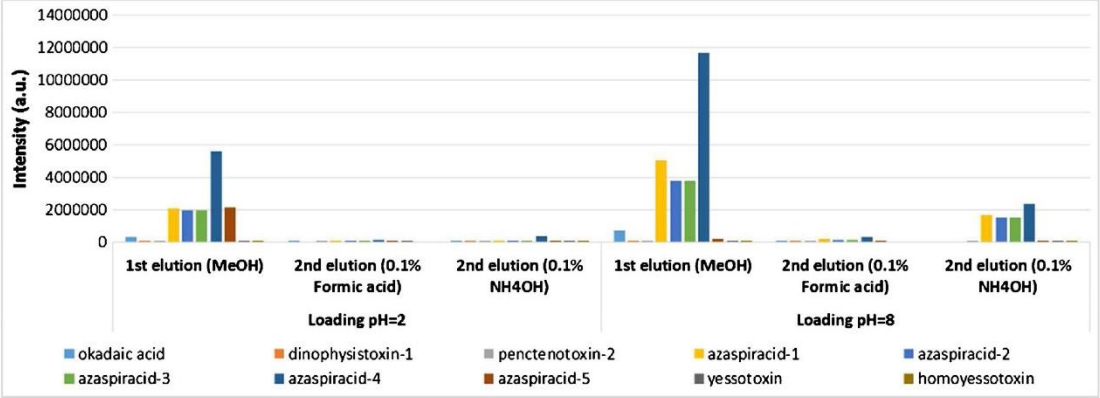

Fig. 2 Amount of marine biotoxin eluted from the cartridge under different loading and elution conditions

cartridges, the optimal volume was set at 500 mL. The effect of pH was also considered, and the sample pH was adjusted to 2 and 8. For most of the compounds, the highest intensity was obtained at pH 8 (Fig. 2). YTX and the related hYTX exhibited slightly higher recoveries when the sample was acidified, but pH 8 was set as the optimal value as a compromise. Also, different elution conditions were tested. Two cycles were tested: the first cycle, with 3 mL of methanol, and the second cycle, with either 3 mL of methanol with 0.1% ammonium hydroxide, or 3 mL of methanol with 0.1% formic acid. As shown in Fig. 2, second elutions with acidified methanol or with basified methanol did not improve significantly the performance of the method. Overall, the best performance was obtained when the seawater samples were loaded at pH 8 and the elution was performed with methanol in two cycles. As can be seen in Table 3, under these conditions, the recovery rates were above 40% for most of the compounds in seawater.

On the other hand, to analyse those biotoxins trapped in the particulate matter during the filtration step, a method based on UAE was optimised. In this case, the use of different solvents was studied, including methanol, methanol with 0.1% ammonium hydroxide, methanol with 0.1% formic acid, and acetonitrile. Each of the solvents tested was used in two extraction cycles of 3 and 30 min, and each with 10 mL of extractant. Two cycle times were tested because although some of the marine biotoxins are degraded by prolonged UAE, other toxins exhibited better recoveries with extraction for 30 min. The recoveries obtained for the extraction from seawater particulate with different solvents for 30 minutes are presented in Fig. 3. For OA and DTX-1, higher recoveries were obtained with use of methanol with 0.1% ammonium hydroxide for the extraction. However, for the rest of the biotoxins studied here, the best recoveries were obtained with methanol. Therefore, methanol was selected as the extraction solvent. As can be seen in Table 3, for most of the compounds, the recovery rates ranged from 43% to 89%, with the exception of AZA-1 and AZA-2, for which they were lower.

Optimisation of the analytical procedure

To optimise the chromatographic separation, different mobile phases composed of water and acetonitrile or water and methanol in different proportions were tested. In Table S1, the tailing factor of each compound is shown under different conditions.

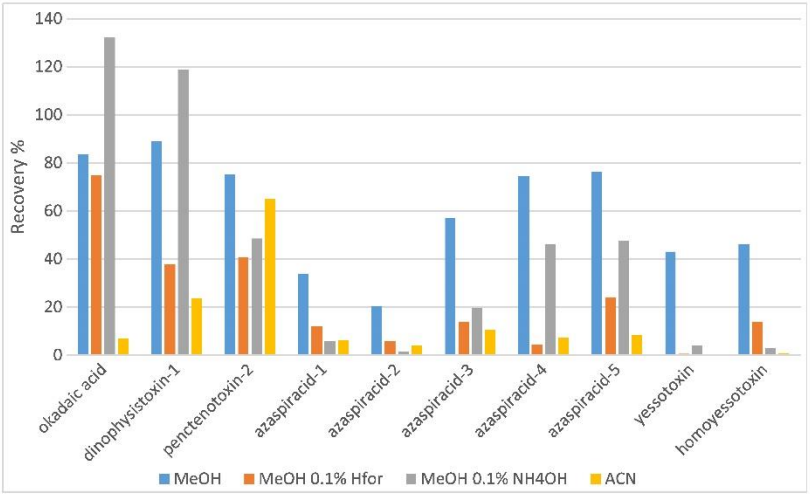

Fig. 3 Absolute recovery for the extraction of the particulate using different solvents: methanol (*MeOH*), MeOH with 0.1% formic acid (*Hfor*), MeOH with 0.1% NH_4OH, and acetonitrile (*ACN*)

The acidic conditions with acetonitrile–water with 0.1% formic acid were selected to give the best shape of the chromatographic peaks for most of the compounds, as in previous studies [18]. Good separation was achieved for the target analytes as presented in the extracted ion chromatograms in Fig. S2.

Toxin standards were directly infused into the HESI source to determine the optimal mass spectrometry conditions. For each analyte, the mass of the corresponding ion obtained was compared with the theoretical mass calculated by Xcalibur 2.1. Mass deviations were found to be below 2 ppm.

The mass spectral characterisation of selected toxins is described in Table 1. Under the optimum working conditions, each compound was identified and several fragment ions were obtained, some of them being in agreement with previous studies [19], and some others being determined during the optimisation.

Identification of the ten lipophilic marine biotoxins was successfully achieved by comparison of the average retention time (Table 2) and the exact mass of the precursor and product ions for each compound (Table 1), both in the matrix and in standard solutions that were analysed under the same experimental conditions.

LODs and LOQs are presented in Table 2. As can be seen, the best instrumental LODs were obtained for AZA toxins, with the method LOQ in the picogram per litre range, showing a high sensitivity of the method, especially for the filtrate portion. The instrumental linearity and sensitivity were estimated for all compounds in the different matrices. For this, calibration curves were prepared in solvent- and matrix-matched extracts of the particulate and filtrate. The concentration range of the calibration curves and Pearson correlation coefficients (R^2) are shown in Table 2 for all analytes in each matrix.

Recoveries were estimated at two concentrations: 588.2 and 58.82 μg/kg for the particulate fraction and 25 and 10 ng/L for the seawater filtrate. Three replicates were used for each matrix and each concentration. Blank samples were treated with the same extraction procedure and then spiked at the same concentration to be used as references.

For the particulate fraction, two different treatments (3 and 30 min of UAE) were required because some of the marine biotoxins can be degraded by prolonged UAE, but prolonged extraction is recommended to be sure of the extraction of toxins from algal cells in suspension. Low recoveries were also

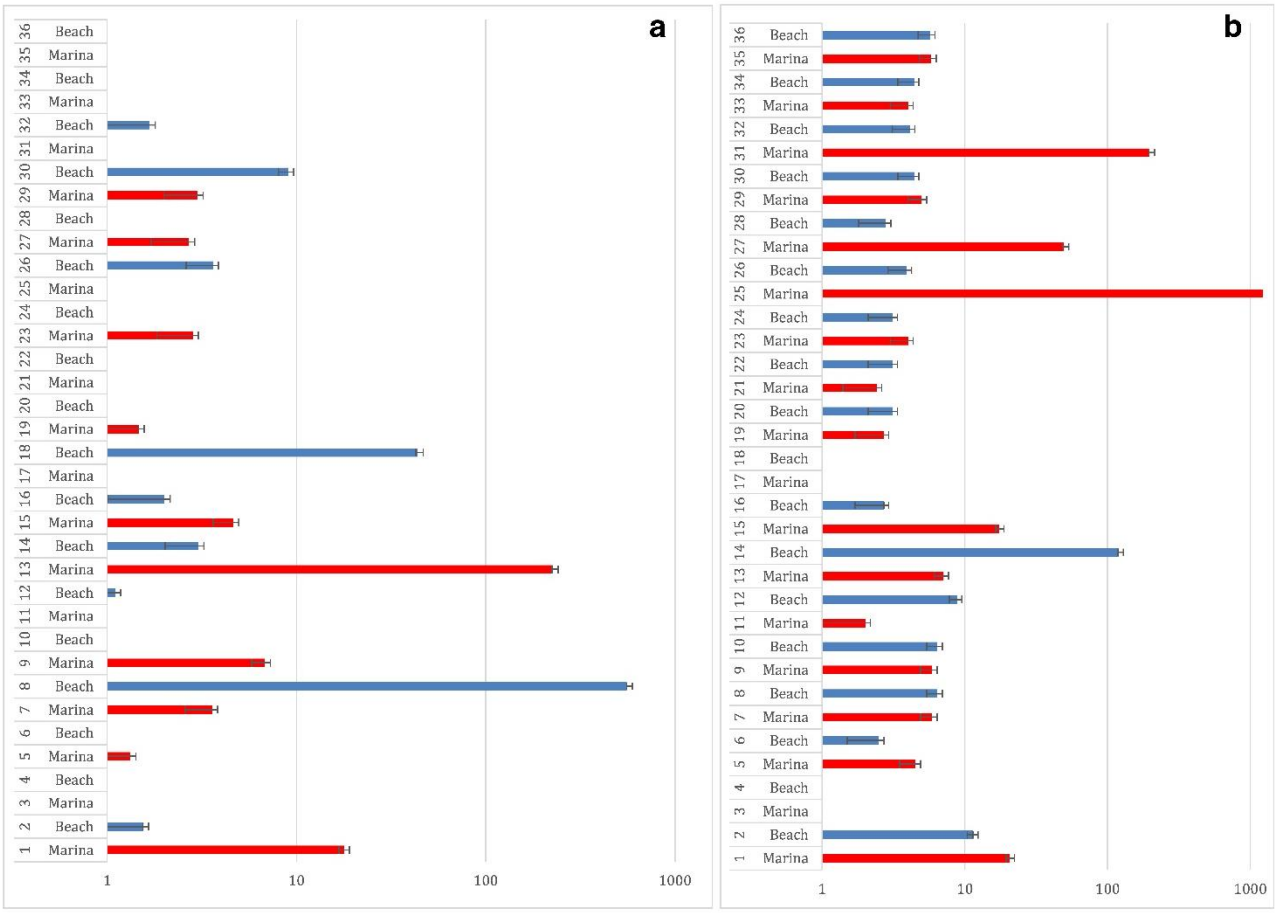

Fig. 4 Occurrence of okadaic acid on a logarithmic scale (**a**) in the particulate fraction expressed as micrograms per gram and (**b**) in seawater expressed as nanograms per litre for the different sampling sites

affected by the low concentrations considered, but in all cases these were corrected by the use of matrix-matched standards.

The matrix effect was calculated for each type of extract by division of the slope of the calibration curve of the particulate and filtrate by the slope of the calibration curve prepared in solvent. The values are summarised in Table 3. In general, matrix enhancement was observed for most of the compounds. In spite of the filtrate fraction being cleaned up by SPE, the effect was greater in this fraction because of the presence of coeluted compounds, as reported previously [20–24]. Previous studies of marine biotoxins also reported matrix enhancement [7, 23], which in our case was compensated for by the use of matrix-matched standards.

Occurrence of the biotoxins on the Catalan coast

Thirty-six surface seawater samples collected according to the description in "Sampling" were analysed. The main result of this study was the presence of a single lipophilic marine biotoxin, OA, which was present in 88% of the samples. The concentrations of OA at each sampling site are presented in Fig. 4. Taking into account the total concentration of OA in both the particulate and the filtrate, the mean value was 730 ng/L and the median value was 71 ng/L. Samples taken near highly urbanised, industrialised or agricultural areas recorded the highest concentrations of OA. The presence of this lipophilic biotoxin has been evidenced in a considerable number of studies related to biota and water from the Mediterranean Sea [26, 27].

The highest concentrations of OA were present in the particulate fraction with a mean partition ratio (K) of 0.298, calculated as the concentration of the filtrate divided by the concentration of the particulate, both in nanograms per litre. For OA, K ranged from 1.00×10^{-3} to 3.82 inside ports and from 1.00×10^{-3} to 0.832 in open coastal areas. Inside ports, the mean of K was 0.405, while in open coastal areas, the mean was 0.166.

The concentrations in suspended material are summarised in Fig. 4a. In the particulate, the concentrations of OA ranged from 0.09 to 560 μg/g. The mean and median concentrations were 24.9 and 1.21 μg/g respectively. Considering only the samples with positives, the mean and median concentrations were 34.5 and 2.36 μg/g respectively. The highest concentration of OA was 560 μg/g, corresponding to site 8—L'Ametlla de Mar beach. This high concentration could be related to high water temperatures accompanied by high amounts of organic matter, partially generated in the aquaculture facilities nearby, that could contribute to the eutrophication, promoting phytoplankton growth. The mean concentration of OA in positive open coastal samples was 52.03 ± 152.5 μg/g, whereas inside ports it was almost half that value, 19.41 ± 59.28 μg/g, although this difference was shown not to be statistically significant ($p > 0.050$) according to the t test and non-parametric tests (Wilcoxon signed rank test).

Related to the occurrence of OA in the filtrate seawater, the concentrations in nanograms per litre are shown in Fig. 4b. In seawater, the concentrations of OA ranged from 2.10 to 1780 ng/L. The mean concentration was 64.0 ng/L and the median concentration was 4.40 ng/L. Considering only the samples with positives, the mean and median concentrations were 72.0 and 4.75 ng/L respectively. The sample with the highest concentration corresponded to site 25—Masnou marina—with 1780 ng/L. Contrary to what happens with the particulate samples in water solution, the highest OA concentrations were found in samples from the interiors of ports.

In addition, no clear tendency was found between the concentration of OA in samples coming from inside marinas and those from open coastal areas. The highest concentration of OA in marinas was 9600 ng/L, whereas in open coastal areas it was 8600 ng/L. The mean concentration inside marinas was 770 ng/L, and the median concentration was 110 ng/L, whereas in open areas the concentrations were 700 and 57 ng/L respectively. No significant correlations were found between the concentrations of OA and measured pH and salinity.

OA is produced by different species of dinoflagellates of the genera *Dinophysis* and *Prorocentrum*, which are present in oceans and seas worldwide [25], including the Mediterranean Sea. The rest of the lipophilic marine biotoxins were not detected in any samples in this study, even those which are produced by species of the same class (Dinophyceae). However, according to our results, the presence of some species of this phylum in the Catalan coast, during the sampling weeks, can be suggested. Some reports have shown that OA is one of the biotoxins that accumulates in higher amounts in Mediterranean shellfish of some areas [27–29].

Conclusions

A multiresidue method has been developed and evaluated for the analysis of ten lipophilic marine toxins in seawater, by our considering two fractions of water and particulate material, showing a solid performance at the parts per trillion level.

The method was assessed with respect to accuracy, specificity, selectivity, repeatability, within-laboratory reproducibility, LOD, LOQ and linearity.

Good performance has been demonstrated, permitting quantitative analysis of selected analytes and fast screening of non-target biotoxins by retrospective screening using its full-scan capabilities.

The method was applied to characterise the occurrence of these contaminants in samples from the western Mediterranean coast. OA was the only biotoxin detected, and this compound was found in most of the samples (88%) in the range between 0.11 and 560 μg/g in the particulate and between 2.1 and 1780 ng/L in the filtrate in positive samples in non-algal bloom conditions. These results support the need for monitoring

programs in Europe, and highlight the importance of further studying the degradation patterns, distribution and chronic toxic effects to properly perform risk assessment studies.

Acknowledgements This work was supported by the European Union through the projects Sea-on-a-Chip (FP7-OCEAN-2013-614168) and BRAAVOO (FP7-OCEAN-2013-614010), the Generalitat de Catalunya (Consolidated Research Groups "2014 SGR 418" Water and Soil Quality Unit) and by the Spanish Ministry of Economy and Competitiveness through the project Integra-Coast (CGL-2014-56530-C4-1-R). The authors express their deepest gratitude to Roser Chaler and Dori Fanjul for their technical support during the high-performance LC–HRMS analyses.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Analysis of lipophilic marine biotoxins by liquid chromatography coupled with high-resolution mass spectrometry in seawater from the Catalan Coast

Cristina Bosch-Orea, Josep Sanchís, Marinella Farré, Damiá Barceló

Table S1 Tailing factor (TF) of the lipophilic MBTs for different chromatographic conditions in the mobile phase

	Acetonitrile/water 0.1% Formic acid	Methanol/water	Methanol/water 10 mM ammonium acetate
Okadaic acid	1.00	1.17	0.89
Dinophysistoxin-1	0.10	1.00	1.41
Penctenotoxin-2	0.71	2.40	0.89
Azaspiracid-1	0.74	1.65	1.23
Azaspiracid-2	0.90	1.50	1.18
Azaspiracid-3	1.00	1.73	1.12
Azaspiracid-4	0.83	1.73	1.11
Azaspiracid-5	0.78	1.92	-
Yessotoxin	0.88	0.79	1.39
Homoyessotoxin	0.83	1.58	1.00

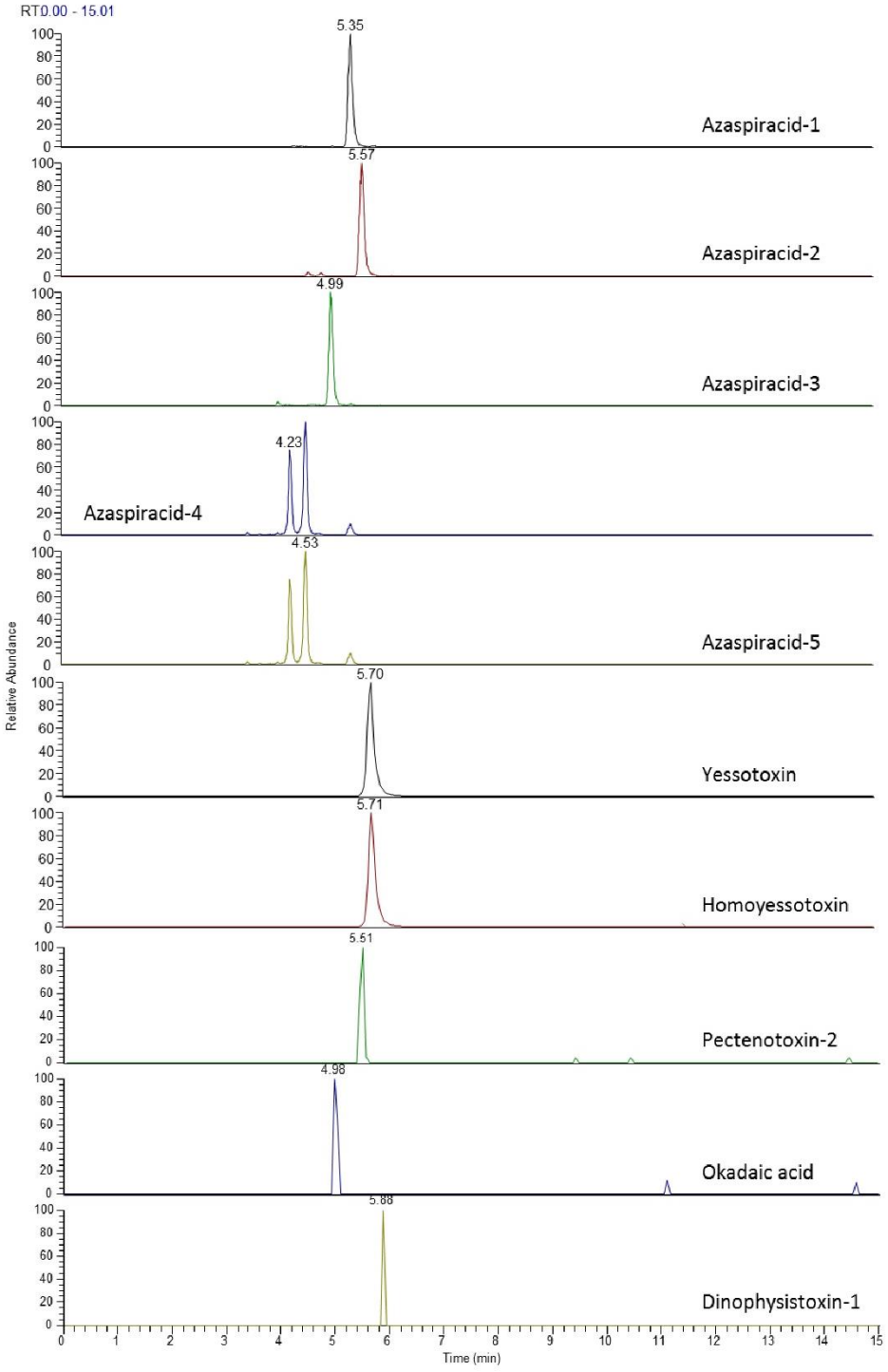

Fig. S2 Extracted ion chromatograms of the 10 lipophilic MBTs

3.2.2 Excitatory amino-acids MBTs: DA

Publication 2: **Ultra-trace determination of domoic acid in the Ebro Delta estuary by SPE-HILIC-HRMS**

DOI: https://doi.org/10.1039/C9AY02617G

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Cite this: DOI: 10.1039/c9ay02617g

Ultra-trace determination of domoic acid in the Ebro Delta estuary by SPE-HILIC-HRMS†

Cristina Bosch-Orea,[a] Josep Sanchís,[bc] Damiá Barceló[ab] and Marinella Farré *[a]

Marine phytoplankton, such as diatoms, are responsible for a considerable part of carbon fixation and form the basis of the marine food web. However, different factors, such as eutrophication, can affect their population growth and induce the production of toxins. During the last few years, the presence of domoic acid (DA) has increased worldwide in coastal areas. Its toxic properties and capacity to bioaccumulate through the food chain have reinforced the need for developing monitoring strategies to ensure the safety and quality of marine resources. In this work, a highly sensitive analytical method has been developed to be used as an early detection and quantification tool for DA in seawater. The method is based on a sample pre-treatment step by solid-phase extraction (SPE) and an instrumental analysis by hydrophilic interaction liquid chromatography coupled with high-resolution mass spectrometry (HILIC-HRMS). The method has been validated, showing good results in terms of sensitivity and repeatability. Limits of detection and quantification were 0.25 and 0.75 ng L^{-1} respectively, and acceptable recovery was in the range of 48–69%. Then, the method was applied to study a real scenario in the Ebro Delta where mariculture activities are relevant for the economy of the area. A total of 34 samples were collected in different campaigns during three seasons covering the two main delta bays (Alfacs and Fangar) and four lagoons (La Tancada, Illa de Buda, l'Encanyissada and Canal Vell). 65% of the samples had concentrations of DA at the ng L^{-1} level, ranging from 0.90 to 69.6 ng L^{-1}. The highest levels were found in samples from Alfacs bay and the summer campaign. In the case of the lagoons, DA was detected only in La Tancada at 8.78 ng L^{-1} in the winter campaign. This study proves the prevalence of DA in highly eutrophic areas, during all seasons, often at trace level concentrations.

Received 6th December 2019
Accepted 27th February 2020

DOI: 10.1039/c9ay02617g

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1. Introduction

The frequency of harmful algal blooms (HABs) has globally increased due to water eutrophication and global warming. This results in negative consequences for ecosystems because of the release of toxins by such harmful algae. Domoic acid (DA) is a potent neurotoxin produced by diatoms of the genus *Pseudo-nitzschia* (present in all coastal areas). DA has bioaccumulation capacity[1] and is the cause of the Amnesic Shellfish Poisoning (ASP) syndrome, known since the first documented intoxication in 1987, through blue mussel consumption in Canada.[2] Since this event, many programs have been established to understand the toxin-release mechanism from algae and to predict and prevent contamination incidents. However, the subject still remains poorly understood; in the case of diatoms, not all species of this genus can produce DA, and the toxicity within the same species can vary even within different strains of the same toxigenic species.[3] Moreover, the presence of other microbial communities can induce the release of DA as a defensive mechanism.[4] In addition, the production of DA depends on miscellaneous factors; the availability of nutrients, temperature and growth phase of the organisms are also relevant parameters. Due to the difficulties in controlling all these abiotic and biotic factors, the assessment of DA has to be done with more selective methodologies to have reliable results.

Liquid chromatography coupled with mass spectrometry (LC-MS) has been proved to be a sensitive and selective technique in the analysis of marine toxins. The European Commission[5] established the use of LC-MS techniques as reference methods for the determination of toxins in shellfish. These techniques, in combination with appropriate sample extraction and clean-up procedures, permit the determination of DA in seafood and other matrices such as phytoplankton and seawater.[6–10] Solid-phase extraction (SPE) is an exceptional technique that provides the isolation of DA from seawater and the elimination of matrix interference, especially the high content of dissolved salts. Moreover, the possibility of concentrating the sample by several orders of magnitude greatly benefits the increase of the overall sensitivity of the method.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9ay02617g

In this study, the development of a sensitive and selective analytical method to determine and quantify DA in seawater is presented. The analytical approach is based on solid-phase extraction (SPE) followed by hydrophilic interaction liquid chromatography (HILIC) coupled with high resolution mass spectrometry (HRMS) using electrospray ionisation (ESI). The analytical approach has been in-house validated and applied to assess DA in the Ebro Delta embayment, which is the major mollusc harvesting area (mussels, oysters and clams) of the Catalan coast. The assurance of the quality and safety of these products is mandatory. Evidence of *Pseudo-nitzschia* spp. has been widely reported in the Ebro Delta bays during the last few years although, to date, the occurrence of *Pseudo-nitzschia* has never been directly related to the presence of DA.[11–14] The developed method has allowed the direct accurate measurement of DA in this area and, consequently, the possibility of studying its spatial and temporal distribution. Three sample campaigns have been conducted in two bays, Fangar and Alfacs, and four lagoons, La Tancada, Illa de Buda, l'Encanyissada and Canal Vell, during three different seasons. Then, the occurrence of DA has been discussed.

2. Materials and methods

2.1 Chemicals and reagents

Domoic acid and C5′-*epi*-domoic acid (a diastereomer of domoic acid) (DA, 44.5 ± 3.1 μg g^{-1}, ≥99% purity and reference CRM-03-DA) were purchased from the Cifga laboratory (Lugo, Spain). Auxiliary reagents such as ammonium formate, HPLC-grade methanol (MeOH), ultra-pure water, acetonitrile (ACN) and formic acid (FA) were supplied by Merck (Darmstadt, Germany). Standard solutions of DA were prepared in MeOH.

2.2 Sampling

Three sampling campaigns were carried out in the Ebro Delta wetland (north-east Spain, Mediterranean Sea) during October 2015, February 2016 and June 2016. Surface seawater was collected from two semi-closed bays, Fangar and Alfacs bays, and from four lagoons, La Tancada, Illa de Buda, l'Encanyissada and Canal Vell. Temperature (°C), salinity and pH (Table S2†) were measured with a multiparameter probe Professional Plus YSI 2030 from Yellow Springs Instruments (Ohio, USA) on each sampling date. Sampling point details are shown in Fig. S1,† and detailed information is provided in Table S1 of the ESI.†

Seawater samples were collected at a 1 m depth using a Niskin bottle and were stored in amber glass bottles at −20 °C until the analyses.

2.3 Sample treatment

The optimized sample treatment (see optimization details in Results section 3.1) was as follows: 500 mL of seawater was extracted by solid-phase extraction (SPE) employing hydrophilic–lipophilic balance cartridges (OASIS HLB, 200 mg) from Waters (Cerdanyola del Vallès, Spain). Cartridge conditioning and equilibration were accomplished with 6 mL of MeOH and 6 mL of ultrapure water with 0.1% FA. Then, the samples were acidified with 0.1% FA, and cartridge loading was performed at a flow rate of 1 mL min^{-1}. After loading, the cartridges were cleaned with 6 mL of ultra-pure water acidified with 0.1% FA. Finally, MeOH was evaporated from the extracts under gentle N_2 flow in a TurboVap from Biotage (Uppsala, Sweden). Extracts were reconstituted with 250 μL of acetonitrile–ammonium formate/FA buffer (9 : 1), which is the mobile phase composition at the beginning of the chromatographic run.

2.4 Instrumental analysis by HILIC-HRMS

The optimal instrumental conditions (see optimization details in Results section 3.1) were as follows: chromatographic separation was achieved by ultra-high performance liquid chromatography (UPLC) using the Acquity Waters (Harwich, Massachusetts, USA) system equipped with a HILIC (HILIC LUNA® 150 mm × 2 mm, 3 μm, 200 Å) column from Phenomenex (Torrance, California, USA). The mobile phase was composed of (A) 90% of acetonitrile and 10% of 50 mM ammonium formate at pH 3.2 and (B) 50% acetonitrile, 40% water and 10% of 50 mM ammonium formate at pH 3. The elution gradient was programmed as follows: 0 min (100% A) – 2 min (100% A) – 8 min (100% B) – 9 min (100% A) – 10 min (100% A). An extra 5 minutes was added between each analysis in order to stabilize the pressure in the column. The flow rate was established to be 0.3 mL min^{-1}. The samples were kept at 10 °C in an auto-sampler, and the injection volume was 20 μL.

Liquid chromatography was coupled with mass spectrometry through a heated electrospray ionization source (HESI-II) from Thermo Fisher Scientific (San Jose, California, USA) operating in positive ion mode. The optimal parameters of the source are as follows: sheath flow gas of 60 a.u., auxiliary gas of 15 a.u., and sweep gas of 2 a.u. Heater and capillary temperatures were set at 350 and 320 °C, respectively. The S-lens RF level was 60% and the spray voltage was 3.5 kV.

Mass spectrometry was performed with a Thermo Scientific Q-Exactive™ mass spectrometer from Thermo Fisher Scientific (San Jose, California, USA) with a hybrid quadrupole-Orbitrap analyser. Data were acquired in full scan mode in the range of 50–800 *m/z* at a resolution of 70 000 [full width at half maximum (FWHM)], and contemporary in the MS/MS spectrum at a resolution of 35 000 FWHM in parallel reaction monitoring (PRM) mode. The normalized collision energy (NCE) was set at 10 a percentage of 30 for the molecular ion $[M + H]^+ = 312.1$ of the target toxin.

Data analysis was carried out using Thermo Xcalibur 3.1.667 software from Thermo Fisher Scientific. Identification of DA was achieved with the retention time and exact mass (never exceeding 2 ppm of error between the accuracy mass and the calculated exact mass) of the precursor ion in the full scan mode and the fragment ions. Using the same criteria, quantification was performed by integrating the area of the most intense product ions (peaks with at least seven scan points) and the confirmation was performed with two other fragment ions following the abundance ratio of each one.

2.5 Validation of the method

Method validation was accomplished with the evaluation of the selectivity, linearity, precision, sensibility, accuracy, limits of

detection (LODs) and limits of quantification (LOQs) using standard solutions of DA and fortified seawater.

2.5.1. Selectivity. For identification purposes, the exact mass of the precursor ion in the full-scan mode, the products ions in the fragmentation pattern, and the retention time of DA in the standard and in the spiked seawater blank samples were compared at a tolerance of + 2.5%. Moreover, in accordance with the 2002/657/EC decision, the relative ion intensities (each product ion area signal *versus* the base product ion area signal) of the spiked samples were compared with the relative ion intensities of the DA standard solutions, at the same concentration levels used for the construction of the calibration curve.

2.5.2. Linearity. The linearity of the measurements in the instrumentation was estimated in the concentration range of 0.5 to 100 μg L^{-1} of DA. Pearson's correlation coefficient (R^2) and the slope of the calibration curve in solvent and matrix were determined to evaluate the sensitivity.

2.5.3. Limits of detection and quantification. The instrumental LOD (iLOD) was experimentally determined by gradual dilutions of the standard solution of domoic acid, starting at 40 μg L^{-1} concentration.

Because of the lack of noticeable instrumental noise, the lowest concentration at which the signal can be measured (fulfilling the required points per peak and selectivity criteria) was considered the iLOD. Consequently, the instrumental LOQ (iLOQ) was calculated, which was 10/3 times the iLOD.

2.5.4. Recovery. Since no certified reference material was available, recovery experiments were carried out using fortified samples in triplicate at two different levels 20 and 50 ng L^{-1}. In all batches of samples experimental blanks were analysed.

The method LOD (mLOD) for each extraction procedure was determined by the analysis of the standard solution in the matrix resulting from the particulate and the filtrate extracts. LODs were established to be the lowest concentration measurable of the matrix-matched calibration curve. The method LOQs (mLOQs) were estimated to be 10/3 times the mLODs.

2.5.5. Intra-day and inter-day precision. The repeatability of the instrument was evaluated with the measurement of a DA solution on the same day ($n = 6$) and consecutive days ($n = 3$), respectively, under the same instrumental conditions.

2.5.6. Accuracy. It was evaluated with the calculation of the DA recovery during the pre-treatment process.

For this, fortified blanks of seawater were subjected to the pre-treatment process. Values obtained were compared with those from the extracts subjected to the same process but fortified for the LC-MS analysis.

The matrix effect was evaluated to determine a possible signal enhancement or ion suppression during the ionization process at source by interferent substances present in seawater. To assess the effects of the matrix, fortified solutions of DA in seawater extracts and pure solvent were compared, and the percentage of effect was calculated according to the following expression:

% Matrix effects $= ([\text{area}]_{\text{seawater}}/[\text{area}]_{\text{solvent}}) \times 100$, where $[\text{area}]_{\text{seawater}}$ is the integrated area of DA in the seawater extracts and $[\text{area}]_{\text{solvent}}$ is the corresponding area in pure solvent.

3. Results and discussion

3.1 Analytical method optimisation

The high polarity of DA increases the difficulty in isolating it from the aqueous phase during extraction. The molecule possesses three carboxylic acid groups and one amino group, with pK_as ranging from 1.85, 4.47, and 4.75 to 10.6.[15] To optimise extraction from seawater different stationary phases and procedures have been studied: (i) the hydrophilic–lipophilic balance using OASIS HLB (500 mg, 6 cm^3) cartridges from Waters; (ii) carbon adsorption using ENVI CARB graphitized charcoal cartridges (500 mg, 6 cm^3), from Merck; and (iii) ionic exchange, in both cation and anion exchange modalities, with Isolute WCX (500 mg, 6 cm^3) and Isolute SAX (500 mg, 6 cm^3) cartridges, respectively, both from Biotage (Uppsala, Sweden).

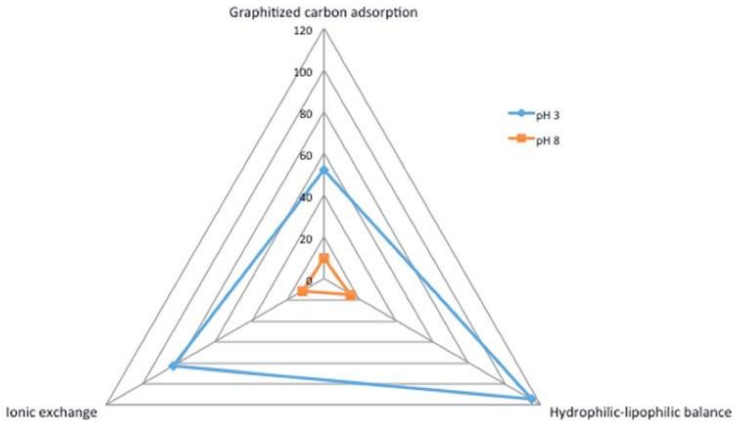

Fig. 1 Values of recovery obtained in the different SPE treatments.

All cartridges were conditioned with 6 mL of methanol and equilibrated with 6 mL of water adjusted at the same pH of the sample. Loading and elution conditions were optimized for each cartridge independently:

For HLB, 100 mL spiked seawater was loaded at pH 3 or pH 8. A washing step after loading was performed with 6 mL of water at pH 3 or pH 8, and elution was carried out with 20 mL of methanol.

For ENVI CARB, 100 mL spiked seawater at pH 3 and pH 8 was loaded into the cartridge. A washing step with 20 mL of water at pH 3 or 8 was added. Elution was performed with 25 mL of a mixture of methanol/acetonitrile/acetone (1 : 1 : 1).

For SAX and WCX, 25 mL of spiked seawater was loaded into the cartridges conditioned at pH 8 for anion exchange and at pH 3 for cation exchange. Elution was carried out with 25 mL of methanol.

All extracts from the elution step were concentrated and reconstituted as explained in Section 2.3 for the subsequent analysis.

Recovery values for different SPE treatments are presented in Fig. 1. As can be seen, the best recovery rates were obtained for the HLB stationary phase when the spiked samples were conditioned at pH 3. The graphitized carbon showed as well acceptable values with the samples conditioned at acidic pH but repeatability was higher with HLB. On the other hand, the elution of DA graphitized carbon required higher volumes of solvent than the HLB. Regarding the cation/anion exchange strategy, the cation exchange allowed better recovery of DA than the anion one, but lower than that with HLB and samples at acidic pH. Considering the results under these conditions HLB cartridges were selected for further optimisation.

The HLB phase was tested by loading different volumes of samples: 100 mL, 250 mL, 500 mL and 1.0 L. A sample volume of 500 mL was selected as the maximum volume without the critical loss of the compounds and the clogging of the cartridge, reaching a value of 69.24 ± 1.14% and 48.09 ± 17% at concentration levels of 20 ng L^{-1} and 50 ng L^{-1}, respectively. Although the recoveries were a bit lower when loading higher volumes of samples, a significant improvement in the mLOQ was obtained with 500 mL of seawater instead of 100 mL because of the high pre-concentration of the sample; thus 500 mL was selected as the optimal volume. For quantification purposes, recovery correction was applied for each sample.

For the chromatographic separation, two different types of chromatography techniques were compared: reversed-phase liquid chromatography (RPLC) using a C18 Synergy (50 mm × 2 mm, 5 µm, 80 Å) analytical column, and hydrophilic interaction liquid chromatography (HILIC) using a HILIC LUNA® (150 mm × 2 mm, 3 µm, 200 Å) analytical column, both from

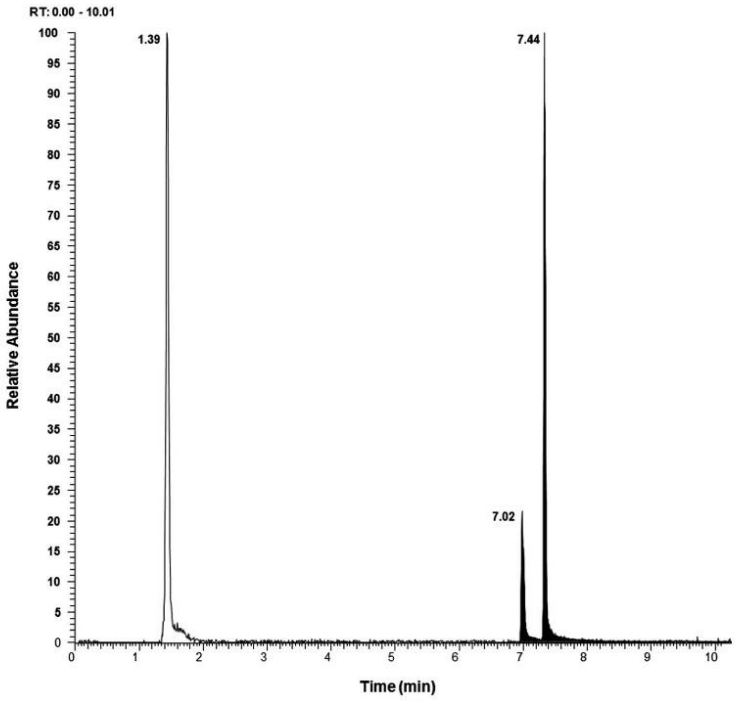

Fig. 2 Extracted ion chromatograms of DA from the two different chromatography techniques: white color corresponds to the reverse-phase column (C_{18}) while the black color corresponds to the hydrophilic interaction column (HILIC) which separates DA (tr 7.44) from its isomer *epi*-DA (tr 7.02).

Phenomenex. For HILIC, the mobile phase was composed of (A) acetonitrile and (B) water with FA and ammonium formate (see the optimised elution gradient in Section 2.4). RPLC was performed using a mobile phase composed of (A) 90% acetonitrile 0.1% FA and (B) 10% water 0.1% FA with an isocratic gradient. The working flow rate was established to be 0.3 mL min^{-1} and the total analysis run was for 10 min.

The extracted ion chromatograms of DA using RPLC and HILIC are shown in Fig. 2. As can be seen, a good peak shape was obtained in both cases, but using RPLC the retention time was 1.40 min. Such a short retention time is favourable in terms of rapid screening, but this retention time is too close to the dead time. While RPLC chromatographic approaches have been used for the analysis of DA in shellfish and plankton in combination with some other lipophilic toxins,[16,17] poor retention of DA may cause its coelution with multiple matrix components that may elute at the beginning of the chromatogram in complex extracts, potentially interfering with its detection and causing matrix effect. Because of this, HILIC has been selected as the optimal column since it increases DA retention and allows the separation of DA from its isomer *epi*-DA with a retention time of 7.40 min. This type of chromatography gives the possibility of combining the analysis of DA with other hydrophilic marine toxins, allowing a high throughput analysis of polar compounds in seawater. In mussel analysis, HILIC columns have already been employed in the determination of DA along with PSP toxins with a suitable resolution.[6,18]

Two mass spectrometer analysers were compared for the determination of DA: a triple quadrupole working in the acquisition mode of Selected Reaction Monitoring (SRM), and an Orbitrap operating in PRM mode. A standard solution of DA in MeOH was directly infused into the mass spectrometers by soft ionization with an electrospray ionization (ESI) source. Optimal conditions were obtained for the ionization of DA and its fragmentation in the two instruments. Fragmentation patterns are shown in Table 1.

Sensitivity, resolution and selectivity were compared for both analysers, and Orbitrap was selected for this study. In terms of sensitivity, there is no significant difference between both mass spectrometer analysers, but working in PRM mode provides extra selectivity than working in SRM, thanks to accurate mass measurements. Also, the PRM mode can be combined with

Table 1 Fragmentation pattern of DA at the optimized collision energies for two different analysers, Orbitrap and triple quadrupole. NCE* corresponds to the normalized collision energy stepped at the value of 10. The mass tolerance error in ppm is shown in parentheses

	Chemical formula	Precursor ion	*m/z*	Collision energy 1	Product ion 1	*m/z*	Collision energy 2	Product ion 2	*m/z*
Orbitrap	$C_{15}H_{21}NO_6$	$[M + H]^+$	312.1442 (1.5 ppm)	30% NCE*	$[C_{14}H_{20}O_4N]^+$	266.1382 (1 ppm)	30% NCE*	$[C_{14}H_{18}O_3N]^+$	248.1275 (1.5 ppm)
QqQ			312	16.6 V		266	17.6 V		248

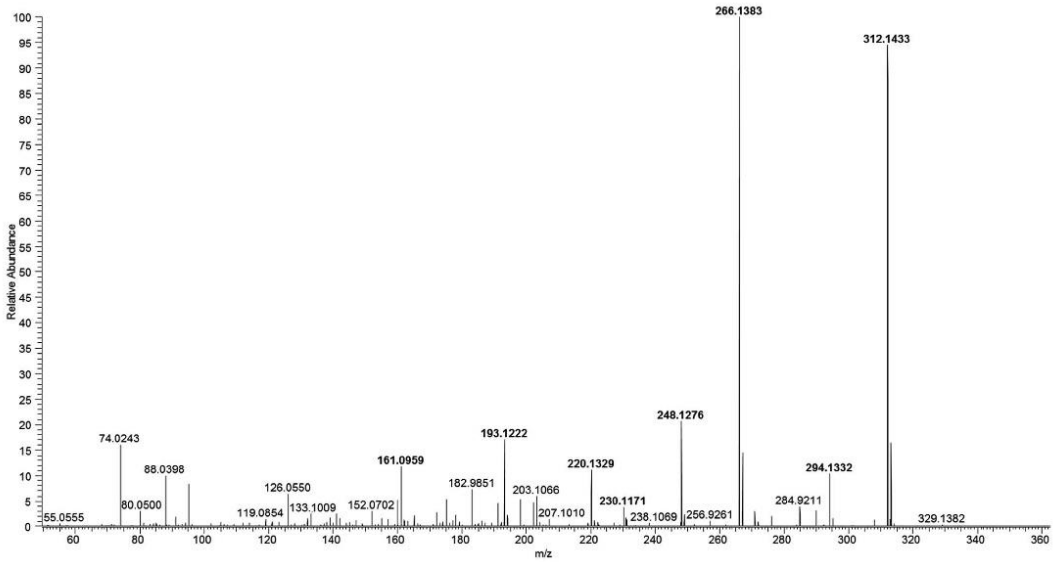

Fig. 3 High resolution mass spectrum of DA obtained with Q Exactive.

parallel full-MS acquisition, which allows the possibility of performing retrospective analysis for the detection of suspected compounds and even non-target analysis at high resolution for the identification of other pollutants. The high-resolution mass spectrum of DA obtained at 30% of NCE is shown in Fig. 3.

In Table 2, the quality parameters of this method are summarized. Good performance of the method has been achieved. The most notable quality is the high sensitivity with the mLOD reaching 0.25 ng L^{-1} for DA. To our knowledge, these are the lowest values reported in the literature. Other sensitive methods based on LC-MS presented mLOD values ranging from 750 ng L^{-1},[8] 30 ng L^{-1} (ref. 7) to the lowest value of 0.3 ng L^{-1}.[19]

3.4 Levels of DA in coastal waters

In Table 3, the DA concentrations detected in 34 seawater samples from Ebro's Delta are summarised. DA was present in 65% of the samples at the ng L^{-1} level. The concentration of DA ranged from 0.90 to 69.6 ng L^{-1} with a mean value of 17.3 ng L^{-1} and a median of 5.59 ng L^{-1}. The presence of DA was prevalent in Alfacs and Fangar bays during the seasons analysed while in the lagoons only La Tancada showed a positive result with a concentration of 8.78 ng L^{-1}. The La Tancada lagoon was similar to the bays in terms of salinity. *Pseudo-nitzschia* spp. are marine and preferably grow in a saline environment.[20] Moreover, the production of DA can be favoured at high salinity.[21] Then, this influence from seawater could explain why DA is present in this lagoon and not in the rest.

The highest concentrations were found in Alfacs bay in the summer season, with a maximum value of 69.6 ng L^{-1}, followed by 67.2 ng L^{-1}, which was detected at the same sampling site during wintertime. This last value was unexpected, considering that during the coldest months the upwelling phenomenon takes place, driving the nutrients from the surface to deeper waters, and the temperatures are lower, having a negative influence on the phytoplankton growth. But these bay areas constantly receive agricultural discharges of freshwater with a high content of nutrients that can disrupt the annual variation of nutrient availability. In other studies, DA has also been detected in shellfish during winter months along the Catalan coast. Giménez-Papiol *et al.* reported concentrations of DA in February and April exceeding the EFSA suggested regulation levels (4.5 mg DA per kg shellfish) and even the maximum permitted in the current legislation (20 mg DA per kg shellfish).[13] These values are in the same range as other studies that determined DA in estuarine areas in the Mediterranean Sea.

Table 2 Analytical parameters for the method validation

Instrumental parameters			
		Intra-day precision	Inter-day precision
iLOD (pg on column)	Linearity range (ng L^{-1}), R^2	10 μg L^{-1} (RSD%, $n = 6$)	10 μg L^{-1} (RSD%, $n = 3$)
1	50–50 000 (0.999)	5.53	2.91

Method parameters				
		Recovery (%, $n = 3$)		
mLOD/mLOQ (ng L^{-1})	Linearity range (ng L^{-1}) and linearity (R^2)	20 ng L^{-1}	50 ng L^{-1}	Matrix effect (%)
0.25/0.75	2–50 (0.995)	69.24 ± 1.14	48.09 ± 7	73

Table 3 Concentration of DA in ng L^{-1} in Ebro's Delta bays and lagoons

		Sampling campaign		
	Coordinates	1 (Oct-15)	2 (Feb-16)	3 (Jun-16)
Alfacs	40.610, 0.724 (beach)	11.6	67.2	14.6
	40.601, 0.610 (shore)	18.4	14.3	69.6
	40.584, 0.579 (open sea)	15.0	21.6	41.7
	40.605, 0.609 (open sea)	No sample	8.08	24.7
Fangar	40.800, 0.700 (beach)	2.20	8.11	5.1
	40.798, 0.713 (shore)	4.16	2.29	11.2
	40.815, 0.743 (open sea)	6.09	0.9	10.1
	40.817, 0.751 (open sea)	No sample	<LOQ	13.9
La Tancada	40.645, 0.742 (lagoon)	8.78	<LOQ	<LOD
Illa de Buda	40.703, 0.841 (lagoon)	<LOD	<LOD	<LOD
L'Encanyissada	40.657, 0.673 (lagoon)	<LOD	<LOD	<LOD
Canal Vell	40.745, 0.788 (lagoon)	<LOD	<LOD	<LOD

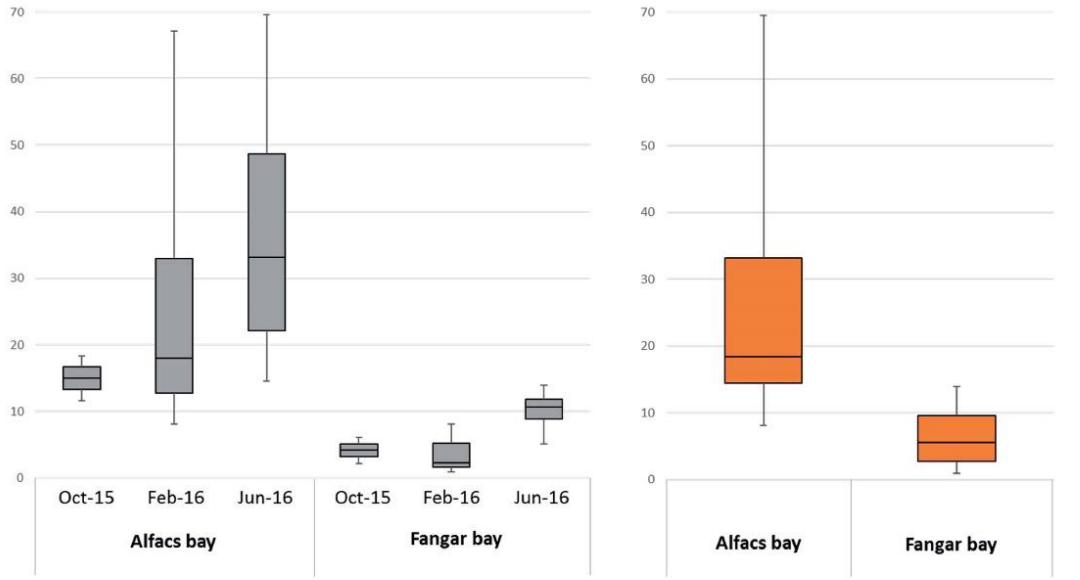

Fig. 4 Box plot graphs of the DA concentration: grey color for the two bays during the three sample campaigns: October 2015, February and June 2016; orange for the total concentration in three campaigns in the two different bays.

For example, Barbaro E. *et al.* registered concentrations ranging from 1.5 to 16.2 ng L^{-1} in the Venice lagoon that is strongly influenced by the seawater from the Adriatic Sea.[19] More focused on the studied area of Ebro's Delta, Busch *et al.* reported concentrations of DA between 3.2 and 18.8 ng L^{-1} in Fangar bay but not in Alfacs.[12]

Comparing the results of the two bays, Alfacs had, in general, higher levels of DA than Fangar. The differences between the total concentration of DA along with the three sampling campaigns in the two bays are displayed in Fig. 4. The location of the bays could be the factor that explains this difference since both have a semi-closed shape but water residence time and stratification differ significantly in both cases: Fangar bay is in the northern part of the delta and influenced by the littoral current coming from the north to the south (Northern current[22]), whereas Alfacs is a bay located in the southern part where marine recirculation of water is slower because of the physical barrier that the delta imposes,[23] and freshwater inputs have longer residence times. Water stratification,[14] the occurrence of inorganic components (*i.e.* silicates and Cu^{2-})[24] and organic nutrients[25] are known to be correlated with the abundance of *Pseudo-nitzschia* spp. (or other algae responsible for their emission, as hypothesized by Busch *et al.*), and all these parameters in the Ebro Delta embayments are substantially affected by the dynamics of irrigation channels. Then, growth of algal phytoplankton could be more favoured in Alfacs, and consequently, the release of DA.

Regarding seasonal variability, a slight tendency can be observed from the lowest DA concentrations in autumn months to the highest in summer months. The differences in the DA concentrations in each bay during different months of sampling are shown in Fig. 4. This tendency can be observed when considering the median values of all the concentrations detected in each bay across different months. More in detail, this tendency seems to be clearer for the samples taken in the open sea than for those samples from a beach and shore, where the concentration of DA did not seem to follow a clear temporary pattern. This tendency can be as well observed in the box-and whisker plot for the tree sampling campaign presented in Fig. S2.† However, the *P*-value was 0.1196 that can be considered insignificant. Therefore, the variation among campaign medians is not significantly greater than expected by chance. The Kruskal–Wallis test was applied because the distribution of the results did not pass the normality test for all sampling campaigns.

As commented previously, these semi-closed bays are constantly influenced by variable freshwater discharges from agricultural activities, leading to no regular values of salinity, nutrients and turbidity in the nearshore.

4. Conclusions

A highly sensitive analytical method for the detection and quantification of DA in seawater has been developed and applied to the analysis of water samples from the marine bays of the Ebro Delta.

For the method development, several stationary phases were tested and Oasis HLB cartridges were selected. Mean recovery values ranging from 48 to 69% were obtained when treating 500 mL samples acidified with FA. A low matrix effect was

observed, indicating the elimination of interference and salt from seawater. Anyway, this effect of ion suppression was assessed by quantification with a matrix-matched calibration curve. High sensitivity has been achieved, thanks to the 2000-fold concentration of the samples, allowing a mLOD and mLOQ of 0.25 and 0.75 ng L^{-1}, respectively.

The high polarity of DA allows the option of using both RPLC and HILIC with suitable resolution and peak shapes. However, HILIC has been selected in this study to determine DA because of its higher retention factor and for a possible combination in the joint analysis of other hydrophilic compounds present in seawater. Moreover, the separation of DA and its isomer *epi*-DA was possible.

The Orbitrap and triple-quadrupole mass spectrometers presented good sensitivity and selectivity. However, the Orbitrap-MS was selected for the method, not only for its highest resolution but also for its capability to acquire comprehensive sample screenings in the full-MS mode at high resolution and the possibility of conducting retrospective analyses and carrying out non-target screening of other MBTs and contaminants.

Final validation of the method showed excellent performance in terms of sensitivity, linearity, repeatability, selectivity and recovery. Application of the method to real samples was successful.

DA was present in 65% of the samples analysed with a mean value of 17.3 ng L^{-1}. The concentration of DA ranged from 0.90 to 69.6 ng L^{-1} and was higher during the summer campaign. Highest levels were found in Alfacs bay in every season. The occurrence of DA was not observed in the lagoons except in La Tancada where DA was detected in autumn and winter, at concentrations of <mLOQ and 8.78 ng L^{-1}.

This study confirms the presence of DA in Ebro's Delta bays during all seasons. Although this marine toxin is present at trace level concentrations, it is essential to monitor and control DA due to its toxic properties and capability to accumulate in feeder filters which are necessary for the mariculture activities of the area.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This study was funded by the Ministry of Economy and Competitiveness through the project PLAS-MED (CTM 2017-89701C3-1-R) and by the Generalitat de Catalunya (Consolidated Research Group 2017 SGR 01404 – Water and Soil Quality Unit). The authors express their deepest gratitude to R. Chaler and D. Fanjul for their technical assistance.

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Electronic Supplementary Material (ESI) for Analytical Methods.

SUPPORTING INFORMATION

ULTRA-TRACE DETERMINATION OF DOMOIC ACID IN THE EBRO DELTA ESTUARY BY SPE-HILIC-HRMS

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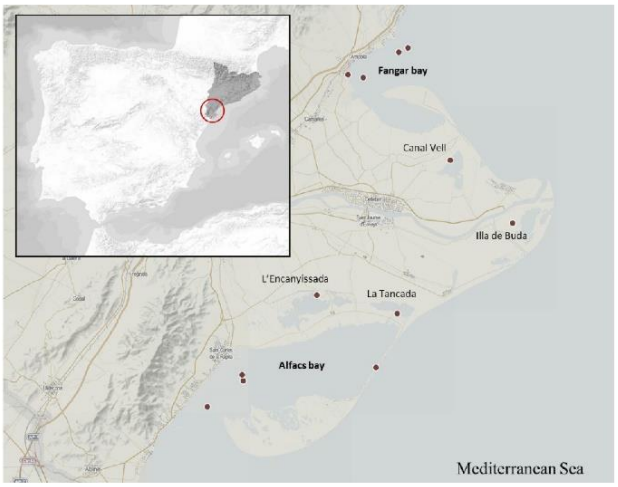

Figure S1. Sampling points in the Ebro's delta estuary for the three campaigns.

3.2.3 Alkaloid MBTs: STXs and TTX.

Publication 3: Analysis of highly polar marine biotoxins in seawater by hydrophilic interaction liquid chromatography coupled to high resolution mass spectrometry.

Submitted to the journal "Methods X"

Reference number: MEX-S-20-01068

Analysis of highly polar marine biotoxins in seawater by hydrophilic interaction liquid chromatography coupled to high resolution mass spectrometry

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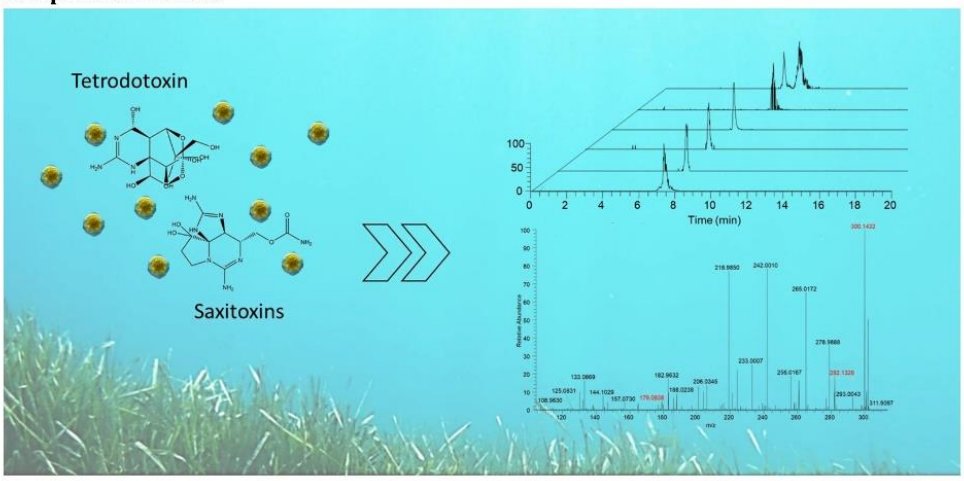
Abstract: The monitoring of marine biotoxins in worldwide coastal areas has become mandatory to ensure the public health. The focus of this surveillance are bivalve molluscs with commercial interest, however some other inconveniences could appear in the environment by the presence of these toxic compounds. The monitoring of MBTs in seawater is presented as an alternative strategy to determine the presence of the toxins and the possible implications in other organisms of the ecosystem. For this, an analytical method based on hydrophilic interaction liquid chromatography coupled to high resolution mass spectrometry has been developed to identify and quantify some hydrophilic marine biotoxins in seawater. The selected toxins are: saxitoxin (STX), decarbamoyl-saxitoxin (dcSTX), neosaxitoxin (NeoSTX), gonaytoxin-2,3 (GTX-2,3) and tetrodotoxin (TTX) and are responsible of gastrointestinal and central nervous system distress in humans when are consumed via seafood. Particulate and filtrate portion were analyzed separately in other to characterize the extracellular toxins dissolved in the water and those present in the particulate. Ultrasound assisted solid-liquid extraction with methanol was used for the isolation of the MBTs from particulate and solid phase extraction using silica cartridges, for the filtrate. Extraction procedure was most critical step during the analytical method due to the high polarity of the toxins and the absolute recoveries obtained ranged from 15 to 47 % in the filtrate and 26 to 71 % in the particulate portions. Limits of detection of the method ranged from 0.5 to 5 µg/l in the filtrate portion and from 3.1 to 62 µg/l in the particulate portion.
The method was applied in 17 real samples from the Murcia's coast, in Mediterranean Sea. Surface seawater was collected during July 2018 and April 2019, but any of the target MBTs was detected nor in particulate not filtrate portion above the mLODs.

Keywords: saxitoxins, paralytic shellfish poisoning, tetrodotoxin, HILIC-HRMS, seawater

Graphical abstract



1. Introduction

The frequency of harmful algal blooms (HABs) has increased in worldwide coastal areas during last decades [1] and consequently the occurrence of marine biotoxins (MBTs) [2]. A subset of algae is able to produce MBTs as secondary metabolites. These compounds have high bio-activity and are able to bio-accumulate along the food chain [3], causing poisoning syndromes in humans when are ingested via seafood [4].

Saxitoxins (STXs) and tetrodotoxins (TTXs) are alkaloid MBTs that act in the nervous system causing muscular paralysis and in the worst cases the death [5]. These substances affect the neuromuscular transmissions by blocking the voltage-gated sodium channel [6] and have caused several intoxication outbreaks [7-9]. The presence of these compounds into the environment supposes a threat to the public health. Fishery and mariculture activities require exhaustive controls of the products in order to assure the safe consume of the marine resources. STXs have been classified as paralytic shellfish poisoning (PSP) toxins and the maximum regulated limits (MRLs) in commercial bivalve molluscs have been stablished at 800 µg STX equivalent kg^{-1} by the CODEX Committee on Fish and Fishery Products (CCFFP) [10]. Even that TTXs share the same mode of action with STXs, these have not been included in PSP classification nor MRLs have been established yet.

Monitoring of shellfish has been extensive to date. However, recent studies have identified new vectors of MBTs propagation such as echinoderms, non-edible molluscs [11] and even marine litter drifting across the oceans [12]. An alternative monitoring is focused on the media where MBTs are produced, the seawater, that could englobe the interaction with the organisms and pollutants present in the ecosystem. Monitoring of MBTs in seawater could be employed as time-integrated indicator for the occurrence of the toxins, and then be used as rapid-alarm system to mitigate its presence.

Chemical methods of analysis based on liquid chromatography coupled to mass spectrometry (LC-MS) have been proved to be the most selective and sensitive for the analysis of MBTs [13]. MS-methods, in particular based on high resolution mass

spectrometry (HRMS), have become powerful tools for structural elucidation of the target toxins and for the characterization of new analogues [14]. Related to LC, the use of traditional reversed-phase has been replaced by hydrophilic interaction liquid chromatography (HILIC) due to the high hydrophilicity of STXs and TTXs [15]. This modality of LC achieves a proper separation of the toxins avoiding long-time processes of derivatisation and the use of ion-pairing agents [16]. On the other hand, seawater pre-treatment is required to eliminate the salt content and the organic matter present in the sample in order to avoid inconveniences during the ionization process in the instrumental analysis [17]. However, due to the polarity of these toxins, its isolation from seawater is not an easy task and diverse strategies have to be considered. Solid phase extraction (SPE) has been widely used in the analysis of MBTs in different matrices of seafood [18-21] and even seawater [22, 23] . SPE is favourable to pre-concentrate the dissolved toxins in seawater and could help to increase its detectability that are assumable at low concentration when algal bloom phenomena are not taking place. Nonetheless, another proportion of the toxins in seawater could be attached to the particulate or remain inside the phytoplankton cells. In this case, solid-liquid extraction assisted with ultrasounds (UAE) is presented as a proper cost effective technique that allows a high throughput extraction of several environmental types samples. It has been widely used in mussel's and algae's extraction [24-26], it has been that sonication achieved the lysis of the phytoplankton cells [27].

In this work is presented the development of an analytical method for the detection and quantification of a subset of hydrophilic MBTs; saxitoxin (STX), decarbamoyl-saxitoxin (dcSTX), neo-saxitoxin (Neo), gonaytoxin-2,3 (GTX-2,3) and tetrodotoxin (TTX). The analysis is carried out by HILIC coupled to HRMS. A prior sample clean-up to isolate the target compounds and eliminate the salt content from the seawater was studied. The pre-treatment consisted in a filtration step of the seawater to separate the particulate from the filtrate portion. Solid-liquid extraction assisted with ultrasounds (UAE) was employed to isolate the toxins from the particulate and solid phase extraction (SPE) with silica cartridges from the filtrate. The method was applied to real samples from the Mediterranean Sea, in the region of Murcia.

2. Material and methods

2.1 Chemicals and reagents

Certified Reference Materials (CRMs) of marine biotoxins were: gonyautoxin-2,3 (22.2 ± 1.5 µg/g and 8.2 ± 0.6 µg/g ≥97 % purity and reference CRM-00-GTX2&3); tetrodotoxin and 4,9-anhydro tetrodotoxin (25.9 ± 1.3 µg/g and 2.99 ± 0.16 µg/g, >96 % purity and reference CRM-03-TTXs); neosaxitoxin dihydrochloride (20.5 ± 1.1 µg/g, ≥99 % purity and reference CRM-00-NEO); decarbamoylsaxitoxin (19.5 ± 1.1 µg/g, ≥99 % purity and reference CRM-00-dcSTX); saxitoxin dihydrochloride (20.5 ± 1.5 µg/g, ≥99 % purity and reference CRM-00-STX), and were purchased from Cifga laboratory (Lugo, Spain). Chemical structures of the certified hydrophilic MBTs of this work represented in the **figure 1**. Solvents and reagents as ammonium formate, ammonium acetate, HPLC-grade methanol, ultra-pure water, acetonitrile, and formic acid were supplied by Merck (Darmstadt, Germany). Cartridges employed for the optimization were Silica 2g-Isolute

from Biotage (Uppsala, Sweden) and OASIS HLB-500mg, Sep-Pak Diol-1g and Sep-Pack Aminopropyl (NH_2)-500mg from Waters (Massachusetts, United States). And 0.2-µm pore size hydrophilic nylon membrane filters Millipore were supplied by Merck (Darmstadt, Germany).

2.2 Sampling

Seawater samples were collected from the surface of different locations in the Murcia's coast in Mar Menor (South-east of Spain, Mediterranean Sea), including harbours and beaches. The sampling took place during two different sampling campaigns in July 2018 and April 2019. Samples were kept frozen at -20ºC in amber glass bottles until the analysis. Details of the sampling points are summarized in the **figure S1** of the supporting information.

2.3 Sample pre-treatment

Pre-treatment of samples were previously optimised and is explained in section 3.1 Optimization of the sample pre-treatment. This treatment consisted in a first step of filtration through a 0.2-µm pore size hydrophilic nylon membrane filter. Particulate and filtrate were extracted and analysed separately. For the particulate in the filters, an UAE was performed using 5-ml methanol during 5-min, in three cycles. The filtrate was subjected to a SPE using 2-g Silica Isolute cartridges. 0.5-ml of the filtered sample was added to 4.5 ml ACN and acidified at pH 3 with formic acid, and then loaded into the cartridge. The washing step was performed with 2 ml of ACN-water (9:1) at pH 3. The elution was carried out by gravity with 12-ml methanol. Final extracts of particulate and filtrate were evaporated under a N_2 stream in a Reacti-Vap III- PIERCE (Rockford, USA) to concentrate the sample and reconstituted to the 250 µl of the initial conditions of the mobile phase, 9:1 acetonitrile-water 5 mM ammonium formate at pH 3.2.

2.4 HILIC-HRMS analysis

Optimization of the instrumental analysis is detailed in section 3.2 Instrument Optimization. Chromatographic separation was carried out in an Acquity™ Ultra High Performance Liquid Chromatograph (UHPLC) system from Waters (Massachusetts, United States). Optimal stationary and mobile phase were previously optimised as can be seen in section 3.2. Then, the stationary phase employed was a HILIC column (HILIC LUNA® 150 mm x 2 mm, 3 µm, 200 Å from Phenomenex (Torrance, United States). Mobile phase was composed by (A) 5 mM ammonium formate-formic acid buffer in acetonitrile and (B) 5 mM ammonium formate-formic acid buffer in water, both with pH adjusted to 3.2. The elution gradient was programmed as following: 90 % A (0-3 min), 50 % A (3-10 % B), 90 % A (10-15 min) and 90 % A (15-20 min). A total time of 20 min was established for the chromatographic run, considering the last 5 min as a stabilisation step of the column. The flow rate was established at 0.3 ml/min and the volume of injection was 20 µl.

A heated electrospray ionisation source HESI-II from Thermo Fisher Scientific (San Jose, CA, USA) was used as interface between liquid chromatography and mass spectrometry. The source worked with the following parameters: sheath gas; 60 a.u., auxiliary gas; 15 a.u., sweep gas; 2 a.u., heater temperature; 350 °C, capillary temperature; 320 °C, S-lens RF level; 60 % and spray voltage; 3.5 kV working in positive mode.

Mass spectrometry was fulfilled in a Thermo Scientific QExactive mass spectrometer from Thermo Fisher Scientific with an Orbitrap analyser. The full scan data acquisition was in the range of 50-800 m/z at 70,000 full width at half maximum (FWHM) of resolution. In parallel, MS/MS spectrum of each compound was recorded at 35,000 FWHM by normalized collision energy (NCE). The most intense fragment was employed for the quantification and the rest as confirmation. Also, the ratio between the fragments of each toxins was used as an extra parameter of confirmation. In **table 1** are summarized the fragmentation pattern of the toxins at the optimal conditions.

2.5 Method validation and QA/QC

The validation of the developed method was accomplished with the evaluation of the selectivity, linearity, precision, detection and quantification limits and recoveries. These parameters are summarized in **table 2** and **table 3**.

Selectivity and linearity of the method were evaluated by analysing the six analytes in solvent and seawater extracts. Linearity was measured in the concentration range of 0.1 to 100 µg/L by the Spearman coefficient R^2.

Repeatability of the instrument was determined as the intra-day and inter-day precision by the consecutive measurements during the same day (n=6) and different days (n=3) at the same instrumental conditions. Limits of detection (LODs) and quantification (LOQs) of the instrument and the final method were estimated as following: ILODs were determined by gradual dilutions of the standard solution mix containing the six biotoxins, starting at 40 µg/l of concentration. Consequently, ILOQs were estimated as 10/3 times the ILODs. MLODs for each extraction method was determined by the analysis of the standard solution mix in the matrix resulting after UAE for the particulate and SPE for the filtrate. Progressive dilutions of concentration starting at 40 µg/l were analysed to determine the limits of detection experimentally. MLOQs were estimated as 10/3 times the MLODs.

Recovery and matrix effect have been the parameters pondered over to evaluate the efficiency of the different pre-treatment processes. For this, fortified samples were processed for both extraction methods and compared with blanks extracts passed by the same processes and fortified in the moment of the analysis. Matrix effect was assessed to determine the interferences during ionization for particulate and filtrate portions. For this, fortified solutions of the target toxins at 40 µg/l were compared by the formula:

$$ME(\%)=([Area]_{extract} / [Area]_{solvent}) \times 100$$

Being $[Area]_{extract}$ the integrated area of fortified blank extracts of seawater and $[Area]_{solvent}$ the integrated area of fortified pure solvent.

3. Results and discussion

3.1 Optimization of the method

UAE of particulate

In order to optimize the extraction of the MBTs from the particulate phase, an ultrasounds bath was employed during the solid-liquid extraction for 5 min. The cycles of extraction were also tested as well as the extraction solvent. Filters were spiked with 100-µl mix toxins at 100 µg/l and extracted with MeOH in three conditions; acidified at 0.1% of formic acid, MeOH acidified at 0.1% of acetic acid and pure MeOH.

In **figure 2** are represented the values of recovery obtained for the particulate extraction via UAE with the different conditions of MeOH. Mean values for all compounds are above the 20% and are no higher than the 45% unless for TTX which has values ranging from the 62% to 145%. In general, there is no a significant difference in using MeOH or MeOH acidified with formic or acetic acid. The extraction of TTX is better when using MeOH at 0.1% FA though the standard deviation is higher than for the other conditions. For GTX-2,3 and STX can be observed slightly higher recoveries when using MeOH 0.1% FA but for the rest, Neo and dcSTX when using neutral MeOH. MeOH seems to be a suitable solvent to extract all compounds at the maximum recovery or with less standard deviation.

In **table 3** are listened the values of recovery for each cycle of extraction assisted with ultrasounds for 5 min with 5-ml MeOH. For most of the compounds, the first cycle removes around the 70% of the total toxin content and the second cycle almost the 100%, unless for GTX-2,3, which are recovered in equal portions during every cycle, making necessary the three cycles extraction. In addition, 5 minutes of cycle extraction is enough time to ensure the lysis of the cells presents in the particulate and to separate the compounds from possible aggregates.

SPE of filtrate

The optimization of this extraction included the test of different stationary phases, the conditioning of the sample and the volume.

The selected stationary phases were; divinylbenzene-pyrrolidone (HLB), silica (Silica), silica bonded with diol groups (Diol) and silica bonded with aminopropyl groups (NH_2). In order to evaluate the retention capacity of the compounds into the stationary phases, a mass balance was carried out with 1-ml spiked sample/ACN (1:9) with 0.1% FA, measuring the content of the target toxins before and after the loading step, and after the elution. The elution was performed with MeOH and the extracts were evaporated and reconstituted to the initial conditions of the chromatography. Stationary phases were prepared in preparative tubes for SPE with 100 mg of each sorbent.

Conditions of the loading sample were tested for raw filtered samples and filtered samples conditioned with ACN (1:9) at 0.1% FA. For both conditions the samples were fortified with the mix of the six standards at 40 µg/L in volumes of 1, 5 and 10 ml.

In **Figure 3** are represented the moiety of the target toxins before and after the loading into the cartridges and after the elution. Results of mass balance confirmed the high hydrophilicity of these compounds and consequently the poor retention into the stationary phases. Best recoveries were obtained when using Silica and NH_2 cartridges, even considering the high loss of the compound. The loss of the compound when using the Silica cartridge goes from the 25% to the 56%, whereas recovery values are ranging from 34 to 58%. In the case of NH_2, higher recoveries for TTX, GTX-2,3 are achieved, 69 and 75%, respectively, but not for the rest of toxins.

According to the conditioning step of the filtrated sample, no recoveries were achieved when the samples were loaded without conditioning with ACN at 0.1% FA.

Acceptable recoveries were obtained in volumes of 1 and 5 ml of conditioned sample, but not for 10-ml samples. Then, the final SPE method selected was the one that includes the

employment of silica cartridges for 5-ml sample conditioned with ACN 0.1 % FA, and the recoveries are shown in **table 3**.

HILIC-HRMS

Optimization of the chromatographic separation, was carried out with a HILIC column as stationary phase; silica surface covered with cross-linked diols groups phase HILIC LUNA® (150 mm x 2 mm, 3 µm, 200 Å) from Phenomenex (Torrance, United States).

For the mobile phase two different buffers of ammonium formate and ammonium acetate in ACN and water were tested; a buffer of ammonium formate adjusted at pH 3.2 with formic acid and the other of ammonium acetate at pH 5.8 with acetic acid. These salts were tested due to the high solubility in ACN and the compatibility with the mass spectrometry. The same mix of the corresponding MBTs was analysed in triplicate in the different conditions of mobile phases and flows.

Better resolution and peak shape were achieved when the mobile phase contained ammonium formate salts and FA. In **figure 4** are represented the extracted ion chromatograms for each toxin at the different conditions of mobile phase. It was important to maintain the pH and the proportion of organic/aqueous phases in the mobile phase in order to avoid displacements in the retention times.

Mass spectrometry conditions were optimized by the direct injection of the standards into the mass spectrometer via an electrospray ionization (ESI) source. Positive and negative mode were tested to determine the best ionization for each analyte. Fragmentation parameters were evaluated for different values of NCE; 10,20,40,60 and 80 %.

Precursor ions type $[M-H]^+$ were the most intense for all the biotoxins and then, positive mode was the selected ionization mode. This group of biotoxins has hydropurine structures with amine functional groups with strongest basic pKas ranging from 9.1 to 9.9. These groups are able to accept protons at the working conditions, with pH of 3, and then, are easily ionisable in positive mode.

The criterion to determine the most appropriated fragmentation pattern was considering the appearance of the maximum fragmentation ions and the precursor ion in the same mass spectrum. In **figure S2** are represented the mass spectra for each biotoxin at the optimized NCE. Some toxins such as TTX required higher collision energies for the fragmentation, while some other such as GTX-2 and GTX-3 experimented fragmentation at the minimum energy employed.

Reconstitution of the final extract equal to the initial conditions of the chromatography was mandatory. Not only to ensure the good peak symmetry but also because ion suppression was observed when the extracts were containing more water content than in the initial conditions of the chromatography 90:10 (ACN/H_2O 0.1% FA). In **figure S3** is represented the loss of intensity when increasing the proportion of water in the injection vial.

3.2 Occurrence of STXs and TTX in real seawater samples

The developed method was successfully applied in 17 seawater samples from the Mar Menor (Mediterranean Sea), in the region of Murcia (South-east of Spain). Any of the

samples presented any trace of the target hydrophilic MBTs nor in particulate nor in filtrate portion above the mLODs.

The existence of these toxins in this area has never been reported before. Last evaluation of the phytoplankton composition determined high density of dinoflagellates from the genus *Gymnodinium* spp. in the region [28]. Even that this genus is related to the production of STXs, not all species are capable to produce it.

4. Conclusions

A selective and sensitive analytical method for the detection and quantification of six hydrophilic MBTs in seawater has been successfully developed.

The method is based on HILIC-HRMS and considers the particulate and the filtrate fractions for specific studies of these MBTs in the medium where are produced, the seawater. Validation of the method showed good sensitivity, selectivity, accuracy and repeatability. However, in terms of recovery, the method presents low absolute recoveries for most of the toxins due to the poor retention of these compounds in the solid phases. Nevertheless, considering the high hydrophilicity of the six MBTs, which present values of log P ranging from -2.62 to -4.59, these values of recovery could be considered as satisfactory. In addition, recoveries present few standard deviation, then, repeatability can be trust even at low values. The matrix suppression effect coming from the high content of salts in the seawater extracts was not completely eliminated during the pre-treatment processes but was compensated through matrix-matched calibration curves of particulate and filtrate extracts.

Moreover, working at HRMS allows the possibility of retrospective analysis in the analysed samples. This is a potent tool that permits the characterization of new marine toxins analogues and other polar pollutants present in seawater.

The developed method was applied in the analysis of 17 real samples from Murcia's coast, in the Mediterranean Sea, but no presence of any of the target compounds was found in this area above the mLODs.

Acknowledgements

The work was supported by the Spanish Ministry of Economy and competitiveness through the project PLAS-MED (CTM2017-89701C3-1-R). The authors would like to express their deepest gratitude to Dr. Roser Chaler and Dori Fanjul, because of their technical support during the HPLC-HRMS analyses.

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Figure 1. Chemical structures of the target hydrophilic MBTs: saxitoxin (STX), decarbamoylsaxitoxin (dcSTX), neosaxitoxin (NeoSTX), gonautoxin-2,3 (GTX-2,3) and tetrodotoxin (TTX).

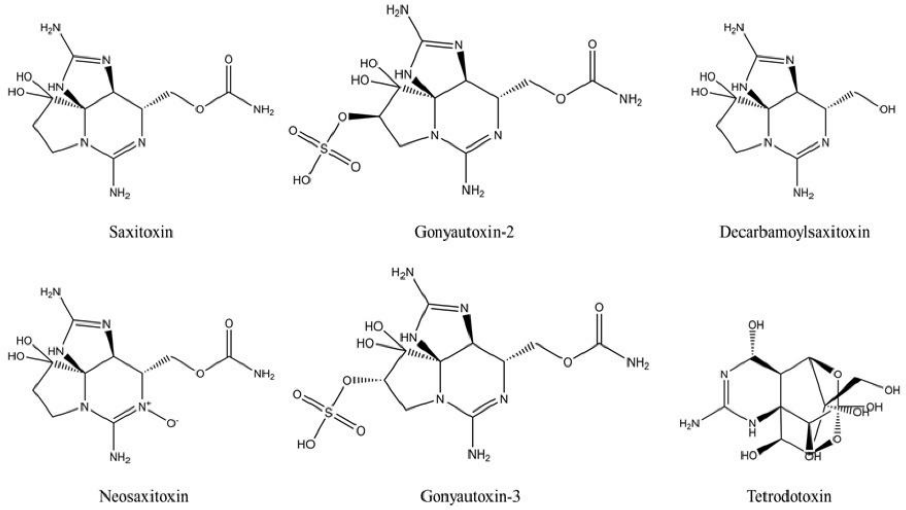

Figure 2. Values of recovery for each MBT when extracted from the particulate using neutral MeOH, MeOH acidified at 0.1% FA and MeOH acidified at 0.1% AA by UAE.

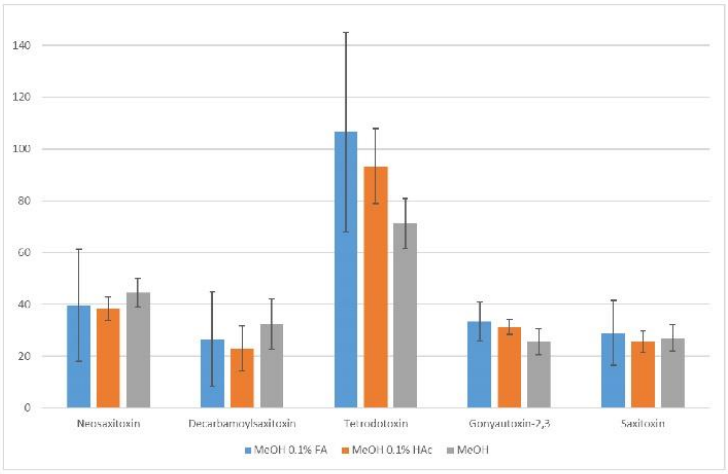

Figure 3. Mass balance of the target toxins for the different stationary phases during the SPE. In blue colour is represented the concentration before the SPE, in orange colour the concentration that has not been retained in the cartridge and grey colour the concentration in the final extract of elution.

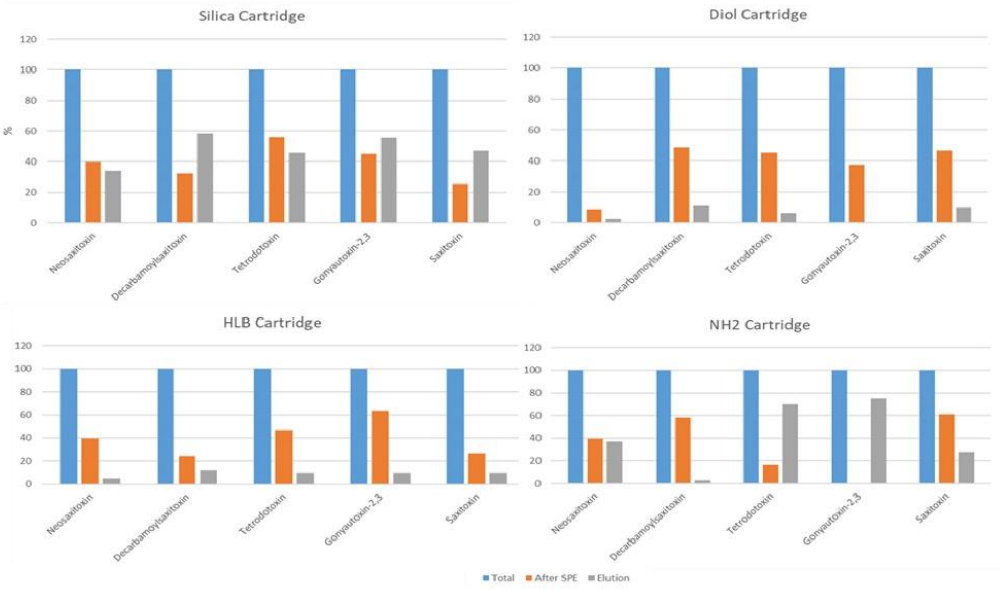

Figure 4. Extracted ion chromatograms of the target MBTs when analysed by HILIC with different mobile phases composition: Ammonium formate salt with FA and ammonium acetate salt with AA.

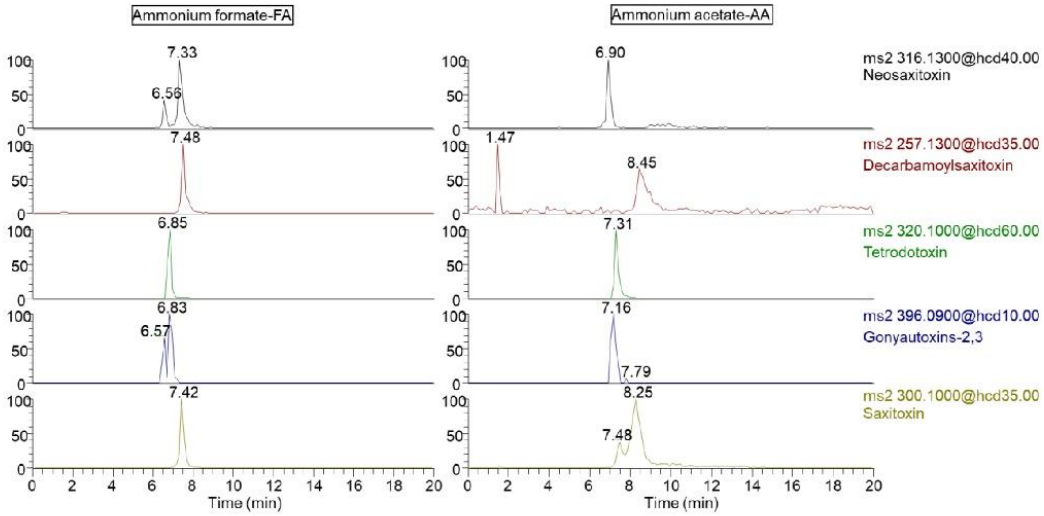

Table 1. Fragmentation pattern of the MBTs studied at optimal normalized collision energy (NCE). The relative standard deviation is given in parentheses.

Compound	Chemical formula	Molecular ion	m/z calculated	Product ion 1	m/z calculated	Relative abundance	Fragment ion ratio	Product ion 2	m/z calculated	Relative abundance	Fragment ion ratio	NCE (%)
Neosaxitoxin	$C_{10}H_{17}N_7O_5$	$[M+H]^+$	316.1364	$[C_{10}H_{16}O_4N_7]^-$	298.1257	30	2.53 (0.292)	$[C_9H_{15}O_2N_6]^-$	237.1093	12	8.84 (3.07)	40
Decarbamoyl saxitoxin	$C_9H_{16}N_6O_3$	$[M+H]^+$	257.1357	$[C_8H_8ON_5]$	126.0662	26	5.53 (1.27)	$[C_9H_{15}O_2N_6]$	239.1247	16	4.66 (1.03)	35
Tetrodotoxin	$C_{11}H_{17}N_3O_8$	$[M+H]^+$	320.1088	$[C_{11}H_{16}O_7N_3]^-$	302.0976	100	0.339 (0.0339)	$[C_8H_8ON_3]^-$	162.0658	38	0.500 (0.0593)	60
Gonyautoxin-2,3	$C_{10}H_{17}N_7O_8S$	$[M+H]^+$	396.0932	$[C_{10}H_{16}O_4N_7]$	298.1254	100	0.192 (0.0581)	$[C_{10}H_{16}O_7N_7S]^+$	378.0817	58	0.375 (0.0789)	10
Saxitoxin	$C_{10}H_{17}N_7O_4$	$[M+H]^+$	300.1432	$[C_{10}H_{16}O3N_7]^+$	282.1326	30	4.82 (0.450)	$[C_9H_{15}O_2N_6]^-$	239.1247	20	20.3 (2.89)	35

Table 2. Analytical parameters for the target hydrophilic MBTs.

Compounds	Instrumental parameters						Particulate		Filtrate	
	Retention time (min) and RDS (n=16)	Linearity (R^2)	Intraday (n=6,%) (20 µg/l)	Interday (n=3,%) (20 µg/l)	iLOD (pg on column)	iLOQ (pg on column)	MLOD (µg/kg)	MLOQ (µg/kg)	MLOD (µg/l)	MLOQ (µg/l)
Neosaxitoxin	7.73 (0.04)	0.5-100 (0.996)	2.41	12.3	1	3	12.5	37.5	0.5	1.5
Decarbamoylsaxitoxin	8.70 (0.07)	0.1-100 (0.995)	1.87	15.0	1	3	3.125	9.375	0.5	1.5
Tetrodotoxin	7.97 (0.06)	1-100 (0.996)	11.02	11.04	1	3	6.25	18.75	5	15
Gonyautoxin-2,3	7.71 (0.04) 7.97 (0.04)	10-100 (0.998)	3.74 8.71	5.14 16.0	1	3	62.5 62.5	187.5 187.5	5 5	15 15
Saxitoxin	7.39 (0.08)	0.5-100 (0.997)	3.8	12.9	1	3	3.125	9.375	0.5	1.5

Table 3. Values of recovery and matrix effect of the target toxins in the particulate and the filtrate portion at 40 µg/l of concentration. All values are expressed in percentage, %.

Compound	Particulate					Filtrate	
	Recovery	Recovery per cycle			Matrix effect	Recovery	Matrix effect
		1st cycle	2nd cycle	3rd cycle			
Neosaxitoxin	44.45 ± 5.48	72.66	23.93	3.41	31.4	23.17 ± 4.66	60.7
Decarbamoylsaxitoxin	32.34 ± 9.62	73.59	24.10	2.30	71.1	15.66 ± 3.03	77.3
Tetrodotoxin	71.18 ± 9.62	74.79	22.83	2.38	70.7	47.14 ± 9.91	79.7
Gonyautoxin-2,3	25.57 ± 4.97	45.20	25.72	29.08	71.0	25.67 ± 5.04	78.9
Saxitoxin	26.97 ± 5.08	63.85	32.75	3.40	43.4	19.81 ± 2.24	90.1

SUPPORTING INFORMATION

Analysis of highly polar marine biotoxins in seawater by hydrophilic interaction liquid chromatography coupled to high resolution mass spectrometry

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Figure S1. Map of the sampling points and geographic coordinates in Manga del Mar Menor, Mediterranean Sea.

	Decimal coordinates (DD)
MM1	37.81214, -0.77692
MM2	37.78137, -0.78288
MM3	37.74318, -0.84474
MM4	37.75337, -0.75878
MM5	37.72759, -0.75137
MM6	37.71499, -0.85229
MM7	37.7019, -0.83382
MM8	37.65878, -0.79364
MM9	37.68303, -0.76357

Figure S2. Mass spectra of the target marine toxins at the optimized normalized collision energy.

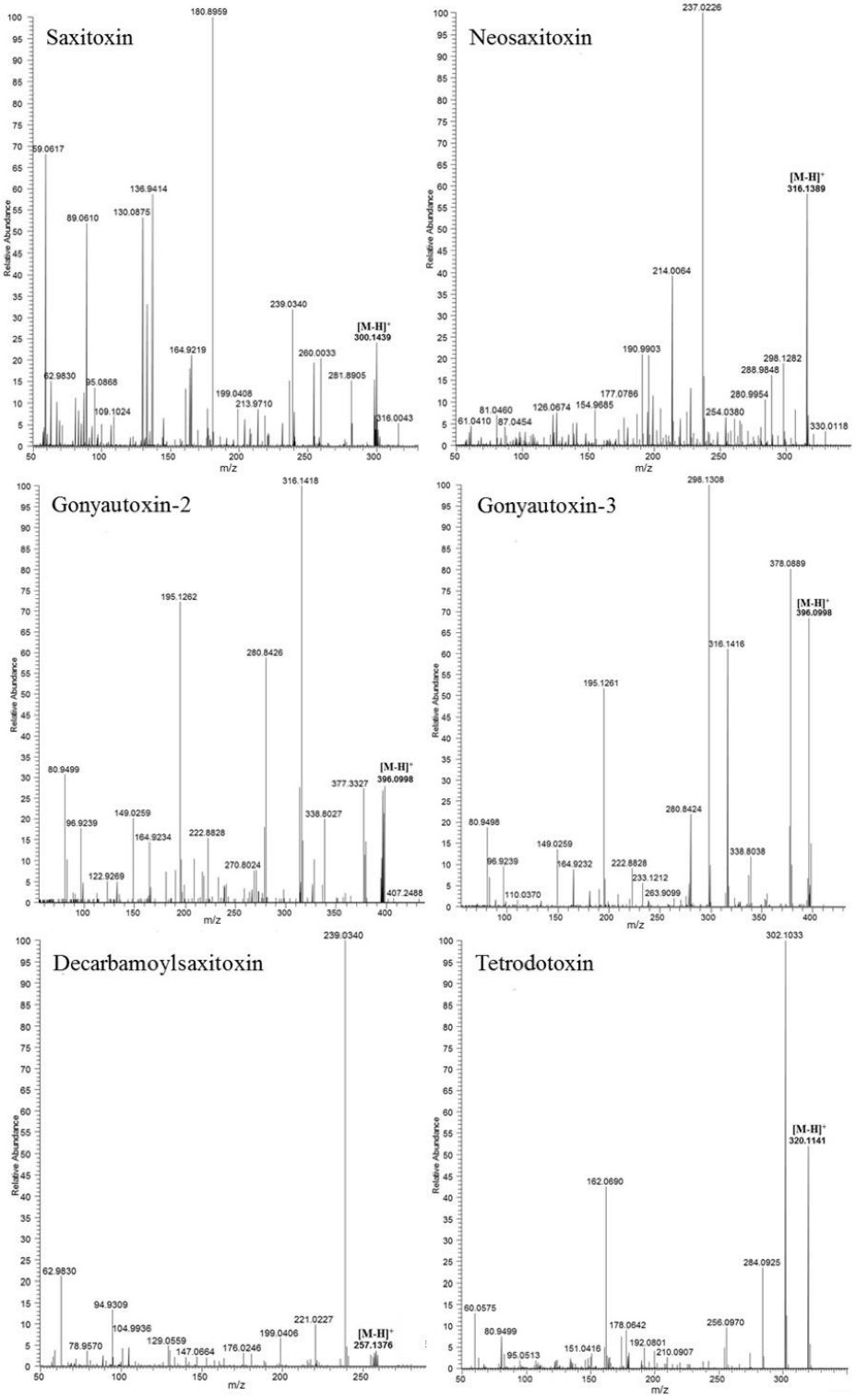
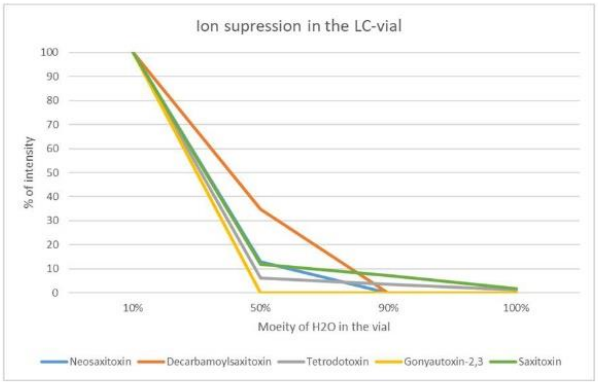


Figure S3. Ion suppression depending on the moiety of aqueous portion in the injection vial.



3.4 Discussion

Due to the assorted properties of the MBTs of the different groups, different methodologies were considered in parallel for their analysis. The chromatographic separation was one of the main challenges to solve during the development, whereas, the most crucial step was the sample pre-treatment, with a particular regard to the extraction of the hydrophilic compounds from seawater.

The different developed methodologies were successfully developed providing high sensitivity and selectivity in the analysis of MBTs in seawater, especially for the most lipophilic compounds and DA. The methods were applied to real samples showing a good performance. The results obtained on the occurrence of MBTs will be discussed in Chapter 4. In the following, some considerations and comments will be discussed related to the validation parameters of the different methods.

- **Selectivity**

Three methods of HPLC-HRMS have been developed in order to accomplish good selectivity for the MBTs analysed. Due to the different polarities of the target toxins different modes of chromatography have been studied. Biotoxins with more lipophilic character were separated using RPLC by employing a C_{18} column, whereas no retention of the hydrophilic MTBs was achieved with this column. Then, for hydrophilic toxins, HILIC was the mode to achieve better retention and resolution. In the case of DA, it was possible to be separated by using RPLC and HILIC, however, retention was better when employing HILIC, and then. In **Figure 17** are some examples of the lack of resolution when aiming to separate hydrophilic compounds by using C_{18} and lipophilic compounds by using HILIC.

C_{18} was suitable for the separation of the lipophilic MBTs in terms of resolution and peak shape. The elution of the toxins followed the polar behaviour, being that DTX-1 was the most lipophilic compound and also the most retained in the column of the compounds, and the less retained compounds were the AZA-4 and the AZA-5 with the latter being less polar. However, YTXs should be the lesser-retained compounds with respect to their polarity for the being the most polar of this class, and retention times are among those retention times of the most lipophilic compounds, DTX-1 and PTX-2.

For hydrophilic MBTs, HILIC was the best option with which to carry out the separation of the compounds, avoiding the drawbacks when working with RPLC that are related to the long time period of analysis and the need to derivatise the compounds prior or posterior to the chromatography. Since Dell'Aversano et al. used HILIC for the first time for the separation of STXs [200], several methods have been developed by employing this technique in the analysis of shellfish, and few methods are based on seawater (**Table 14**). Nonetheless, the mechanism of retention in HILIC is complex and involves different interactions between analytes, mobile phase and stationary phase: adsorption, ion exchange and hydrophobic interactions [279]. Salt and pH variations in the mobile phase influence the separation of the analytes. In agreement with Aversano et al., few variations in the pH changed the retention of the toxins in more than one minute [200]. For this reason, it is so important to keep the pH constant in the mobile phase by the use of salt

buffers. Ammonium formate was chosen for giving better resolution and peak shape that ammonium acetate, and because it is really soluble in ACN and provides a low pH that is optimal for working in positive mode ionisation. Related to the stationary phase, there are different modalities based on bare silica with or without bonded diol, zwitterionic, carbamoyl or amide groups, that can highly influence the separation of the compounds [279]. The stationary phase that was selected for this research thesis, the diol type, was appropriated for the separation of the toxins. The elution of the compounds started with TTX and GTX-2,3, which are those with more polar behaviour and the last compound to be eluted was the dcSTX.

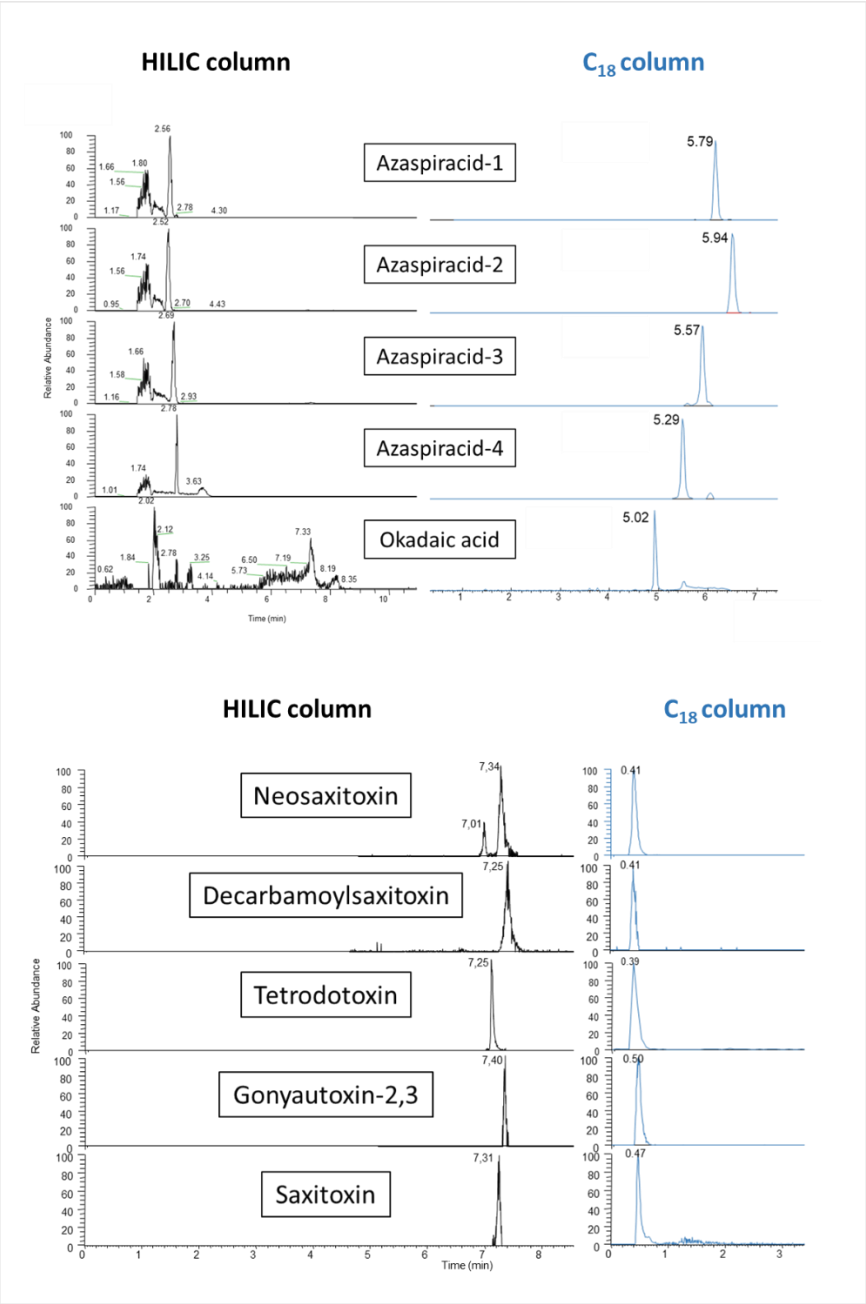


Figure 17. Examples of extracted ion chromatograms (XICs) for some lipophilic and hydrophilic MBTs separated by HILIC and RPLC employing a HILIC and a C_{18} column.

DA was separated by both types of chromatography, but the elution in RPLC is close to the elution front because it is not well retained in the C_{18} column. This fact would be considered as an advantage for a rapid analysis of DA. However, better resolution is achieved when the separation takes place using HILIC, and the compound is more retained in the stationary mode. Moreover, the separation of DA and its isomer epi-DA, which is present in the standard, was only resolved when using HILIC.

Positive ionisation was selected for most of the compounds, with the exception of YTXs. All of the compounds were ionised in both negative and positive modes, however, and YTXs were only acceptably ionised in negative mode. Relatives of the acidic compounds, such as OA, DTX-1, AZAs and DA, were analysed in positive mode. It is believed that ionisation was favoured by the sodium adducts for OA and DTX-1. In the case of PTX-2, the sodium adducts were favouring the ionisation while in other methods the ionisation is favoured by the formation of ammonium adducts [224, 227]. Other authors obtained better ionisation while working in negative mode for OA and DTX-1, [276, 280, 281] and PTX-2 [280], however, in this research thesis, ionisation in positive mode was sufficient, and better sensitivity was obtained. In addition to AZAs the positive mode is considered in most of the LC-MS methods [192, 224, 282-284]. DA has been found in different modalities, but due to the easier ionisation in positive mode at acidic pH, the most reported is in the positive mode [176, 192, 234, 276].

Hydrophilic MBTs has been ionised in positive mode in agreement with all methods in the body of literature [179, 237, 276]. The structure of STXs and TTX is consists of a hydropurine with some other functional groups such as an amine. The strongest basic pKas for these compounds was in the range of 9.1 to 9.9, which means that at the working pH (pH 3) they are accepting protons, and are easily ionised in positive mode.

High resolution mass spectrometry was employed in the same way for all of the compounds in this research. Full scan at high resolution 70000 FWHM was combined with Parallel Reaction Monitoring (PRM) to fragment the target toxins at medium resolution of 35000 FWHM. An example of this mode of acquisition is shown in **Figure 18** for the analysis of TTX. The full scan of the whole sample can be acquired at the same time that the fragmentation spectrum shows the different ion fragments, which can be integrated separately for each fragment. Using the combination of chromatography with HRMS, with a 2-ppm mass accuracy in the MS/MS spectrum, the unequivocal identification of the toxins has been carried out.

An additional advantage in the use of HPLC-HRMS with the target MBTs is that the lack of purity in the standards is overcome. Furthermore, the greatest advantage of using HRMS is the simultaneous analysis of the target MBTs and non-target compounds, that can be tentatively identified by retrospective analysis.

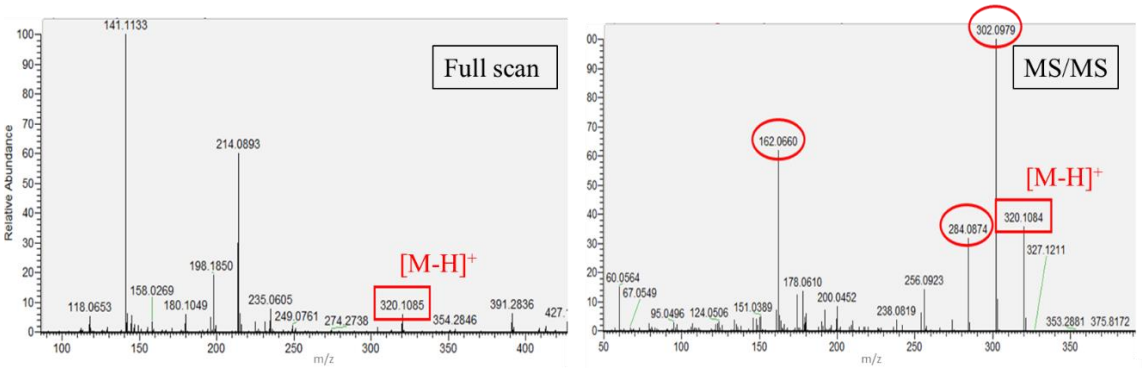


Figure 18. Full scan and MS/MS spectrum of TTX analysed by HILIC-ESI-HRMS.

- **Sensitivity and linearity**

The sensitivity of the developed methods was determined by the mLODs of each toxin that were really low, ranging from ng/l to pg/l in lipophilic MBTs and from µg/l to ng/l for the hydrophilics. MLODs were determined experimentally to obtain more reliable values of the detectability of the instrument for the different matrices. Additionally, to calibration curves were added low concentrations points below the LOQ were added to the calibration curves to ensure the sensitivity of the equipment. LODs and LOQs for all MBTs have been compared with those in the body of literature and are represented in **Figure 19**. Working with high resolution provided good sensitivity: high values of S/N were measured due to the lack of noise. Moreover, the concentration of the toxins from big volumes of sample to small volumes for analysis allowed us to improve the sensitivity, in the case of lipophilic MBTs and DA.

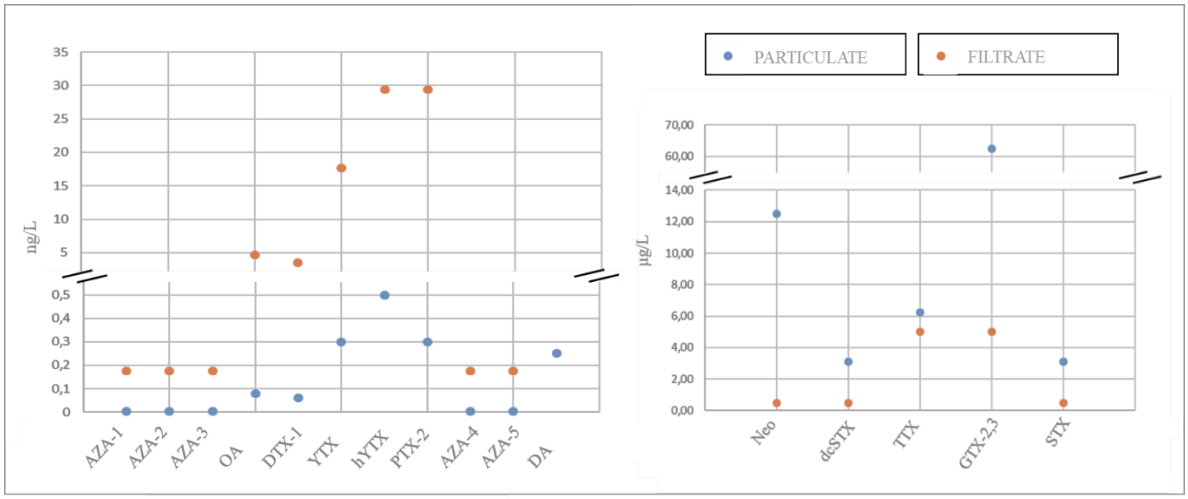


Figure 19. Limits of detection of the MBTs at the different LC-MS developed methods.

MLODs of the lipophilic MBTs were considerably low compared with those in the body of literature. As previously reported, there are few LC-MS methods for the analysis of MBTs in seawater, especially for the most hydrophilic compounds.

The LODs of OA and DTX-1 achieved with our LC-MS/MS methodology were satisfactorily low and were only were improved by other methods which were developed

afterwards. Zhang et al. achieved LODs of OA and DTX-1 equal to 0.2 ng/L [285] and He et al. reached 18 pg/L and 30 pg/L, respectively [286]. Compared to biochemical methods, our LC-MSMS achieved better sensitivity. Petropolous et al. developed an automatised ELISA with LODs of 50 ng/L for OA [287] and Molinero et al. developed a sophisticated enzyme-sensor with LODs of 2.7 ng/L [288].

In the case of AZAs, our LODs ranged from 2 to 3 pg/L and are the lowest reported in the literature, to the best of our knowledge. The lowest LODs found for the analysis of these compounds in seawater were from Zhang et al., and He et al., that were 20 pg/L and 30 pg/L, respectively [285, 286].

In addition to DA, the LOD achieved by our LC-MS method, was the lowest on the body of literature, to the best of our knowledge. Other methods detect LODs of DA in seawater at levels ranging from 20 ng/L [289] to 9 ng/L [193]. Further different methods working with HRMS did not obtain lower LODs for DA, such as in the work of Gagez et al. that reported 0.75 ng/L of LOD [176] but has not overtaken our LOD of 0.25 ng/L.

In contrast, LODs for the hydrophilic MBTs were not as low as documented in the literature. As aforementioned, few LC-MS methods exist for the analysis of MBTs in seawater, especially for the most hydrophilic compounds. In this case, to the best of our knowledge, only Riccardi et at. have published a LC-MS/MS methodology for the analysis of STXs in seawater, with low LODs of 66 ng/L for STX, 81 ng/L for GTX-2,3, 79 ng/L for dcSTX and 68 ng/L for Neo [276]. These LODs are only improved by biochemical assays such as, for example, the commercial kits from Abraxis Bioscience S.L. that have a detectability of 15 ng/L of STX equivalents in seawater [290]. The automatised ELISA developed by Petropoulous et al., reached LODs of STX equal to 10 ng/L [287] and Jin et al., recently developed an immunosensor with a sensitivity of 1.2 ng/L [291].

Sensitivity was also determined by comparing the slopes of the different calibrations curves for each toxin and the Spearman correlation (R^2). In **Figure 20** are represented the values of the calibration curves for the toxins in pure solvent and in extracts from the particulate and the filtrate. In general, lipophilic MBTs have better sensitivity than hydrophilic MBTs, with the exception of DTX-1 and YTXs. Better sensitivity was achieved in the filtrate fraction for DA and the lipophilic toxins, with the exception of DTX-1, whereas the sensitivity was higher in pure solvent for the hydrophilic toxins. In addition, R^2 was only considered for calibration curves with values of 0.99 onwards.

- **Precision and trueness**

Precision was, in general, very good for the three developed methods. Instrumental precision was measured for all of the toxins and the values of relative standard deviation (RSD) were always lower than 20%, except for the interday measurements for PTX-2 which had a RSD of 27.5 %. In general, values ranged from 1.89 % to 10.8 % and 2.91 % to 16 % of RSD in the intraday and interdays analyses, respectively.

In order for estimates to represent the trueness of the analysis, the recoveries and the matrix effects were evaluated for being some of the largest contributors to misleading

information in the results. The corresponding correction in the recovery values were applied for the quantification of the samples and matrix-matched calibration curves were used to compensate the matrix effect.

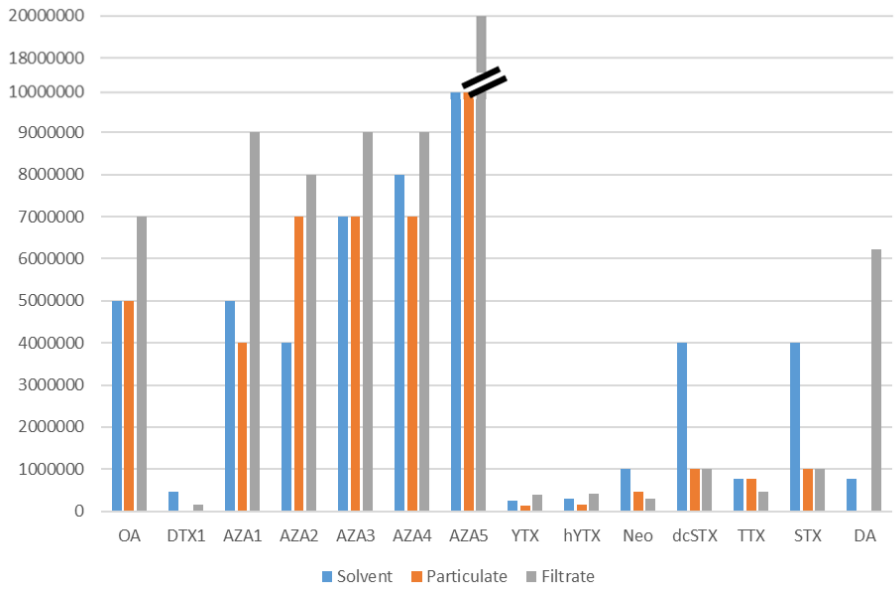


Figure 20. Slopes of the calibration curves for all the MBTs prepared in solvent, particulate extracts and filtrate extracts.

Related to contamination during the pre-treatment or the analysis, no interferences, contamination or carryover were detected in the blanks. Instrumental blanks were the marine-biotoxins-free solvent, MeOH; blanks that were analysed at the beginning of the run. The extraction blanks were marine-biotoxin-free ultrapure water that was extracted and analysed together with the samples. Procedural blanks were blanks subjected to the sample pre-treatment of each method and the instrumental analysis.

Recoveries The most challenging step during the method development was the selection of a proper pre-treatment method that would be capable of eliminating the maximum interferences in the matrix, but without losing the analytes. The complexity of the matrix and the nature of the toxins, made impossible the idea of having just one pre-treatment method with which to extract all the MBTs. Then, different strategies were adopted which taking into consideration the polarity of the compounds. Another drawback in the development of the extraction methods is the low concentration of the available standards and its elevated cost. This fact limits the type and number of tests. The different sample pre-treatments for every method that was developed in this research thesis are summarised in **Table 16**.

For the most lipophilic MBTs, the pre-treatment was established by employing UAE to extract the toxins from the particulate and SPE with HLB cartridges from the filtrate, in agreement with the work of other authors in the body of literature [171, 174, 274].

However, for the most hydrophilic toxins, the high affinity for the aqueous phase added difficulties in the extraction of these compounds. Only UAE was considered for the extraction of particulate, due to its being easy to use and effective.

Table 16. Scheme of the pre-treatments employed in the different methods and the range of recovery obtained for the different MBTs.

Toxins	**OA, DTX-1, AZAs, YTXs, PTX-2**		**DA**	**STXs, TTX**	
Seawater volume	500 ml		500 ml	500 ml	
Filtration	0.45 um nylon filter		No	0.22 um nylon filter	
Fractions	Particulate	Filtrate	No	Particulate	Filtrate
Extraction	UAE	SPE	SPE	UAE	SPE
	3 min 20 ml MeOH	OASIS HLB cartridge C: 3 ml MeOH E: 3 ml water L: 500 ml W: 3 ml water E: 6 ml MeOH	OASIS HLB cartridge C: 6 ml MeOH E: 6 ml water 0.1% FA L: 500 ml sample acidified 0.1% FA W: 3 ml water 0.1% FA E: 6 ml MeOH	5 min 5 ml MeOH x 3	Silica cartridge C: 6 ml MeOH E: 6 ml water 0.1%FA L: 5 ml ACN/sample (1:9) 0.1% FA W: 2 ml of ACN/water (9:1) 0.1%FA E: 6 ml MeOH
Concentration	500 µl	500 µl	250 µl	250 µl	250 µl
Recovery (%)	35-107	24-97	48-69	26-71	16-47

C: Conditioning, E: Equilibration, L: Loading of the sample, W: Washing and E: Elution

Notwithstanding, while it is thought that these toxins are mainly dissolved in the water due to its high polarity, it was interesting to study the possible moiety in particulate. We considered that the toxins could remain intracellular in the producer algae or in their predators or attached in suspended material, that can be extracted from the particulate fraction.

However, the extraction from the filtrate fraction was more complicated. Prior to the use of silica as the stationary phase of SPE, we tested several other matrices. Firstly, HLB-type cartridges were tested with different pore diameters, for different volumes of sample and with different conditionings. Also, ion exchange-mode cartridges were used, for both cationic and anionic retention. The results were not satisfactory for the extraction of all the compounds. In some cases, some toxin was recovered with an acceptable value, but the rest were completely lost. For example, when using weak cationic exchange (WCX) cartridges, TTX was recovered above the 100%, but none of the other toxins were recovered. These results could be taken as advantageous in the development of a single method of extraction for TTX.

Then, the possibility of using carbon-type cartridges was studied. Activated charcoal is employed as supportive medical assistance for poisoned victims of PSP toxins and TTX [55]. The toxins are effectively absorbed in the stomach by the charcoal, then, this material was considered for the extraction process from the seawater. Moreover, other authors reported the extraction with carbon-based cartridges of SPE of these toxins from shellfish [178] and even seawater [276] with satisfactory recoveries. Further, ENVI-Carb cartridges were used by conditioning the samples at different volumes and pHs. This type

of SPE provided better results in terms of recovery and elimination of salts, however, the results were not reproducible.

Therefore, we considered the possibility of working directly with the activated charcoal and doing a dispersive SPE assisted with ultrasounds. For this, we passed conditioned samples at pH 3 and 8 through a filter covered with 1 g of activated charcoal, the conditioned samples at pH 3 and 8. Afterwards, the filter with the carbon was extracted with MeOH assisted by ultrasounds. Preliminary results were encouraging; the recovery ranged from 50 to 105% for Neo, GTX-2,3 and STX but 11 to 19% for dcSTX and TTX. After some tests and optimisations, the conclusions were similar to those for the Carbon-based SPE: the recoveries were not reproducible.

Finally, and taking into account that toxins were good retained during the HILIC, we decided to use a cartridge that was made with the same stationary phase as our chromatographic column. Then, we tried different silica-based cartridges, with different functional groups and conditioning the sample with an organic modifier. Recovery was only obtained when the loading of the sample was 9:1 ACN/sample. **Figure 21** summarises the best recoveries obtained for every MBT in the different seawater fractions by using the different methodologies.

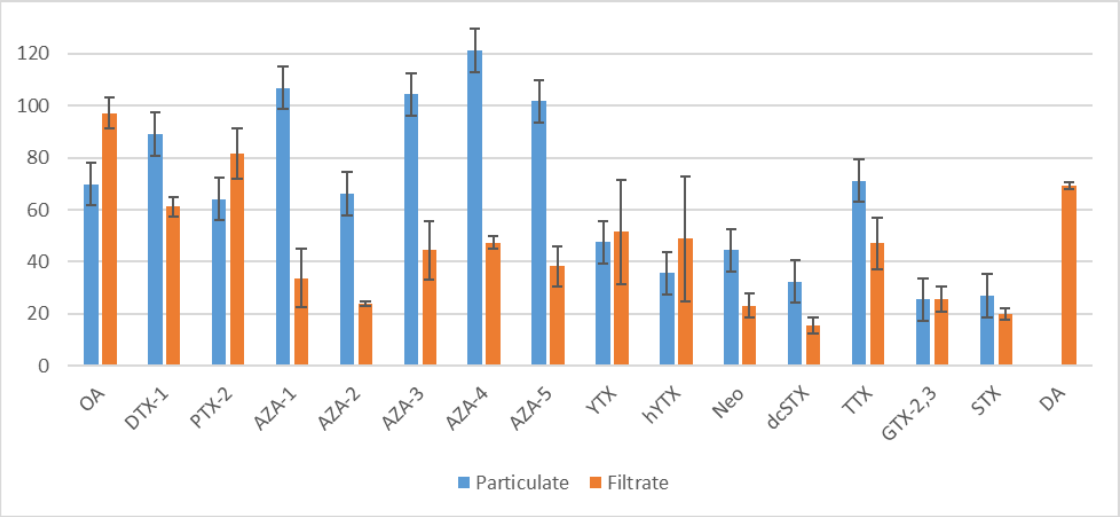


Figure 21. Recovery of the MBTs extracted from the particulate by UAE and from the filtrate by SPE.

Good results of recovery were obtained for the most lipophilic compounds and DA in the filtrate fraction. AZAs and YTXs had the lowest values of recovery, nevertheless, the ranges were in agreement with other authors in the body of literature who worked with the same compounds in seawater. Li et al. had values of recovery from seawater ranging from 52 to 70% for OA, YTX and PTX-2 [174], and Barbaro et al. had recoveries of DA from seawater in the range of 57 to 69% [193], both using SPE-LC-MS methods. In addition, Chen et al. had values of recovery ranging from 55 to 83% for OA, DTXs, AZAs with the exception of AZA-2 that recovered only the 30%. The production of methyl esters when AZAs are dissolved in MeOH has been reported by Jaufrais et al. who recommend the use of Acetone for the extraction of AZAs [292]. Our low recoveries of AZAs in the filtrate could be explained by this undesirable formation of esters. However,

only low values of recovery are obtained for the filtrate fraction and not for the particulate fraction, in which MeOH is also the extraction solvent. Then, the explanation should be related to SPE. AZAs have pka values of around 4 for the carboxylic acid and 9 for the amine groups. Working at the seawater pH of around 8 the molecule should be ionised and this fact could make the retention of the compounds more difficult in the SPE cartridges and, consequently, the obtaining of lower recoveries. Probably, conditioning the sample at lower pH would improve the recovery of AZAs.

Taking into account working at low pHs, it was fundamental for DA. DA has three carboxylic acids and an amine group with pka values of 1.85, 4.47, 4.75 and 10.6. In order to decrease the polarity of the molecule to achieve a better retention in the cartridge, the value of pH was established at 3. Other authors also work at low pH for the extraction of DA by SPE from seawater [176, 193].

In contrast, STXs and TTX extracted from seawater are only reported by Riccardi et al. who achieved successful recovery when working with a big volume of seawater, 50 ml, compared with that of this thesis research, and with the combination of a C_{18} cartridge coupled to a carbon-type. Recoveries of STXs were in the range of 77-111% [276].

Related to particulate, the recoveries are acceptable for all the compounds. The lowest values were for the most hydrophilic compounds. Comparing these values with those reported in the body of literature, only data for lipophilic MBTs are available. Chen et al. presented values of recovery ranging from 84 to 110% for OA, DTX-1, AZAs, PTX-2 and YTX, extracting the particulate during 30 minutes by UAE with MeOH [274]. Even then our values were lower and reproducible.

The last chance to improve the extraction was by adding a final step of dialysis at the samples. In order to eliminate the salts via osmosis, a semi-permeable membrane dialysis unit made of cellulose ester (CE) with symmetrical pores was employed. The smallest molecular weight cut-off (MWCO): 100-500 Da membrane was selected for the hydrophilic MBTs that are in the range of 250-400 Da. Seawater blanks were fortified with the standards and were exposed in the membrane during 30 min, 2 and 8 hours in pure water. The water was replaced every 2 hours and the extracts were evaporated and reconstituted to the initial condition of the chromatography. Unfortunately, the elimination of the salt was accomplished, but this was not so for the recovery of the toxins. Only Neo was recovered at the 70% and TTX at the 4% during the first 30 minutes. Thus, this possibility was discarded.

Matrix effect Seawater is a complex matrix that is mainly composed of salts and dissolved organic matter and, consequently, the removal or reduction of these interferences is mandatory for the posterior analysis by LC-ESI-MS. Interferences from this matrix are hardly affecting the ionisation of the analytes producing suppression or enhancement in the intensity of the signal.

The matrix effect of all the MBTs that were analysed in the different seawater extracts are summarised in **Figure 22**. In general, lipophilic MBTs experienced ion enhancement and especially in the filtrate fraction. In the particular case of the toxin DTX-1 a strong

ion suppression was observed with a similar intensity for both matrices. OA, AZA-3 and AZA-5 did not show any matrix effect when analysed in the particulate fraction. In contrast, most of the hydrophilic MBTs were affected by ion suppression in both fractions. In the particulate fraction the suppression was much higher than in the filtrate. Particulate extracts carry higher suspended solids and this could be the main cause of producing higher ion suppression during the ESI process. Finally, DA was only measured in the filtrate fraction, and some ion suppression was observed, but not significantly.

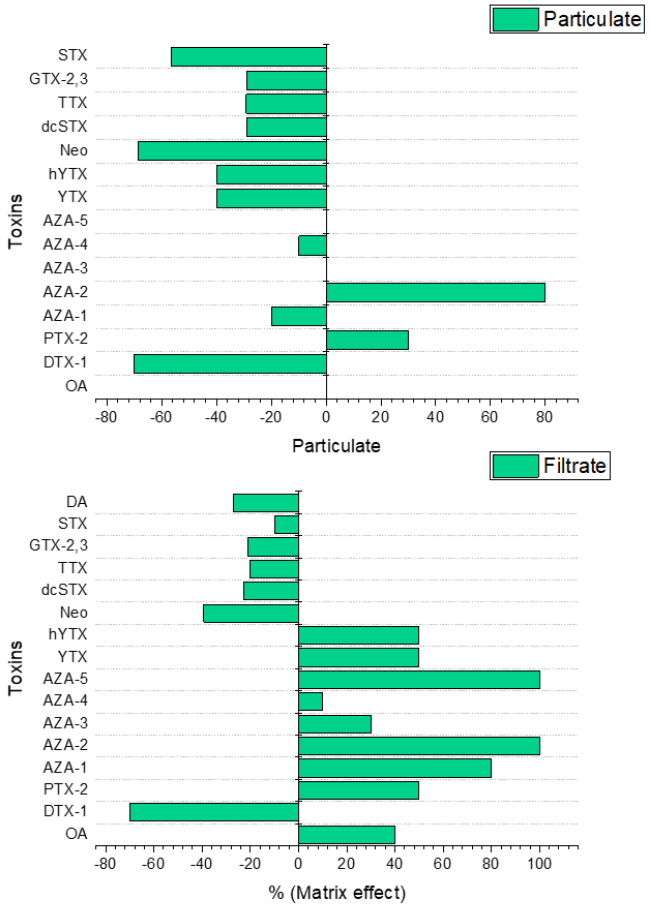


Figure 22. Matrix effect of the MBTs in the particulate and filtrate fractions after the pre-treatment of sample.

Riccardi et al. experimented with a severe signal suppression when analysing some lipophilic MBTs and STXs in seawater, even though they used a double system of cartridges for the SPE treatment [276]. In contrast, Zhang et al. used a dispersive micros-SPE based on a polymeric ion exchange material for the extraction of lipophilic MBTs from seawater that significantly reduced the matrix content, having matrix effects ranging in the range of -16% to 8% [285]. Other authors, such as Li et al., showed study results suggesting that lipophilic MBTs had less matrix effect when the SPE was carried out using HLB cartridges rather than C_{18}-type cartridges [174].

Matrix effects were overcome by the use of matrix-matched calibration curves of particulate and filtrate fractions. Even though the correction by matrix-matched calibration provides the proper accuracy in the qualitative and quantitative analyses, it is

important to reduce the matrix content in order to avoid technical difficulties. Precipitation of the salts during the analysis could be one of the most common undesirable difficulties, causing obstruction of the equipment or damage to the chromatographic columns due to overpressure. The methods employed for the MBTs extraction were convenient to eliminate matrix interferences and concentrate the toxins content. However, these pre-treatments were not efficient enough to completely eliminate the interferences of the seawater, particularly regarding the salts.

Extraction of the particulate by UAE and elution in SPE were carried out with MeOH solutions. Solubility of salts in MeOH is high, thus methanolic extracts contained dissolved salts. In the case of lipophilic MBTs analysis, the initial conditions of the mobile phase in RPLC content higher aqueous moiety. Methanolic extracts are evaporated and reconstituted with Water/ACN (9:1), but then the dissolved salts are still present, especially in the filtrate fraction. These salts favour the ionisation of the most lipophilic MBTs, with the exception of DTX-1 and YTXs in the particulate. In contrast, in the case of the analysis of hydrophilic MBTs analysis, the initial mobile phase condition in HILIC has a higher content of organic solvent, and ACN in particular. Salts are not soluble in ACN and its precipitation takes place with the changing of the solvents in the final extracts after evaporation. The precipitate is rejected after a centrifugation and the collection of the supernatant for the analysis, thus, precipitation during the analysis was prevented.

Other authors experimented with undesirable precipitations in the analysis of STXs from shellfish extracts. Thomas et al. concluded that SPE was not completely effective in the elimination of the polar interferences in the matrix, and also experimented with precipitation upon injection in the HILIC analysis, which began due to the high organic content at the initial conditions [273]. Li et al. and Chen et al. alleviated the matrix effect but derived difficulties from seawater extracts by having a chromatographic run time of 50 minutes [171, 174, 274].

These facts reinforce that matrix effect strongly depends on the matrix nature and the election of the pre-treatment method will determine the effects during ionization process and undesirable physical obstruction in the analysis.

4. APPLICATION OF LC-MS IN THE ANALYSIS OF MBTs

4.1 Introduction to the use of the LC-MS technique for the analysis of MBTs

The implementation of LC-MS techniques in the analysis of MBTs during recent years has provided many advantages and solutions in the study of these compounds into the environment. Several applications have been conducted, from the determination of MBTs in routine analysis of seafood to specific characterisation of the compounds in the ecosystem.

In this chapter are presented two applications of LC-MS for the analysis of MBTs in seawater with different purposes. The first application is related to the use of LC-MS for the validation of immunoassays of the ELISA type that are able to detect five pollutants in seawater, including DA. The other application has the aim of studying the presence of MBTs in seawater from the occidental area of the Mediterranean Sea.

4.1.1 ELISA validation

HPLC-UV is the official method for the analysis of DA in shellfish by the European Commission Regulation EU 2019/627 [255]. However, ELISA is also included as the official method in AOAC OMA 2006.02 [256] for the rapid screening of DA. This immunoassay is becoming popular in the analysis of MBTs due to the advantages that it provides in terms of high throughput analysis and rapid response. Several studies on the presence of DA have been conducted using different formats of ELISA. The most employed format is the direct competitive ELISA, in which DA extracted from the samples competes with peroxidase labelled-DA for the specific binding with the antibodies. However, the indirect format is generally more sensitive.

Nowadays, there are numerous commercial kits of ELISA available for the analysis of DA in different matrices. The kits provide high sensitivity, with LODs reaching the 10 ng/L in the best cases and high specificity for DA, without significant cross reactivity with other compounds. The application of these kits goes from routine analysis of seafood to research studies, such as the determination of DA in rat serum and rat brain [293] or in fetal fluids from sea lions of California, USA in order to study the transfer of the toxin to the new-born [294] and in the lastest studies, to determine whether DA of other biotoxins is caused by the massive dying-off of seabirds in Alaska, USA [295].

However, even though ELISA provides an efficient and rapid analysis, the development of these methods entails a long period of time, due to the production and selection of the proper immunoreagents and the optimal conditions for the reaction. Another difficulty comes from the small size of DA, that requires the conjugation to a protein to carry it for a proper conjugation. Moreover, the antigen-antibody reaction has to be very specific to avoid cross reactivity with other compounds that are structurally similar. Then, the proper validation of these immunoassays is required to ensure the good performance of the bioassay. HPLC-UV methods are usually applied for this purpose, being the official methods [296, 297]. In some other cases, more rigorous validation is required, and inter-laboratory studies for the same samples are employed [298]. Also, due to the high

selectivity and sensitivity that LC-MS provides, the combination of these techniques is considered for the proper validation of the ELISA [294, 299].

In this thesis research, HPLC-HRMS was employed for the validation of a multianalyte ELISA that is able to detect and quantify DA in seawater samples in combination with other pollutants.

4.1.2 Occurrence of MBTs in seawater from Mediterranean seawater

The Mediterranean Sea is an almost closed sea with a surface area of 2,500,000 km^2 that is connected to the Atlantic Ocean. It is estimated that the water volume of the Mediterranean Sea reaches 3,750,000 km^3, with an average depth of 1,500 m. It is considered to be an oligotrophic sea, nonetheless, it is home to a high diversity of organisms. To date, several toxigenic species of phytoplankton and their associated MBTs have been reported during outbreaks of intoxication.

Over the years, the implementation of different methodologies has made possible the accurate identification of MBTs without the need for determining the producer-species. The application of LC-MS techniques in these analyses has provided many improvements, in both routine screening and research studies. Numerous MBTs are supervised through the official monitoring programmes and also characterised in new transmission of vectors. Moreover, new emerging analogues are being discovered in the Mediterranean Sea in agreement with the HRMS [277, 284, 300-302].

The largest family of MBTs in the Mediterranean Sea belongs to the lipophilic group. OA, DTXs, AZAs, YTXs and PTXs are included as lipophilic MBTs by the European Commission on Regulation, the method of analysis employed for its detection in shellfish is LC-MS [255], and these toxins form part of the studies of this thesis research. In the same way, DA and the most hydrophilic MBTs such as STXs have also been abundantly present in the Mediterranean Sea, and as part of the monitoring programmes much data are available in relation to its occurrence in seafood. Nevertheless, TTX is not considered in the monitoring programmes because the commercialisation of the fishes that carry the toxin is forbidden in Europe. However, its similarities with STXs have made our analyses possible by using similar methodologies and the fact that this toxin has been found in commercial shellfish.

Some of the latest reported levels of these MBTs in the Mediterranean Sea are summarised in **Table 17**.

However, scant data are available on the levels of MBTs in the seawater of the Mediterranean Sea compared to those levels that have been reported in shellfish matrices. In order to give some contributions of the in situ occurrence of MBTs in seawater, the application of the developed LC-MS methodologies has been used in this research thesis in the analysis of seawater samples from real scenarios of the western Mediterranean Sea. These cases of study are:

Table 17. Occurrence of lipophilic MBTs in Mediterranean Sea during last years.

Toxins	Concentration range	Matrix	Mediterranean Sea	REF
OA PTX-2	592 µg/kg 61 µg/kg	Mussels	Catalan-Balearic Sea	[24]
OA	1.06-1,480 mg/kg	Mussels	Tyrrhenian Sea	[303]
OA PTX-2 YTXs	45.9-758 µg/kg 53.2-93.5 µg/kg 0.063-0.49 µg/kg	Mussels	Adriatic Sea	[224]
OA YTXs AZAs	2.7-62.4 µg/kg 29-4,715.5 µg/kg 1.2-7.1 µg/kg	Mussels	Adriatic Sea	[300]
DA	0.15- 3.88 mg/kg	Oysters	Southeast Mediterranean	[232]
DA	170-770 µg/kg	Cockle	Adriatic Sea	[233]
STX	10.82–98.04 µg/kg	Cockle	Adriatic Sea	[233]
TTX	25.0-222.9 µg/kg	Shellfish	Aegean Sea	[304]
TTX	0.07- 52.07 µg/g	Fish	Northeast Mediterranean	[305]
OA DTX-2 PTXs YTX	3.74 ng/L 4.96 ng/L 0.53-4.53 ng/L 1.11 ng/L	Plankton	Catalan-Balearic Sea	[37]
OA DTX-1 PTX-2	17.7-50.1 µg/L 12.5-47.1 µg/L 7.1-22.8 µg/L	Seawater (SPATT collection during 36 weeks)	French Sea	[306]
OA PTX-2	94 ng/g resin per day 42 ng/g resin per day	Seawater (SPATT)	Catalan-Balearic Sea	[24]

1) Determination of the occurrence of lipophilic MBTs in waters from the Catalan coast. The littoral of Catalonia is located in the Northwest region of Mediterranean Sea, with a total extension of approximately 827 km. The sampling of the seawater took place from the localities of Roses (Costa Brava, northeast Spain) to Sant Carles de la Rapita (southwest of the Ebro Delta which is southwest of Catalonia, northeast Spain) during the period of February to March of 2015. Samples were collected from the shoreline of marinas and beaches. Details of the sampling are in **Scientific Publication 1**.
2) Seasonal and spatial variability of DA in Ebro delta wetland. Located in the south of Catalonia and formed around the Ebro river estuary, as aforementioned, this area has a total size of 320 km^2. The most important mollusc farming of the Catalan region is located there, distributed in the two semi-enclosed embayments that forms of the Ebro Delta, Alfacs Bay and Fangar Bay. The sampling took place in these bays and four lagoons during the months of October 2015, February 2016 and June 2016. Details of the sampling conditions are in **Scientific Publication 2**.
3) Analysis of hydrophilic MBTs in Mar Menor (Menor Sea). Located in the Southeast of Murcia, Mar Menor is a coastal saltwater lagoon with an area of 170

km^2 and with a maximum depth of 7 m. In this area mainly recreational activities take place. The collection of the samples was carried out during the months of July 2018 and April 2019 in different sites along the edge of the lagoon. Detailed information about the sampling is documented in **Scientific Publication 3**.

4.2 Results

The experimental results of this chapter are presented in the following scientific publications.

4.2.1 Validation of ELISA

Scientific publication 4 details the application of HPLC-HRMS techniques for the validation of a multianalyte immunoassay type ELISA in which on the target compounds is one of the MBTs studied in this thesis, the DA.

Scientific publication 4: Sanchis, A., Bosch-Orea, C., et al., Development and validation of a multianalyte immunoassay for the quantification of environmental pollutants in seawater from the Catalonian coastal area. Analytical and Bioanalytical Chemistry, 2019. 411 (22): p.5897-5907.

This work has been supported by the frame of the European project Sea-on-a-chip (FP7-KBBE-OCEAN2013.1 grant no. 614168) with the collaboration of the Nanobiotechnology for Diagnostics (Nb4D) group from the department of chemical and biomolecular nanotechnology at the Institute for Advanced Chemistry of Catalonia (IQAC) of the Spanish Council for Scientific Research (CSIC).

4.2.2 Occurrence of MBTs in seawater from the Mediterranean Sea

The three groups of MBTs were studied in three different regions of the Mediterranean Sea by the application of the developed methods of LC-MS.

Lipophilic MBTs (OA, DTX-1, PTX-2, AZAs, and YTXs) were studied along the Catalan coast as reported in **Scientific publication 3**.

DA was studied in waters from Ebro delta wetland during three sampling campaigns and the results appear in **Scientific publication 2.**

Finally, the most hydrophilic MBTs, Neo, dcSTX, TTX, GTX-2,3 and STX, were analysed in waters from the Menor Sea of Murcia as documented in **Scientific publication 3**.

4.2.1 Validation of ELISA

Publication 4: Development and validation of a multianalyte immunoassay for the quantification of environmental pollutants in seawater samples from the Catalonia coastal area

DOI: https://doi.org/10.1007/s00216-019-01971-3

Analytical and Bioanalytical Chemistry
https://doi.org/10.1007/s00216-019-01971-3

RESEARCH PAPER

Development and validation of a multianalyte immunoassay for the quantification of environmental pollutants in seawater samples from the Catalonia coastal area

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Received: 11 April 2019 / Revised: 23 May 2019 / Accepted: 7 June 2019

Abstract
Five different enzyme-linked immunosorbent assays (ELISAs) have been developed and applied for the detection of five representatives of important families of chemical pollutants in seawater: Irgarol 1051® (triazine biocide), sulfapyridine and chloramphenicol (antibiotics), 17β-estradiol (hormone), and domoic acid (algae toxin). The assays were validated by high-performance liquid chromatography (HPLC) coupled with high-resolution mass spectrometry (HRMS) showing good correlation between both immunochemical and chemical techniques. A process of extraction and clean-up was added prior to the analysis based on solid-phase extraction (SPE). The multianalyte platform presented good specificity for each compound and adequate sensitivity, with limits of detection (LOD) after the SPE treatment of 0.124 ± 0.006, 0.969 ± 0.09, 0.20 ± 0.05, 1.11 ± 0.012, and 1.39 ± 0.09 ng L^{-1} for Irgarol 1051®, sulfapyridine, chloramphenicol, 17β-estradiol, and domoic acid, respectively. No matrix effects were noticed in working with the seawater extracts. Afterward, seawater samples from the Mediterranean Sea (coastal area of Catalonia) were analyzed by both techniques and only one sample presented one contaminant, 17β-estradiol, in the concentration of 0.011 ± 0.04 μg L^{-1}.

Keywords ELISA · Multianalyte immunoassay · HPLC · Environmental analysis · Validation

Introduction

During last decades, there have been many efforts in understanding the origin and levels of pollutants in aquatic environments, since they are considered both reservoirs of biodiversity and areas with great economic potential [1]. Considering, in particular, coastal areas as regions that are exposed to contaminants coming from many different sources, the need for controlling the occurrence of contaminants becomes essential. Some groups of contaminants such as endocrine disruptor chemicals (EDC) can cause severe negative effects due to its accumulation in water and soil samples, or aquatic organisms; also, EDC may affect the hormonal balance of fishes and other organisms [2, 3]. Furthermore, some examples of antibiotics can be highlighted as interesting contaminants to be monitored considering its massive use for combating infections and infestations in aquaculture facilities [4]. Most of the pollutants found in the environment are directly generated by humans; however, there is an increasing concern about the marine biotoxins which are being produced by algal blooms which can produce harmful effects, both affecting the environment and causing economic losses [5].

As a result of this scenario and during the last years, several global and regional monitoring programs have been proposed [6, 7] aiming for the establishment of early warning systems and screening methods that could provide information about multiple contaminants with high sensitivity and selectivity. Several environmental

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Published online: 19 June 2019

quality standards (EQS) have been set by the European Commission in order to control the allowed concentration of certain chemical species that could be related to environmental or human health risks (established in the Environmental Quality Standards Directive 2008/105/EC as amended by the Priority Substances Directive 2013/39/EU).

Is within these initiatives that the development of multianalyte and/or multiplexed platforms able to measure simultaneously several analytes from a single sample became a hot topic. Several multiresidue or multiplexed analytical procedures for the analysis of a huge number of chemicals in environmental samples have been reported in the last years based on chromatographic techniques [8–10]; however, due to its features of being rapid, simple, and low-cost methods altogether with their high specificity, potential portability, and high-throughput capabilities, immunochemical techniques have been considered great candidates for environmental monitoring at low concentration levels. Several examples have been presented in the literature regarding multianalyte or multiplexed systems aiming to detect pollutants in environmental samples. Microbial based assays [11, 12] and enzymatic assays [13] have been described using different configuration such as microarrays [14, 15], or biosensors [16, 17] and detection systems for instance, based on fluorescence [18, 19].

For the development of this work, some representative compounds pertaining to different family groups of contaminants and with different chemical natures, commonly present in seawater, have been considered. These compounds are as follows: the biocide Irgarol 1051® (Irg), two antibiotics sulfapyridine (Spy) and chloramphenicol (CAP), the hormone 17β-estradiol (E2), and the marine biotoxin domoic acid (DA). The analysis of the selected compounds has been carried out through a multianalyte ELISA platform and then, the results have been compared and validated by a reference analytical technique such as high-performance liquid chromatography (HPLC) coupled with high-resolution mass spectrometry (HRMS).

Experimental section

Reagents, immunoreagents, and equipment

The chemical reagents and immunoreagents used were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) and from Sigma Chemical Co. (St. Louis, MO, USA). Domoic acid was purchased from Sigma Chemical Co. as well as the estradiol hapten; β-estradiol 6-(O-carboxymethyl)oxime (6E2). The preparation of the described bioconjugates and antibodies has been performed with the support of the ICTS "NANBIOSIS", more specifically by the Custom Antibody Service (CAbS, CIBER-BBN, IQAC-CSIC). The immunoreagents for Irgarol (Irg), sulfapyridine (SPy), and chloramphenicol (CAP) detection used for the development of the competitive assays in the microarray platform have been described before [20, 21]. The preparation of the bioconjugate competitors for 17β-estradiol and domoic acid has been reported [22]. The monoclonal antibody used on the estradiol assay was purchased from Fitzgerald Industries International. The monoclonal antibody for the detection of domoic acid has been kindly provided by Prof. Chris Elliot from Queen's University [23]. The analytes used in this platform were Irgarol (Irg), sulfapyridine (SPy), chloramphenicol (CAP), 17β-estradiol (E2), and domoic acid (DA) (Fig. 1), all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of each analyte were prepared at 10 mM concentration in DMSO and stored at 4 °C until its use.

The pH and the conductivity of all buffers and solutions were measured with the pH meter pH 540 GLP and the conductimeter LF 340, respectively (WTW, Weilheim, Germany). Dilution plates were purchased from Nirco (Barberà del Vallés, Spain). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were performed on a Biotek ELx405HT (Biotek Inc.). Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA, USA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v4.7 (Molecular Devices) and

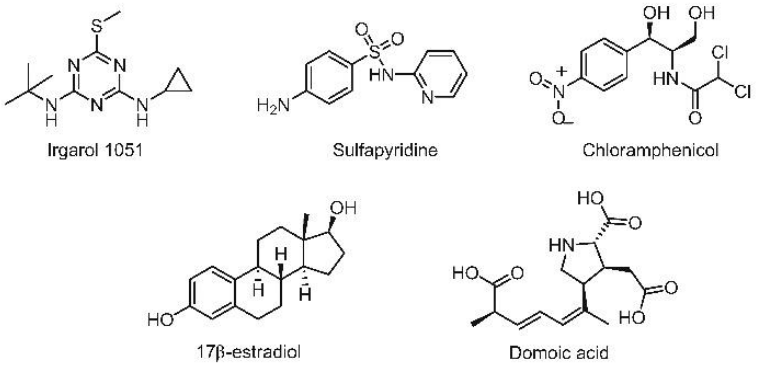

Fig. 1 Chemical structure of the 5 different analytes of this work

GraphPad Prism 5.03 (GraphPad Software Inc., San Diego, CA, USA). For the matrix studies, sea salts used to prepare the artificial seawater (aSW) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and prepared at 40 mg mL^{-1} in ultrapure water.

Buffers

Unless otherwise indicated, phosphate-buffered saline (PBS) is 0.01 M phosphate buffer in a 0.8% saline solution at pH 7.5. Coating buffer is a 0.05 M carbonate-bicarbonate buffer, pH 9.6. PBST is PBS with 0.05% of Tween 20. PBT2x is PB (0.02 M phosphate buffer, no saline solution) with 0.1% Tween 20 at pH 7.5. Citrate buffer is 0.04 M sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer. Borate buffer is 0.2 M boric acid/sodium borate, pH 8.7.

Sampling

Surface seawater samples were collected from 6 different locations along the Catalan coast (NE of Spain, Mediterranean Sea) during the 18th–25th of May 2017; Llafranc (41° 53′ 35.5″ N 3° 11′ 35.7″ E), Palamos (41° 50′ 53.2″ N 3° 06′ 47.2″ E), Platja d'Aro (41° 48′ 59.7″ N 3° 04′ 10.5″ E), Sant Feliu de Guixols (41° 47′ 26.9″ N 3° 03′ 09.5″ E), Badalona (41° 26′ 17.0″ N 2° 14′ 40.9″ E), Forum-Sant Adria del Besos (41° 25′ 14.6″ N 2° 14′ 01.0″ E). Samples were stored in amber glass bottles and preserved at − 20 °C until the analysis.

Sample pre-treatment

Solid-phase extraction (SPE) was employed for the general cleanup of water samples. With minor modifications, the extractions were proceeded following the previously developed and optimized methods [24–28] with recoveries ranging from 76 to 98%. Briefly, cartridges were conditioned with 6 mL of methanol and equilibrated with 6 mL of pure water. For Irgarol 1051, ISOLUTE® ENV+ (220 mg, 6 mL) cartridges were employed, whereas cartridges of OASIS HLB (200 mg, 6 mL) were used for the rest of compounds. Five hundred milliliters of different seawater samples was loaded into the cartridges at approximately 1 mL min^{-1}. Cartridges were then washed with 6 mL of pure water to remove the interferences and salts and dried under vacuum for 20 min. Finally, 6 mL of methanol was used for the elution. All extracts were concentrated through a flow of N_2 steam of an evaporator Reacti–Therm III of Pierce (Rockford, IL, USA) and then reconstituted to 0.8 mL of solvent. The extracts analyzed by ELISA were reconstituted with DMSO and diluted 5 times in PBST (20% of final DMSO). Under these conditions, it would allow achieving a preconcentration factor of 625/5 = 125 times; however, this value should be corrected with the real recovery obtained after the SPE column treatment (see Table 1). The extracts analyzed by HPLC-HRMS were reconstituted with the initial chromatographic conditions of mobile phase (9:1, methanol/water).

Competitive ELISA assay

Microtiter plates were coated with the proper concentration of each antigen (in coating buffer, 100 µL/well), overnight at 4 °C and covered with adhesive plate sealers. The next day, the plates were washed four times with PBST (300 µL $well^{-1}$), using a Biotek ELx405HT microplate washer form Biotek Inc. (Winooski, Vermont, USA). Then, each analyte was added at different concentrations for each analyte (50 µL $well^{-1}$, in PBST, aSW or PBST 20% DMSO, 50 µL $well^{-1}$) followed by the specific antibody at their corresponding concentration (50 µL $well^{-1}$, in PBST or PBT2x for the individual assays, or the cocktail of antisera for the multiplexed analysis). After 30 min at r.t., the plates were washed as mentioned before, and a solution of anti-IgG-HRP (single or a mixture of anti-mouse and anti-rabbit IgGs for the multiplexed assays, 1/6000 in PBST) was added to the wells (100 µL $well^{-1}$) and incubated for 30 min at r.t. The plates were washed again, and the substrate solution was added (100 µL $well^{-1}$). Color development was stopped after 30 min at r.t. with 4 N H_2SO_4 (50 µL $well^{-1}$), and the absorbance was read at 450 nm, with a SpectramaxPlus microplate spectrophotometer from Molecular Devices (Sunnyvale, CA, USA). The standard curves were fitted to a four-parameter equation according to the following formula: $y = (A - B/[1 - (x/C)^D] + B)$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. For this, the software SoftmaxPro v4.7 of Molecular Devices (Sunnyvale, CA, USA) and GraphPad Prism 5.03 of GraphPad Software Inc. (San Diego, CA, USA) were employed. Unless otherwise indicated, data presented correspond to the average of at least two wells replicates.

Matrix effects

Matrix studies were performed in single-analyte measurements with aSW prepared at 40 mg/mL in ultrapure water. Non-specific interferences produced by the parameters associated with the matrix of interest were studied by preparing a standard curve directly in seawater, or in buffer with an appropriate percentage of solvent for the multianalyte assays. Measurement of blank sample extracts after the SPE process made possible the study of the hypothetic matrix effect.

Table 1 SPE recoveries and LOD obtained for the different pollutants

Pollutant	SPE cartridge	Recovery (%)	Preconcentration factor[a]	mLOD[b] (μg L^{-1})
Irgarol 1051®	ISOLUTE® ENV+	96.7	120.8	0.004
Sulfapyridine	OASIS HLB	99	123.8	1.09
Chloramphenicol	OASIS HLB	76	95	0.10
Estradiol	ISOLUTE® ENV+	72	90	0.02
Domoic acid	OASIS HLB	98	122.5	5

[a] The preconcentration factor was calculated as the theoretical preconcentration factor 625, divided by 5 (due to the 1/5 dilution in PBST) and corrected by the recovery value of each analyte

[b] mLOD means limit of detection of the HPLC-HRMS method for each pollutant

Quality parameters

In order to assess the reproducibility of the assays, these were carried out three times within three different days, with three replicates for each one. The main features of the final assay were described as the mean of all the replicates. The accuracy of each ELISA assay for its specific analyte was assessed by analyzing 5 blind SW extracts, including 4 extracts spiked at different concentrations within the working range of each curve's analyte and one blank. The results were fitted to a linear regression curve between the spiked concentrations and the measured ones.

The possible cross-reactivity between the pairs of antibody-competitors used during this work was previously studied, showing in any case significative non-specific interactions [22].

Immunoassay validation

The multianalyte ELISA format was validated with HPLC-HRMS. The accuracy of the multiplexed ELISA assay for all analyte concentrations was assessed by analyzing 6 different real SW samples altogether with 3 more extracts which contained all the analytes spiked at three different levels of concentration (high, medium, and low) within the working range of each curve's analyte. Samples analyzed by ELISA were preserved in pure DMSO and for its analysis were diluted five times in PBST, obtaining final samples of only a 20% of solvent. All the samples were analyzed in parallel by high-performance liquid chromatography (HPLC) coupled with high-resolution mass spectrometry (HRMS) as a reference technique.

HPLC-HRMS

An Acquity™ ultra-high-performance liquid chromatograph (UHPLC) from Waters (MA, USA) was employed for the chromatographic separation. A reversed-phase column C18 Synergy (50 mm × 2 mm and 5 μm, 80 Å) from Phenomenex (Torrance, USA) was used as stationary phase. Methanol (A) and water (B) were merged into the mobile phase with an elution gradient program of 10 min in total: 0 min (10% A)–5 min (95% A)–7 min (95% A)–10 min (10% A). The flow corresponded to 0.3 mL min^{-1}, and the volume of injection was 20 μL for each sample.

A Thermo Scientific QExactive mass spectrometer was coupled with the chromatography employing a heated electrospray ionization probe (HESI), both acquired from Thermo Fisher Scientific (San Jose, CA, USA). The HESI was working with the following parameters: sheath flow gas, auxiliary gas, and sweep gas were 60, 20, and 2 of arbitrary units, respectively. The S-lens RF level was 60%, the spray voltage 3 kV, and the temperatures for the heater and the capillary were 320 and 300 °C.

The analysis by HRMS was performed with the acquisition working in the mode of data-dependant MS^2, obtaining at the same time the full scan from 100 to 700 *m/z* and the MS/MS spectrum for each compound at the resolution of 70,000 and 35,000 full width at half maximum (FWHM), respectively. In

Table 2 Precursor and fragments ions of the 5 compounds at the optima normalized collision energy (NCE)

	Chemical formula	Molecular ion	*m/z* precursor ion	*m/z* fragmentation ions		NCE[a] (%)	Retention time (min)
Irgarol 1051®	$C_{11}H_{19}N_5S$	$[M+H]^+$	254.1425	198.0805	108.0554	65	6.2
Sulfapyridine	$C_{11}H_{11}N_3O_2S$	$[M+H]^+$	250.0635	156.0109	184.0865	35	3.6
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	$[M-H]^-$	321.0042	152.0337	194.0446	20	4.6
17β-Estradiol	$C_{18}H_{24}O_2$	$[M-H]^-$	351.1261	271.1695	79.9555	35	5.9
Domoic acid	$C_{15}H_{21}NO_6$	$[M+H]^+$	312.1430	266.1377	248.1271	30	0.6

[a] NCE was established at 10 and stepped at different percentages for each compound + retention time

Table 3 Analytical parameters for all the target assays in an ELISA format, performed individually in PBST and in a multiplexed ELISA format (PBST 10% DMSO). The results shown are the average a standard deviation of assays performed in three different days. On each day, the assays were run using three-well replicates. The values of LOD* were obtained correcting with the real preconcentration factor (i.e., 120.8 for Irgarol 1051®; 123.8 for sulfapyridine; 95 for chloramphenicol; 90 for 17β-estradiol; and 122.5 for domoic acid) the values of each individual assay ($N = 3$)

	Irgarol 1051®			Sulfapyridine			Chloramphenicol		
ELISA	4eBSA/As87			SA2BSA/As155			CA6BSA/As226		
Condition	PBST	aSW	Multianalyte ELISA	PBST	aSW	Multianalyte ELISA	PBST	aSW	Multianalyte ELISA
[CA], μg mL^{-1}	0.25	0.25	0.078	0.25	0.25	0.156	0.0625	0.0625	0.0625
[As] dilution	1/16000	1/16000	1/32000	1/16000	1/16000	1/8000	1/64000	1/32000	1/32000
Abs_{min}	0.03 ± 0.02	0.04 ± 0.004	0.108 ± 0.02	0.03 = 0.02	0.019 ± 0.015	0.076 = 0.05	0.030 = 0.01	0.03 ± 0.02	0.102 ± 0.03
Abs_{max}	1.47 ± 0.09	1.46 ± 0.17	1.65 ± 0.22	1.34 = 0.08	0.839 ± 0.097	1.64 = 0.16	0.809 ± 0.02	0.984 = 0.1	1.39 ± 0.22
Slope	− 0.98 + 0.22	− 1.06 + 0.06	− 1.21 − 0.16	− 0.784 + 0.09	− 0.95 − 0.15	− 0.729 + 0.11	− 0.60 + 0.05	− 0.82 − 0.1	− 0.736 + 0.04
IC_{50} (nM)	0.58 = 0.19	0.43 ± 0.11	3.47 ± 0.11	6.58 ± 1.73	7.41 ± 1.6	10.7 ± 2.64	0.59 = 0.05	0.94 ± 0.07	2.16 ± 0.16
IC_{50} (μg/L ^{1})	0.145 ± 0.05	0.111 ± 0.03	0.879 ± 0.16	1.43 _ 0.23	1.84 ± 0.39	2.64 _ 0.66	0.192 ± 0.09	0.324 _ 0.09	0.699 ± 0.05
LOD (μg/L^{-1})	0.012 ± 0.007	0.015 ± 0.007	0.106 ± 0.02	0.08 = 0.02	0.12 ± 0.07	0.115 ± 0.06	0.004 = 0.01	0.019 ± 0.01	0.034 = 0.01
LOD* (ng L^{-1})	–	–	0.124 + 0.006	–	–	0.969 − 0.09	–	–	0.20 + 0.05

	17β-Estradiol			Domoic acid		
ELISA	6E2BSA/MAb_E2			DABSA/MAb_DA		
Condition	PBST	aSW	Multianalyte ELISA	PBST	aSW	Multianalyte ELISA
[CA], μg mL^{-1}	0.3	0.3	0.3	0.6	0.6	0.6
[As] dilution	1/64000	1/64000	1/64000	1/16000	1/16000	1/16000
Abs_{min}	0.013 ± 0.003	0.012 ± 0.006	0.125 ± 0.09	0.038 = 0.01	0.07 ± 0.001	0.128 ± 0.05
Abs_{max}	1.36 ± 0.20	0.902 ± 0.16	1.17 ± 0.19	0.935 _ 0.12	1.08 ± 0.16	0.826 ± 0.01
Slope	− 1.01 ± 0.07	− 0.958 ± 0.04	− 1.07 ± 0.02	− 0.866 = 0.13	− 0.788 ± 0.15	− 1.24 ± 0.13
IC_{50} (nM)	4.01 + 0.74	2.84 + 0.05	4.20 + 0.30	9.69 + 1.05	9.77 − 0.87	6.48 + 0.36
IC_{50} (μg/L ^{1})	1.09 ± 0.20	1.02 ± 0.02	1.06 ± 0.08	3.02 ± 0.33	2.82 = 0.14	2.02 ± 0.11
LOD (μg/L^{-1})	0.11 = 0.03	0.10 ± 0.007	0.149 ± 0.002	0.24 = 0.02	0.17 ± 0.07	0.40 ± 0.03
LOD* (ng L^{-1})	–	–	1.11 − 0.012	–	–	1.39 + 0.09

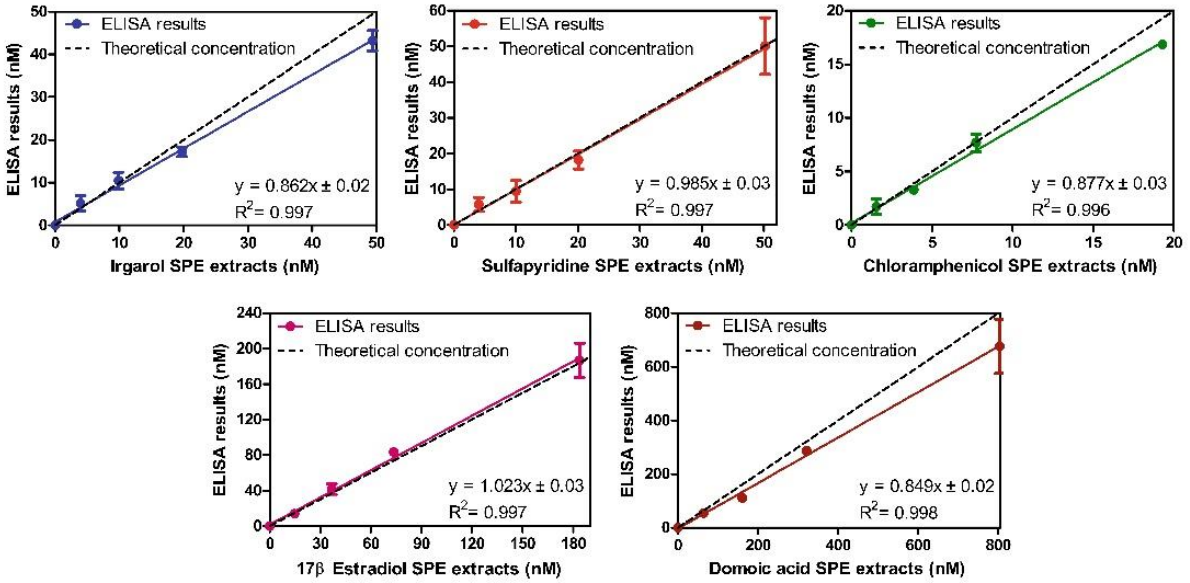

Fig. 2 Results from the accuracy study of the single-analyte assays performed in SPE extracts of real seawater samples spiked at different concentrations. The graph shows the correlation between the spiked and measured concentration values. The dotted line corresponds to a perfect correlation ($m = 1$). The data correspond to the average of at least three-well replicates from 2 different days

Table 2, the corresponding precursor and fragment ions at the optimized collision energy for each compound are summarized. Quantification was carried out considering the most intense signal of the fragment ion and confirmation with the second most intense.

Results and discussion

Recently, it has been reported two examples of platforms designed as proposals for screening and monitoring different pollutants in the environment, one based on a fluorescent multiplexed microarray [22] and another based on an amperometric sensor [29], and each with their own advantages. In the case of the microarray format, the assay protocol is faster, because the secondary antibodies are labelled with a fluorophore, not needing a substrate for obtaining the final signal like in the case of an ELISA protocol [22]. Furthermore, not only the volume of sample is reduced but also the analysis of all the pollutants can be done simultaneously in a single well. In the case of an electrochemical sensor, not only they are considered cheap options for monitoring organic contaminants such the ones selected for this work but are usually more fitted to be used for on-site measures; however, no multiplexation nor multianalyte format was addressed [29].

In order to exploit most of the advantages related to immunoassays, the platform chosen for developing this work was an ELISA configuration. Although the assay time is a bit longer than in microarray options, the equipment needed for all the protocol is more accessible, relying on absorbance readings instead of fluorescence. The high-throughput capabilities along with the easy protocols related to ELISA, and finally the possibility to develop it in a multianalyte format allowing the detection of several pollutants altogether makes the multianalyte ELISA a great candidate for screening contamination of natural environmental waters.

Table 4 Results of the blind's samples quantification by each single-analyte assay. The ELISA results were obtained as a result of three different assays in different days

Blind sample	Measured concentration (μg L^{-1})	
	Nominal concentration	ELISA measurement
Irgarol 1051®	3	4.2 ± 0.7
Sulfapyridine	3	4.1 ± 0.4
Chloramphenicol	6	2.2 ± 0.2
17β-Estradiol	14	17.1 ± 2.5
Domoic acid	70	50.6 ± 1.5

Evaluation of immunoreagents in an ELISA format with different matrix conditions and reproducibility of the assays

One of the challenges of detecting and monitoring marine contaminants is related to the low concentration in which they

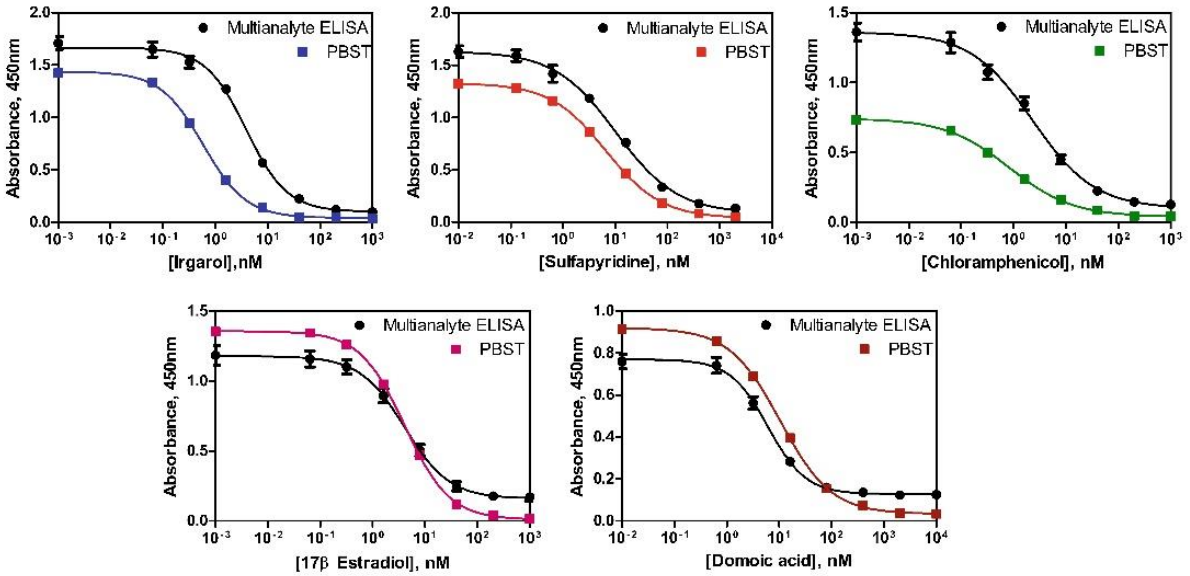

Fig. 3 Multianalyte ELISA assay results obtained for each analyte (corresponding to the average of 3 assays performed on different days using 3-well replicates) and the comparison with the calibration curves in buffer conditions (PBST) using each specific antibody against its coating antigen associated (corresponding to the average of 3 assays performed on different days using 2-well replicates)

are found in the environment. For that reason, great sensible techniques are required, and therefore, the possibility to include pre-treatment or preconcentration stages during the quantification of the samples could improve the sensitivity of already established platforms.

Aiming to achieve enough sensitivity to the analysis of seawater samples, a SPE cleanup step was performed prior to immunoassay, according to the procedure described before. Therefore, the target samples of this study are seawater samples that have been pre-treated by SPE before its analysis, in order to, on the one hand, pre-concentrate the possible pollutants present in it and, on the other hand, to eliminate possible interferences like other pollutants and salts. That means, that a certain percentage of the elution

Table 5 Results of the accuracy quantification of the different seawater samples spiked with all the target analytes simultaneously. The ELISA results shown are the average of three-well replicates and the experiments were repeated 3 days. For HPLC-HRMS measurements, the results shown corresponds to three replicates within the same day

	Blind sample	Measured concentration (μg L^{-1})		
		Nominal concentration	ELISA quantification	HPLC quantification
High level	Irgarol 1051®	1.25	1.25 ± 0.01	1.82 ± 0.04
	Sulfapyridine	2.5	2.48 ± 0.08	2.94 ± 0.02
	Chloramphenicol	2.5	2.58 ± 0.17	2.44 ± 0.21
	17β-Estradiol	2.5	2.49 ± 0.11	2.24 ± 0.14
	Domoic acid	12.5	13.16 ± 0.82	17.2 ± 0.84
Medium level	Irgarol 1051®	1	1.01 ± 0.01	1.46 ± 0.01
	Sulfapyridine	1.25	1.34 ± 0.20	1.00 ± 0.09
	Chloramphenicol	1	1.10 ± 0.06	1.61 ± 0.75
	17β-Estradiol	1.25	1.23 ± 0.12	1.61 ± 0.52
	Domoic acid	10	10.72 ± 0.58	15.8 ± 1.19
Low level	Irgarol 1051®	0.5	0.56 ± 0.05	2.00 ± 0.04
	Sulfapyridine	1	1.12 ± 0.07	0.81 ± 0.19
	Chloramphenicol	0.5	0.58 ± 0.20	0.39
	17β-Estradiol	1	0.95 ± 0.03	1.22 ± 0.59
	Domoic acid	5	5.89 ± 0.82	5.72 ± 0.57

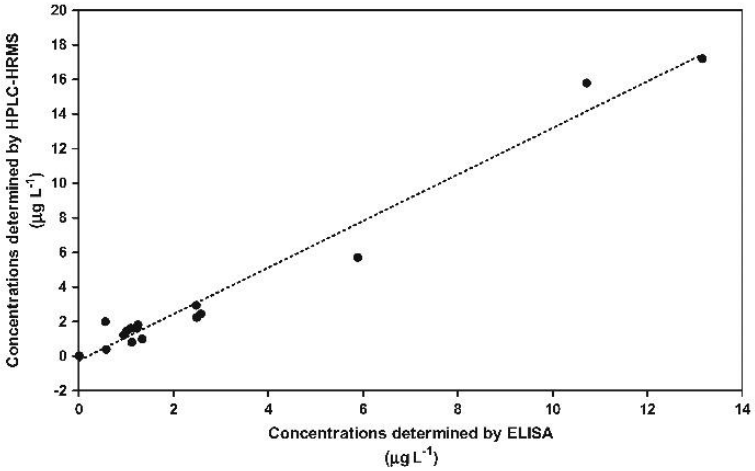

Fig. 4 Correlation of all samples analyzed by ELISA and HPLC-HRMS

solvent used in the SPE process will be present in the sample, and accordingly, the evaluation of this solvent in the final multianalyte platform and its comparison with the initial buffer conditions was needed. Both the addition of the solvent and the use of an antibody cocktail was studied and compared with the buffer conditions in terms of detectability, sensitivity, and limit of detection (LOD).

The effect of the addition of up to 20% DMSO in the final assay was tested previously for all the analytes (data not shown). In all the cases, most of the analytical parameters were maintained, although in some cases the maximum signal slightly decreased, and therefore the antibody concentration was adjusted. The possible cross-reactivity between the pairs of antibody-competitors used during this work was previously studied, showing in any case significative non-specific interactions [22]. In parallel, the reproducibility of all the assays, in both single-analyte and multianalyte format, was approached. In Fig. 3, it can be observed that when multiplexed (and even in the presence of a 20% of DMSO), all the assays maintained the levels of detectability and sensitivity (see Table 3), achieving LOD of 0.106 ± 0.02, 0.115 ± 0.06, 0.034 ± 0.01, 0.149 ± 0.002, and 0.40 ± 0.03 ppb for Irgarol 1051®, sulfapyridine, chloramphenicol, 17β-estradiol, and domoic acid, respectively. Even though the concentration of the pollutants selected is low in the environment, thanks to the preconcentration step of the SPE, the detectability could be improved with a factor of 500. All the results correspond to the average of 3 assays performed on different days using three-well replicates. The slight differences between the multianalyte ELISA and the individual assays can be explained, on the one hand, by the presence of a solvent in the assay buffer in the case of the multianalyte ELISA, and, on the other hand, by the use of a cocktail of antibodies that can modify lightly the parameters of each assay.

Furthermore, the parameters of IC_{50} and LOD obtained with the multianalyte ELISA (without the SPE preconcentration) are maintained in the same range as the ones reported for the multiplexed microarray already mentioned in the "Results and discussion" section (except in the case of domoic acid in which the multiplexed ELISA is slightly better). However, the advantage of the platform presented is the improvement of up to 125 times the detectability levels once the SPE treatment is performed; for instance, the final LOD found for Irgarol 1051, sulfapyridine, chloramphenicol, 17β-estradiol, and domoic acid were 0.124 ± 0.006, 0.969 ± 0.09, 0.20 ± 0.05, 1.11 ± 0.012, and 1.39 ± 0.09 ng·L^{-1}, respectively, compared with the LOD obtained with its corresponding individual assay in seawater that were found in the microgram per liter range. According to these results, the detectability achieved will be sufficient to reach the environmental levels usually found for most of the analytes selected.

Accuracy studies in the individual ELISA assays

Once the different ELISA assays were established in the desired matrix, the accuracy assays were performed in order to determine the reliability of each assay to determine the specific pollutant selected. Different extracts of seawater previously treated were spiked at 4 different concentrations for each pollutant and were analyzed aiming to perform an accuracy assay for each contaminant separately. The matrix effect was also studied analyzing a blank sample for each assay, in order to explore if the composition of the seawater treated could have the potential to affect the correct quantification of the target analytes. A matrix effect for all the blank samples tested was not observed. As represented in Fig. 2, the linear regression analysis showed slopes near 1 in all cases (being $m = 1.00$ the perfect correlation) with good regression coefficients ($R^2 > 0.95$), indicating the great accuracy of the different assays.

Table 6 Comparison of quantification results of the real seawater samples by both platforms, multianalyte ELISA and chromatographic reference technique (HPLC-HRMS). Seawater samples collected from different locations in the Catalonian Coast were passed through the SPE module, eluted with DMSO and the eluate split in two parts, one for analysis with the multianalyte ELISA and the other for the analysis by HPLC-HRMS. For the case of the ELISA, the results shown are the average of three-well replicates and the experiments were repeated 3 days. For HPLC-HRMS measurements, the results shown corresponds to only one replicate

Measured concentration (μg L^{-1})													
	Palamós		Sant Feliu		Llafranc		Fórum		Badalona		Platja d'Aro		
	ELISA quantification	HPLC quantification	ELISA quantification	HPLC quantification	ELISA quantification	HPLC quantification	ELISA quantification	HPLC quantification	ELISA quantification	HPLC quantification	ELISA quantification	HPLC quantification	
Irgarol 1051®	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	
Sulfapyridine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	
Chloramphenicol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
17β-Estradiol	nd	nd	nd	nd	0.011 ± 0.04	0.012	<LOQ	nd	nd	nd	<LOQ	<LOQ	
Domoic acid	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	

In parallel, 5 blind samples were analyzed for each specific ELISA aiming to demonstrate the feasibility of applying the different tools developed for the monitoring of seawater samples. The quantification results are summarized in Table 4, showing good levels of accuracy and precision in comparison with the nominal values of those samples. These results demonstrate that the different single-analyte ELISA assay developed can be presented as good candidates for analyte detection in seawater extracts.

HPLC validation of multiplexed ELISA results: accuracy assays and real sample evaluation

The development of the multianalyte ELISA platform was performed using a cocktail of all the specific antibodies required for the individual assays, for analyzing the different study samples proposed. The features of all the assays are summarized in Table 3, whereas the comparison between the multianalyte assays and the individual ones can be visualized in Fig. 3. Validation of the ELISA platform was assessed with accuracy studies of seawater extracts fortified at 3 different concentration levels of all the target analytes (within the working range of each curve's analyte) and with the analysis of 6 real samples. The quantification with the multianalyte platform and the HPLC technique had the goal to explore the potential of the ELISA platform developed in analyzing complex samples with a combination of the target analytes. In Table 5 and Fig. 4, the three spiked levels of concentration (high, medium, and low) are compared between the two quantification techniques selected. The HPLC-MS technique presents worse accuracy than the ELISA, which could be explained as the ion suppression during the ionization process because of the presence of salts in the extracts even after the SPE. On the contrary, it does not affect the response of the immunoassay that does not present matrix effect in using directly seawater within the buffers, as it has been seen in the "Evaluation of immunoreagents in an ELISA format with different matrix conditions and reproducibility of the assays" section. Finally, 6 seawater samples were obtained from different locations over the Catalan coast. These samples were, as in the previous accuracy assay, pre-treated as commented in the "Sampling" section to obtain concentrated extracts, which were analyzed by ELISA and HPLC simultaneously. As shown in Table 6, only one of the samples gave a positive response of 17β-estradiol in the location of Llafranc and this was good correlated by ELISA and HPLC-HRMS.

A Wilcoxon matched-pairs signed-ranks test was employed to determine the correlation between the results obtained by both techniques. The nonparametric Spearman coefficient (r) was equal to 0.812 resulting in a significant correlation between the values of concentration determined by ELISA and HPLC-HRMS, at the 0.01 value of confidence. In Fig. 4, the correlation of all samples measured by ELISA and HPLC-

HRMS can be observed. Then, the quantification by the immunoassay option showed good levels of correlation with both the spiked value and the HPLC measurements, demonstrating that the presence of other analytes is no impediment for the correct quantification of each pollutant.

Conclusions

The 5 ELISA platforms developed for the analysis of Irgarol 1051®, sulfapyridine, chloramphenicol, 17β estradiol, and domoic acid in complex matrices as seawater presented good response in terms of accuracy, sensitivity, and reproducibility. No matrix effect and non-specific responses by other structurally similar compounds were interfering the functioning of the immunoassays. The validation of the immunoassays through HPLC-HRMS indicated a proper correlation between both immunochemical and analytical techniques. This fact demonstrates the capability of the platform developed for its application in monitoring the levels of several pollutants present in real environmental samples, even at low concentrations. Due to this fact and the urge to obtain multi-residual analytical methods for the detection of contaminants in environmental matrixes and the demonstration of its possible implementation by reference techniques, this article reports a successful multianalyte ELISA platform for the detection of the five pollutants in seawater.

Acknowledgments The Nb4D group (formerly Applied Molecular Receptors group, AMRg) is a consolidated research group (Grup de Recerca) of the Generalitat de Catalunya and has support from the Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya (expedient: 2014 SGR 1484). CIBER-BBN is an initiative funded by the Spanish National Plan for Scientific and Technical Research and Innovation 2013-2016, Iniciativa Ingenio 2010, and Consolider Program; CIBER Actions are financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. The ICTS "NANOBIOSIS", and particularly the Custom Antibody Service (CAbS, IQAC-CSIC, CIBER-BBN), is acknowledged for the assistance and support related to the immunoreagents used in this work.

Funding information This work has been funded by SEA-on-a-CHIP project (FP7-OCEAN-2013, no. 614168).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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4.3 Discussions

4.3.1 Validation of the ELISA for the detection and quantification of DA in seawater by LC-MS

The multianalyte ELISA for the detection and quantification of five pollutants in seawater has been successfully validated and confirmed by the LC-HRMS method. With regards to the DA, the correlation between the analytical technique and the immunoassay results shows the good agreement of both techniques. In the following, the different parameters are going to be discussed.

Sensitivity The SPE-ELISA method provides high sensitivity in the detection of DA in seawater. The LOD of the assay was 0.40 µg/L and with the application of the SPE as sample pre-treatment, the concentration of the toxin content made an increase in the sensitivity to 1.35 ng/L. Some of the ELISA methods that were employed in the analysis of DA at different matrices are summarised in **Table 18**. As can be seen, the developed SPE-ELISA provides higher sensitivity compared to those techniques that are employed in the shellfish analysis. This is an important fact, considering that pollutants in seawater are at low concentrations. DA has been reported in seawater at trace concentrations in the range of 0.2 pg/L to 220 ng/L in the Alaska's Gulf of the Pacific Ocean [124] or in the range of 1.5 to 16.2 ng/L in the Adriatic Sea [193] and even reaching levels of 13 µg/L during a *Pseudo-nitzschia* spp. bloom with concentrations of 10^6 cells/L on the Atlantic coastline of the Gulf of Mexico [307].

In the case of the ELISAs that were employed for the detection of DA in shellfish, LODs are higher than those of the developed ELISA and the commercial kits. However, these ELISAs are sensitive enough for the detection of DA in shellfish under the MRLs that are 20 mg DA equivalent/kg.

Table 18. DA-analysis by ELISA in different matrices and the corresponding LODs.

Sample matrix	ELISA format	LODs (µg/kg)	REF
Bird tissue	-	1	[308]
Shellfish	Direct competitive	3	[123]
	Direct competitive	25	[296]
	Indirect competitive	0.006	[126]
	-	720	[309]
Seawater	Direct competitive	0.01* (µg/L)	[124]

Specificity Non-specific responses were noted in the analysis of DA. An important parameter to define the proper performance of the analysis by ELISA is the specificity of the antigen–antibody (Ag-Ab) binding. The monoclonal Abs and the corresponding

bioconjugates that were prepared by Traynor et al. presented high affinity in their single analysis [310]. Moreover, the specificity was maintained in the multiplexed assay and no cooperative phenomena appeared with other bioconjugates and antibody competitors. This proved the efficiency in the DA-antibody recognition of the corresponding antigen and DA, even working with the same bioconjugate procedure for the recognition of different analytes. Furthermore, during the development of the immureagents, cross reactivity was tested with other toxins such as STX, other structurally similar acids, kainic acid and L-aspartic acid, and different isomers of DA. The results showed significant cross reactivity with the major isomer of DA but not with the rest of the compounds [310]. This fact demonstrates the efficiency of the assay, which is capable of detecting any of the conformations of DA that could be present in the seawater.

Cross reactivity is present in the application of ELISA for the analysis of other MBTs within the same group [311-313]. The epitope, the structural fragment of the antigen which is recognised by the Ab, is the same for all of the toxins in the same family. For example, STXs have a tetrahydropurine common structure with different additional functional groups for each congener. The specific Ag-Ab recognition is the same for all of the congeners, however, the interaction will be different and the response will also be different. However, it is not possible to have the distinction of each compound, and additional confirmatory techniques will be required in order to identify and quantify the specific biotoxin. Although this lack of selectivity could be solved by using LC-MS, ELISA provides the rapid identification of STXs in a sample, regardless of the nature of congener.

Matrix effect No significant matrix effect was detected in the seawater analysis by ELISA. The working range of the immunoassay can be affected by the presence of matrix interferences due to the interaction of the Ag-Ab and can be hampered by soft or strong unspecific molecular interactions. In this case, after the SPE treatment, most of the organic matter that is present in the sample was removed or reduced. Nonetheless, the high content of salts from seawater was not completely removed, but this did not significantly affect the immunoassay. The immunoreaction takes place in a medium of phosphate buffers, while not being modified by the presence of salts. In contrast, LC-MS is more affected by the presence of salts. The matrix effect that was estimated in the analysis of DA from seawater by the method developed in the **Scientific publication 2** of this thesis research was 73%. This method is based on SPE-HILIC-HRMS and even though the SPE reduces the matrix interferences content, the presence of salts complicates the ionisation process, thus causing the suppression of the ions signal. Some more examples in which LC-MS analysis are subjected to experimentation of more matrix effect than ELISA are explained by Frame et al. In that study, DA was analysed in several mammal samples by both techniques. LC-MS was not able to detect the lowest range of DA concentration in mammal faeces, urine and serum samples whereas ELISA was able [314].

Therefore, both analytical and immunochemical methods provide good accuracy, sensitivity, repeatability and selectivity for the analysis of DA in seawater. Selecting one of these techniques will be determined by the specific requirements of its application.

While LC-MS provides major selectivity, resolution, accuracy and robustness, some other disadvantages are presented, such as the elevated cost, the requirement of sample-pre-treatment and the time consumption for the achievement of the results. The main advantages and limitations of each technique are exposed in **Table 19**.

Table 19. Advantages and limitations of ELISA and LS-MS for the detection and quantification of DA in seawater.

Limitations	Advantages
ELISA	
- Long time for the development - Difficulties to achieve immunoreagents - Immunoreagents are less stable - Few selectivity in the same class of toxins	- High sensitivity - Economic - Rapid response - High throughput analysis - Portability - Specificity - Multianalyte analysis
LC-MS	
- High cost of the instrument and the maintenance - Pre-treatment of sample is required - Long-time of analysis response - Requires specialized personnel	- High selectivity - High sensitivity - Robustness - Multianalyte analysis - Non-target analysis

Once the ELISA is optimised, the analysis of DA is fast and cost-effective. However, one of the main limitations of this technique is the long time period that is required for the development of the immunoassay. The obtaining of antibodies can be done by the synthesis of an animal product. The modelling and synthesis of the proper structure with high affinity to DA is complex and expensive. The most employed methodology is the immunisation of mouse [315], rabbits [316], sheeps [317], chicken [318], etc. to the toxin for the extraction of specific monoclonal or polyclonal antibodies that are naturally produced. However, in the case of DA causes amnesic syndromes and death in the worst intoxication cases are caused, thus, the dose of inoculation has to be highly controlled. However, the small size of DA requires its conjugation with a bigger molecule, a protein carrier such as ovalbumin (OVA), bovine serum albumin (BSA), or human gamma globulin (HGG). After the production of immunoreagents, the purification and estimation of the haptens and bioconjugates density is required by the use of instrumental analytical techniques such as MALDI-TOF-MS [319]. Finally, it has to be tested under the proper conditions for the specific reaction of Ag-Ab.

In conclusion, the preparation of the immunoassay is a long process but once it is resolved, the application is rapid and effective. Another great advantage is the possibility to make the analysis automatic or even portable by the incorporation into a biosensor. Some examples of these implementations are the combination of CE-enzyme immunoassay (CE-EIA) with electrochemical detection that allows the analysis of DA in shellfish in just 5 minutes with a LOD equal to 0.02 µg/L [320]. Further, the Analytical

System for Marine Algal Toxins (ASMAT) that was developed by Petropolous et al. and that made the integration of an enzyme-linked immune-magnetic colorimetric (ELIMC) assay with an automated system that is capable of Online monitoring of DA and other MBTs in seawater [287].

4.3.2 Occurrence of MBTs in seawater from Mediterranean seawater

4.3.2.1 Lipophilic MBTs in the Catalan littoral

In the thirsty-six surface seawater samples that were collected from the Catalan littoral only OA was detected and quantified. The toxigenic phytoplankton that produce OA are dinoflagellates of the genus Dinophysis and Prorocentrum which are ubiquitously present in coastal waters [321]. More than 170 species of these genus are reported to produce OA, DTXs and PTXs [204]. Many of the reported species in the Mediterranean waters are *D. acuminata, D. acuta, D caudata, D sacculus and D. fortii* [24, 303, 322] which are capable of producing OA, DTXs or PTXs or different moieties of these [204]. The occurrence of OA has been widely reported, especially in shellfish, with the co-occurrence of other lipophilic toxins such as DTXs, PTXs, YTXs, or AZAs (**table 17**). To our surprise, no other lipophilic MBT was detected in the samples, not even PTXs nor DTXs, that are normally co-occurring with OA. Different explanations could answer these controversies. Firstly, the presence of other toxins could exist in the samples but at levels below the LODs, then it was not possible to detect it at such low concentrations. Another possibility could yield on the fact that the co-occurrence of OA, DTXs and PTXs is mainly reported in shellfish and some SPATT, because they are accumulated during a longer time period. Then, the co-occurrence of the toxins could take place in these matrices but not simultaneously in the same waters. But this fact could not explain why no other toxins have been detected in the particulate fraction. The co-occurrence of OA and other lipophilic toxins was confirmed in both filtrate and particulate fractions of the waters that were collected in the mariculture bay of Jiaozhou in China, being DTX-1 and PTX-2 as mainly predominant [278]. Then, the last hypothesis, is that the toxigenic species that produce OA, DTX-1 and PTX-2 only produced OA in the moment close to our sampling or also produced PTXs and DTXs, but at lower concentrations than our LODs. The production of toxins can widely vary within the same specie depending on the surrounding conditions and the stage of the phytoplankton growth [323, 324]. For example, *Dinophysis accuta* produces OA, DTXs and PTXs [204] but it has been reported that some strains from western Spain are only producing PTX-2 [325]. In another analysis from the body of literature of lipophilic MBTs that was carried out on the Catalan coast, OA, DTX-2 and PTX-2 were quantified from a plankton sample in concentrations of 3.73, 4.96 and 4.52 ng/L respectively, whereas in a closer area, only PTX-2 was quantified at the concentration of 3.81 ng/L [37]. Another example of this variability are the results that were obtained from the analysis of lipophilic toxins in seawaters from the Gulf of Guinea, Nigeria. The most abundant toxins were OA and PTX-2 in concentrations ranging from 1 to 100 ng/L, whereas DTX-1 was detected occasionally at trace levels [306]. Thus, the dominance of a unique toxin can take place in determined situations even though the algae can produce more diversity. Finally, in accordance with our owndata for

this research thesis, some strains of *Dinophysis acuminata*, for example, have been reported to only produce OA, in western Spain, Portugal and even in Denmark [326]. Hence, it can be concluded that the moieties of toxins produced by a toxigenic-specie is widely variable and depends on several factors. For this reason, the analysis of the toxins is the best strategy to determine the toxicity of a sample rather than characterisation of the species.

In relation to the concentrations of OA that were determined in the waters and their spatial distribution, the highest concentrations were quantified in samples that were collected close to urban areas in concentration levels of ng/L in the filtrate fraction and µg/kg in the particulate fraction. The concentration ranges, mean and median of OA for the particulate and filtrate fractions in marinas and beaches are summarised in **Table 20**.

Table 20. Summary of the results of OA in seawater from **Scientific publication 1.**

	Particulate (µg/kg)			Filtrate (ng/L)			Particulate + filtrate (ng/L)		
	Total	Marina	Beach	Total	Marina	Beach	Total	Marina	Beach
Conc. range	0.03-560	0.03-224.8	0.09-560	2.10-1780	2.10-1780	2.5-119.2	2-9586	2.0-9586	3.1-8584
Mean	34.5	19.41	52.03	72	131	12	732	770	700
Median	2.36	2.74	1.54	4.75	5.8	4.6	71.3	110	57

In comparing our results with the results from other studies that were carried out along the Catalonian coast, we found that our concentrations were higher. Rivetti et al. analysed OA in the particulate of Ebro Delta bays. The concentrations ranged from 2.40 to 9.16 µg/kg [327], which are much lower in comparison to our own results, however, it is in agreement with the median value of OA in the particulate of our samples and still higher compared with the mean. As previously noted, there is scant information that is related to MBTs in Mediterranenan seawater, hence the other regions have to be compared. The analysis of lipophilic MBTs at was carried out in the mariculture area of Qingdao, China, determined a constant concentration of OA ranging from 6.2 to 6.6 ng/L in the filtrate fraction in three samples that were collected every week. However, two samples that were collected weekly by SPATT had a range of concentration of 7.9-64 ng/g per day [328]. In addition, Chen et al. determined the occurrence of lipophilic MBTs in seawater from Haizhou Bay in the Yellow Sea, China. Concentrations of OA in the particulate ranged from 0.33 to 4.25 µg/kg with a mean of 1.67 µg/kg, whereas in the filtrate concentrations were higher, ranging from 11.47 to 55.85 ng/L with a mean value of 27.29 ng/L [171]. This range of concentrations are still lower than those in the present study.

As discussed in the **Scientific publication 1,** there were no significant differences in the concentrations of OA that were determined inside marinas and on open beaches, including particulate, filtrate and in total. We expected to find the highest concentrations inside the marinas, assuming that the resilience time of the water is higher, then the organic matter is more accumulated and consequently the proliferation of algae would be higher and the probability of more toxins occurring is more elevated. Then, taking into account the

succession strategy of *Dinophysis* spp. as defined by Margalef's mandala [329], their survival could be favoured by the low turbulence of a the marina. However, the high content of nutrients in the marina would not promote the proliferation of the dinoflagellates. In addition, the presence of the toxigenic algae, does not ensure the production of MBTs, hence the association of more phytoplankton and more toxins cannot always be assumed. Notwithstanding, there is an absence of data related to the turbulence and nutrients is missing, as well as the detection of the species, hence no further detailed explanations can be deduced.

However, no significant differences were found in the concentration levels of OA in the particulate and in the filtrate fractions, in contrast to our own preliminary hypothesis. We expected to find higher concentrations in the particulate fraction due to the lipophilic character of OA, however, many other factors are involved. Liu et al. studied the presence of lipophilic MBTs in seawater and sediments of the coastal area of Daya Bay, South China Sea. OA was present in the filtrate but not on the particulate, at the concentration of 15.55 ng/L and in sediments was present in a range of 0.095–3.94 µg/kg [175]. This fact suggests that the absence of OA in the particulate fraction could be because the possible toxin dissolved in water tends to move towards the sediment at the sea bottom and accumulate in the sediments. In addition, as previously commented, Chen et al. determined higher values of OA in the dissolved fraction and not in the particulate of the samples from Haizhou Bay, China [171].

4.3.2.2 DA in Ebro Delta wetland

DA was analysed in 34 surface seawater samples from the Ebro Delta wetland, and was present in the 65% of the concentrations at low ng/L levels. The DA-producer species *Pseudo-Nitzschia* spp., are cosmopolitan and have been widely documented in the Ebro Delta. During recent years, the abundance of these diatoms has increased in the area, thus forming recurrent blooms [330]. The hydrography of the area is fundamental for the high primary production that takes place in the two semi-enclosed bays (Fangar Bay and Alfacs Bay). This fact makes the growth of the molluscs propitious and that is the reason why the main harvesting farms are located there. On the one hand, due to river sedimentation, the depth in the bays is not really great. And on the other hand, the freshwater inclusion is from the river but also is especially from the irrigation channels of the rice cultivation fields which carry over high amounts of nutrients and dilutes the salinity in both bays bays (Fangar Bay and Alfacs Bay). These factors, leave the water bodies in a semi-permanent state of stratification [331]. The notorious detection of DA in the upper water samples, suggests that the light irradiance and stratification would be favourable for the DA production or at least for the diatoms habitats [37]. The abundance of *Pseudo-Nitzschia* spp. has been reported in the area with higher values during the summer and autumn seasons [332].

Since several factors are involved in the production of toxins, different parameters have been studied separately. One such research work is the study of the organic nutrients in the influence of HABs development. Loureiro et al. carried out some experiments with samples that were collected from Alfacs Bay during a bloom of *Pseudo-Nitzschia* spp.

After the four days of experiments in which different conditions of nitrates, phosphates, dissolved organic matter (DOM), high molecular weight dissolved organic matter (HMWDOM) and bacteria were controlled, the conclusions were that organic nutrient has a potential significance for the *Pseudo-nitzschia* spp., that grows in this area [330].

With the aim of establishing possible relations between the occurrence of DA and the environmental parameters, concentration of nitrates, phosphates and chlorophyll-a were determined additionally, by the application of the colorimetric technique using different protocols. According to the approved protocols for the USA-based National Pollutants Discharge Elimination System (NPDES) nitrates were measured by the brucine method 352.1 and phosphate by the ascorbic acid method 365.3 [333]. Chlorophyll-a was determined by the tri-chromatic method by acetone extraction [334]. Results are summarised in **Table 21**.

Table 21. Concentrations of nitrates, phosphates and chlorophyll-a in the analysed samples from Ebro Delta wetland.

		Nitrates NO_3^- (mg/L)			Phosphates PO_4^{3+} (µg/L)			Chlorophyll-a (µg/L)		
		Oct-15	Feb-16	Jun-16	Oct-15	Feb-16	Jun-16	Oct-15	Feb-16	Jun-16
Alfacs	**P1**	3.6	0.9	2.8	97.2	<LOD	146.6	n.m.	47.7	1.4
	P2	2.2	3.6	34.9	<LOD	64.3	n.m.	n.m.	10.3	19.0
	P3	1.6	24.2	<LOD	142.1	<LOD	<LOD	4.469	37.9	46.6
	P4	n.s.	2.5	2.0	n.s.	113.7	<LOD	n.s.	<LOD	142.9
Fangar	**P5**	22.5	2.3	10.6	146.6	<LOD	47.8	21.618	4.8	21.4
	P6	1.7	0.6	7.6	64.3	163.1	<LOD	1.625	0.9	14.3
	P7	0.7	3.2	10.9	463.2	31.3	<LOD	1.034	12.5	20.1
	P8	n.s.	0.4	6.8	n.s.	n.m.	<LOD	n.s.	12.8	11.9
La Tancada	**P9**	4.1	14.0	42.6	441.2	225.2	<LOD	0.757	34.1	23.6
Illa de Buda	**P10**	7.0	1.9	1.3	64.3	920.2	n.m.	5.507	6.8	4.1
L'Encanyissada	**P11**	4.4	0.7	2.3	47.8	47.7	441.2	1.361	n.m.	16.5
Canal Vell	**P12**	5.2	29.2	4.9	308.3	225.2	97.2	52.940	35.1	7.8

Being n.s. - not sample available, n.m. - non measured sample, <LOD – below LOD

Regarding the obtained values, none of them seem to follow the seasonal pattern. Hence, these values been compared with the DA occurrence in addition to the physico-chemical parameters that were measured in the waters during the sampling, with respect to temperature, pH, salinity and dissolved O_2 (Supporting information is in **Scientific publication 2**). In order to have a global comparison between all of these parameters together, the results were compared with a Principal Components Analysis (PCA), but no relation was attributed.

However, the relation between the concentration of DA and the ratio of nitrates and phosphates N/P has also been compared separately too, and the results are shown in **Figure 23**. At the range value of N/P going from 0 to 16, the concentration of DA varies from 0 to 15. At the N/P range from 16 to 80, the concentration of DA is highly variable, from non-detection to a value of 14 ng/L. Further, for a ratio of N/P higher than 80, the presence of DA seems to be less abundant. However, any clear tendency could be described by comparing the N/P ratio with the concentration of DA.

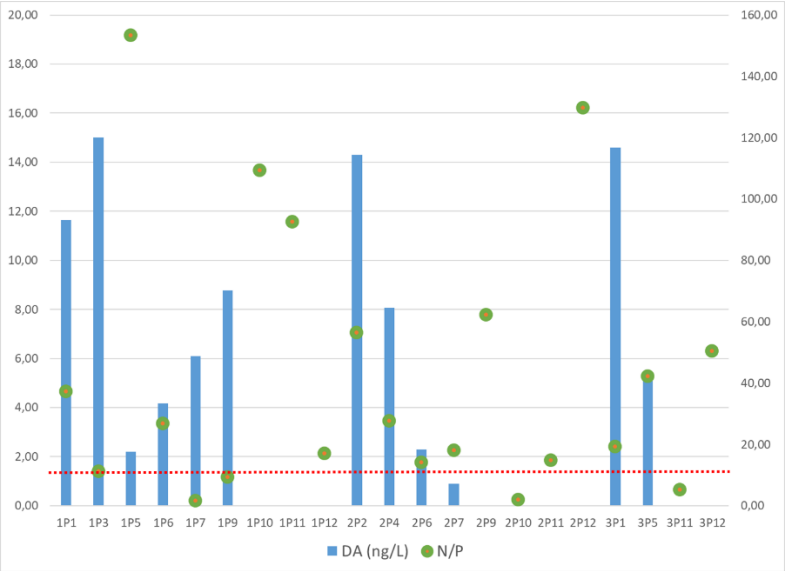

Figure 23. Relations between the DA concentration and the relation between the nitrates and phosphates N/P.

Finally, the data exposed from this research thesis are scant for determining the influence of the DA production in relation with the environmental parameters and also with respect to determining the influence of the phytoplankton dynamics. However, the high sensitivity that this SPE-LC-MS method provides could contribute some information that is related to the concentration of DA which could be employed in mathematical/statistical modelling to better understand the dynamics and production of DA.

4.3.2.3 Hydrophilic MBTs in Mar Menor

The results from the application of the developed LC-MS method in the samples from Mar Menor (Menor Sea) were negative regarding the presence of STXs and TTX. No occurrence of these toxins was determined above the LODs in this reseach thesis.

The occurrence of these toxins in Mar Menor has never been never reported before, to the best of our knowledge. Until 1970, STXs had not been reported in the Mediterranean waters [335], but after this date, they have been the responsible for several outbreaks [336-338]. The identification of the causative species of Alexandrium spp. has been reported for the western Mediterranean, however, the data were not always including STXs [37]. Gymnodinium spp. were identified in the latest report of phytoplankton composition in the Mar Menor [339], however, as previously commented several times, the presence of the toxigenic producer cannot be directly associated to the production of toxins. In a similar way, TTX has been defined as being produced by several ubiquitous marine bacteria, but its occurrence in seawater has not yet been reported, to the best of our knowledge.

The LC-MS method that was developed for the analysis of hydrophilic MBTs analysis is less sensitive compared to those methods that were developed in this research thesis for the analysis of lipophilic MBTs and DA, for which LODs are in the range of ppb-ppts. However, scant information concerning the concentration levels of STXs and TTXs in seawater can be expected. Few methodologies have been developed for the detection of

these compounds in seawater, nevertheless, its application to the study of the levels in real ecosystems is almost inexistent, to the best of our knowledge. One of the few studies in which STX is determined in seawater is by Lefebvre et al., who quantified STX at concentrations of µg/L in samples that were collected from Sequim Bay, Washington , USA [340]. Thus, our LC-MS method should be sensitive enough to detect and quantify STX at this level of concentration.

5. CONCLUSIONS

The final conclusions of the developed work during this thesis research are listened as follows:

1) The development of three analytical methodologies for the detection and quantification of MBTs in seawater have been carried out during this thesis research by using HPLC-HRMS techniques. The sample pre-treatment included a filtration of the seawater through a micrometer mesh filter and the particulate and filtrate fraction were extracted and analysed separately. Extraction of the toxins from the particulate fraction was accomplished by UAE and from the filtrate fraction by SPE, which permitted the removal of interferences and the concentration of the analytes. Due to the high sensitivity and selectivity that the methods provide, these can be used as early warning tools for the unequivocal detection and quantification of MBTs. Moreover, the developed HPLC-HRMS methods permit the quantitative analysis of the target MBTs and the screening and characterisation of new MBTs analogues or other pollutants occurring in the same samples via retrospective non-target analysis.
   The developed methods are:

- Polyketide MBTS. The proper separation of these lipophilic MBTs was carried out via RPLC. OA, DTX-1, PTX-2, AZA-1, AZA-2, AZA-3, AZA-4, AZA-5, YTX and hYTX were successfully isolated from particulate via UAE and from filtrate fraction by SPE using HLB mode cartridges. The method showed good performance and high sensitivity, with LODs reaching pg/L.

- Alkaloid MBTs. The use of HILIC was suitable for the separation of most polar MBTs that were nor even retained in the chromatographic column in RPLC. The extraction of STX, dcSTX, NeoSTX, GTX-2,3 and TTX from the particulate and filtrate fraction was carried out via UAE and SPE employing silica cartridges, respectively. However, recovery values were quite low due to the difficulties in extracting these polar compounds from seawater. But, the repeatability was good, hence the recoveries were considered to be acceptable.

- Excitatory amino acid MBT. DA was analysed by both HILIC and RPLC, however, and better resolution was achieved with HILIC. The extraction process was accomplished by using SPE with HLB cartridges that allowed the concentration of the compound to be 2000 times. Due to the DA versatility, this compound could be incorporated into the instrumental analysis of the polyketides or alkaloids MBTs

2) The occurrence of MBTs in seawater from the Mediterranean Sea has been assessed by the application of the developed HPLC-HRMS methodologies. From the different studies carried out in the coastal waters of the Catalan littoral, the wetland of the Ebro Delta and the marine lagoon of Mar Menor in Murcia, both located in Spain.

- Lipophilic MBTs were analysed along the Catalan littoral, OA, DTX-1, PTX-2, AZA-1,2,3,4,5, YTX and hYTX. The samples were collected from inside marinas and open coastal areas. Only OA was present in a frequency of the 88 %. The levels were in agreement with similar studies, within which the presence of lipophilic MBTs was determined, but at a higher range of concentrations. Total concentration of OA in seawater ranged from 2 ng/L to 9.5 µg/L taking into account the particulate and the filtrate fractions. There were no significant differences related to the occurrence of OA inside marinas and open beaches.

- Seasonal variability of DA was studied in the Ebro Delta wetland during three seasons. Sixty-five percent (65%) of the samples contained DA in concentrations ranging from 0.9 to 69.6 ng/L. The highest concentrations were found during the summer months in Alfacs Bay.

- Hydrophilic MBTs were analysed in surface seawater samples from Mar Menor in Murcia, Spain, including Neo, dcSTX, TTX, GRX-2,3 and STX. The sampling was carried out during two seasons along the edge of the lagoon. None of the toxins were detected in the samples.

- Physico-chemical parameters of seawater were measured during the sampling campaigns in order to find relations with the MBTs occurrence. Temperature, salinity, dissolved oxygen, and pH were measured *in situ* during sampling and concentrations of nitrates, phosphates and chlorophyll were determined in the laboratory for all of the samples that were collected in the Ebro Delta. No relations were directly associated between the environmental parameters and the presence of OA or DA.

3) The application of an LC-MS technique for the analysis of MBTs in seawater has been successfully conducted to validate a multianalyte SPE-ELISA. The immunoassay was capable of detecting and quantifying five pollutants in seawater, including DA. The ELISA showed good agreement related to LC-MS, high sensitivity, high specificity for DA without cross-reactivity with other structurally similar compounds and the matrix effect was not observed.

- The proposed ELISA method is a valuable alternative for the rapid screening of DA in seawater and the other four pollutants. The high sensitivity, with a LOD of 1.39 ng/L and the high specificity that the assay provides, make it a powerful tool for the rapid analysis of the early warning signs of DA presence into the environment. The incorporation of other MBTs could implement the monitoring of more hazardous compounds.

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