

Marisa Pinto da Silva

Emergent toxins in North Atlantic temperate waters and New Vectors

Supervisor: Vitor Vasconcelos

Co-supervisor: Luis M. Botana



Dissertation presented to the Faculty of Sciences from the University of Porto to obtain the PhD in Biology

Dissertação apresentada à Faculdade de Ciências da Universidade do Porto para obtenção do grau de Doutor em Biologia

Porto, 2015

I dedicate this work to my grandparents Alberto e Glória Pinto, Alfredo Torrão and to my uncle Manuel Pinto.

Acknowledgements

There once was a girl, who carried by her father to dive in the tip of Sagres, had her first encounter with a small octopus, which her father had put upon her left arm. Where it rested briefly, it let her caress him and slowly resumed its journey, slipping into the seabed. Thus was born a passion in itself has latent, genetically induced in family ties.

Later, marked by this event, there is the dream of studying "an extremely poisonous little octopus", the blue-ringed octopus, making a bridge with an interest in the handling and investigation of toxins.

First I would like to thank my advisor, Professor Vitor Vasconcelos, for believing in me and for his trust. Thank you for encouraging me always and ever-present stimulus to scientific growth;

I would like to thank my co-advisor, Professor Luis Botana, for the always warm welcome in his team that I proudly was part of, for his prompt availability and encouragement;

Blue Biotechnology and Ecotoxicology Laboratory, Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, and Departamento de Farmacologia and Departamento de Química Analítica, Universidade de Santiago de Compostela for the installation and equipment facilities;

Professor Ana Botana for orientation, pleasing conversations and incentive to move forward;

Professor Amparo Alfonso, for enthusiasm, guidance and availability;

Fundação para a Ciência e Tecnologia (FCT), through my PhD fellowship

(SFRH/BD/73269/2010) and by funds under INTERREG IV projects Atlantox and Pharmatlantic

Project MARBIOTECH (reference NORTE-07-0124-FEDER-000047) within the SR & TD Integrated Program MARVALOR—Building research and innovation capacity for improved management and valorization of marine resources, supported by the Programa Operacional Regional do Norte (ON.2—O Novo Norte) and NOVOMAR through the European Regional Development Fund, co-financed by POPH/FSE, and the European Regional

Development Fund (ERDF) through the COMPETE – Operational Competitiveness

Programme under the project PEst-C/MAR/LA0015/2013;

All my colleagues in BBE, in Departamento de Farmacologia and in Departamento de Química analítica, especially to: Joana Martins, Ana Regueiras, José Carlos Martins, Cristiana Moreira, Rui Borges, Cidália Gomes, Aldo Barreiro, Verónica Rey, Maria Fraga, Silvia Ascariz, Paula Rodriguez, Paz Otero, Sheila Lopez, Andrea Fernandez thank you for all your support, great exchange of ideas, great companionship and friendship.

I especially thank my parents, thank you for your love, for your advice, because you lived it all with me and for being my biggest supporters and role models.

A special thanks to all my closest family and friends, especially to Joana Capelo, Susana Correia, Mina Fonseca and Marco Silva, you make everything better and I bring you always with me, in my heart. Last but not least, I thank Joana Saiote for all support and challenge to new experiences, you did the final step of this journey much easier to overcome.

Previous note

This thesis, was made with paragraph 2 of Article 4 of the General Regulation of Third Cycle Studies, University of Porto and Article 31 of Decree 74/2006, of March 24, with new wording introduced by Decree 230 / 2009 of September 14, the total utilization of a coherent set of research papers already published or submitted for publication in international journals indexed and peer review, which comprise some of the chapters of this thesis was made. Given that such work was done in collaboration with other authors, the candidate clarifies that, in all of them actively participated in its design, obtaining, analysis and discussion of results, as well as in preparing its published form. The presented study was carried out CIIMAR (Interdisciplinary Centre for Marine and Environmental Research), specifically in Blue Biotechnology and Ecotoxicology (BBE), in Departamento de Farmacologia and Departamento de Quimica Analítica of the University of Santiago de Compostela.

List of Published Papers

Articles published in peer-reviewed scientific journals, resulting from the present work:

Silva M., Azevedo J., Rodriguez P., Alfonso A., Botana L.M., Vasconcelos V. New Gastropod Vectors and Tetrodotoxin Potential Expansion in Temperate Waters of the Atlantic Ocean. *Marine Drugs*. 2012; 10(4):712-726.

Silva, M.; Barreiro, A.; Rodriguez, P.; Otero, P.; Azevedo, J.; Alfonso, A.; Botana, L. M.; Vasconcelos, V. New Invertebrate Vectors for PST, Spirolides and Okadaic Acid in the North Atlantic. *Marine Drugs* 2013, 11, 1936-1960.

Silva, M.; Pratheepa, V.K.; Botana, L.M.; Vasconcelos, V. Emergent Toxins in North Atlantic Temperate Waters: A Challenge for Monitoring Programs and Legislation. *Toxins* 2015, 7, 859-885.

Silva, M., Rodriguez, I., Barreiro, A., Kaufmann, M., Neto, A., Hassouani, M., Sabour, B., Alfonso, A., Botana, L., Vasconcelos, V. First Report of Ciguatoxins in two starfish species: *Ophidiaster ophidianus* and *Marthasterias glacialis*. *Toxins* 2015, 7, 3740-3757.

List of Manuscripts Submitted

Articles submitted to peer-reviewed scientific journals, resulting from the present work:

Silva, M., Rey, V., Botana, A. M., Vasconcelos, V. and Botana, L.M. Determination of gonyautoxin-4 in echinoderms and gastropod matrices by conversion to neosaxitoxin using 2-mercaptoethanol and post-column oxidation liquid chromatography with fluorescence detection.

Silva, M., Rodriguez, I., Barreiro, A., Kaufmann, M., Neto, A., Hassouani, M., Sabour, B., Alfonso, A., Botana, L., Vasconcelos, V. New invertebrate vectors of Okadaic acid from the North Atlantic Waters - Portugal (Azores and Madeira) and Morocco.

This work was supported by the Foundation for Science and Technology (FCT) through the award of doctoral fellowship (SFRH/BD/73269/2010).

Sumário

As alterações climáticas em conjunto com a influência antropogénica promoveram nos últimos anos o aparecimento de toxinas emergentes, típicas de águas mais quentes em ecossistemas mais temperados. Estas biotoxinas, representam um desafio, tanto em termos de legislação como em termos de monitorização, uma vez que o conhecimento é limitado, sendo pertinente a otimização de métodos de análise e de amostragem. Os planos de monitorização de toxinas já legisladas na União Europeia demonstram que os incidentes de envenenamento podem ser minimizados e até evitados e quando acontecem geralmente é por desrespeito das proibições de apanha de moluscos bivalves vivos ou por ingestão de organismos recolhidos pelos próprios consumidores. No que toca às toxinas emergentes, este problema está acentuado, devido à inexistência de antídotos, e falta de conhecimento por parte dos profissionais de saúde, relativamente à sintomatologia provocada por ingestão destas toxinas.

Neste trabalho, a costa Portuguesa, as ilhas da Madeira, São Miguel nos Açores e a costa noroeste de Marrocos foram rastreadas para diferentes tipos de toxinas emergentes (Tetrodotoxinas, Ciguatoxinas, Iminas Cíclicas) e legisladas (Saxitoxina e Ácido Ocadaico). Estas biotoxinas foram analisadas com o auxílio de diferentes técnicas cromatográficas, em 33 espécies diferentes de organismos bentónicos (bivalves, gastrópodes, equinodermes, crustáceos) e peixes com o objetivo de procurar novos vetores destas fitotoxinas, e tentar perceber a sua dinâmica ao longo da cadeia alimentar. Outro objetivo importante, prende-se com a otimização de protocolos de extração e análise destas toxinas, pois uma deteção, rápida, fiável e económica.

Reportamos 29 novos vetores detetados nos diferentes grupos de toxinas analisados, havendo evidências de bioacumulação ao longo da cadeia alimentar para Ácido Ocadaico e Iminas Cíclicas, e também pela primeira vez foram detetadas Ciguatoxinas em equinodermes, o ponto mais a norte de deteção de Tetrodotoxina, (Angeiras, Matosinhos) e a primeira deteção da presença de Iminas cíclicas na costa Portuguesa.

Considerando todos os resultados, podemos afirmar, que a monitorização de biotoxinas baseada apenas em bivalves é redutora, uma vez que nem todos os produtores, se encontram em suspensão na coluna de água. Novos vetores, muitos deles comestíveis são reportados neste trabalho, sugerindo que o risco para a saúde pública está subavaliado. Esperamos que o nosso contributo, seja o ponto de partida para o aprofundamento desta temática na nossa costa, e que sirva de impulsionador para a continuidade deste tipo de estudos para melhor proteger a população.

Palavras-chave: Toxinas Emergentes; Toxinas Legisladas; Novos Vetores; Monitorização; Saúde Pública; Atlântico Norte.

Summary

Climate change together with anthropogenic influence promoted in the recent years the appearance of emerging toxins, typical of warmer waters in more temperate ecosystems. These biotoxins, represent a challenge in terms of both legislation and monitoring, since not much known about them, being relevant the optimization of both analysis and sampling methods. Monitoring plans of legislated toxins in the European Union show that poisoning incidents can be minimized and even avoided. Poisoning incidents generally happen when infringing the prohibition of live bivalve mollusks or by consumption of organisms directly collected by consumers. Regarding emerging toxins this problem is highlighted by the absence of antidotes and lack of knowledge from the health professionals regarding the symptoms caused by ingestion of these toxins.

In this work, the Portuguese continental coast, the islands of Madeira, São Miguel in the Azores and northwest coast of Morocco were screened for different types of emerging toxins (Tetrodotoxins, Ciguatoxins, Cyclic Imines) and those legislated (Saxitoxin and Okadaic Acid). These biotoxins were analyzed using several chromatographic techniques, in 33 different species of benthic organisms (bivalves, gastropods, echinoderms, crustaceans) and fish in order to seek for new vectors for these phycotoxins, and try to understand their dynamics along the food chain. Another important objective is the optimization of protocols for extraction and analysis of these toxins using fast, reliable and economical methods.

We reported 29 new vectors in different groups of analyzed toxins, with evidences of bioaccumulation in the food chain of okadaic acid and cyclic Imines. We also reported for the first time Ciguatoxins in echinoderms, the most northerly point of Tetrodotoxin detection (Angeiras, Matosinhos) and the detection of the presence of Cyclic imines for the first time in the Portuguese Coast.

Considering all the results we can say that monitoring of biotoxins in bivalves alone is reductive, since not all toxin producers are in suspension in the water column. New vectors are reported in this study, many of them are edible, suggesting that the risk to public health is undervalued. We hope that our contribution is the starting point for the further development of this issue on our coast and to serve as an incentive for the continuation of this type of studies to better protect the population.

Keywords: Emerging toxins; Legislated Toxins; New Vectors ; Monitoring; Public Health; North Atlantic.

Resumo

O cambio climático xunto coa influencia antropoxénica promoveron nos últimos anos a aparición de toxinas emerxentes, típicas de augas máis quentes propias de ecosistemas máis mornos. Estas biotoxinas, representan un desafío tanto en termos de lexislación como en termos de monitorización, posto que pouco se sabe sobre elas, sendo pertinente a optimización de métodos de análise como de mostraxe. Plans de seguimento de toxinas xa lexisladas na Unión Europea, demostran que os incidentes de envenenamento poden ser minimizados e ata evitados. Cando acontecen xeralmente é por non respetar as prohibicións de recolla de moluscos bivalvos vivos ou por consumo por motu propio. No que toca ás toxinas emerxentes este problema está acentuado debido á inexistencia de antidotos e falta de coñecemento por parte dos profesionais da saúde en relación coa sintomatoloxía provocada por inxestión destas toxinas.

Neste traballo, a costa Portuguesa, as illas de Madeira, San Miguel nas Azores e a costa noroeste de Marrocos foron rastrexadas para distintos tipos de toxinas emerxentes (Tetrodotoxinas, Ciguatoxinas, Iminas cíclicas) e lexisladas (Saxitoxina e Ácido Ocadaico). Estas biotoxinas foron analizadas mediante diferentes técnicas cromatográficas, en 33 especies diferentes de organismos bentónicos (bivalvos, gasterópodos, equinodermos, crustáceos e peixes) co obxectivo de atopar novos vectores de fitotoxinas, e intentar entender a dinámica ao longo da cadea alimentaria. Outro obxectivo importante foi a optimización dos protocolos de extracción e análise destas toxinas, pois unha detección, rápida, fiable e económica é crucial.

Detectamos 29 novos vectores nos distintos grupos de toxinas analizados, habendo evidencias de bioacumulación ao longo da cadea alimentaria para Ácido Ocadaico e Iminas cíclicas. Informamos tamén por primeira vez da presenza de Ciguatoxinas en equinodermos, do punto máis ao norte de detección de tetrodotoxina (Angeiras, Matosinhos) e da presenza de Iminas cíclicas na costa Portuguesa por primeira vez.

Considerando todos os resultados podemos afirmar que a monitorización de biotoxinas baseada só en bivalvos é escasa, xa que non todos os produtores se atopan en suspensión na columna de auga aberta. Novos vectores, moitos deles comestibles, son reportados neste traballo, o que suxire que o risco para a saúde pública está subestimado. Esperamos que a nosa contribución sexa o punto de partida para a profundización nesta temática na nosa costa e que sirva de impulsor para a continuidade deste tipo de estudos para mellorar a protección da poboación.

Palabras clave: Toxinas Emerxentes; Toxinas Lexisladas; Novos Vectores; Monitorización; Saúde Pública; Atlántico Norte.

Index

Contents

Acknowledgements	VI
Previous note	IV
Sumário	IV
Summary	III
Resumo	III
Index.....	III
Contents	III
Table Index	III
Figure Index.....	III
Abbreviations and Symbols.....	III
First Section	1
Introduction – Thesis structure.....	1
<i>Toxins</i> 2015, 7, 859-885.....	1
Emergent Toxins in North Atlantic Temperate Waters: A Challenge for Monitoring Programs and Legislation	1
Introduction.....	2
Analytical Methods.....	10
Monitoring and Legislation Challenges.....	11
Conclusions	15
Acknowledgments.....	16
Author Contributions	16
Conflicts of Interest	16
References	16
Objectives	36
Second Section.....	37
Abstract.....	38

Introduction	39
Results and Discussion	40
Experimental Section	45
Conclusions	48
Acknowledgements	48
Author Contributions	48
Conflicts of Interest	48
References	49
<i>Marine Drugs</i> 2012, 10, 712-726	52
New Gastropod Vectors and Tetrodotoxin Potential Expansion in Temperate Waters of the Atlantic Ocean.....	52
Introduction	53
Results and Discussion	53
Experimental Section	60
Conclusions	63
Acknowledgments	64
References	64
<i>Marine Drugs</i> 2013, 11, 1936-1960	71
New Invertebrate Vectors for PST, Spirolides and Okadaic Acid in the North Atlantic	71
Introduction	72
Results and Discussion	74
Experimental Section	82
Conclusions	91
Acknowledgments	91
References	91
New Invertebrate Vectors of Okadaic Acid from the North Atlantic Waters - Portugal (Azores and Madeira) and Morocco.....	100
Introduction	101
Results and Discussion	104
Experimental Section	107
Conclusions	110

Acknowledgments.....	110
Author Contributions	111
Conflict of Interest.....	111
References and Notes	111
<i>Toxins</i> 2015, 7, 3740-3757	116
First Report of CIGUATOXINS in two starfish species: <i>Ophidiaster ophidianus</i> and <i>Marthasterias glacialis</i>	116
Introduction.....	117
Results and Discussion.....	119
Experimental Section	126
Conclusions	130
Acknowledgments.....	130
Author Contributions	131
Conflicts of Interest	131
References and Notes	131
Third Section.....	137
General discussion.....	138
References	142
General Conclusions	145

Table Index

Table1.1 Detection of emergent poisoning incidents in the Mediterranean Sea and North Atlantic Ocean.	9
Table1.2 Limit of detection/quantification of Emergent toxins (CTX, PTX, CI and TTX) by using different methods. (LOD—Limit of Detection, LOQ—Limit of Quantification, LD50—Lethal Dose 50%, SM—Shellfish Meat, mL—Milli liter, kg—Kilogram, µg—Microgram, ng—Nanogram	12
Table1.3 Summary of information of current and emergent toxins regarding: acute reference dosage (ARfD), median lethal dosage (LD50), legal limits in the European Union (EU) and standard availability, and comparison with regulated toxins PSP's and OA group (DSP'	14
Table2.1 . % recovery of NEO after reaction of GTX1 and GTX4 with 2-ME 1M in phosphate buffer 0.1M, pH 7.4 (average n=3, and RSD %).	41
Table2.2 Tested samples and results before the reaction with 2-ME.	43
Table2.3 - TTX and analogues levels (µg/g) in marine gastropods from Portugal (pw-present work and [21]), China and Taiwan.	58
Table2.4 - Average number of specimens to set a composed sample and number of samples collected since July 2009 until the end of 2010. Availability of animals is dependent on their geographical distribution and ecology.	60
Table2.5 - Average number of specimens to set a composed sample and number of samples collected since July 2009 until the end of 2010. Availability of animals is dependent on their geographical distribution and ecology.	73
Table2.6 - Species' OA concentration pairwise comparisons using post hoc Tukey test.	79
Table2.7 - Species' SPX concentration pairwise comparisons using post hoc Tukey test.	81
Table2.8 - Pairwise comparisons between factor levels of sampling site and species, using the three groups of toxins as dependent factors.	82
Table2.9 - Average number of specimens comprising a pooled sample and number of samples collected since July 2009 till 2010. Availability of animals is dependent on their geographical distribution and ecology.	83
Table2.10 - Limits of detection (LODs) and quantification (LOQs) of the method for each toxin (expressed in ng/mL).	88
Table2.11 - Toxic equivalent factors for OA and its analogs.	102
Table2.12 - Species sampled and their trophic level, average number of specimens comprising a pooled sample (AvNr) and number of samples collected (NrPSamples) on Madeira Island in September 2012, São Miguel Island, Azores, in June 2013 and Morocco in July 2013,	103
Table2.13 - Results of the binomial regression model for OA occurrence with “organism” as factor.	106
Table2.14 - Results of the gamma regression model for OA occurrence with organism as factor. ...	106

Table2.15 - Species sampled and their trophic level, average number of specimens comprising a pooled sample (AvNr) and number of samples collected (NrP Samples) on Madeira Island in September 2012, São Miguel Island, Azores, in June 2013 and Morocco in July 2013 and their edibility. Availability of animals is dependent on their geographical distribution and ecology.	120
Table2.16 - Screened CTX's UPLC-MS in Positive Mode.	120
Table2.17 - Presumed CTX detected in UPLC-MS (FW-Fresh weight).	124
Table2.18 - Results of the gamma hurdle model for CTX occurrence with sampling site as factor. .	126
Table2.19 - Results of the gamma hurdle model for CTX occurrence with starfish species as factor.	126
Table2.20 - Sampling Sites and respective geographical coordinates, surveyed during September of 2012 and June and July of 2013.	127
Table 3.1. Tested species versus detected toxins (TTX- Tetrodotoxin; PSTs – Paralytic Shellfish toxins; OA- Okadaic Acid; CI – Cyclic Imines; CTX- Ciguatoxins).....	138

Figure Index

Figure 1.1 -Tetrodotoxin (TTX) structure modified from Noguchi 2008 [55].	4
Figure 1.2 Palytoxin structure modified from Ramos and Vasconcelos 2010 [66].	5
Figure 1.3 Structures Caribbean (C) and Pacific (P) CTX-group toxin. The energetically less favored epimers, P-CTX-2 (52-epi P-CTX-3), P-CTX-4A (52-epi P-CTX-4B) and C-CTX-2 (56-epi C-CTX-1) are indicated in parenthesis. Modified image from Lewis, 2001 [81]. Copyri.....	6
Figure 1.4 Gymnodimines (GYM) structure modified form Cembella and Krock 2008 [123]. Copyright 2008, Taylor & Francis. Spirolide (SPX) structure modified from Otero et al. 2010 [124]. Copyright 2010, Wiley. Pinnatoxin (PnTX) structure modified from Selwood et al. 2	8
Figure 2.1 - PSTs elution pattern in spiked mussel with standards.....	40
Figure 2.2 -GTX1,4 (1.6 nmol) incubated with 1M 2-ME in 0.1M phosphate buffer, pH 7.4 during 30 minutes in water bath. a) Before incubation; b) After incubation.....	42
Figure 2.3 -a) Sample 477 (<i>S. haemostoma</i>) before transformation, b) sample 477 (<i>S. haemostoma</i>) after transformation.....	43
Figure 2.4 -a) Sample 454 (<i>Cerithium vulgatum</i>) before transformation, b) sample 454 (<i>Cerithium vulgatum</i>) after transformation.	43
Figure 2.5 - Location of the sampling points in the North Atlantic Portuguese coast: 1 Viana do Castelo; 2 Esposende; 3 Póvoa do Varzim; 4 Angeiras; 5 Memória; 6 Valadares; 7 Aguda; 8 São Martinho do Porto; 9 São Torpes; 10 Porto Côvo; 11 Monte Clérigos; 12 Vila Nova	54
Figure 2.6 - Mass chromatograms of the LC-ESI-CID-MS/MS obtained under MRM operation of the TTX standard and naturally-contaminated samples of <i>Charonia lampas</i> and <i>Lagocephalus sceleratus</i> . (A) MRM of TTX standard (1500 ng/mL), m/z 320 > 302; (B) Extracted Ion Chrom.....	55
Figure 2.7 - Mass chromatograms of the UPLC-MS/MS obtained under MRM operation of the TTX standard. TIC (total ion chromatogram). TTX standard 1000 ng/mL, m/z 320 > 302/162.....	56
Figure 2.8 - Mass chromatograms of the LC-ESI-CID-MS/MS obtained under MRM operation of the positive sample of <i>Gibbula umbilicalis</i> for the analogue monodeoxyTTX (m/z 304 > 286/176). XIC extracted ions chromatogram.....	57
Figure 2.9 - Mass chromatograms of the UPLC-MS/MS obtained under MRM operation of the positive samples of <i>Monodonta lineata</i> and <i>C. lampas</i> for (A): TTX (m/z 320 > 162/302), 4-epiTTX (m/z 320 > 162/302); and (B): 5,6,11-trideoxyTTX (m/z 272 > 254/162).....	57
Figure 2.10 - Paralytic shellfish toxin (PST) content in saxitoxin (STX) 2 HCl equivalent ng/g fresh weight (fw) in all groups: (A) bivalves— <i>Mytilus galloprovincialis</i> ; (B) echinoderms— <i>Marthasterias glacialis</i> and <i>Paracentrotus lividus</i> ; (C) gastropods— <i>Nucella lapill</i>	75

Figure 2.11 - Box and whisker plots for PST concentrations [SXT HCl 2 equivalents (ng/g....fw)] found in each species (C.la.- C. lampas; G. um.- G. umbilicalis; M.ga.- M. galloprovincialis; M. gl.- M. glacialis; M. li.- M. lineata; M. tu.- M. turbinata;	76
Figure 2.12 - PST profiles in each group screened.....	76
Figure 2.13 - Okadaic acid (OA) positive results (ng/g fw) for all sampled groups of organisms: (A) bivalves; (B) echinoderms; (C) gastropods. Y-axis in logarithmic scale. Limit value (LV) established in Europe is 160 ng OA equivalents (eq)/g shellfish meat (s.m.).	78
Figure 2.14 - Box and whisker plots of OA concentrations (ng/g fw) found in each species (C. la.- C. lampas; G. um.- G. umbilicalis; M. ga.- M. galloprovincialis; M. gl.- M. glacialis; M. li.- M. lineata; M. tu.- M. turbinata; N. la.- N. lapillus; P. in.- P. interm.....	79
Figure 2.15 - Trophic relation between M. galloprovincialis, N. lapillus and M. glacialis.....	80
Figure 2.16 - SPX positive results (ng/g fw) for all sampled groups of organisms.....	80
Figure 2.17 - Box and whisker plots of SPX concentration (ng/g fw) found in each species (C. la.- C. lampas; G. um.- G. umbilicalis; M. ga.- M. galloprovincialis; M. gl.- M. glacialis; M. li.- M. lineata; M. tu.- M. turbinata; N. la.- N. lapillus; P. in.- P. interm.....	81
Figure 2.18 - Location of the sampling points on the Atlantic Portuguese coast: 1: Viana do Castelo; 2: Esposende; 3: Póvoa do Varzim; 4: Angeiras; 5: Memória; 6: Valadares; 7: Aguda; 8: São Martinho do Porto; 9: São Torpes; 10: Porto Côvo; 11: Monte Clérigos; 12:	83
Figure 2.19 - Chromatograms of the PSTs standards and a positive sample for <i>M. glacialis</i> , hydroxylated toxins are oxidized with periodate and non-hydroxylated toxins with peroxide. (A) Standards injected with periodate oxidation, peak GTX _{1,4} (1) and GTX _{1,4} (3) are secondary oxidation products of GTX _{1,4} . Peaks NEO(1) and NEO(3) are secondary oxidation products of NEO. (B) Standards injected with peroxide oxidation. (C) Chromatogram of a positive sample in <i>M. glacialis</i> injected with periodate oxidation. (D) Chromatogram of a positive sample in <i>M. glacialis</i> injected with peroxide oxidation....	87
Figure 2.20 -Mass chromatograms of the LC-ESI-CID-MS/MS obtained under multiplex reaction monitoring (MRM): (A) extracted ion chromatogram (XIC) of OA standard (200 ng/mL), <i>m/z</i> 803.5 > 255.5/113.5; (B) XIC of 13-desmethyl SPX C standard (200 ng/mL), <i>m/z</i> 692.4 > 674.4/444.4; (C) XIC of a positive sample for OA in <i>N. lapillus</i> (<i>m/z</i> 803.5 > 255.5/113.5); (D) XIC of a positive sample for 13-desmethyl SPX C in <i>N. lapillus</i> (<i>m/z</i> 692.4 > 674.4/444.4).	89
Figure 2.21 - Mass chromatograms of the UPLC-MS/MS obtained under MRM operation: (A) total ion chromatogram (TIC) of OA standard 200 ng/mL, <i>m/z</i> 803.5 > 255.5/113.5; (B) TIC of a positive sample for OA in <i>M. glacialis</i> , <i>m/z</i> 803.5 > 255.5/113.5	90
Figure 2.22 - Chemical structures of OA, DTX1&2.	101
Figure 2.23 - Location of the sampling points: (A) São Miguel island coast, Azores archipelago: 1- Cruzeiro; 2- Mosteiros; 3- Étar; 4- São Roque; 5- Lagoa; 6- Caloura; (B) Madeira island coast: 1- Reis Magos; 2- Caniçal; (C)) Northwestern Moroccan coast: 1- Casablanca Corniche; 2- El Jadida Haras; 3- El Jadida Sâada; 4- Sidi Bouzid; 5- Mrizika; 6- Oualidia.....	104

Figure 2.24 - OA ($\mu\text{g}/\text{Kg}$ fw) for all sampled groups of organisms in Azores, Madeira and Morocco. Numbers correspond to different sampling locations in Morocco: 1- Casablanca Corniche; 2- Sidi Bouzid; 3- El Jadida Saâda; 4- Mrizika; 5- Oualidia.....	105
Figure 2.25 -Mass chromatograms of the UPLC- MS/MS obtained under multiple reaction monitoring (MRM) in negative mode. (A) Total ion chromatogram (TIC) of OA standard (100 ng/mL), m/z 803.5 > 255.2/113.2, DTX2 standard (100 ng/mL), m/z 803.5 > 255.5/113.5 and DTX1 standard (100 ng/mL), m/z 817.5 > 255.2/113. (B) TIC of a sample with quantifiable OA contents in <i>Mytilus</i> spp. (m/z 803.5 > 255.5/113.5).....	109
Figure 2.26 - Structures Caribbean (C) and Pacific (P) CTX-group toxin. The epimers, P-CTX-2 (52-epi P-CTX-3), P-CTX-4A (52-epi P-CTX-4B) and C-CTX-2 (56-epi C-CTX-1) are indicated in parenthesis.	118
Figure 2.27 - Location of the sampling points: (A) São Miguel island coast, Azores archipelago: 1, Cruzeiro; 2, Mosteiros; 3, Étar; 4, São Roque; 5, Lagoa; and 6, Caloura. (B) Madeira island coast: 1, Reis Magos and 2, Caniçal. (C) Northwestern Moroccan coast: 1, Casablanca Corniche; 2, El Jadida Haras; 3, El Jadida Sâada; 4, Sidi Bouzid; 5, Mrizika; and 6, Oualidia..	119
Figure 2.28 - Selected Ion Monitoring (SIM) chromatogram (A) and mass spectrum (B) of standard CTX-3C. SIM obtained by UPLC-MS/MS and spectrum obtained by UPLC-MS-IT-TOF.	122
Figure 2.29 - MS2 spectrum of CTX analogue at m/z 1111.5 $[\text{M}^+\text{H}]^+$ (A); CTX analogue at m/z 1109.5 $[\text{M}^+\text{H}]^+$ (B); and CTX analogue m/z 1123.5 $[\text{M}^+\text{H}]^+$ (C). Spectra obtained by Ultra Performance Liquid Chromatography-Mass Spectrometry-Ion Trap-Time of Flight UPLC-MS-IT-TOF.....	124
Figure 2.30 - CTX purification scheme.	128

Abbreviations and Symbols

2-ME	2-mercaptoethanol
AOAC	Association of Official Analytical Chemists
ARfD	Acute Reference Dose
ASP	Amnesic Shellfish Poisoning
C-CTX	Caribbean-Ciguatoxin
CI	Cyclic Imines
CIEIA	Competitive Inhibition Enzyme Immunoassay
CID	Collision-induced Dissociation
CTX	Ciguatoxin
dc	decarbamoyl
DSP	Diarrheic Shellfish Poisoning
DSTs	Diarrheic Shellfish Toxins
DTX	Dinophysis Toxin
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electrospray Ionization
EU	European Union
EURLMB	European Union Reference Laboratory for Marine Biotoxins
FAO	Food and Agricultural Organization of the United Nations
FLD	Fluorimetric detection
FW	Fresh weight
GC	Gas Chromatography
GSH	Gluthione
GTX	Gonyotoxin
GYMs	Gymnodimines
HABs	Harmfull Algal Blooms
HCl	Hydrochloridric Acid
HPLC	High Performance Liquid Chromatography
I-CTX	Indian-Ciguatoxin
IOC	Intergovernmental Oceanographic Commission of UNESCO
i.p.	Intraperitoneal injection
IT	Ion Trap
LC	Liquid Chromatography
LD ₅₀	Lethal Dose 50%,
LDH	Lactate Dehydrogenase

LV	Limit value
LOD	Limit of Detection
LOEL	Lowest Observed Effect Level
LOQ	Limit of Quantification
mAChR	Muscarinic acetylcholine receptors
MIA	Immunobead Assay
MU	Mouse Unit
MBA	Mouse Bioassay
MBMP	Marine Biotoxin Management Plan
monodeoxyTTX	monodeoxyTetrodotoxin
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular Weight
nAChR	Nicotinic Acetylcholine Receptors
Nav	Voltage Gated Sodium Channels
NEO	Neosaxitoxin
NMR	Nuclear magnetic resonance
NRC	National Research Council
NRL	National Reference Laboratories
OA	Okadaic Acid
P-CTX	Pacific Ocean-Ciguatoxin
PnTX	Pinnatoxins
PSP	Paralytic Shellfish Poisoning
PSTs	Paralytic Shellfish Toxins
PtTX	Pteriatoxins
PTX	Palytoxin
RBA	Receptor Binding Assays
RT	Retention Time
SIM	Selected Ion Monitoring
SM	Shellfish Meat
SPE	Solid Phase Extraction
SPR	Surface Plasmon Resonance
SPX	Spirolide
SXT	Saxitoxin
TCA	Trichloroacetic Acid
TEFs	Toxicity Equivalence Factors
TIC	Total Ion Chromatogram
TOF	Time-of-flight Mass Spectrometry

TTX	Tetrodotoxin
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet detection
WHO	World Health Organization
XIC	Extracted Ion Chromatogram

First Section

Chapter 1. Introduction

Thesys Structure

Paper 1

- Emergent Toxins in North Atlantic Temperate Waters: A Challenge for Monitoring Programs and Legislation
- Published: Toxins2015, 7: 859-885.

Chapter 2. Objectives

Introduction – Thesis structure

This thesis began under the project ATLANTOX, whose main aim was the screening of new toxins in the North Atlantic and the development of new analytical techniques. Climate change combined with anthropological intervention allowed the potential migration of toxins, considered restricted to tropical environments, to more temperate regions.

Benthic organisms are generally poorly studied regarding their role as potential toxins vectors, with exception of bivalves due to their economical relevance. Several species of benthos were surveyed in this study, along the Portuguese coast, Azores and Madeira Islands and the Moroccan northwestern coast. Were screened: gastropods (sea-snails, sea-slugs and limpets), bivalves, echinoderms (sea-stars and sea-urchins), fish (pufferfish) and crustaceans (barnacles). The study of these organisms is pertinent, since they are an important part of the food-chain that tops up in humans, but also because they are good indicators of potential changes in the ecosystem. One of our main aims was to gain knowledge regarding the prevalence of these toxins in the Atlantic Ocean, believing that legislation could be revised and updated based on our results and since it is reductive to think that monitoring bivalves alone is enough for protecting public health. Due to intensive sampling and availability of technical resources and in order to maximize the study, was decided to widen the search when it comes to toxins groups screened,: Tetrodotoxin, Saxitoxin group (PSTs – Paralytic Shellfish Toxins), Okadaic Acid group (DSTs – Diarrheic Shellfish Toxins), Yessotoxins (YTXs), Azaspiracids (AZAs), from the Cyclic Imine group, Spirolides (SPX), Palytoxin (PLT) and the Ciguatoxin group (CTX).

With this, the prior objectives of the thesis had to be adapted, that will be described in the objectives section.

This work will be exposed in the format of compilation of papers, first with a review paper for contextualization of the study, followed by a paper on methodology optimization. The 3rd and 4th sections of the thesis are dedicated to monitoring work performed in the Portuguese coast, islands and northwestern Moroccan coast.

Toxins 2015, 7, 859-885

Emergent Toxins in North Atlantic Temperate Waters: A Challenge for Monitoring Programs and Legislation

Marisa Silva^{1,2}, **Vijaya K. Pratheepa**¹, **Luis M. Botana**³ and **Vitor Vasconcelos**^{1,2,*}

CIIMAR/CIMAR—Interdisciplinary Center of Marine and Environmental Research, University of Porto, Rua dos Bragas 289, Porto 4050-123, Portugal;

E-Mails: marisasilva17@gmail.com (M.S.); vpratheepa@gmail.com (V.K.P.)

Faculty of Sciences, University of Porto, Rua do Campo Alegre, Porto 4169-007, Portugal

Department of Pharmacology, Faculty of Veterinary, University of Santiago of Compostela, Lugo 27002, Spain; E-Mail: luis.botana@usc.es

Abstract: Harmful Algal Blooms (HAB) are complex to manage due to their intermittent nature and their severe impact on the economy and human health. The conditions which promote HAB have not yet been fully explained, though climate change and anthropogenic intervention are pointed as significant factors. The rise of water temperature, the opening of new sea canals and the introduction of ship ballast waters all contribute to the dispersion and establishment of toxin-producing invasive species that promote the settling of emergent toxins in the food-chain. Tetrodotoxin, ciguatoxin, palytoxin and cyclic imines are commonly reported in warm waters but have also caused poisoning incidents in temperate zones. There is evidence that monitoring for these toxins exclusively in bivalves is simplistic and underestimates the risk to public health, since new vectors have been reported for these toxins and as well for regulated toxins such as PSTs and DSTs. In order to avoid public health impacts, there is a need for adequate monitoring programs, a need for establishing appropriate legislation, and a need for optimizing effective methods of analysis. In this review, we will compile evidence concerning emergent marine toxins and provide data that may indicate the need to restructure the current monitoring programs of HAB.

Keywords: Emergent toxins; monitoring; new vectors; legislation

Introduction

1.1. Harmful Algal Blooms: General Description

Phytoplankton may develop blooms in marine coastal waters with seasonal, regional and species-specific features [1]. Several factors, which are not yet entirely understood, promote these blooms, but in recent decades these occurrences have tended to be more frequent, persistent and intense [2–6]. Climate change, eutrophication and cysts, together with alien species transported in ballast waters, are noted as important contributors [7]. Blooms can be classified as benign or harmful according to their impact on the ecosystem, on public health and on the economy. Benign algal blooms lead to an increase of primary producers boosting the richness of the ecosystem, whereas Harmful Algal Blooms (HAB) have adverse consequences [8,9]. So far, about 5000 species of phytoplankton have been distinguished, 300 of which form blooms, and are reported as toxic, noxious or as being a nuisance [1,10–12].

A phytoplankton bloom is a complex community that can be monospecific or composed of several different species [13]. In both cases, harmful species may or may not be present. Toxic blooms produce secondary metabolites that may help them outcompete similar species or have deleterious effects on predators [14–17]. These toxins can accumulate in the food-chain and cause poisoning incidents to humans through harvested shellfish or other seafood present in the bloom area [18,19]. HABs affect the fishing and aquaculture industries by causing high mortalities in fish and invertebrates through mechanical damage due to their spiny conformation or mucilage production, and by causing lesions or obstruction of the gills [20,21]. As an example, the diatoms *Chaetoceros concavicornis* and *C. convolutus*, can cause fish mortalities at the very low concentration of 5 cells/mL [22].

A bloom can also create anoxic zones when it is very extensive and enters into senescence, thereby causing mortalities or deviation of fish migration routes [23,24]. An example of this is in the Gulf of Mexico, where the Mississippi River delivers heavy loads of urban and agricultural runoff leading to an increase in nitrogen and phosphorus levels and fueling phytoplankton growth. This influx causes extensive blooms whose decomposition eliminates oxygen faster than it can be replaced thereby forming dead zones [24].

HABs may cause huge economic losses in the tourism sector even when blooms are not a risk for humans or other organisms by producing foams, mucilage, repellent odors or altering the water color [25–30]. They can also affect an entire ecosystem by creating regions of anoxia, causing death by mechanical block preventing micro invertebrates to feed, affecting the reproduction of predators, benthic anoxia, sea grass die-off, and the alteration of food web function [23,31–34].

Regarding public health, a need for guidelines led to the establishment of international regulations resulting in mandatory and frequent monitoring of the most common syndromes: Paralytic Shellfish Poisoning (PSP), Amnesic Shellfish Poisoning (ASP) and Diarrheic Shellfish Poisoning (DSP) [35–37].

Nowadays, owing to these regulations, the cases of human intoxications are sporadic and are mostly due to illegal harvest and/or confusion of toxic species with non-toxic ones, *i.e.*, failure of harvest and consumption prohibitions implemented by national health authorities [38,39].

The establishment of guideline values for marine toxins follows some procedures that take into account the toxicity values produced through laboratory assays, data on incidence, prevalence, seasonal variation and vectors of the toxin obtained through field work. The monitoring of biotoxins is usually evaluated through phytoplankton counting and the testing of bioaccumulation of toxins in bivalves. However, there can be a lack of important data since some toxins are produced by bacteria (Tetrodotoxin (TTX)) and others are produced by benthic dinoflagellates (Ciguatoxin (CTX), Palitoxin (PTX)). Moreover, vector species that are not normally monitored, such as gastropods, crustaceans and fish, should be included in risk assessments since other toxin uptake routes, apart from filter feeding, are present in marine ecosystems [40,41]. This risk analysis is the key for the proposal of new guideline values and this procedure has to be done in accordance with international guidelines and institutions, such as the European Union Reference Laboratory for Marine Biotoxins (EURLMB) and the European Food Safety Authority (EFSA), coordinated by United Nations Organizations, such as the Food and Agricultural Organization of the United Nations (FAO), Intergovernmental Oceanographic Commission of UNESCO (IOC) and the World Health Organization (WHO). The EURLMB coordinates the activities of a network of the National Reference Laboratories (NRL) which is established in each EU Member State, regarding the methodologies applied to control marine biotoxins in shellfish in order to protect public health and guarantee a maximum level of food safety. The EFSA is the keystone of European Union (EU) risk assessment regarding food and feed safety. In close collaboration with national authorities and in open consultation with its stakeholders, EFSA provides independent scientific advice and clear communication on existing and emerging risks.

In this review we explore the challenges of HABs, more specifically the problem of the emergent toxins, as evidence for their presence in temperate waters has become more substantive resulting in the need for new monitoring programs and the development of more sensitive and rapid analysis methods associated with a revised legislation in order to avoid social and economic consequences.

1.2. Emergent Toxins

There is urgency in the study of emergent toxins as the rise of water temperature, together with anthropogenic impacts, may allow for the dispersion and the establishment of new populations of highly toxic organisms [3,6,42–44]. These phycotoxins have many routes of uptake in humans, the most common one being via ingestion. The majority of these toxins are heat-stable, whereby cooking processes do not affect their structure or function. Dermal and respiratory exposure also has to be considered as some biotoxins can form aerosols. This is the case with PTX, which causes the development and aggravation of lung diseases affecting mainly coastal and fishermen populations [45–

48]. In the following paragraphs, we will describe the chemical structure of TTX, PTX, CTX and Ciclic Imines (CI), action modes and symptoms in humans.

1.2.1. Tetrodotoxin

TTX is a non-proteinaceous neurotoxin with a molecular weight of 319.3 Da. TTX was first isolated in 1950 by Yokoo as a crystalline prism, from the toxic puffer fish and named after the puffer fish family Tetraodontidae [49,50]. The structure of TTX (Figure 1.1), was identified after the independent findings by several researchers, namely Goto *et al.* (1965), Tsuda *et al.* (1964) and Woodward (1964) [51–53]. TTX is a colorless, crystalline-weak basic substance with a molecular formula of C₁₁H₁₇O₈N₃ and has 30 analogues or derivatives which have been separated from puffer fish, newts, frogs and other TTX bearing organisms [54]. It is found in phylogenetically different marine and terrestrial organisms from six different phyla [55]. The widespread occurrence indicates that the origin of TTX may be exogenous [56–58]. The structure of TTX is characterized by a positively charged guanidinium group and a pyrimidine ring that may help TTX to work as a specific blocker of voltage gated sodium channels. Intoxication of TTX occurs within hours and may progress from localized numbness at the mouth shortly after ingestion to vomiting, strong headache, muscle weakness, respiratory failure, hypotension and even death [59]. As there is no antidote available, the main objective is to keep the patient alive in the first 24 h after intoxication of TTX occurs, with ventilator and hemodynamic support, as well as the correction of any possible cardiac arrhythmias, resulting in the mandatory stay in an intensive care unit [59–61].

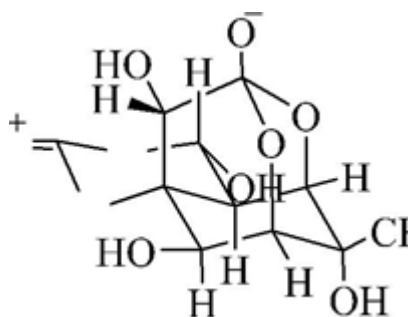


Figure 1.1 -Tetrodotoxin (TTX) structure modified from Noguchi 2008 [55].

1.2.2. Palytoxin

PTX is a non-proteinaceous marine toxin which is mainly produced by marine zoanthids (soft corals) of the genus *Palythoa* [62]. Initially they were found only in Hawaii and Japan but the occurrence of PTX and its analogues is reported worldwide [63–65]. PTX is also produced by dinoflagellates (*Ostreopsis* spp.) and found in other organisms, such as fish [66,67]. Its structure was first described in 1981 [63,68].

PTX has a polyketide structure (Figure 1.2) with both lipophilic and hydrophilic moieties. The general chemical formula of PTX is $C_{129}H_{233}N_3O_{54}$ consisting in a long, partially unsaturated aliphatic backbone, containing cyclic ethers, 64 chiral centers, 40-42 hydroxyl and 2 amide groups. Many different analogues of PTX, such as isobaric PTX, ostreocin-D, ovatoxin (a to f), mascarenotoxins, ostreotoxin-1 and 2, homopalytoxin, bishomopalytoxin, neopalytoxin, deopalytoxin and 42-hydroxypalytoxin are known and the molecular weights vary depending on the species from which they are produced, ranging from 2659 to 2680 Da [69–72]. PTX has ultraviolet absorption at a wavelength of 233 and 263 nm and is heat-stable [69,73]. Palytoxin causes intoxication called clupeotoxism due to the consumption of clupeoid fish, such as sardines, herrings and anchovies [74]. Symptoms of PTX-group toxins include vasoconstriction, hemorrhage, myalgia, ataxia, muscle weakness, ventricular fibrillation, ischemia and death [75,76]. Moreover, Rhabdomyolysis syndrome is pointed out as being the most commonly reported complication after a poisoning incident with PTX [77]. This life threatening condition consists of a loss of intracellular contents into the blood plasma, causing injury to the skeletal muscle, with the worst cases resulting in renal failure and disseminated cardiovascular coagulation. Staying well-hydrated is strongly advised for the prevention of this condition [78].

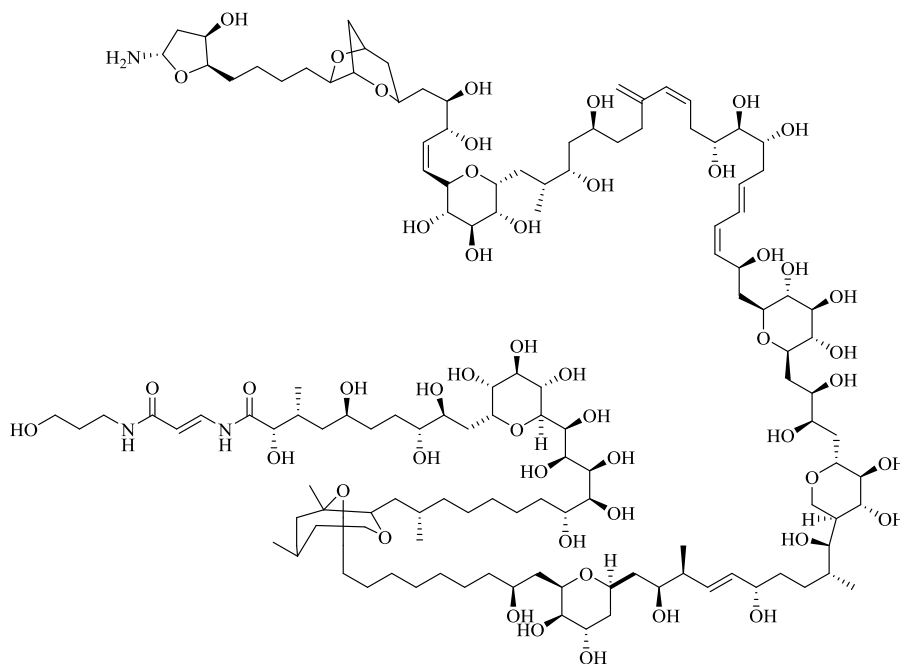


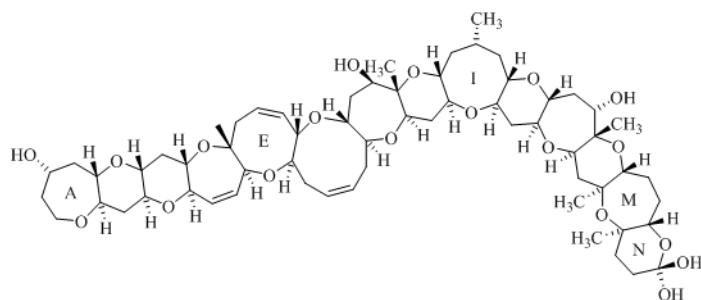
Figure 1.2 Palytoxin structure modified from Ramos and Vasconcelos 2010 [66].

1.2.3. Ciguatoxin

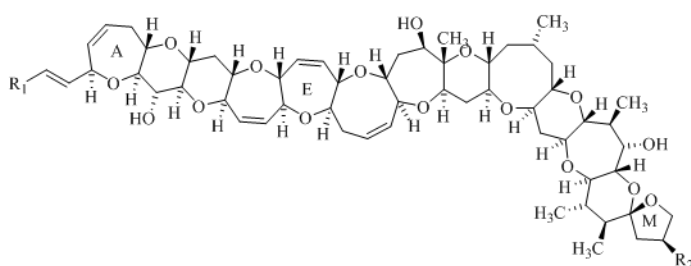
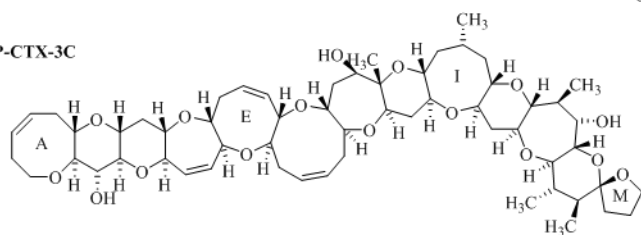
CTXs are reef toxins produced by the dinoflagellate *Gambierdiscus* spp. in warm, tropical or subtropical waters [79]. A three letter code with prefix is used to distinguish structurally different Caribbean (C-CTX), Indian (I-CTX) and Pacific Ocean (P-CTX) congeners. Even though they differ structurally, the common features that integrate these group of toxins is the long semi-rigid architecture that comprises

trans/syn-fused ether ring with a molecular weight of 1023-1157 Da (Figure 1.3). Chemical structures of P-CTX [80–86] and C-CTX [87,88] are well-studied. They are heat-stable, highly oxygenated, lipid soluble cyclic polyethers. More than 20 analogues of P-CTX have been reported, with the main toxin groups being P-CTX-1, P-CTX-2 and P-CTX-3. Among these, P-CTX-1 is the most potent and thought to be responsible for the majority of neurological symptoms associated with ciguatera in the Pacific [81]. Ten analogues of C-CTX were identified by Pottier *et al.* [87]. C-CTX is the major analog group among the CTX toxin group. Four I-CTX toxin groups have been identified. I-CTX-1 & I-CTX-2 are the most common ones in comparison to I-CTX-3&I-CTX-4. The I-CTX-1 & I-CTX-2 have the same molecular weight (1140 Da) as C-CTX-1, with a closely related structure [89]. CTX poisoning occurs due to the ingestion of tropical reef fishes, which bioaccumulate the toxin from the dinoflagellate *Gambierdiscus* [90]. The CTX group causes cellular toxicities by elevating intracellular calcium concentration and by the binding and opening of non-selective, non-voltage activated ion channels, resulting in neurologic symptoms, such as hyperesthesia, paresthesia and dysesthesia which may appear from a few hours to two weeks after ingestion of a toxic specimen. Acute symptoms result in gastrointestinal and cardiovascular distress [91,92].

C-CTX-1/ C-CTX-2



P-CTX-3C



	R ₁	R ₂
P-CTX-1	¹ CH ₂ OHCHOH	OH
P-CTX-3 /P-CTX-2	¹ CH ₂ OHCHOH	H
P-CTX-4B/ P-CTX-4A	¹ CH ₂ CH	H

Figure 1.3 Structures Caribbean (C) and Pacific (P) CTX-group toxin. The energetically less favored epimers, P-CTX-2 (52-epi P-CTX-3), P-CTX-4A (52-epi P-CTX-4B) and C-CTX-2 (56-epi C-CTX-1) are indicated in parenthesis. Modified image from Lewis, 2001 [81]. Copyri

1.2.4. Cyclic Imines

Cyclic imines (CI) are a group of toxins which include spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs) produced by dinoflagellates. These toxins are macrocyclic compounds which share an imine functional group within their chemical structure (Figure 1.4) [93,94]. These cyclic imines are known as “fast-acting” toxins because they induce rapid death in the intraperitoneal mouse bioassay [95,96]. SPXs and GYMs are the largest group of CIs that are well-characterized. At present, 14 SPXs analogues have been isolated, whereby 13-desmethyl SPX-C is the most commonly one found in shellfish. In 1995, SPX was discovered in the Atlantic coast of Nova Scotia, Canada from mussels (*Mytilus edulis*) and scallops (*Placopecten magellanicus*) during the routine monitoring of lipophilic toxic compounds [97]. The spirolides toxin producing dinoflagellates, *Alexandrium ostenfeldii* and *A. peruvianum* were later described. Spirolides A–D are fast-acting toxins in mouse bioassay [98,99]. Spirolide E and F are biologically inactive with a keto-amine structure, which are the hydrolysis products of the Spirolides A–D [100–102]. This shows that imine group is important for the biological activity [100]. GYMs are produced by the dinoflagellate, *Karenia selliformis*. The structure of GYMs was first reported in 1995 by Seki and later confirmed by Stewart in 1997 by X-ray crystallographic analysis [103,104]. GYMs were first isolated from oysters (*Tiostrea chilensis*) coming from the South Island of New Zealand. The molecular mass of GYMs is 504.704 g/mol with a molecular weight of C₃₂H₄₅NO₄. GYM-A has also been reported in Tunisia [105]. GYM-B and GYM-C were isolated from the coast of New Zealand as well. The structure of GYM-B is similar to GYM-A, but contains an exocyclic methylene at the C-17 position and an allylic hydroxyl group at the C-18 position, while GYM-C is an oxidized analog of GYM-A and was found to be isomeric with GYM-B at the C-18 position [106,107]. PnTX and ptTXs are closely related to the chemical structure of SPXs. The pinnatoxin also contains a number of analogues (PnTXs A-G). The first of these to be discovered was pinnatoxin A from the digestive gland extract of *Pinna attenuata* in China and Japan. Pinnatoxins B, C and D were isolated from viscera of the *Pinna muricata* [108–110]. Pinnatoxins E and F were found in the Pacific oysters (*Crassostrea gigas*) from Ranganau Harbour, Northland, New Zealand [111]. Pinnatoxin G was also isolated from the Norwegian blue mussel (*M. edulis*) [112]. Pinnatoxins E, F and G have also been isolated from Pacific oysters and razorfish (*Pinna bicolor*) from South Australia [113,114]. The organism responsible for pinnatoxins (the dinoflagellate, *Vulcanodinium rugosum*), was discovered only after the analysis of sediment samples from Rangaunu Harbour and the French Mediterranean coast. The species was also found in South Australia, China, Spain, Hawaii and Japan [115–120]. Pteriatoxins (A, B and C) were isolated in 2001 by Uemura and co-workers from *Pteria penguin*. Pteriatoxins A, B and C have the same polyether macrocycles as in pinnatoxin A. These CIs are fast-acting neurotoxins in laboratory animals which inhibit the nicotinic and muscarinic acetylcholine receptors (mAChR and nAChR, respectively) in the central and peripheral nervous system and at the neuromuscular junction causing death [121]. The lack of reports of acute intoxications caused by the consumption of contaminated sea products may be due to poor recognition of the adverse symptoms

of a mild intoxication, such as tachycardia or gastric distress [122]. Moreover, the chronic effects are not yet fully understood, therefore this matter should be treated with caution and efforts should be made to disclose CIs' acute and long term effects.

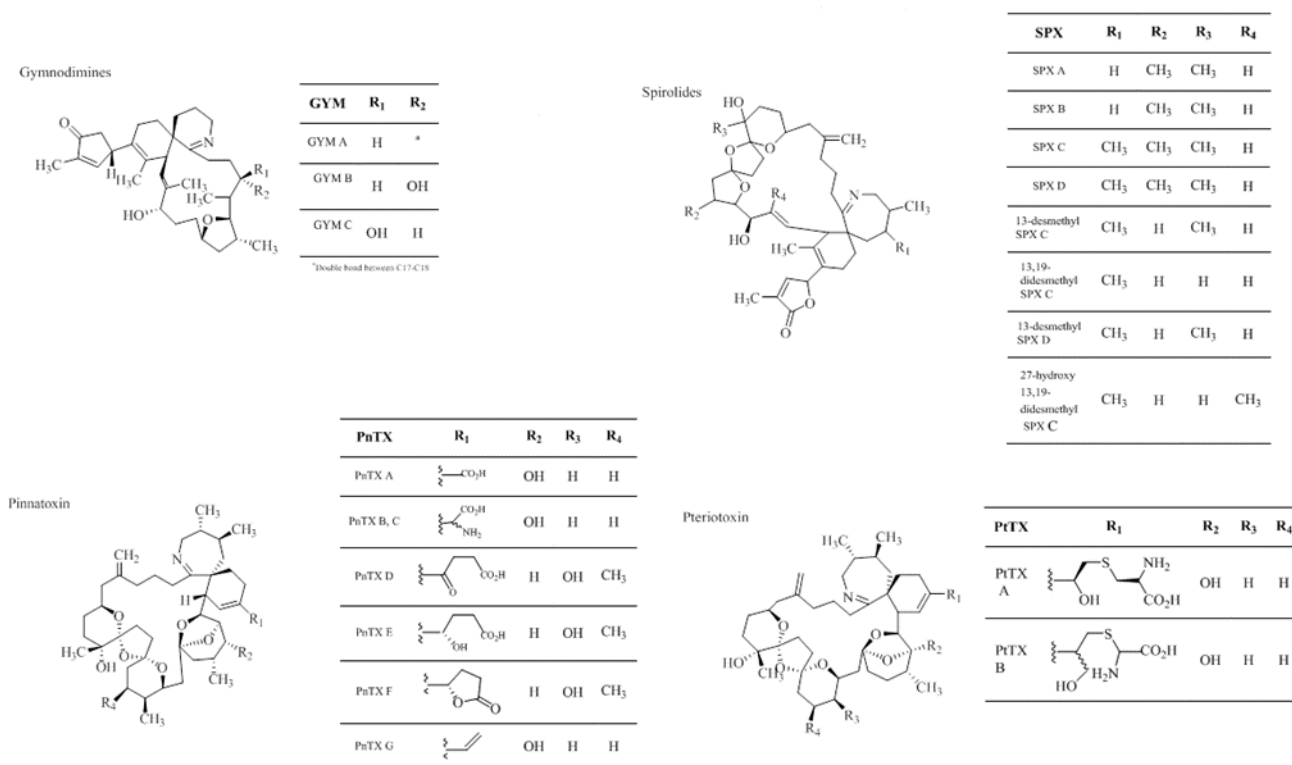


Figure 1.4 Gymnodimines (GYM) structure modified from Cembella and Krock 2008 [123]. Copyright 2008, Taylor & Francis. Spirolide (SPX) structure modified from Otero et al. 2010 [124]. Copyright 2010, Wiley. Pinnatoxin (PnTX) structure modified from Selwood et al. 2

The presence of emerging toxins in temperate coastal waters has recently been reported and episodes of human poisoning usually follow [19,39,125,126]. Due to the lack of systematic data detecting these new toxins, a more comprehensive research strategy which better assesses the risk of public health is required. Some recent human intoxication episodes have alerted our attention. In October 2007, a Spanish man who consumed a trumpet shell (*Charonia lampas*) collected in the south of Portugal (Algarve) was severely intoxicated requiring hospital care. Analysis revealed the occurrence of TTX and 5,6,11-trideoxyTTX analogue in sublethal concentrations [19]. Ciguatoxin is a common toxin from Indo-Pacific and Caribbean waters that was first reported in Europe in 2003 in Greece. This toxin is produced by a dinoflagellate from the genus *Gambierdiscus* [127]. In July 2008, the intoxication of 11 crew members who ate carnivorous fish caught off the Madeira archipelago revealed the presence of CTX [39]. CIs are neurotoxic phycotoxins which were first reported in shellfish aquaculture in Nova Scotia, Canada in 1992 [97,100,102]. Their origin has been tracked to the dinoflagellates *Alexandrium ostenfeldii* and *A. peruvianum* [128]. Though they have acute neurotoxicity in mice, no human poisoning incidents have been reported to date [117,129]. CIs were reported along the North Atlantic and some groups of these biotoxins are confined to the Pacific Ocean [40,103,129–133]. PTX was first reported

in Hawaii and Japan and their origin has been tracked to marine zoanthids, belonging to the genus *Palythoa*, and in dinoflagellates of the genus *Ostreopsis* [63,64]. Currently, blooms of *Ostreopsis* spp. have been reported in southern Europe indicating that the number of producers of this group of biotoxins is probably increasing from the Mediterranean Sea to the North Atlantic Ocean [134]. Also, since PTXs can form aerosols, several poisoning incidents have been reported among Mediterranean coastal populations as mild skin and respiratory disorders after exposure to high concentrations of *Ostreopsis* sp., luckily with no fatal outcomes [29].

These episodes suggest that there is an emergent phenomenon, indicating that marine toxins from tropical and subtropical ecosystems are most likely increasing their prevalence in temperate waters (Table 1.1). Multiple causes contribute to this phenomenon, such as the warming of coastal waters attributable to climate change and the increasing use of artificial waterways (*i.e.*, the Suez Canal) that allow for colonization and the establishment of exotic species in the Mediterranean Sea and the Atlantic Ocean [42,135–137]. Eutrophic areas of the Mediterranean Sea contribute to the formation of seed banks that provide favorable conditions for the establishment and migration of tropical organisms in more temperate areas of the North Atlantic.

Table 1.1 Detection of emergent poisoning incidents in the Mediterranean Sea and North Atlantic Ocean.

Toxin	Report location	Year	Vector/uptake route	No poisoning cases	Ref
TTX	Egypt/Israel	2005/2007/2008	<i>Lagocephalus sceleratus</i> (ingestion)	13	[136]
	Spain	2007	<i>Charonia lampas</i> (ingestion)	1	[19]
	Italy	2005/2006	<i>Ostreopsis ovate</i> (aerosol)	228	[138]
	Spain	2010	<i>Ostreopsis</i> sp. (aerosol)	2	[139]
PTX	France	2006–2009	<i>Ostreopsis</i> sp. (aerosol/Dermic)	47	[29]
CTX	Canary Islands	2004	<i>Seriola rivoliana</i> (ingestion)	5	[140]
	Madeira Island	2008	<i>Seriola</i> sp. (ingestion)	11	[39]
CI	-	-	-	-	-

Analytical Methods

Detection and quantification of the emergent toxins such as TTX, CTXs, CI and PTX have been based on different approaches (Table 1.2). Mouse bioassay (MBA) is the simplest method used for screening the total toxicity of the sample. In order to assess the toxicity, purified toxin samples or biological extracts are injected intraperitoneally and then animals are monitored for 24–48 h. The results are based on the biological response of mice and the toxicity of the sample is calculated in mouse units (MU). The relationship between time and lethal dose is used for estimation of the toxicity of the sample. This assay gives the total toxicity of a sample [141–144]. For screening and monitoring the toxins, many rapid, sensitive and specific assays (*i.e.*, cytotoxicity assay, immunological and receptor binding assays) have been developed. Cytotoxicity assay is used as the alternative method replacing the whole animal assays. This assay is based on the changes in the morphology of cells by the toxin. Cytotoxicity can be measured either through the lactate dehydrogenase (LDH) release assay or the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] assay in the living cells at pg concentrations [143,145,146]. Immunoassays are antibody based assays used for the detection of toxins. These methods are very sensitive and allow for the detection of toxins in pg concentrations [147–150]. Receptor binding assays (RBA) are based on the principle of the affinity of the toxin to a specific binding site. The toxin can be measured by the binding reaction between a radiolabelled toxin and a non-radiolabelled toxin that binds specifically to the receptor. Mouse bioassay, receptor binding assay and immunological methods have been used for the analysis of these emergent toxins. It has been found that these methods are successfully used for the identification of toxins, but they fail to analyze the analogues or derivatives of these toxins. The immunological assay addressed for the analysis of the toxins involves the use of expensive antibodies and there are some ethical issues regarding the use of live animal for bioassays [54,151–153]. Analytical methods such as HPLC/MS, LC/MS/MS, GC-MS, LC-FLD and NMR have been developed, which are helpful for the identification of the structure and analogues of the toxin. The LC-FLD and GC-MS are not a good choice because those methods have difficulties in the quantification of toxins and its analogues, due to a large variation in the absorbance intensities. The non-volatile nature of some toxins should also be derived in the case of GC-MS analysis. LC-UV method does not provide proper selectivity for the toxins which lack chromophore structure. Therefore, LC-LC/MS is regarded as the best choice for the determination of emergent toxin and its analogues. Improvement in these methods and sample preparation decrease the limit of detection and quantification of the toxin [54,144,151–153].

Monitoring and Legislation Challenges

Safety management practices are required for shellfish due to the unpredictable nature of blooms [5,122]. Monitoring became the official strategy to control harvesting of shellfish areas throughout the world to prevent health and economical losses [122].

Monitoring is an essential, labour-intensive and costly activity. As a result of the Joint of FAO/IOC/WHO ad hoc Expert Consultation on biotoxins in bivalve mollusks held in 2004, guidelines for the organization of the marine biotoxin management plan (MBMP) were proposed. MBMP is based on several action plans that encompass an efficient sample strategy comprising periodicity and frequency, sample size and composition, and also, which analysis methods and managing action plans based on expert judgment of the results are the best and most effective [122].

Toxic phytoplankton species monitoring by itself is insufficient and strongly discouraged since it faces various inherent difficulties. It does not reflect the toxin content in shellfish species due to their intra and inter-specific differences nor their irregular distribution in the water column. Toxins can accumulate in bivalve species after the bloom has entered into a senescence state. Moreover, some toxin producing species may not be in the water suspension [154]. A good example is bacterial origin toxin TTX [154].

Table 1.2 Limit of detection/quantification of Emergent toxins (CTX, PTX, CI and TTX) by using different methods. (LOD—Limit of Detection, LOQ—Limit of Quantification, LD50—Lethal Dose 50%, SM—Shellfish Meat, mL—Milli liter, kg—Kilogram, µg—Microgram, ng—Nanogram)

Assay	CTX	PTX	CI	TTX	Refs
MBA	LOD _{P-CTX-1} = 0.2 µg/kg SM LOD _{C-CTX-1} = 3.0 µg/kg	LD50 = 150–720 ng/µL	LOD _{GYMA} = 77 µg/kg SM. <i>i.p.</i> LD _{50PnTx E, F and G} = 12.7–57 µg/kg SM	LOD _{TTX} = 0.2 µg	[114,129,142–144,155–158]
Citotoxicity assay					
Haemolysis assays	LOD = 50 µg/mL	LOD = 1.6 ng/kg SM 0.005 pg/µL–1 pg/mL	-	LOD = 5.0 µg/mL	[73,74,146,159,160]
Fluorimetric method	LOQ _{C-CTX-1} = 0.039 ng/g	-	-	-	-
Receptor-binding assays	LOQ _{P-CTX-3Ceq} = 15.5 fg/cul for algal samples 0.155 ng/g in fish samples	-	-	-	[161]
RBA with Neuroblastoma	LOQ _{C-CTX-1} = 0.039 ng/g	-	-	-	[162]
Fluorescence polarization					
Microsphere flow cytometry	-	-	LOQ _{GYMA} = 50–80 µg/kg LOQ _{13-desMeC} = 50–85 µg/kg SM LOQ _{13,19-didesMeC} = 40 µg/kg SM LOD _{13-desMeC} = 10 µg/kg SM	-	[163–168]
Chemiluminescence method	-	-	LOD _{Spirolides} = 50 µg/kg SM	-	-
Assays with MCF-7 cells	-	LOD = 0.5 ng/mL	-	-	[169]
Assays with neuroblastoma cells	-	LOD = 5 ng/mL	-	LOD = 3.2–160 ng/mL	[145,170,171]
Immunoassays					
Immunobead assay (MIA)	LOD _{P-CTX-1} = 32 ng/kg fish flesh	-	-	-	[147,172,173]
CIEIA	-	-	-	LOD = 10 ng/mL	[149]
ELISA	LOD = 0.28 ng/mL	LOD = 0.5 pg/mL	-	LOD = 5–50 ng/mL	[148,150,160,174,175]
Surface plasmon resonance (SPR)	-	-	-	100 µg/kg	[176]
Chemical methods					
HPLC-FLD/LC-FLD	LOD = 0.5–1.0 ng	LOD = 0.75 ng	-	LOD = 0.07 pmol–0.4 pmol	[73,177–182]
HPLC/MS	LOD _{P-CTX-1} = 4 ng/g	-	-	LOD = 2 ng/mL	[183,184]
HPLC-UV/LC-UV	-	LOD = 0.1–2 µg	LOD _{GYM} = 5 ng/mL	LOD = 10 ng/mL	[185,186]

MBMP on harvesting shellfish areas should be based on a combination of phytoplankton and shellfish to best assess the risk. It should be done periodically in order to timely detect the increase in toxin content in shellfish caused by the seasonal and spatial shifting in phytoplankton community. Samples should be representative of the area with adequate location and number of sampling sites that are reachable in all weather conditions. A good alternative to obtaining information about dissolved toxin content in water is through passive sampling techniques. Different resins can cover different toxins, reducing the cost and human effort and simplifying the analytical analysis [187]. This is due to the fact that the matrix effects are diminished, *i.e.*, phytoplankton matrices are less complex than shellfish meat [188]. However, there are some downsides to this methodology, as it does not cover all biotoxins. Firstly, it is not effective in the screening of toxins with bacterial origin and of benthic dinoflagellates. Moreover, toxins can be metabolized in shellfish and therefore the real risk for human consumers is not accurately measured [114,189,190]. In order to ensure representativeness, sampling must comply with important factors: samples should be gathered throughout the cultivation area, samples should represent all depths when a toxic event is in progress, shellfish must be gathered in all marketed sizes to address variability in toxin uptake, and samples should be in sufficient number in order to perform all the analyses needed. MBMPs ought to also gather atmospheric and hydrographic parametric information of the area along with an in-depth understanding of impending factors and their interactions. In order to see what the favorable conditions for the formation of a toxic phenomenon are, predictive modeling should be in place as well [122]. Finally, HABs can be also region specific, one good example of that is CTX [156]. Local and historical knowledge should be taken in consideration since it is often useful for targeting baseline studies prior to setting up a monitoring program, not only for CTX but for other potential emerging toxins.

Good practices are required for standardizing procedures. Emergent toxins pose a great challenge and answers are needed to address the issue of spreading to new temperate environments and trophic chains with unknown consequences. Standardization of the analytical procedures is urgently needed because contrary to other toxins like PSPs and DSPs, there is limited knowledge for emergent toxin routes, biochemical paths and standard reference materials. All of these contribute to the difficulties of monitoring and planning strategies for risk assessment.

The MBA is the most common method to assess phycotoxins in shellfish, although there are inherent difficulties with this method such as the ethics of using test animals and supply. The MBA also lacks specificity; identification of a toxin and its analogues or a mixture of toxins is not possible. Moreover, performing the test relies on toxin routes that are not extrapolated to humans. The purified extract of toxins is administrated via intraperitoneal injection, a different route from the common ones (oral, dermal or inhalation). Furthermore, extrapolating these results to humans relies on inter-specific errors. Given this, EFSA recommended the use of analytical methods such as the LC-MS. These procedures avoid the ethical issues. They are able to identify a toxin, in addition to its derivatives, in a mixture with a high degree of sensitivity, but as a downside analytical methods depend on reference standards for calibration. In terms of new emergent toxins, the need for standards is urgent. Currently only a few

standards are available, meaning that the detection and quantification of these “new” toxins lack accuracy and are estimated according to their response factor. Data regarding acute reference dosage, median lethal dosage, legal limits in the European Union and standard availability on legislated toxins and on each group of emergent toxins will be described in more detail in Table 1.3.

Table 1.3 Summary of information of current and emergent toxins regarding: acute reference dosage (ARfD), median lethal dosage (LD50), legal limits in the European Union (EU) and standard availability, and comparison with regulated toxins PSP's and OA group (DSP)

Toxin group	Reference material	ARfD	LD50 mice	Legal limits in EU	Antidote	Refs
		µg/kg bw	µg/kg bw			
PSP	Yes (NRCC/Cifga)	0.5 STX	eq.10	0.8 µg SXT eq/g SM	N.A.	[191]
OA	Yes (NRCC/Cifga)	0.3	192	0.16 µg OA eq/g SM	N.A.	[192,193]
TTX	Lacking analogues (Cifga) No certified material	2 *	9	2 µg of TTX eq/g SM *	N.A.	[194,195]
PTX	available No certified material	0.2	0.15–0.72	30 µg PLT eq/kg SM **	N.A.	[151,191]
CTX	available Lacking analogues	N.A.	0.25	***	N.A.	[80,129,156]
CI	(NRCC/Cifga)	N.A.	5–8	400 µg CI/kg SM ****	N.A.	[152,196]

* Legislated limits for TTX are regarding the Japanese Government; ** EFSA recommends this value; *** EFSA recommends this value to cover all CTX-toxins; **** guidance value proposed by the EURLMB.

Analytical methods should comprise the whole animal but due to their high sensitivity, complex matrix interference may mask the results [41,197]. In order to overcome this problem, only the most affected organs are screened in some cases. This difficulty depends on the analyzed species, leading to another controversial issue: finding a species that can be used as an indicator. The commonly analyzed shellfish species are filter-feeders (mussels, scallops, cockles, oysters), and choosing a specific species, although it is advantageous in terms of cost reduction, is not effective since each species has different filtering and depuration rates. In addition to that, some studies showed that marine toxins risk assessment based on bivalves alone is redundant and misleading since some emergent toxins are not produced by phytoplankton [40,41]. These studies proved the possibility of bioaccumulation phenomena along the food-chain and reported new vectors for TTX and CIs from gastropods (*Monodonta lineata*, *Gibbula umbilicalis*, *Nucella lapillus*, *Aplysia depilans*, *Pattela intermedia*), to echinoderms (*Marthasterias glacialis* and *Paracentrotus lividus*) [40,41]. Likewise, CTXs have been detected from mollusks (ex: Turbinidae family) to top predator fish (ex: barracuda—Sphyraenidae family) occurring in the latter in higher concentrations suggesting that biotransformation and biomagnification could occur along the food-chain [122]. PTX poisoning incidents vary since all three exposure routes can occur (ingestion, inhalation and dermal) though there is a lack of proper reporting due to the difficulties inherent to identification/quantification owing to the absence of reference material

[125,152]. This data shows that we are not analyzing the whole food-chain effectively, underestimating the risk for consumers.

As described in Table 3, certified material for emergent toxins is lacking. Regarding TTX, neither the toxin nor its analogs are regulated. There is only the Regulation (EC) No. 853/2004, which prevents the entry of products and derivatives belonging to the Tetraodontidae fish family in the EU [198]. Guidelines values and legislation only exist in Japan and Korea, where this family of fish is well appreciated. Chefs must be certified and the Japanese Ministry of Health and Welfare established the limit value of 2 µg of TTX equivalents/g SM [195].

In terms of PLT, there are no regulations globally. In 2009, EFSA recommended that PLT plus derivatives should not exceed 30 µg/kg SM [191]. There are also no regulations for CTX, only recommended values [156], and in some countries, fish with toxic provenance are prohibited from entering the market. That is true for the EU [198], Fiji, American Samoa, French Polynesia, Hawaii and Miami [199]. For CIs, though there is some toxicological information about SPX and GIM, there is still a lack of information on the other toxins of this group. Regarding certified material, there are only two groups that are characterized. Based on this, EFSA does not have enough information to establish the ARfD, since SPX is the only CI totally characterized so far [152]. Therefore, there is need to study the other groups in order to reach solid conclusions for creating safety measures.

Not much is known about the chronic effects of emergent toxins. Their appearance in temperate systems is quite recent and physicians may not be prepared to deal with these new symptoms of poisoning incidents. When a poisoning case appears, it is advised to query the patient, if possible, whether or not sea products had been consumed. If so, the patient will need to be admitted into hospital for gastric cleaning, ventilator and fluid support as there is no antidote available yet. Gastric content should be analyzed for confirmation of the poisoning agent.

Few epidemiologic reports exist which are crucial to understanding emergent toxin health risks. For that reason, it is also crucial to develop faster, more accurate and reliable methods of identification and qualification of these poisons to better help health professionals in their diagnosis and treatment.

Conclusions

The efficiency of risk assessment of marine toxins relies on the monitoring of HABs and risk evaluation of phycotoxins in fish and shellfish. Detailed epidemiological studies are needed to better evaluate safety levels and to promote regulations updates that will protect human health and reduce economic losses. An international effort must be made to share information, to optimize certified materials and to explore more expeditious and sensitive methods, such as chromatographic and molecular ones. All this becomes more relevant and urgent in the case of new emergent toxins like TTX, PTX, CI and CTX. In comparison to toxins, which are regulated (DSPs and PSPs), emergent toxins demonstrate higher lethality, with the exception of the CI, posing a potentially higher human health risk and thus requiring further research.

Acknowledgments

This research was partially supported by the European Regional Development Fund (ERDF) through the COMPETE—Operational Competitiveness Programme—and national funds through FCT—Foundation for Science and Technology—under the project PEst-C/MAR/LA0015/2013 and by the projects MARBIOTECH (reference NORTE-07-0124-FEDER-000047) within the SR&TD Integrated Program MARVALOR—Building research and innovation capacity for improved management and valorization of marine resources, supported by the Programa Operacional Regional do Norte (ON.2-O Novo Norte) and NOVOMAR. MS and PV acknowledge also FCT for the grant SFRH/BD/73269/2010 and SFRH/BPD/78269/2011 respectively.

Author Contributions

M.S. and V.V. conceived the idea, M.S., V.P., L.B and V.V. wrote the paper, L.B. and V.V. funded.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Smayda, T.J. What is a bloom? A commentary. *Limnol. Oceanogr.* 1997, 42, 1132–1136.
2. Hallegraeff, G.M. Algal blooms are not a simple toxic broth. *Search* 1993, 24, 179.
3. Anderson, D.M.; Glibert, P.M.; Burkholder, J.M. Harmful Algal blooms and eutrophication: Nutrient sources, composition, and consequences. *Estuaries* 2002, 25, 704–726.
4. Glibert, P.; Seitzinger, S.; Heil, C.A.; Burkholder, J.M.; Parrow, M.W.; Codispoti, L.A.; Kelly, V. The role of eutrophication in coastal proliferation of harmful algal blooms: New perspectives and new approaches. *Oceanography* 2005, 18, 198–209.
5. Hallegraeff, G.M. Ocean climate change, phytoplankton community responses, and harmful algal blooms: A formidable predictive challenge. *J. Phycol.* 2010, 46, 220–235.

6. Heisler, J.P.; Gilbert, J.; Burkholder, J.; Anderson, D.; Cochlan, W.; Dennison, W.; Dortch, Q.; Gobler, C.J.; Heil, C.; Humphries, E.; et al. Eutrophication and harmful algal blooms: Scientific consensus. *Harmful Algae* 2008, 8, 3–13.
7. Burkholder, J.M.; Glibert, P.M.; Skelton, H.M. Mixotrophy, a major mode of nutrition for harmful algal species in eutrophic waters. *Harmful Algae* 2008, 8, 77–93.
8. Thomas, A.; Carr, M.; Strub, P. Chlorophyll variability in eastern boundary currents. *Geophys. Res. Lett.* 2001, 28, 3421–3424.
9. Benemann, J.R. Microalgae aquaculture feeds. *J. Appl. Phycol.* 1992, 4, 233–245.
10. Peperzak, L. Future increase in harmful algal blooms in the North Sea due to climate change. *Water Sci. Technol.* 2005, 51, 31–36.
11. Davis, T.W.; Berry, D.L.; Boyer, G.L.; Gobler, C.J. The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. *Harmful Algae* 2009, 8, 715–725.
12. Moore, S.K.; Trainer, V.L.; Mantua, N.J.; Parker, M.S.; Laws, E.A.; Backer, L.C.; Fleming, L.E. Impacts of climate variability and future climate change on harmful algal blooms and human health. *Environ. Health* 2008, 7, S4.
13. Touzet, N.; Farrell, H.; Ní Rathaille, A.; Rodriguez, P.; Alfonso, A.; Botana, L.M.; Raine, R. Dynamics of co-occurring *Alexandrium minutum* (Global Clade) and *A. tamarense* (West European) (Dinophyceae) during a summer bloom in Cork Harbour, Ireland (2006). *Deep Sea Res. II* 2010, 57, 268–278.
14. Schmidt, L.; Hansen, P. Allelopathy in the prymnesiophyte *Chrysochromulina polylepis*: Effect of cell concentration, growth phase and pH. *Mar. Ecol. Prog. Ser.* 2001, 216, 67–81.
15. Cembella, A. Chemical ecology of eukaryotic microalgae in marine ecosystems. *Phycologia* 2003, 42, 420–447.
16. Anderson, D.; Cembella, A.; Hallegraeff, G.M. Physiology and bloom dynamics of toxic *Alexandrium* species, with emphasis on life cycle transitions. In *Physiological Ecology of Harmful Algal*

Blooms; Anderson, D., Cembella, A., Hallegraeff, G.M., Eds.; Springer-Verlag: Berlin, Germany, 1998; pp. 29–48.

17. Calbet, A.; Vaque, D.; Felipe, J.; Vila, M.; Sala, M.; Alcaraz, M.; Estrada, M. Relative grazing impact of microzooplankton and mesozooplankton on a bloom of the toxic dinoflagellate *Alexandrium minutum*. *Mar. Ecol. Prog. Ser.* 2003, 259, 303–309.

18. Correia, F.S. Um caso raro de intoxicação alimentar colectiva. *Bol. Inst. Super. Hig. Doutor Ricardo Jorge* 1946, 3, 216–221.

19. Rodriguez, P.; Alfonso, A.; Vale, C.; Alfonso, C.; Vale, P.; Tellez, A.; Botana, L.M. First Toxicity Report of Tetrodotoxin and 5,6,11-trideoxyTTX in the Trumpet Shell *Charonia lampas lampas* in Europe. *Anal. Chem.* 2008, 80, 5622–5629.

20. Moestrup, Ø. *Haptophyt Algae*; Clarendon Press: Oxford, UK, 1994; Volume 51.

21. Davidson, K.; Miller, P.; Wilding, T.A.; Shutler, J.; Bresnan, E.; Kennington, K.; Swan, S. A large and prolonged bloom of *Karenia mikimotoi* in Scottish waters in 2006. *Harmful Algae* 2009, 8, 349–361.

22. Yang, C.Z.; Albright, L.J. Effects of the harmful diatom *Chaetoceros concavicornis* on respiration of rainbow trout *Oncorhynchus mykiss*. *Dis. Aquat. Org.* 1992, 14, 105–114.

23. Conley, D.J.; Björck, S.; Bonsdorff, E.; Carstensen, J.; Destouni, G.; Gustafsson, B.G.; Hietanen, S.; Kortekaas, M.; Kuosa, H.; Meier, H.E.M.; et al. Critical review: Hypoxia-related processes in the Baltic Sea. *Environ. Sci. Technol.* 2009, 43, 3412–3420.

24. National Oceanic and Atmospheric Administration, NOAA. Dead Zones: Hypoxia in the Gulf of Mexico. Available online: http://www.noaanews.noaa.gov/stories2009/pdfs/new%20fact%20sheet%20dead%20zones_final.pdf (accessed on 31 March 2014).

25. Morgan, K.L.; Larkin, S.L.; Adams, C.M. Firm-level economic effects of HABS: A tool for business loss assessment. *Harmful Algae* 2009, 8, 212–218.

26. Garcés, E.; Masó, M.; Camp, M. A recurrent and localized dinoflagellate bloom in a Mediterranean beach. *J. Plankton Res.* 1999, 21, 2373–2391.

27. Degobbi, D.; Umani, S.F.; Franco, P.; Malej, A.; Precali, R.; Smolaka, N. Changes in the northern Adriatic ecosystem and hypertrophic appearance of gelatinous aggregates. *Sci. Total Environ.* 1995, 165, 43–48.

28. Hoagland, P.; Scatasta, S. Ecology on harmful algae. In *The Economic Effect of Harmful Algal Blooms*; Graneli, E., Turner, T., Eds.; Springer: Berlin, Germany, 2006; Volume 189, pp. 391–402.
29. Tichadou, L.; Glaizal, M.; Armengaud, A.; Grosseil, H.; Lemée, R.; Kantin, R.; Lasalle, J.-L.; Drouet, G.; Rambaud, L.; Malfait, P.; et al. Health impact of unicellular algae of the *Ostreopsis* genus blooms in the Mediterranean Sea: Experience of the French Mediterranean coast surveillance network from 2006 to 2009. *Clin. Toxicol.* 2010, 48, 839–844.
30. Anderson, D.M.; Alpermann, T.J.; Cembella, A.D.; Collos, Y.; Masseret, E.; Montresor, M. The globally distributed genus *Alexandrium*: Multifaceted roles in marine ecosystems and impacts on human health. *Harmful Algae* 2012, 14, 10–35.
31. Mitra, A. A multi-nutrient model for the description of stoichiometric modulation of predation in micro- and mesozooplankton. *J. Plankton Res.* 2006, 28, 597–611.
32. Jones, R.H.; Flynn, K.J. Nutritional status and diet composition affect the value of diatoms as copepod prey. *Science* 2005, 307, 1457–1459.
33. Geraci, J.R.; Anderson, D.M.; Timperi, R.J.; Aubin, D.J.S.; Early, G.A.; Prescott, J.H.; Mayo, C.A. Humpback whales (*Megaptera novaeangliae*) fatally poisoned by dinoflagellate toxin. *Can. J. Fish. Aquat. Sci.* 1989, 46, 1895–1898.
34. Scholin, C.A.; Gulland, F.; Doucette, G.J.; Benson, S.; Busman, M.; Chavez, F.P.; Cordaro, J.; DeLong, R.; Vogetaere, A.D.; Harvey, J.; et al. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 2000, 403, 80–84.
35. Dale, B.; Yentsch, C.M. Red tide and paralytic shellfish poisoning. *Oceanus* 1978, 2, 41–49.
36. Vale, P.; Sampayo, M.A.M. Domoic acid in Portuguese shellfish and fish. *Toxicon* 2001, 39, 893–904.
37. Vale, P.; Sampayo, M.A.D.M. First confirmation of human diarrhoeic poisonings by okadaic acid esters after ingestion of razor clams (*Solen marginatus*) and green crabs (*Carcinus maenas*) in Aveiro lagoon, Portugal and detection of okadaic acid esters in phytoplankton. *Toxicon* 2002, 40, 989–996.
38. Sabrah, M.M.; El-Ganainy, A.A.; Zaky, M.A. Biology and toxicity of the pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from the Gulf of Suez. *Egypt. J. Aquat. Res.* 2006, 32, 283–287.

39. Otero, P.; Pérez, S.; Alfonso, A.; Vale, C.; Rodríguez, P.; Gouveia, N.N.; Gouveia, N.; Delgado, J.; Vale, P.; Hiram, M.; et al. First toxin profile of ciguateric fish in Madeira Archipelago (Europe).

Anal. Chem. 2010, 82, 6032–6039.

40. Silva, M.; Barreiro, A.; Rodriguez, P.; Otero, P.; Azevedo, J.; Alfonso, A.; Botana, L.M.; Vasconcelos, V. New invertebrate vectors for PST, spirolides and okadaic acid in the North Atlantic. Mar. Drugs 2013, 11, 1936–1960.

41. Silva, M.; Azevedo, J.; Rodriguez, P.; Alfonso, A.; Botana, L.M.; Vasconcelos, V. New gastropod vectors and tetrodotoxin potential expansion in temperate waters of the Atlantic Ocean. Mar. Drugs 2012, 10, 712–726.

42. Lima, F.P.; Wethey, D.S. Three decades of high-resolution coastal sea surface temperatures reveal more than warming. Nat. Commun. 2012, 3, 704.

43. Hallegraeff, G.M. A review of harmful algal blooms and their apparent global increase. Phycologia 1993, 32, 79–99.

44. Burkholder, J.M. Implications of harmful microalgae and heterotrophic dinoflagellates in management of sustainable marine fisheries. Ecol. Appl. 1998, 8, 37–62.

45. Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Forino, M.; Magno, G.S.; Tartaglione, L.; Grillo, C.; Melchiorre, N. The Genoa 2005 outbreak. Determination of putative palytoxin in Mediterranean *Ostreopsis ovata* by a new liquid chromatography tandem mass spectrometry method.

Anal. Chem. 2006, 78, 6153–6159.

46. Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Forino, M.; Tartaglione, L.; Grillo, C.; Melchiorre, N. Putative palytoxin and its new analogue, ovatoxin-a, in *Ostreopsis ovata* collected along the Ligurian coasts during the 2006 toxic outbreak. J. Am. Soc. Mass Spectrom. 2008, 19, 111–120.

47. Fleming, L.E.; Katz, D.; Bean, J.A.; Hammond, R. Epidemiology of seafood poisoning. In Foodborn Disease Handbook: Seafood and Environmental Toxins; Hui, Y.H., Kitts, D., Stanfield, S., Eds.; Marcel Dekker: New York, NY, USA, 2001; Volume 4, pp. 288–306.

48. Casabianca, S.; Casabianca, A.; Riobo, P.; Franco, J.M.; Vila, M.; Penna, A. Quantification of the toxic dinoflagellate *Ostreopsis* spp. by qPCR assay in marine aerosol. Environ. Sci. Technol. 2013, 47, 3788–3795.

49. Yokoo, A. Study on chemical purification of tetrodotoxin—Purification of spheroidine. *J. Chem. Soc. Jpn.* 1950, 71, 590–592.
50. Tahara, Y.; Hirata, Y. Studies on the puffer fish toxin. *J. Pharm. Soc. Jpn.* 1909, 29, 587–625.
51. Goto, T.; Kishi, Y.; Takahashi, S.; Hirata, Y. Tetrodotoxin. *Tetrahedron* 1965, 21, 2059–2088.
52. Tsuda, K.; Ikuma, S.; Kawamura, M.; Tachikawa, R.; Sakai, K. Tetrodotoxin. VII. On the structure of tetrodotoxin and its derivatives. *Chem. Pharm. Bull.* 1964, 12, 1356–1374.
53. Woodward, R.B. The structure of tetrodotoxin. *Pure Appl. Chem.* 1964, 9, 49–74.
54. Bane, V.; Lehane, M.; Dikshit, M.; O’Riordan, A.; Furey, A. Tetrodotoxin: Chemistry, toxicity, source, distribution and detection. *Toxins* 2014, 6, 693–755.
55. Noguchi, T.; Arakawa, O. Tetrodotoxin—Distribution and accumulation in aquatic organisms, and cases of human intoxication. *Mar. Drugs* 2008, 6, 220–242.
56. Chau, R.; Kalaitzis, J.A.; Neilan, B.A. On the origins and biosynthesis of tetrodotoxin. *Aquat. Toxicol.* 2011, 104, 61–72.
57. Pratheepa, V.; Vasconcelos, V. Microbial diversity associated with tetrodotoxin production in marine organisms. *Environ. Toxicol. Pharm.* 2013, 36, 1046–1054.
58. Noguchi, T.; Narita, H.; Maruyama, J.; Hashimoto, K. Tetrodotoxin in the starfish *Astropecten polyacanthus*, in association with toxification of a trumpet shell, “Boshubora”, *Charonia sauliae*. *Bull. Jpn. Soc. Sci. Fish.* 1982, 48, 1173–1177.
59. Noguchi, T.; Ebesu, J.S.M. Puffer poisoning: Epidemiology and treatment. *J. Toxicol. Toxin Rev.* 2001, 20, 1–10.
60. Hommel, D.; Hulin, A.; Saignavong, S.; Desbordes, J.M. Intoxication par poisson-coffre (tetrodotoxine). A propos d'une intoxication familiale. *Méd. D’Afrique Noire* 1992, 39, 146–148.
61. Ravaonindrina, N.; Andriamaso, T.H.; Rasolofonirina, N. Puffer fish poisoning in Madagascar: Four case reports. *Arch. Inst. Pasteur Madag.* 2001, 67, 61–64.
62. Moore, R.E.; Scheuer, P.J. Palytoxin—New marine toxin from a coelenterate. *Science* 1971, 172, 495–498.

63. Moore, R.E.; Bartolini, G. Structure of palytoxin. *J. Am. Chem. Soc.* 1981, 103, 2491–2494.
64. Deeds, J.R.; Schwartz, M.D. Human risk associated with palytoxin exposure. *Toxicol* 2010, 56, 150–162.
65. Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Forino, M.; Grauso, L.; Tartaglione, L.; Florio, C.; Lorenzon, P.; de Bortpo, M.; et al. 1851–1859, Stereostructure and biological activity of 42-hydroxy-palytoxin: A new palytoxin analogue from Hawaiian *Palythoa* subspecies. *Chem. Res. Toxicol.* 2009, 22, 1851–1859.
66. Ramos, V.; Vasconcelos, V. Palytoxin and analogs: Biological and ecological effects. *Mar. Drugs* 2010, 8, 2021–2037.
67. Ukena, T.; Satake, M.; Usami, M.; Oshima, Y.; Naoki, H.; Fujita, T.; Kan, Y.; Yasumoto, T. Structure elucidation of ostreocin D, a palytoxin analog isolated from the dinoflagellate *Ostreopsis siamensis*. *Biosci. Biotechnol. Biochem.* 2001, 65, 2585–2588.
68. Uemura, D.; Ueda, K.; Hirata, Y.; Naoki, H.; Iwashita, T. Further-studies on palytoxin. II. Structure of palytoxin. *Tetrahedron Lett.* 1981, 22, 2781–2784.
69. Tan, C.H.; Lau, C.O. Seafood and freshwater toxins. In *Pharmacology, Physiology and Detection*; Botana, L.M., Ed.; Marcel Dekker: New York, NY, USA, 2000; p. 533.
70. García-Altres, M.; Tartaglione, L.; Dell'Aversano, C.; Carnicer, O.; De la Iglesia, P.; Forino, M.; Diogène, J.; Ciminiello, P. The novel ovatoxin-g and isobaric palytoxin (so far referred to as putative palytoxin) from *Ostreopsis cf. ovata* (NW Mediterranean Sea): Structural insights by LC-high resolution MSn. *Anal. Bioanal. Chem.* 2015, 407, 1191–1204.
71. Lenoir, S.; Ten-Hage, L.; Turquet, J.; Quod, J.P.; Bernard, C.; Hennion, M.C. First evidence of palytoxin analogs from an *Ostreopsis mascarenensis* (Dinophyceae) benthic bloom in Southwestern Indian Ocean. *J. Phycol.* 2004, 40, 1042–1051.
72. Mercado, J.A.; Viera, M.; Escallona de Mota, G.; Tosteson, T.R.; González, I.; Silva, W. An extraction procedure modification changes the toxicity, chromatographic profile and pharmacologic action of *Ostreopsis lenticularis* extracts. *Toxicol* 1994, 32, 256.

73. Riobó, P.; Paz, B.; Franco, J.M. Analysis of palytoxin-like in *Ostreopsis* cultures by liquid chromatography with precolumn derivatization and fluorescence detection. *Anal. Chim. Acta* 2006, 566, 217–223.
74. Onuma, Y.; Satake, M.; Ukena, T.; Roux, J.; Chanteau, S.; Rasolofonirina, N.; Ratsimaloto, M.; Naoki, H.; Yasumoto, T. Identification of putative palytoxin as the cause of clupeotoxism. *Toxicon* 1999, 37, 55–65.
75. Gleibs, S.; Mebs, D. Distribution and sequestration of palytoxin in coral reef animals. *Toxicon* 1999, 37, 1521–1527.
76. Hirata, Y.D.U.; Ohizumi, Y. Chemistry and pharmacology of palytoxin. In *Handbook of Natural Toxins: Marine Toxins and Venoms*; Tu, A.T., Ed.; Marcel Drecker: New York, NY, USA, 1988; pp. 241–258.
77. Wiles, J.S.; Vick, J.A.; Christensen, M.K. Toxicological evaluation of palytoxin in several animal species. *Toxicon* 1974, 12, 427–433.
78. Taniyama, S.; Arakawa, O.; Terada, M.; Nishio, S.; Takatani, T.; Mahmud, Y.; Noguchi, T. *Ostreopsis* sp., a possible origin of palytoxin (PTX) in parrotfish *Scarus ovifrons*. *Toxicon* 2003, 42, 29–33.
79. Yasumoto, T.; Nakajima, I.; Bagnis, R.; Adachi, R. Finding of a dinoflagellate as a likely culprit of ciguatera. *Bull. Jpn. Soc. Sci. Fish.* 1977, 43, 1021–1026.
80. Lewis, R.J.; Sellin, M.; Poli, M.A.; Norton, R.S.; MacLeod, J.K.; Sheil, M.M. Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). *Toxicon* 1991, 29, 1115–1127.
81. Lewis, R.J. The changing face of ciguatera. *Toxicon* 2001, 39, 97–106.
82. Murata, M.; Legrand, A.M.; Ishibashi, Y.; Fukui, M.; Yasumoto, T. Structures and configurations of ciguatoxin from the moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. *J. Am. Chem. Soc.* 1990, 112, 4380–4386.
83. Lehane, L.; Lewis, R.J. Ciguatera: Recent advances but the risk remains. *Int. J. Food Microbiol.* 2000, 61, 91–125.

84. Satake, M.; Fukui, M.; Legrand, A.M.; Cruchet, P.; Yasumoto, T. Isolation and structures of new ciguatoxin analogs, 2,3-DihydroxyCTX3C and 51-HydroxyCTX3C, accumulated in tropical reef fish. *Tetrahedron Lett.* 1998, 39, 1197–1198.
85. Satake, M.; Murata, M.; Yasumoto, T. Gambierol: A new toxic polyether compound isolated from the marine dinoflagellate *Gambierdiscus toxicus*. *J. Am. Chem. Soc.* 1993, 115, 361–362.
86. Satake, M.; Murata, M.; Yasumoto, T. The structure of CTX3C, a ciguatoxin congener isolated from cultured *Gambierdiscus toxicus*. *Tetrahedron Lett.* 1993, 34, 1975–1978.
87. Pottier, I.; Vernoux, J.P.; Jones, A.; Lewis, R.J. Characterization of multiple Caribbean ciguatoxins and congeners in individual specimens of horse-eye jack (*Caranx latus*) by high performance liquid chromatography/mass spectrometry. *Toxicon* 2002, 40, 929–939.
88. Lewis, R.J.; Vernoux, J.P.; Brereton, I.M. Structure of Caribbean ciguatoxin isolated from *Caranx latus*. *J. Am. Chem. Soc.* 1998, 120, 5914–5920.
89. Hamilton, B.; Hurbungs, M.; Jones, A.; Lewis, R.J. Multiple ciguatoxins present in Indian Ocean reef fish. *Toxicon* 2002, 40, 1347–1353.
90. Lewis, R.; King, G. *Ciguatera (fish poisoning)*; University of New South Wales: Sydney, Australia, 1996.
91. Pottier, I.; Vernoux, J.P.; Jones, A.; Lewis, R.J. Analysis of toxin profiles in three different fish species causing ciguatera fish poisoning in Guadalupe, French West Indies. *Food Addit. Contam.* 2002, 19, 1034–1042.
92. Lu, X.Z.; Deckey, R.; Jiao, G.L.; Ren, H.F.; Li, M. Caribbean maitotoxin elevates $[Ca^{2+}]_i$ and activates non-selective cation channels in HIT-T15 cells. *World J. Diabetes* 2013, 4, 70–75.
93. Krock, B.; Tillmann, U.; John, U.; Cembella, A. LC/MS-MS aboard ship: Tandem mass spectrometry in the search for phycotoxins and novel toxigenic plankton from the North Sea. *Anal. Bioanal. Chem.* 2008, 392, 797–803.
94. Molgó, J.; Girard, E.; Benoit, E. Cyclic imines: An insight into this emerging group of bioactive marine toxins. In *Phycotoxins: Chemistry and Biochemistry*; Botana, L.M., Ed.; Blackwell Publishing: Ames, IA, USA, 2007; pp. 319–335.
95. EFSA Report on Data Collection: Future Directions; EFSA Journal: Parma, Italy, 2010; p. 1533.

96. Otero, A.; Chapela, M.J.; Atassanova, M.; Vieites, J.M.; Cabado, A.G. Cyclic imines: Chemistry and mechanism of action: A review. *Chem. Res. Toxicol.* 2011, 24, 1817–1829.
97. Hu, T.; Curtis, J.M.; Oshima, Y.; Quilliam, M.A.; Walter, J.A.; Watson-Wright, W.M.; Wright, J.L.C. Spirolides B and D, two novel macrocycles isolated from the digestive glands of shellfish. *J. Chem. Soc. Chem. Commun.* 1995, 20, 2159–2161.
98. Cembella, A.D.; Lewis, N.I.; Quilliam, M.A. Spirolide composition of micro-extracted pooled cells isolated from natural plankton assemblages and from cultures of the dinoflagellate *Alexandrium ostenfeldii*. *Nat. Toxins* 1999, 7, 197–206.
99. Richard, D.; Arsenaulf, E.; Cembella, A.; Quilliam, M. Investigations into the toxicology and pharmacology of spirolides, a novel group of shellfish toxins, in harmful algal blooms. In *Proceedings of the Ninth International Conference on Harmful Algal Blooms*, Hobart, Australia, 7–11 February 2000; Hallegraef, G.M., Blackburn, S.I., Bolch, C.J., Lewis, R.J., Eds.; Intergovernmental Oceanographic Commission of UNESCO: Paris, France, 2001; pp. 383–390.
100. Hu, T.; Curtis, J.M.; Walter, J.A.; Wright, J.L.C. Characterization of biologically inactive spirolides E and F: Identification of the spirolide pharmacophore. *Tetrahedron Lett.* 1996, 37, 7671–7674.
101. Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Forino, M.; Tartaglione, L.; Boschetti, L.; Rubini, S.; Cangini, M.; Pigozzi, S.; Poletti, R. Complex toxin profile of *Mytilus galloprovincialis* from the Adriatic sea revealed by LC-MS. *Toxicon* 2010, 55, 280–288.
102. Hu, T.; Burton, I.W.; Cembella, A.D.; Curtis, J.M.; Quilliam, M.A.; Walter, J.A.; Wright, J.L. Characterization of spirolides A, C, and 13-desmethyl C, new marine toxins isolated from toxic plankton and contaminated shellfish. *J. Nat. Prod.* 2001, 64, 308–312.
103. Seki, T.; Satake, M.; Mackenzie, A.L.; Kaspar, H.F.; Yasumoto, T. Gimnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium* sp. *Tetrahedron Lett.* 1995, 36, 7093–7096.
104. Stewart, M.; Blunt, J.W.; Munro, M.H.; Robinson, W.T.; Hannah, D.J. The absolute stereochemistry of the New Zealand shellfish toxin gymnodimine. *Tetrahedron Lett.* 1997, 38, 4889–4890.

105. Bire, R.; Krys, S.; Fremy, J.M.; Dragacci, S.; Stirling, D.; Kharrat, R. First evidence on occurrence of gymnodimine in clams from Tunisia. *J. Nat. Toxins* 2002, 11, 269–275.
106. Miles, C.O.; Wilkins, A.L.; Stirling, D.J.; MacKenzie, A.L. New analogue of gymnodimine from a *Gymnodinium* species. *J. Agric. Food Chem.* 2000, 48, 1373–1376.
107. Miles, C.O.; Wilkins, A.L.; Stirling, D.J.; MacKenzie, A.L. Gymnodimine C, an isomer of gymnodimine B, from *Karenia selliformis*. *J. Agric. Food Chem.* 2003, 51, 4838–4840.
108. Zheng, S.Z.; Huang, F.L.; Chen, S.C.; Tan, X.F.; Zuo, J.B.; Peng, J.; Xie, R.W. The Isolation and Bioactivities of Pinnatoxin. *Chin. J. Mar. Drugs* 1990, 9, 33–35.
109. Chou, T. Relative stereochemistry of pinnatoxin A, a potent shellfish poison from *Pinna muricata*. *Tetrahedron Lett.* 1996, 37, 4023–4026.
110. Takada, N.; Umemura, N.; Suenaga, K.; Chou, T.; Nagatsu, A.; Haino, T.; Yamada, K.; Uemura, D. Pinnatoxins B and C, the most toxic components in the pinnatoxin series from the Okinawan bivalve *Pinna muricata*. *Tetrahedron Lett.* 2001, 42, 3491–3494.
111. Rhodes, L.; Adamson, J.; Suzuki, T.; Briggs, L.; Garthwaite, I. Toxic marine epiphytic dinoflagellates, *Ostreopsis siamensis* and *Coolia monotis* (Dinophyceae), in New Zealand. *N. Z. J. Mar. Freshw. Res.* 2000, 34, 371–383.
112. Rundberget, T.; Aasen, J.A.B.; Selwood, A.I.; Miles, C.O. Pinnatoxins and spirolides in Norwegian blue mussels and seawater. *Toxicon* 2011, 58, 700–711.
113. McNabb, P.; Rhodes, L.; Selwood, A. Results of Analyses for Brevetoxins and Pinnatoxins in Rangaunu Harbour Oysters, 1993–2008. Prepared for New Zealand Food Safety Authority, Northland, New Zealand, 2008; Cawthron Report No. 1453, p. 18.
114. Selwood, A.; Miles, C.; Wilkins, A.; van Ginkel, R.; Munday, R.; Rise, F.; McNabb, P. Isolation, structural determination and acute toxicity of pinnatoxins E, F and G. *J. Agric. Food Chem.* 2010, 58, 6532–6542.
115. Nézan, E.; Chomérat, N. *Vulcanodinium rugosum* gen. et sp. nov. (Dinophyceae), un nouveau dinoflagellé marin de la côte méditerranéenne française. *Cryptogam. Algol.* 2011, 32, 3–18.

116. Rhodes, L.; Smith, K.; Selwood, A.; McNabb, P.; Molenaar, S.; Munday, R.; Wilkinson, C.; Hallegraeff, G. Production of pinnatoxins E, F and G by scrippsielloid dinoflagellates isolated from Franklin Harbour, South Australia. *N. Z. J. Mar. Freshw. Res.* 2011, 45, 703–709.
117. Rhodes, L.; Smith, K.; Selwood, A.; McNabb, P.; van Ginkel, R.; Holland, P.; Munday, R. Production of pinnatoxins by a peridinoid dinoflagellate isolated from Northland, New Zealand. *Harmful Algae* 2010, 9, 384–389.
118. Satta, C.T.; Anglès, S.; Lugliè, A.; Guillén, J.; Sechi, N.; Camp, J.; Garcés, E. Studies on dinoflagellate cyst assemblages in two estuarine Mediterranean bays: A useful tool for the discovery and mapping of harmful algal species. *Harmful Algae* 2013, 24, 65–79.
119. Smith, K.; Rhodes, L.; Suda, S.; Selwood, A. A dinoflagellate producer of pinnatoxin G, isolated from sub-tropical Japanese waters. *Harmful Algae* 2011, 10, 702–705.
120. Zeng, N.; Gu, H.; Smith, K.; Rhodes, L.; Selwood, A.; Yang, W. The first report of *Vulcanodinium rugosum* (Dinophyceae) from the South China Sea with a focus on the life cycle. *N. Z. J. Mar. Freshw. Res.* 2012, 46, 511–521.
121. Gill, S.; Murphy, M.; Clausen, J.; Richard, D.; Quilliam, M.; MacKinnon, S.; LaBlanc, P.; Mueller, R.; Pulido, O. Neural injury biomarkers of novel shellfish toxins, spirolides: A pilot study using immunochemical and transcriptional analysis. *NeuroToxicology* 2003, 24, 593–604.
122. Lawrence, J.; Loreal, H.; Toyofuku, H.; Hess, P.; Karunasagar, I.; Ababouch, L. Assessment and Management of Biotoxin Risks in Bivalve Mollusks; No. 551; FAO: Rome, Italy, 2011; p. 337.
123. Cembella, A.; Krock, B. *Cyclic Imine Toxins: Chemistry, Biogeography, Biosynthesis and Pharmacology*; CRC Press (Taylor and Francys Group): Boca Raton, FL, USA, 2008.
124. Otero, P.; Alfonso, A.; Alfonso, C.; Vieytes, M.R.; Louzao, M.C.; Botana, A.M.; Botana, L.M. New protocol to obtain spirolides from *Alexandrium ostenfeldii* cultures with high recovery and purity. *Biomed. Chromatogr.* 2010, 24, 878–886.
125. Tubaro, A.; Durando, P.; Del Favero, G.; Ansaldi, F.; Icardi, G.; Deeds, J.R.; Sosa, S. Case definitions for human poisonings postulated to palytoxins exposure. *Toxicon* 2011, 57, 478–495.
126. Schmitt, C.; de Haro, L. Clinical marine toxicology: A European perspective for clinical toxicologists and poison centres. *Toxins* 2013, 5, 1343–1352.

127. Aligizaki, K.; Nikolaidis, G. Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. *J. Biol. Res. Thessalon.* 2008, 9, 75–82.
128. Touzet, N.; Franco, J.M.; Raine, R. Morphogenetic diversity and biotoxin composition of *Alexandrium* (Dinophyceae) in Irish coastal waters. *Harmful Algae* 2008, 7, 782–797.
129. Munday, R. Toxicology of cyclic imines: Gymnodimine, spirolides, pinnatoxins, pteriatoxins, prorocontrolide, spiro-prorocentrimine, and symbioimines. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, 2nd ed.; Botana, L.M., Ed.; CRC Press (Taylor and Francys Group): Boca Raton, FL, USA, 2008; Volume 173, pp. 581–594.
130. Aasen, J.A.B.; MacKinnon, S.L.; LeBlanc, P.; Walter, J.A.; Hovgaard, P.; Aune, T.; Quilliam, M.A. Detection and identification of spirolides in Norwegian shellfish and plankton. *Chem. Res. Toxicol.* 2005, 18, 509–515.
131. Ciminiello, P.; Dell’Aversano, C.; Fattorusso, E.; Magno, S.; Tartaglione, L.; Cangini, M.; Pompei, M.; Guerrini, F.; Boni, L.; Pistocchi, R. Toxin profile of *Alexandrium ostenfeldii* (Dinophyceae) from the Northern Adriatic Sea revealed by liquid chromatography-mass spectrometry. *Toxicon* 2006, 47, 597–604.
132. Amzil, Z.; Sibat, M.; Royer, F.; Masson, N.; Abadie, E. Report on the first detection of pectenotoxin-2, spirolide a and their derivatives in French shellfish. *Mar. Drugs* 2007, 5, 168–179.
133. Álvarez, G.; Uribe, E.; Ávalos, P.; Mariño, C.; Blanco, J. First identification of azaspiracid and spirolides in *Mesodesma donacium* and *Mulinia edulis* from Northern Chile. *Toxicon* 2010, 55, 638–641.
134. David, H.; Laza-Martinez, A.; Orive, E.; Silva, A.; Moita, T.; Mateus, M.; de Pablo, H. First bloom of *Ostreopsis cf. ovata* in the continental Portuguese coast. *Harmful Algae News* 2012, 45, 12–13.
135. Lasram, F.B.; Mouillot, D. Increasing southern invasion enhances congruence between endemic and exotic Mediterranean fish fauna. *Biol. Invasions* 2009, 11, 697–711.
136. Bentur, Y.; Ashkar, J.; Lurie, Y.; Levy, Y.; Azzam, Z.S.; Litmanovich, M.; Golik, M.; Gurevych, B.; Golani, D.; Eisenman, A. Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean. *Toxicon* 2008, 52, 964–968.

137. Katikou, P.; Georgantelis, D.; Sinouris, N.; Petsi, A.; Fotaras, T. First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece). *Toxicon* 2009, 54, 50–55.
138. Durando, P.; Ansaldi, F.; Oreste, P.; Moscatelli, P.; Marensi, L.; Grillo, C.; Gasparini, R.; Icardi, G. *Ostreopsis ovata* and human health: Epidemiological and clinical features of respiratory syndrome outbreaks from a two-year syndromic surveillance, 2005–2006 in north-west Italy. *Euro Surveill.* 2007, 12, 191–193.
139. Vila, M.; Arin, L.; Battochi, C.; Bravo, I.; Fragac, S.; Penna, A.; Reñé, A.; Riobó, P.; Rodriguez, F.; Sala, M.M.; et al. Management of *Ostreopsis* blooms in recreational waters along the Catalan Coast (NW Mediterranean Sea): Cooperation between a research project and a monitoring program. *Cryptogam. Algol.* 2012, 33, 143–152.
140. Pérez-Arellano, J.L.; Luzardo, O.P.; Brito, A.P.; Cabrera, M.H.; Zumbado, M.; Carranza, C.; Alfonso, A.M.; Dickey, R.W.; Boada, L.D. Ciguatera fish poisoning, Canary Islands. *Emerg. Infect. Dis.* 2015, 11, 1981–1982.
141. Lewis, R.J.; Sellin, M. Recovery of ciguatoxin from fish flesh. *Toxicon* 1993, 31, 1333–1336.
142. Munday, R.; Towers, N.R.; Mackenzie, L.; Beuzenberg, V.; Holland, P.T.; Miles, C.O. Acute toxicity of gymnodimine to mice. *Toxicon* 2004, 44, 173–178.
143. Noguchi, T.; Mahmud, Y. Current methodologies for detection of tetrodotoxin. *J. Toxicol. Toxin Rev.* 2001, 20, 35–50.
144. Riobó, P.; Franco, J.M. Palytoxins: Biological and chemical determination. *Toxicon* 2011, 57, 368–375.
145. Gallacher, S.; Birkbeck, T.H. A tissue culture assay for direct detection of sodium channel blocking toxins in bacterial culture supernates. *FEMS Microbiol. Lett.* 1992, 71, 101–107.
146. Shimojo, R.Y.; Iwaoka, W.T. A rapid hemolysis assay for the detection of sodium channel-specific marine toxins. *Toxicology* 2000, 154, 1–7.
147. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerford, J.M.; Hokama, Y.; Dickey, R.W.; Granade, H.R.; Lewis, R.; Yasumoto, T.; Wekell, M.M. Detection of sodium-channel toxins—Directed cytotoxicity

assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *J. AOAC Int.* 1995, 78, 521–527.

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

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

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

151. Alexander, J.; Benford, D.; Boobis, A.; Ceccatelli, S.; Cravedi, J.P.; Di Domenico, A.; Doerge, D.; Dogliotti, E.; Edler, L.; Farmer, P.; et al. EFSA Panel on Contaminants in the Food Chain (CONTAM); Scientific Opinion on marine biotoxins in shellfish—Palytoxin group. *EFSA J.* 2009, 1393, 1–38.

152. Alexander, J.; Benford, D.; Boobis, A.; Ceccatelli, S.; Cravedi, J.P.; Di Domenico, A.; Doerge, D.; Dogliotti, E.; Edler, L.; Farmer, P.; et al. Scientific Opinion on marine biotoxins in shellfish—Cyclic imines (spiroclides, gymnodimines, pinnatoxins and pteriatoxins). *EFSA J.* 2010, 1628, 1–39.

153. Caillaud, A.; Iglesia, P.; Darius, H.T.; Pauillac, S.; Aligizaki, K.; Fraga, S.; Chinain, M.; Diogène, J. Update on methodologies available for ciguatoxin determination: Perspectives to confront the onset of ciguatera fish poisoning in Europe. *Mar. Drugs* 2010, 8, 1838–1907.

154. Miyazawa, K.; Noguchi, T. Distribution and origin of tetrodotoxin. *J. Toxicol. Toxin Rev.* 2001, 20, 11–33.

155. Dickey, R.W. Ciguatera toxins: Chemistry, toxicology, and detection. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*; CRC Press, Taylor and Francis Group: Boca Raton, FL, USA, 2008; pp. 479–500.

156. Alexander, J.; Benford, D.; Boobis, A.; Ceccatelli, S.; Cravedi, J.P.; Di Domenico, A.; Doerge, D.; Dogliotti, E.; Edler, L.; Farmer, P.; et al. EFSA Panel on contaminants in the food chain; Scientific opinion on marine biotoxins in shellfish—Emerging toxins: Ciguatoxin group. *EFSA J.* 2010, 1627, 1–38.

157. Munday, R.; Selwood, A.I.; Rhodes, L. Acute toxicity of pinnatoxins E, F and G to mice. *Toxicon* 2012, 60, 995–999.
158. Hess, P.; Abadie, E.; Hervé, F.; Berteaux, T.; Séchet, V.; Aráoz, R.; Molgó, J.; Zakarian, A.; Sibat, M.; Rundberget, T.; et al. Pinnatoxin G is responsible for atypical toxicity in mussels (*Mytilus galloprovincialis*) and clams (*Venerupis decussata*) from Ingril, a French Mediterranean lagoon. *Toxicon* 2013, 75, 16–26.
159. Aligizaki, K.; Katikou, P.; Nikolaidis, G.; Panou, A. First episode of shellfish contamination by palytoxin-like compounds from *Ostreopsis* species (Aegean Sea, Greece). *Toxicon* 2008, 51, 418–427.
160. Bignami, G.S. A rapid and sensitive hemolysis neutralization assay for palytoxin. *Toxicon* 1993, 31, 817–820.
161. Darius, H.T.; Ponton, D.; Revel, T.; Cruchet, P.; Ung, A.; Fouc, M.T.; Chinain, M. Ciguatera risk assessment in two toxic sites of French Polynesia using the receptor-binding assay. *Toxicon* 2007, 50, 612–626.
162. Dechraoui, M.Y.B.; Tiedeken, J.A.; Persad, R.; Wang, Z.; Granade, H.R.; Dickey, R.W.; Ramsdell, J.S. Use of two detection methods to discriminate ciguatoxins from brevetoxins: Application to great barracuda from Florida Keys. *Toxicon* 2005, 46, 261–270.
163. Otero, P.; Alfonso, A.; Alfonso, C.; Araoz, R.; Molgo, J.; Vieytes, M.R.; Botana, L.M. First direct fluorescence polarization assay for the detection and quantification of spirolides in mussel samples. *Anal. Chim. Acta* 2011, 701, 200–208.
164. Fonfría, E.S.; Vilariño, N.; Molgó, J.; Aráoz, R.; Otero, P.; Espiña, B.; Louzao, M.C.; Alvarez, M.; Botana, L.M. Detection of 13,19-didesmethyl C spirolide by fluorescence polarization using Torpedo electrocyte membranes. *Anal. Biochem.* 2010, 403, 102–107.
165. Fonfría, E.S.; Vilariño, N.; Espiña, B.; Louzao, M.C.; Álvarez, M.; Molgó, J.; Aráoz, R.; Botana, L.M. Feasibility of gymnodimine and 13-desmethyl C spirolide detection by fluorescence polarization using a receptor-based assay in shellfish matrixes. *Anal. Chim. Acta* 2010, 657, 75–82.
166. Rodríguez, L.P.; Vilariño, N.; Molgó, J.; Aráoz, R.; Louzao, M.C.; Taylor, P.; Talley, T.; Botana, L.M. Development of a solid-phase receptor-based assay for the detection of cyclic imines using a microsphere-flow cytometry system. *Anal. Chem.* 2013, 85, 2340–2347.

167. Rodríguez, L.P.; Vilariño, N.; Molgó, J.; Aráoz, R.; Botana, L.M. High-throughput receptor-based assay for the detection of spirolides by chemiluminescence. *Toxicon* 2013, 75, 35–43.
168. Vilariño, N.; Fonfría, E.S.; Molgó, J.; Aráoz, R.; Botana, L.M. Detection of gymnodimine-A and 13-desmethyl C spirolide phycotoxins by fluorescence polarization. *Anal. Chem.* 2009, 81, 2708–2714.
169. Bellocchi, M.; Ronzitti, G.; Milandri, A.; Melchiorre, N.; Grillo, C.; Poletti, R.; Yasumoto, T.; Rossini, G.P. A cytolytic assay for the measurement of palytoxin based on a cultured monolayer cell line. *Anal. Biochem.* 2008, 374, 48–55.
170. Ledreux, A.; Krys, S.; Bernard, C. Suitability of the Neuro-2a cell line for the detection of palytoxin and analogues (neurotoxic phycotoxins). *Toxicon* 2009, 53, 300–308.
171. Espiña, B.; Cagide, E.; Louzao, M.C.; Fernandez, M.M.; Vieytes, M.R.; Katikou, P.; Villar, A.; Jaen, D.; Maman, L.; Botana, L.M. Specific and dynamic detection of palytoxins by in vitro microplate assay with human neuroblastoma cells. *Biosci. Rep.* 2009, 29, 13–23.
172. Hokama, Y.; Takenaka, W.E.; Nishimura, K.L.; Ebeso, J.S.M.; Bourke, R.; Sullivan, P.K. A simple membrane immunobead assay for detecting ciguatoxin and related polyethers from human ciguatera intoxication and natural reef fishes. *J. AOAC Int.* 1998, 81, 727–735.
173. Campora, C.E.; Hokama, Y.; Yabusaki, K.; Isobe, M. Development of an enzyme-linked immunosorbent assay for the detection of ciguatoxin in fish tissue using chicken immunoglobulin Y. *J. Clin. Lab. Anal.* 2008, 22, 239–245.
174. Tao, J.; Wei, W.J.; Nan, L.; Lei, L.H.; Hui, H.C.; Fen, G.X.; Jun, L.Y.; Jing, Z.; Rong, J. Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of south-east China. *Food Addit. Contam. Part A* 2010, 27, 1589–1597.
175. Wang, X.; Yu, R.; Luo, X.; Zhou, M.; Lin, X. Toxin-screening and identification of bacteria isolated from highly toxic marine gastropod *Nassarius semiplicatus*. *Toxicon* 2008, 52, 55–61.
176. Campbell, K.; Barnes, P.; Haughey, S.A.; Higgins, C.; Kawatsu, K.; Vasconcelos, V.; Elliott, C.T. Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood. *Anal. Bioanal. Chem.* 2013, 405, 7753–7763.

177. Lewis, R.; Holmes, M.; Alewood, P.; Jones, A. Ionspray mass spectrometry of ciguatoxin-1, maitotoxin-2 and -3, and related marine polyether toxins. *Nat. Toxins* 1994, 2, 56–63.
178. Dickey, R.W.; Bencsath, F.A.; Granade, H.R.; Lewis, R.J. Liquid chromatographic mass spectrometric methods for the determination of marine polyether toxins. *Bull. Exot. Pathol. Society* 1992, 85, 514–515.
179. Shoji, Y.; Yotsu-Yamashita, M.; Miyazawa, T.; Yasumoto, T. Electrospray ionization mass spectrometry of tetrodotoxin and its analogs: Liquid chromatography/mass spectrometry, tandem mass spectrometry, and liquid chromatography/tandem mass spectrometry. *Anal. Biochem.* 2001, 290, 10–17.
180. Nakagawa, T.; Jang, J.; Yotsu-Yamashita, M. Hydrophilic interaction liquid chromatography electrospray ionization mass spectrometry of tetrodotoxin and its analogs. *Anal. Biochem.* 2006, 352, 142–144.
181. Jang, J.; Yotsu-Yamashita, M. Distribution of tetrodotoxin, saxitoxin, and their analogs among tissues of the puffer fish *Fugu pardalis*. *Toxicon* 2006, 48, 980–987.
182. Mebs, D.; Arakawa, O.; Yotsu-Yamashita, M. Tissue distribution of tetrodotoxin in the red-spotted newt *Notophthalmus viridescens*. *Toxicon* 2010, 55, 1353–1357.
183. Lewis, R.; Jones, A. Characterization of ciguatoxins and ciguatoxin congeners present in ciguateric fish by gradient reverse-phase high-performance liquid chromatography/mass spectrometry. *Toxicon* 1997, 37, 159–168.
184. Kawatsu, K.; Hamano, Y.; Yoda, T.; Terano, Y.; Shibata, T. Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin. *Jpn. J. Med. Sci. Biol.* 1997, 50, 133–150.
185. Marrouchi, R.; Dziri, F.; Belayouni, N.; Hamza, A.; Benoit, E.; Molgó, J.; Kharrat, R. Quantitative Determination of Gymnodimine-A by High Performance Liquid Chromatography in Contaminated Clams from Tunisia Coastline. *Mar. Biotechnol.* 2010, 12, 579–585.
186. Jen, H.C.; Lin, S.J.; Lin, S.Y.; Huang, Y.W.; Liao, I.C.; Arakawa, O.; Hwang, D.F. Occurrence of tetrodotoxin and paralytic shellfish poisons in a gastropod implicated in food poisoning in southern Taiwan. *Food Addit. Contam.* 2007, 8, 902–909.

187. Caillaud, A.; Iglesia, P.; Barber, E.; Eixarch, H.; Mohammad-Noor, N.; Yasumoto, T.; Diogène, J. Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: Application to *Gambierdiscus pacificus* in culture. *Harmful Algae* 2011, 10, 433–446.
188. Rundberget, T.; Gustad, E.; Samdal, I.A.; Sandvik, M.; Miles, C.O. A convenient and cost-effective method for monitoring marine algal toxins with passive samplers. *Toxicon* 2009, 53, 543–550.
189. Louzao, M.C.; Espiña, B.; Cagide, E.; Ares, I.R.; Alfonso, A.; Vieytes, M.R.; Botana, L.M. Cytotoxic effect of palytoxin on mussel. *Toxicon* 2010, 56, 842–847.
190. Beaumont, S.; Ilardi, E.A.; Tappin, N.D.C.; Zakarian, A. Marine toxins with spiroimine rings: Total synthesis of pinnatoxin A. *Eur. J. Org. Chem.* 2010, 2010, 5743–5765.
191. Alexander, J.; Auðunsson, G.A.; Benford, D.; Cockburn, A.; Cravedi, J.P.; Dogliotti, E.; Di Domenico, A.; Fernández-Cruz, M.L.; Fink-Gremmels, J.; Fürst, P.; et al. Scientific opinion of the panel on contaminants in the food chain on a request from the european commission on marine biotoxins in shellfish—saxitoxin group. *EFSA J.* 2009, 1019, 1–76.
192. Alexander, J.; Auðunsson, G.A.; Benford, D.; Cockburn, A.; Cravedi, J.P.; Dogliotti, E.; Di Domenico, A.; Fernández-Cruz, M.L.; Fink-Gremmels, J.; Fürst, P.; et al. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish—Okadaic acid and analogues. *EFSA J.* 2008, 589, 1–62.
193. Aune, T.; Larsen, S.; Aasen, J.A.; Rehmann, N.; Satake, M.; Hess, P. Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* 2007, 49, 1–7.
194. Islam, Q.T.; Razzak, M.A.; Islam, M.A.; Bari, M.I.; Basher, A.; Chowdhury, F.R.; Sayeduzzaman, A.B.M.; Ahasan, H.A.M.N.; Faiz, M.A.; Arakawa, O.; et al. Puffer fish poisoning in Bangladesh: Clinical and toxicological results from large outbreaks in 2008. *Trans. R. Soc. Trop. Med. Hyg.* 2011, 105, 74–80.
195. Kawabata, T. The manual for the methods of food sanitation tests. In Bureau of Environmental Health, Ministry of Health and Welfare; Japan Food Hygienic Association: Tokyo, Japan, 1978; Volume II, p. 232.

196. CRLMB (Community Reference Laboratory for Marine Biotoxins). Report on toxicology working group meeting, Cesenatico, Italy, 24–25 October 2005. Available online: <http://www.aesan.msps.es/en/CRLMB/web/home.shtml> (accessed on 9 April 2014).
197. Botelho, M.J.; Vale, C.; Mota, A.M.; Rodrigues, S.M.; Costa, P.R.; Simões Gonçalves, M.L.S. Matrix effect on paralytic shellfish toxins quantification and toxicity estimation in mussels exposed to *Gymnodinium catenatum*. *Food Addit. Contam. Part A* 2010, 27, 1724–1732.
198. Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 Laying Down Specific Hygiene Rules for Food of Animal Origin OJ L 139, 30.4.2004. Available online: <http://eur-lex.europa.eu> (accessed on 15 April 2014).
199. De Fouw, J.C.; van Egmond, H.P.; Speijers, G.J.A. Ciguatera fish poisoning: A review. Available online: <http://www.rivm.nl/bibliotheek/rapporten/388802021.pdf> (accessed on 10 May 2014).

Objectives

Initially the objectives were related to the study of TTX:

- 1 – Screening of TTX in several systematic groups in the Portuguese waters.
- 2- Optimization and Validation of the extraction methods used previously.
- 3 - Detection and dynamics of the TTX in gastropods.
- 4 – Bioaccumulation and biomagnification of TTX via food.

As the study evolved and new toxin groups were added, the objectives had to be adapted to:

1. Screening of regulated and emergent toxins in several systematic groups not monitored regularly.
2. Optimization and Validation of the extraction methods used previously.
3. Detection and dynamics of the screened toxins in benthic and subtidal species.

Second Section

Chapter 3. Emergent toxins in the Portuguese Coast, Islands and the northwestern Moroccan Coast

Paper 2

New Method of conversion for GTX1,4 using thiol compounds in Starfish and Gastropod Matrices
Submitted

Paper 3

New Gastropod Vectors and Tetrodotoxin Potential Expansion in Temperate Waters of the Atlantic Ocean.

Published: Marine Drugs, 2012; 10(4):712-726

Paper 4

New Invertebrate Vectors for PST, Spirolides and Okadaic Acid in the North Atlantic.

Published: Marine Drugs 2013; 11, 1936-1960.

Paper 5

New Invertebrate Vectors of Okadaic Acid from the North Atlantic Waters - Portugal (Azores and Madeira) and Morocco.

Submitted

Paper 6

First Report of CIGUATOXINS in two starfish species: *Ophidiaster ophidianus* and *Marthasterias glacialis*.

Published: Toxins 2015; 7, 3740-3757.

Submitted

Determination of gonyautoxin-4 in echinoderms and gastropod matrices by conversion to neosaxitoxin using 2-mercaptoethanol and post-column oxidation liquid chromatography with fluorescence detection

Marisa Silva^{1,2†}, Verónica Rey^{3†}, Ana M. Botana³, Vitor Vasconcelos^{1,2} and Luis M. Botana⁴

¹Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, Porto 4619-007, Portugal

²Interdisciplinary Center of Marine and Environmental Research–CIMAR/CIIMAR, University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

³ Department of Analytical Chemistry, Science Faculty, University of Santiago of Compostela, 27002 Lugo, Spain

⁴ Department of Pharmacology, Veterinary Faculty, University of Santiago de Compostela, Lugo 27002, Spain

† These authors contributed equally for this work.

Abstract

Paralytic Shellfish Toxins blooms are common worldwide which makes their monitoring crucial in the prevention of poisoning incidents. These toxins can be monitored by a variety of techniques, including mouse bioassay, receptor binding assay, and liquid chromatography with either mass spectrometric or pre- or post-column fluorescence detection. The post-column oxidation liquid chromatography with fluorescence detection method, used routinely in our laboratory, has been shown to be a reliable method for monitoring paralytic shellfish toxins in mussel, scallop, oyster and clam species. However, due to its high sensitivity to naturally fluorescent matrix interferences, when working with unconventional matrices, there may be problems to identify toxins because of naturally fluorescent interferences that co-elute with the toxin peaks. This can lead to erroneous identification. In this work, to overcome this challenge in echinoderm and gastropod matrices, we optimized the conversion of Gonyautoxins 1 and 4 to Neosaxitoxin with 2-mercaptoethanol. We present a new and fast method with a good recovery (82.2%, RDS 1.11%, n=3), requiring only a single reaction step.

Keywords: Paralytic shellfish poisoning; Toxins; Postcolumn oxidation method; interfering matrix peaks; thiol compounds.

Introduction

Paralytic Shellfish Toxins (PSTs) are neurotoxic alkaloids responsible for the Paralytic Shellfish Poisoning syndrome (PSP), produced mainly by three dinoflagellates genera: Alexandrium, Pyrodinium and Gymnodinium. This biotoxin group is composed by saxitoxin (STX) and its analogues, to date more than 57 known saxitoxin analogues, the three main subgroups are: STX group- saxitoxin (STX), neosaxitoxin (NEO), decarbamoyl saxitoxin (dcSTX), GTXs group - gonyautoxins 1-5 (GTX1, GTX2, GTX3, GTX4 and GTX5), decarbamoyl gonyautoxins 2-3 (dcGTX2, dcGTX3) and C group- N-sulfocarbamoyl-gonyautoxins 1-4 (C3, C1, C2 and C4) [1,2]. PSTs were first reported in 1920's in the USA but till nowadays have been reported worldwide, affecting the ecosystem, causing economical losses and human health through poisoning incidents, some of them with fatal outcomes [3,4]. PSTs are fast acting toxins, affecting skeletal muscle, by binding specifically to voltage gated sodium channels, inhibiting cell communication, causing paralysis [1,5]. The most common route of intoxication is through the ingestion of contaminated seafood, but due to monitoring programs and legislation, poisoning events have been scarce in the past 20 years [3,4]. Though these measures are only focused in bivalve mollusks, PSTs have already been reported in other vectors, from crustaceans to gastropods and echinoderms and updated risk assessment is currently required [6]. For many years the mouse bioassay (MBA), has been the reference method for the detection and quantitation for monitoring purposes [7]. Actually, due to ethical and technical issues associated with the MBA [8], chemical methods have gained preference and replaced this for regulatory monitoring in most countries, such as is required by EU legislation [7,9]. These methods depend on standard reference materials, in the case of PSTs there are 13 (STX, NEO, GTX1, GTX4, GTX2, GTX3, GTX5, dcSTX, dcGTX2, dcGTX3, C1, C2 and dcNEO) reference standards for calibration available by Cifga. In 2006, a pre-column oxidation high performance liquid chromatography (HPLC) with fluorescence detection (FLD) method (Lawrence method) was integrated in the European Directives as a reference method [9]. Latter in 2011, a post-column oxidation high performance liquid chromatography (HPLC) with fluorescence detection (FLD) method (PCOX method) was validated by the Association of Official Analytical Chemists (AOAC International) [10]. The PCOX method presents several advantages regarding routine analysis in comparison to the Lawrence method [11], though its high sensitivity to naturally fluorescent matrix interferences can pose some challenges [12]. The PCOX method is characterized by dividing the toxins in two groups: C group (C1, C2, C3 and C4) and GTXs and STX group (GTX1, GTX2, GTX3, GTX4, GTX5, dcGTX2, dcGTX3, NEO, dcSTX and STX). While to visualize the toxins of C group, the standard is diluted in milliQ water, the same doesn't occur for GTXs and STX group, where the standards are diluted in PSP free shellfish tissue [13], and the elution pattern of the toxins starts with GTX4 and ends in STX (Figure 2.1).

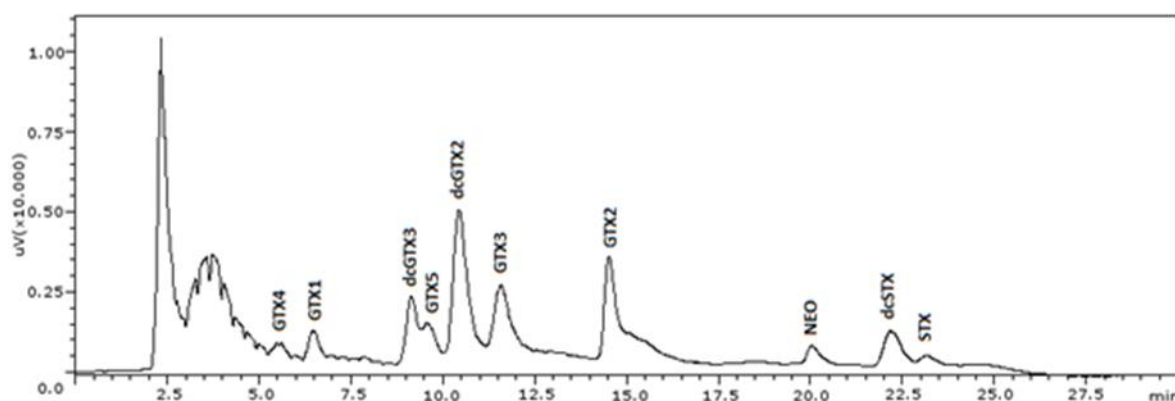


Figure 2.1 - PSTs elution pattern in spiked mussel with standards..

In this study, we worked with several matrices - bivalves, gastropods, echinoderms, crustaceans and fish. However, as seen in previous work with scallop and oyster matrices [11], there is an overlapping effect issue, due to GTX4 standard that in some matrices (starfish and gastropod) overlaps with the naturally fluorescent matrix compounds.

To overcome this issue, the aim of this work was the conversion of GTX4 into NEO using thiol compounds, to quantify GTX4 in several samples that belong to different species of starfish and gastropods, in which it is suspected that GTX4 overlaps with matrix peaks.

In this study, we worked with several different matrices - bivalves, gastropods, echinoderms, crustaceans and fish. However, as seen in previous work with some scallop and oyster matrices [12,14], there can be naturally fluorescent interferences that co-elute with GTX4. Naturally fluorescent interferences were found in this study in echinoderms and gastropods.

Some of the GTXs have been shown to be transformed reductively into STX or NEO by biologically available thiol reagents such as glutathione (GSH) [15]. This activity was also found in another thiol compound, 2-mercaptoethanol (2-ME) [16]. These thiols compounds convert the mixtures of GTX2 and GTX3 to STX and mixtures of GTX1 and GTX4 to NEO [16].

To overcome this issue of naturally fluorescent matrix interferences we converted GTX1 and GTX4 in NEO using 2-mercaptoethanol in samples which were suspected to have naturally fluorescent interferences.

Results and Discussion

The reaction of toxins with thiol reagents (especially GSH, but also 2-ME) seems to be involved in the bioconversion of GTX1 and GTX4 to NEO in shellfish, as observed by several researchers [15,17]. These thiols selectively reduce O-sulfate group at C11 of GTX1 and GTX 4 to form NEO.

Sakamoto et al. [16] converted GTXs to STXs with thiol compounds: GTX1 and GTX4 (4 nmol) were mixed with 1 mL of 8 mM 2-ME in 0.1M phosphate buffer, pH 7.4. Then this mixture was incubated at 70 °C for 2 h. These conditions used by Sakamoto et al. were replicated in our laboratory, and it seemed that GTX1 and GTX4 disappeared but without NEO formation, as a result of the reaction. The theoretically yielded amount of NEO after this reductive reaction should be 0.6 nmol of NEO (15% of conversion) as it was described [16]. This values are below the PCOX detection limit [12], and for this reason NEO is not seen. The low conversion rate is due to the formed conjugates between GTXs and 2-ME in the reaction course. To complete the reaction, a second step is needed which consists of an incubation with 1M 2-ME at 100 °C for 10 min, so that conjugates of GTX1, GTX4 and 2-ME are converted into NEO. Here the percentage of recovery obtained was lower than expected, being only 40% of NEO whereas circa 90% was expected according to Sakamoto et al. [16]. To ensure the effectiveness of the transformation reaction there must be an excess of the thiols molecules. Therefore, for the second step of the reaction different concentrations of 2-ME in 0.1M buffer phosphate were assayed: 1M, 3M and 5M; no differences were observed with these variations in 2-ME concentration. Taking into account what happened, a different way to optimize reaction conditions was tried being a one-step reaction where incubation temperature and time were varied according to the following: 4 nmol of GTX1 and GTX4 were mixed with 1M 2-ME in 0.1M phosphate buffer, pH 7.4. The mixture was heated in a water bath at 100°C and different incubation times were tested: 10, 15, 20, 30 and 40 minutes (Table 2.1).

Table 2.1 . % recovery of NEO after reaction of GTX1 and GTX4 with 2-ME 1M in phosphate buffer 0.1M, pH 7.4 (average n=3, and RSD %).

Time (minutes)	100°C water bath		100°C incubator	
	Average %	RSD %	Average%	RSD%
10	59.5	0.8	64.5	2.8
15	55.3	0.3	58.4	1.7
20	62.6	1.0	58.9	1.0
30	82.2	1.1	50.5	1.4
40	64.8	1.3	31.0	2.4

The same procedure was made in an incubator at 100°C, and the times tested were the same: 10, 15, 20, 30 and 40 minutes; the results were lower in most cases than those obtained with the water bath (Table 2.1), and although for 10 and 15 minutes the recovery percentages are a bit higher there is a remarkable difference in recovery for 30 minutes in the water bath, which has been taken as the optimal value. In general the obtained results showed that the reaction is more effective with wet heat compared to dry heat, and higher percentage of transformed NEO was obtained after 30 minutes (Table 2.1).

The reduction reaction had been replicated (n=3). Regarding repeatability, the average conversion percentage was 82.2% with RSD 1.11%. Although the results were not higher than previous works [16], the reaction is now simpler as it has changed from two steps to only one as showed in Figure 2.2a (before reductive reaction) and 2.2b (after reductive reaction).

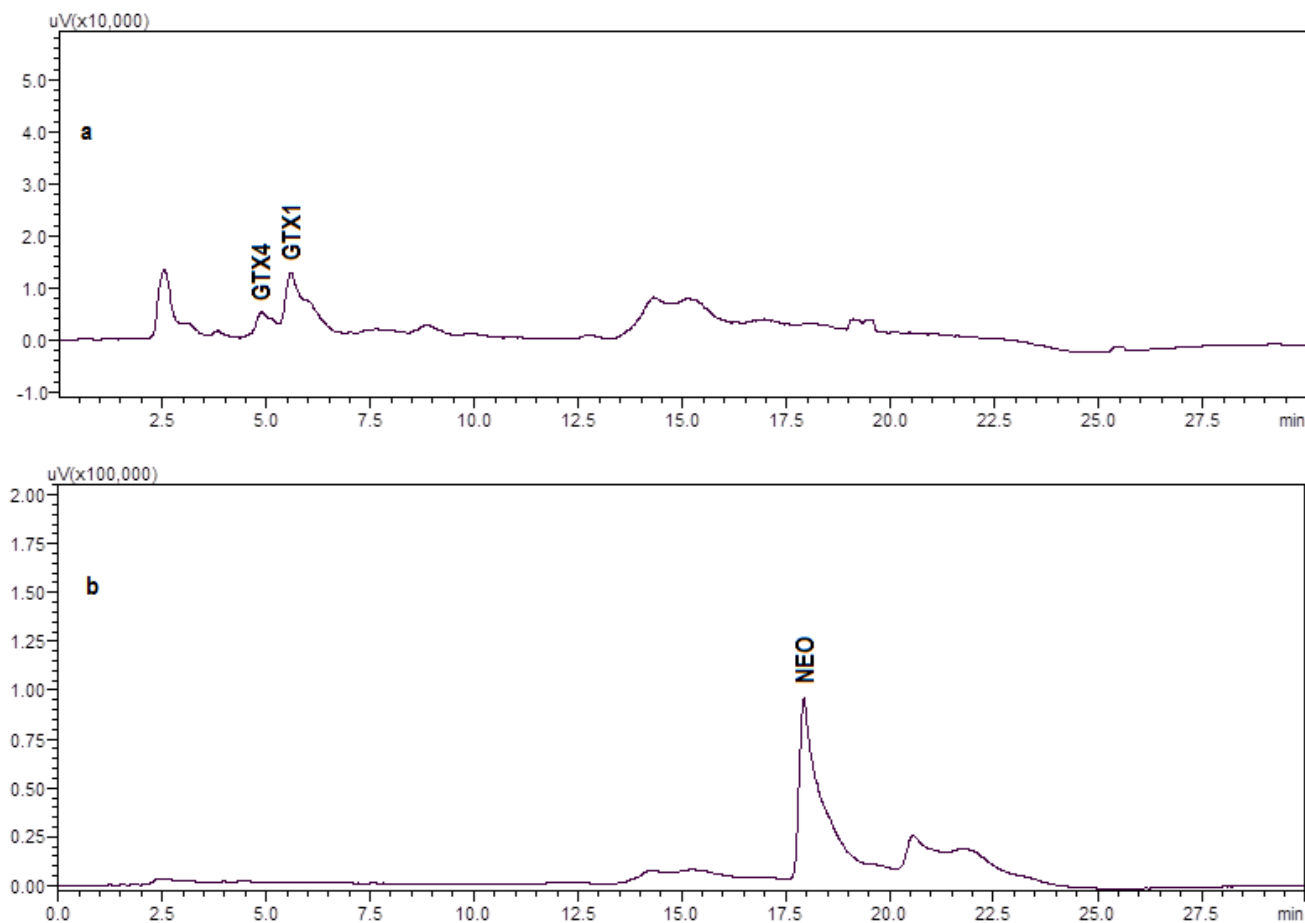


Figure 2.2 -GTX1,4 (1.6 nmol) incubated with 1M 2-ME in 0.1M phosphate buffer, pH 7.4 during 30 minutes in water bath. a) Before incubation; b) After incubation.

Suspected samples of containing GTX4 or a co-eluting naturally fluorescent interference, were subjected to 2-ME reduction as described in the sample preparation and transformation section Table 2.2 shows tested samples and results. It was found that when the peak was due to GTX4, it disappeared and it was reduced to NEO; however if the peak was due to natural fluorescent components present in the sample, the size of the peak before and after the reductive reaction did not change as described in the experimental section. Figure 2.3 shows the chromatograms of sample 477 (*S. haemostoma*) before incubation with 2-ME (Figure 3a) and after incubation with 2-ME (Figure 2.3b), this sample had a chromatographic peak for GTX4 which was suspected to be a natural fluorescent matrix interference. After exposure to 2-ME this peak was not reduced and there was no production of NEO confirming that this peak was due to naturally fluorescent matrix interference.

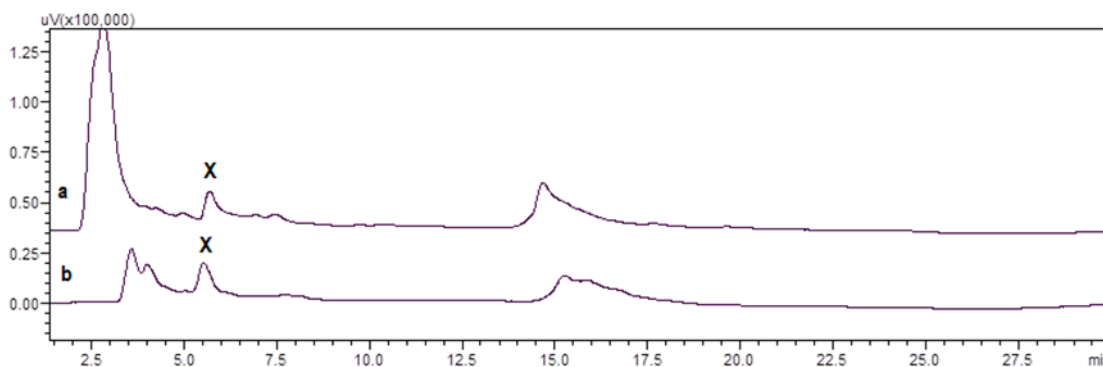


Figure 2.3 -a) Sample 477 (*S. haemostoma*) before transformation, b) sample 477 (*S. haemostoma*) after transformation.

Figure 2.4 shows a positive sample, 454 (*Cerithium vulgatum*), before and after transformation (2.4a and 2.4b, respectively). The chromatographic peak for GTX4 decreased but was not completely eliminated after the reaction and NEO was produced by the transformation, indicating that the peak was likely due to a combination of both GTX4 and naturally fluorescent matrix interference. When NEO was produced by the reduction, the GTX4 peak was reduced; when NEO was not produced by the reduction, no change was observed to the GTX4-interference peak in the reduced sample, making interpretation easier.

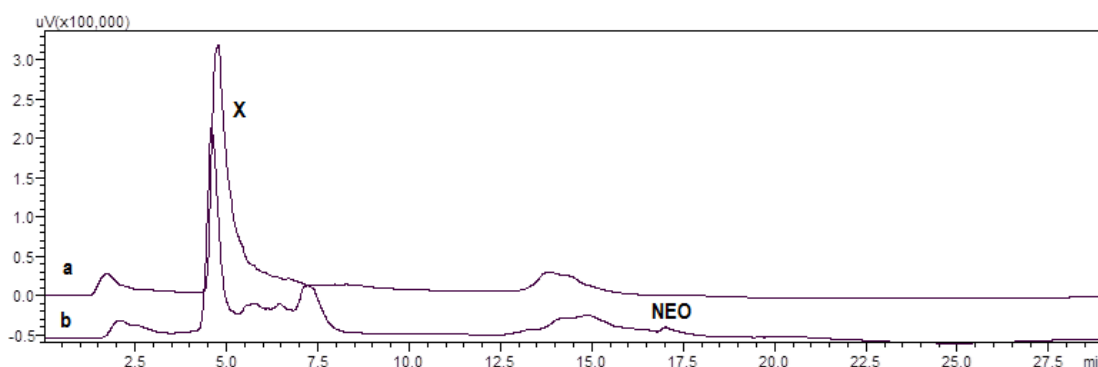


Figure 2.4 -a) Sample 454 (*Cerithium vulgatum*) before transformation, b) sample 454 (*Cerithium vulgatum*) after transformation.

In Table 2.2 summarized all the samples tested with code, specie and results before and after transformation.

After, optimizing the conversion reaction conditions using 2-ME as a reducing agent, the same settings were tested with Glutathione (GSH). A solution of 0.2 M GSH in 0.1 M phosphate buffer, pH 7.4; was used to achieve transformation and it was found that after heating the mixture in water bath during 30 min, GTX1 and GTX4 wasn't reacted with GSH, so the reaction wasn't occurred.

Table2.2 Tested samples and results before the reaction with 2-ME.

Code	Specie	Before transformation (µM)		After transformation (µM)	
		GTX4	GTX1	NEO	GTX4

351	<i>Umbraculum umbraculum</i>	1.69	1.11	1.27	0.16
353	<i>Echinaster sepositus</i>	0.77	1.81	2.31	0.51
354	<i>Charonia lampas</i>	90.48	3.78	29.30	25.52
412	<i>Ophidiaster ophidianus</i>	17.29	3.67	4.36	0.68
424	<i>O. ophidianus</i>	17.00		4.55	4.55
428	<i>M. glacialis</i>	42.84	18.92	29.85	10.93
440	<i>O. ophidianus</i>	26.43			
443	<i>Stramonita haemostoma</i>	428.55			
454	<i>Cerithium vulgatum</i>	121.56		0.39	0.39
470	<i>M. lineata</i>	3.86		2.54	2.54
474	<i>Onchidela celtica</i>	6.91			
475	<i>C. lampas</i>	8.44			
477	<i>S. haemostoma</i>	2.50			
483	<i>G. umbilicalis</i>	13.34			

While previous works [15,16] demonstrated that both thiol reagents are able to transform GTXs in STXs, in this case GSH did not have the same results as 2-ME.

There is evidence that the action of intestinal bacteria mediate the transformation of GTXs analogs into STXs through GSH [17-19]. This conversion increases the toxic content of PSP-bearers, and consequently increasing the toxicity in the food chain [6].

In this work, the transformation reaction was used to confirm the presence and to quantify GTX4 in several samples.

The PCOX method, currently used for monitoring of PSP in our laboratory, requires the use of toxins-free shellfish extract to prepare the calibration solution to overcome the matrix effect and also to facilitate data interpretation [13]. The post-column method pose some challenges relatively to the co-eluting of matrix peaks with toxin ones, hindering data analysis [12]. The PCOX method was optimized and validated for mussel, clam, scallop and oyster [13], although initially these four matrixes showed no naturally fluorescent matrix interferences, later works [12,14] showed that both scallop and oyster have naturally fluorescent matrix interference. GTX1 and GTX4 elute early off the chromatographic column [13], and this region has since been shown to contain naturally fluorescent peaks in some matrices [12,14].

As toxin-free shellfish extracts of these matrices (echinoderm and gastropod) were not available, the performance of the method for these new matrices was not able to be assessed or validated. The

conversion of GTX1 and GTX4 to NEO made it possible to detect and quantify GTX4 when it co-eluted with a naturally fluorescent matrix chromatographic peak. It is important because even though this method is only validated for bivalves, PSTs have been reported in other vectors [6] could present a risk to the health of consumers.

It was observed that the transformation of GTX1 and GTX4 into NEO was the most cost-effective at a concentration of 1M 2-ME in 0.1M phosphate buffer (pH 7.4), as increasing the concentration did not increase the yield. This means that this is a two-step reaction and excess molecules of thiols are necessary to release NEO [16].

In this work, the conditions of the conversion of GTX4 to NEO were optimized with an 82.2% of recovery (n=3, RSD. 1.11%). Although results of this conversion are not as high as those shown in previous studies [16], is important to note that this study represents an optimization of the protocol to only one step reaction using an unique solution with thiol compounds. The naturally fluorescent matrix peak was not affected by the reduction allowing confirmation of non-detectable GTX4 when NEO was not produced. This protocol represents a faster, easier and more economical way to overcome the challenge of co-eluting peaks in new shellfish matrices.

Experimental Section

Chemical and solutions

HPLC grade acetonitrile, ortho-phosphoric acid 85% (v/v), periodic acid, sodium hydroxide, nitric acid 65% (v/v), hydrochloric acid 37% (v/v), sodium dihydrogen phosphate (NaH₂PO₄) and disodium hydrogen phosphate (Na₂HPO₄) were acquired from Panreac Quimica S.A. (Barcelona, Spain). Heptane sulfonate, ammonium hydroxide 30% (v/v), trichloroacetic acid (TCA), 2-mercaptoethanol (2-ME) and glutathione (GSH) were purchased from Sigma Aldrich (Madrid, Spain).

Standards of NEO, were purchased from CIFGA S.A. (Lugo, Spain) and GTX1 and GTX4 was provided by NRC Certified Reference Material Program (Institute for Marine Biosciences, Halifax, Canada) for the identification of each toxin.

A quality control standard of GTX1 and GTX4 from CIFGA S.A. was used for transformation reactions.

Sample preparation

Shellfish homogenate was extracted according to the procedure indicated in PCOX method [13]. The method involves a sample extraction with 0.1 M HCl with heating (adjust the pH previously if required between 2 and 4, preferably 3), an extract deproteization with TCA and a neutralization with NaOH to bring the mixture pH between 2 and 4, preferably 3. Then the extract was filter with 0.2 µm syringe filter.

The toxin profile was analyzed by PCOX method. The samples which have the naturally fluorescent interferences were analyzed again after reductive transformation as described below.

Mixtures of GTX1 and GTX4 (4 nmol from both) were mixed with 1 M 2-ME in 0.1 M phosphate buffer, pH 7.4. An aliquot of this mixture was analyzed by HPLC before reductive reaction to direct comparison with an aliquot after reductive reaction. These mixtures were heated at 100 °C for 30 minutes in water bath. An aliquot of reductive reaction mixture was analyzed for toxin components by HPLC. Shellfish sample extracts had the same treatment and were analyzed by HPLC for toxin profile determination.

HPLC toxins identification

A modification of PCOX method was used [14] to identify PSTs. STX and GTXs group were separated using a Zorbax Bonus-RP column (15 cm x 4.6 mm i.d., 3.5 µm particle size, part number 863668-901) from Agilent Technologies (Madrid, Spain), with column oven at 30 °C. Solvent A was composed by 8.25 mM heptane sulfonate and 5.5 mM H₃PO₄ aqueous solution adjusted to pH 7.1 using NH₄OH 28-30%. Solvent B composition was 8.25 mM heptane sulfonate, 16.5 mM H₃PO₄ in 11.5% MeCN, pH 7.1 using NH₄OH 28-30%. The gradient used was 0% B over 8.4 min, then 100% B at 8.5 min for 10 min, 0% B for 9 min before the next injection. The injection volume was 10 µl. The running time was 30 min, with a flow rate adjusted at 0.8 ml/min. The column eluate was combined using a tee connector with the oxidant: 100 mM H₃PO₄, 5 mM H₅IO₆ aqueous solution adjusted to pH 7.8 with 5 M NaOH; the oxidant flow was set at 0.5 ml/min. The resulting mix is heated while passing through a knitted reaction coil (teflon tube, 5 m x 0.50 mm i.d.) from Supelco (Madrid, Spain) immersed in a water bath at 80 °C. The eluate from the reaction coil was combined using a tee connector with 0.1 M nitric acid at a flow rate of 0.3 ml/min, to reach a pH outflow ranging between 5-7 [20]. Finally, the fluorescent eluted derivatives were monitored using a fluorescence detector at 330 and 395 nm excitation/emission wavelengths, respectively.

Calculate the concentration of GTX4

Identification of PSTs was done comparing retention times between the samples and standards.

GTX1 was quantified directly, the reductive reaction was not needed for quantification. Consequently, the concentration was calculated using the linear regression of the calibration curve for this toxin and was corrected the results for the method dilution [13]. The equation for calculation of final concentration is:

$$\mu\text{MGTX1} = (\mu\text{M} * (\text{Fvol}/\text{Ext.vol})^2) [1]$$

Where μMGTX1 = $\mu\text{mol/L}$, final concentration of GTX1 in the extract; μM = $\mu\text{mol/L}$, concentration obtained to calibration curve; F_{vol} = final volume of the deprotenized extract (560 μl); Ext.vol = volume of crude extract used (500 μl); 2 = dilution factor of the extraction method: 5 g of tissue diluted to 10 ml. The concentration of GTX4 was calculated after reduction reaction, from the amount of NEO formed. Determination of the linear regression curve (peak area versus concentration in μM) from the results for injections of the standards solutions of NEO. Calculation of the amount (μM) of NEO in the samples extracts using the linear regression of the calibration curve.

The amount of NEO formed in the reduction reaction is equal to the amount of GTX1 combined with the initial concentration of GTX4 for each sample. Therefore:

$$\mu\text{MNEO} = \mu\text{M for GTX1} + \mu\text{M for GTX4} \quad [2]$$

The reduction reaction involves a dilution of the sample, 22 μl of sample to 200 μl of final volume. Besides the conversion is not complete, only 82.2% is transformed into NEO. These two factors should be used to correct the amount of NEO. The amount must be corrected again with dilutions factors inherent PCOX method as showed in equation 1.

$$\mu\text{MNEO} = (\mu\text{M} * (F_{\text{re}} / \text{Ext.re}) * (100 / 82.2)) * (F_{\text{vol}} / \text{Ext.vol}) * 2 \quad [3]$$

Where μMNEO = $\mu\text{mol/L}$, final concentration of NEO in the sample; μM = $\mu\text{mol/L}$, obtained using the linear regression of the calibration curve; F_{re} = final volume of the reaction mixture (200 μl); Ext.re = volume of extract used in the reaction (22 μl); 100/82.2 = recovery of reduction reaction; F_{vol} = final volume of the deprotenized extract (560 μl); Ext.vol = volume of extract used (500 μl); 2 = dilution factor of the extraction method: 5 g of tissue diluted to 10 ml.

Combining equations 2 and 3 it is obtained as result:

$$\mu\text{MGTX1} + \mu\text{MGTX4} = (\mu\text{M} * (F_{\text{re}} / \text{Ext.re}) * (100 / 82.2)) * (F_{\text{vol}} / \text{Ext.vol}) * 2 \quad [4]$$

If in the sample was previously detected GTX1 it was necessary to subtract its concentration to reduce the potential for overestimation of GTX4:

$$\mu\text{MGTX4} = ((\mu\text{M} * (F_{\text{re}} / \text{Ext.re}) * (100 / 82.2)) * (F_{\text{vol}} / \text{Ext.vol}) * 2) - \mu\text{MGTX1} \quad [5]$$

If in the sample was no detected GTX1, the concentration obtained in equation 4 correspond only of GTX4.

Conclusions

In summary, each matrix is different and influences greatly toxins elution pattern. Regarding routine monitoring analyses this different chromatographic behaviors pose some issues of erroneous toxin identification or quantification. In this work it is proposed a GTX4 reductive reaction to NEO. This new protocol is a quicker and economical alternative to solve the co-eluteing peak problem between natural fluorescence matrix interference and toxin peaks.

Acknowledgements

The USC research leading to these results has received funding from the following FEDER cofunded-grants. From CDTI and Technological Funds, supported by Ministerio de Economía y Competitividad, AGL2012-40185-CO2-01, AGL2014-58210-R, and Consellería de Cultura, Educación e Ordenación Universitaria, GRC2013-016, and through Axencia Galega de Innovación, Spain, ITC-20133020 SINTOX. From CDTI under ISIP Programme, Spain, IDI-20130304 APTAFOOD.

From the European Union's Seventh Framework Programme managed by REA – Research Executive Agency (FP7/2007-2013) under grant agreement 312184 PHARMASEA. This research was partially funded at CIIMAR by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Program - and national funds through FCT - Foundation for Science and Technology - under the project PEst-C/MAR/LA0015/2013 and by the projects MARBIOTECH (reference NORTE-07-0124-FEDER-000047) within the SR&TD Integrated Program MARVALOR - Building research and innovation capacity for improved management and valorization of marine resources, supported by the Programa Operacional Regional do Norte (ON.2-O Novo Norte) and NOVOMAR. Marisa Silva acknowledge also FCT for the grant SFRH/BD/73269/2010.

Author Contributions

M.S., V.R.conceived the idea, M.S. performed the sampling, M.S. and V.R. held sample analyzes and paper writing.

A.B. contributed on experimental design.

V.V. and L.M. contributed in funding, materials and analyses tools.

All: proof reading of the manuscript

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Alexander, J.; Auðunsson, G.; Benford, D.; Cockburn, A.; Cravedi, J.; Dogliotti, E.; Di Domenico, A.; Fernández-Cruz, M.; Fürst, P.; Fink-Gremmels, J., Cross-contamination of non-target feedingstuffs by monensin authorised for use as a feed additive. *The EFSA Journal* 2008, 592, 1-40.
2. Rourke, W.A.; Murphy, C.J.; Pitcher, G.; van de Riet, J.M.; Burns, B.G.; Thomas, K.M.; Quilliam, M.A., Rapid postcolumn methodology for determination of paralytic shellfish toxins in shellfish tissue. *Journal of AOAC International* 2008, 91, 589-597.
3. Rodrigues, S.M.; de Carvalho, M.; Mestre, T.; Ferreira, J.J.; Coelho, M.; Peralta, R.; Vale, P., Paralytic shellfish poisoning due to ingestion of gymnodinium catenatum contaminated cockles--application of the aoac hplc official method. *Toxicon* 2012, 59, 558-566.
4. Meyer, K.; Sommer, H.; Schoenholz, P., Shellfish poisoning. *Medicine* 1928, 2, 365.
5. Bandem, D.; Trainer, V., Mode of action of toxins of seafood poisoning. In *Algal toxins in seafood and drinking water*, Falconer, I.R., Ed. Academic Press: San Diego, California, 1993; p 49.
6. Silva, M.; Barreiro, A.; Rodriguez, P.; Otero, P.; Azevedo, J.; Alfonso, A.; Botana, L.M.; Vasconcelos, V., New invertebrate vectors for psp, spirolides and okadaic acid in the north atlantic. *Marine Drugs* 2013, 11, 1936-1960.
7. Anon, A., Official method 959.08. Paralytic shellfish poison. Biological method. Final action. *AOAC Official Methods for Analysis* 2005, 79-80.
8. Alexander, J.; Benford, D.; Boobis, A.; Ceccatelli, S.; Cravedi, J.-P.; Di, A.; Domenico, D.D.; Dogliotti, E.; Edler, L.; Farmer, P., Marine biotoxins in shellfish—summary on regulated marine biotoxins scientific opinion of the panel on contaminants in the food chain. *The EFSA Journal* 2009, 1306, 1-23.
9. Anon, A., Official method 2005.06 quantitative determination of paralytic shellfish poisoning toxins in shellfish using pre-chromatographic oxidation and liquid chromatography with fluorescence detection. *AOAC International*, Gaithersburg, MD, USA 2005.
10. Anon, A., Official method 2011.02 determination of paralytic shellfish poisoning toxins in mussels, clams, oysters and scallops. Post-column Oxidation Method (PCOX). First Action 2011 *AOAC International*, Gaithersburg, MD, USA 2011.
11. DeGrasse, S.L.; van de Riet, J.; Hatfield, R.; Turner, A., Pre-versus post-column oxidation liquid chromatography fluorescence detection of paralytic shellfish toxins. *Toxicon* 2011, 57, 619-624.
12. Rey, V.; Alfonso, A.; Botana, L.M.; Botana, A.M., Influence of different shellfish matrices on the separation of psp toxins using a postcolumn oxidation liquid chromatography method. *Toxins* 2015, 7, 1324-1340.
13. Van De Riet, J.; Gibbs, R.S.; Muggah, P.M.; Rourke, W.A.; MacNeil, J.D.; Quilliam, M.A., Liquid chromatography post-column oxidation (pcox) method for the determination of paralytic shellfish toxins in mussels, clams, oysters, and scallops: Collaborative study. *Journal of AOAC International* 2010, 94, 1154-1176.

14. Li, A.; Ma, J.; Cao, J.; Wang, Q.; Yu, R.; Thomas, K.; Quilliam, M., Analysis of paralytic shellfish toxins and their metabolites in shellfish from the north yellow sea of china. *Food Additives & Contaminants: Part A* 2012, 29, 1455-1464.
15. Sato, S.; Sakai, R.; Kodama, M., Identification of thioether intermediates in the reductive transformation of gonyautoxins into saxitoxins by thiols. *Bioorganic & Medicinal Chemistry Letters* 2000, 10, 1787-1789.
16. Sakamoto, S.; Sato, S.; Ogata, T.; Kodama, M., Formation of intermediate conjugates in the reductive transformation of gonyautoxins to saxitoxins by thiol compounds. *Fisheries science* 2000, 66, 136-141.
17. Oshima, Y., Chemical and enzymatic transformation of paralytic shellfish toxins in marine organisms. In *Harmful marine algal blooms*, Lassus, P.; al., e., Eds. Lavoisier Intercept Ltd.: Paris, 1995; p 475.
18. Asakawa, M.; Takagi, M.; Iida, A.; Oishi, K., Studies on the conversion of paralytic shellfish poison (psp) components by biochemical reducing agents. *Eisei Kagaku* 1987, 33, 50-55.
19. Shimizu, Y.; Yoshioka, M., Transformation of paralytic shellfish toxins as demonstrated in scallop homogenates. *Science* 1981, 212, 547-549.
20. Vale, C.; Alfonso, A.; Vieytes, M.R.; Romarís, X.M.; Arévalo, F.; Botana, A.M.; Botana, L.M., In vitro and in vivo evaluation of paralytic shellfish poisoning toxin potency and the influence of the pH of extraction. *Analytical chemistry* 2008, 80, 1770-1776.

Marine Drugs 2012, 10, 712-726

New Gastropod Vectors and Tetrodotoxin Potential Expansion in Temperate Waters of
the Atlantic Ocean

Marisa Silva^{1,2}, **Joana Azevedo**^{1,3}, **Paula Rodriguez**⁴, **Amparo Alfonso**⁴, **Luis M. Botana**⁴ and
Vítor Vasconcelos^{1,2,*}

Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4619-007 Porto, Portugal; E-Mails: marisasilva17@gmail.com (M.S.); joana_passo@hotmail.com (J.A.)

Center of Marine and Environmental Research—CIMAR/CIIMAR, University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

Department of Chemical and Biomolecular Sciences, School of Health and Technology of Porto, Vila Nova de Gaia, 4400-330 Vila Nova de Gaia, Portugal

Department of Pharmacology, Faculty of Veterinary, University of Santiago of Compostela, 27002 Lugo, Spain; E-Mails: paula.rodriguez17@rai.usc.es (P.R.); amparo.alfonso@usc.es (A.A.); Luis.Botana@usc.es (L.M.B.)

Abstract: Tetrodotoxin is a potent low weight marine toxin found in warm waters, especially of the Indian and Pacific Oceans. Intoxications are usually linked to the consumption of the puffer fish, although TTX was already detected in several different edible taxa. Benthic organisms such as mollusks and echinoderms, with different feeding habits, were collected monthly along the Portuguese coast from the summer of 2009 until the end of 2010. The extraction and analysis techniques were optimized and TTX and some analogues were detected for the first time in two intertidal gastropod species—*Gibbula umbilicalis* and *Monodonta lineata* by LC-MS/MS and UPLC-MS/MS. Although the levels are low, these findings suggest that monitoring of TTX and analogues in North Atlantic species should be implemented so as to detect potentially new toxin vectors and seasonal and/or geographical patterns.

Keywords: tetrodotoxin; new vectors; gastropods; North Atlantic Waters

Introduction

Tetrodotoxin (TTX) is a low weight potent neurotoxin, named after the Tetradontidae fish family from where it was first isolated in 1909 by Tahara and Hirata [1]. TTX is an interesting toxin, since it was reported in several taxa genetically not close related; from bacteria; marine invertebrates; terrestrial and marine vertebrates [2]. Neither its biochemical path nor its true origin is fully clarified, since three hypotheses point to its origin: endogenous [3,4], through food-chain [5–8] or through symbionts [9–12]. TTX is an extremely potent toxin, it binds specifically to site 1 of the voltage-gated sodium channels (Nav), occluding the external pore blocking the cellular communication and causing death by cardio-respiratory paralysis [13–16]. Several poisoning incidents have occurred, especially in Asia, with Japan being the most affected country and where Fugu is considered a delicacy. Japan is the only country to have guideline values for TTX [17]. Although TTX-bearers are typical of warm waters, recent studies report the possible migration of these toxic species from the Red Sea to the Mediterranean Sea through the Suez Canal [18–20]. This may happen due the opening of new corridors allied to the increase of water temperature as a result of climate change. These factors all together probably influenced the bidirectional migration of species between the Red Sea and the Mediterranean Sea, resulting in the increase of poisoning incidents, especially due to the ingestion of toxic alien species, among them TTX-bearers [18–22]. A good example of intoxication incidents caused by TTX-bearers was the ingestion of the elongated puffer, *Lagocephalus sceleratus*. Its presence in the Mediterranean was first reported in 2003, causing several poisoning incidents in Egypt in the end of 2004 and in Israel between 2005 and 2008. Fortunately all patients recovered [18,20,23].

In October 2007 a case of TTX poisoning occurred in Malaga, Spain, due to the ingestion of a specimen of *Charonia lampas*, an autochthonous predatory gastropod from Atlantic and Mediterranean waters, caught in the southern Portuguese waters [21]. This episode was the first report TTX occurrence in autochthonous species in Atlantic and Mediterranean waters and triggered our investigation to monitor different invertebrate species in several sites of our coast [21].

In this work our goal was to detect the presence of TTX and some analogues in several marine invertebrate species collected along the continental Portuguese coast, by using UPLC-MS/MS and LC-MS/MS.

Results and Discussion

In this study, 134 samples were collected in a monthly sampling program, from July 2009 until November 2010, in 13 sites distributed along the Portuguese coast (Figure 2.5). The collected species

belonged to different taxa and included gastropods (*Monodonta lineata*, *Monodonta turbinata*, *Gibbula umbilicalis*, *Gibbula magus*, *Littorina littorea*, *Littorina saxatilis*, *Nucella lapillus*, *Ocenebra erinacea*, *Calliostoma zizyphinum*, *Patella intermedia*, *Charonia lampas*), bivalves (*Mytilus galloprovincialis*), sea-urchins (*Paracentrotus lividus*) and sea-stars (*Marthasterias glacialis*). The naturally contaminated *Charonia lampas* and *Lagocephalus sceleratus*, obtained in former works [21,24], were used as standards in the LC-MS/MS analysis and provided us with the retention times of TTX and of the other analogues as follows; TTX (21.14 min), 4-*epi*TTX (20.4 min), 5,6,11-trideoxyTTX (13.7–14.2 min), monodeoxyTTX (18.08–18.80 min), 11-norTTX-6-ol (19.0–19.7 min) and 4-anhydroTTX (18.9 min) (Figure 2.6).

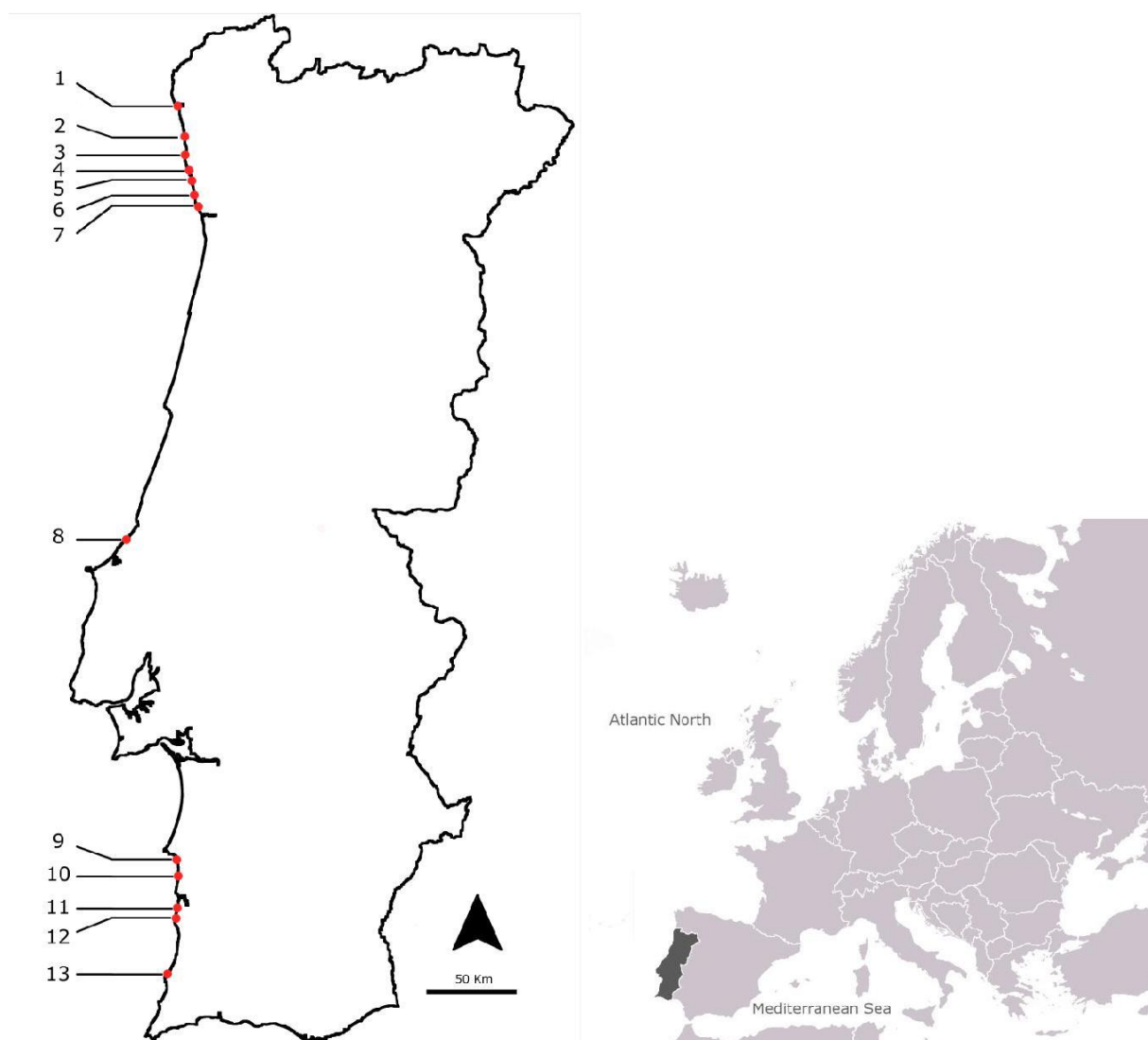


Figure 2.5 - Location of the sampling points in the North Atlantic Portuguese coast: 1 Viana do Castelo; 2 Esposende; 3 Póvoa do Varzim; 4 Angeiras; 5 Memória; 6 Valadares; 7 Aguda; 8 São Martinho do Porto; 9 São Torpes; 10 Porto Côvo; 11 Monte Clérigos; 12 Vila Nova

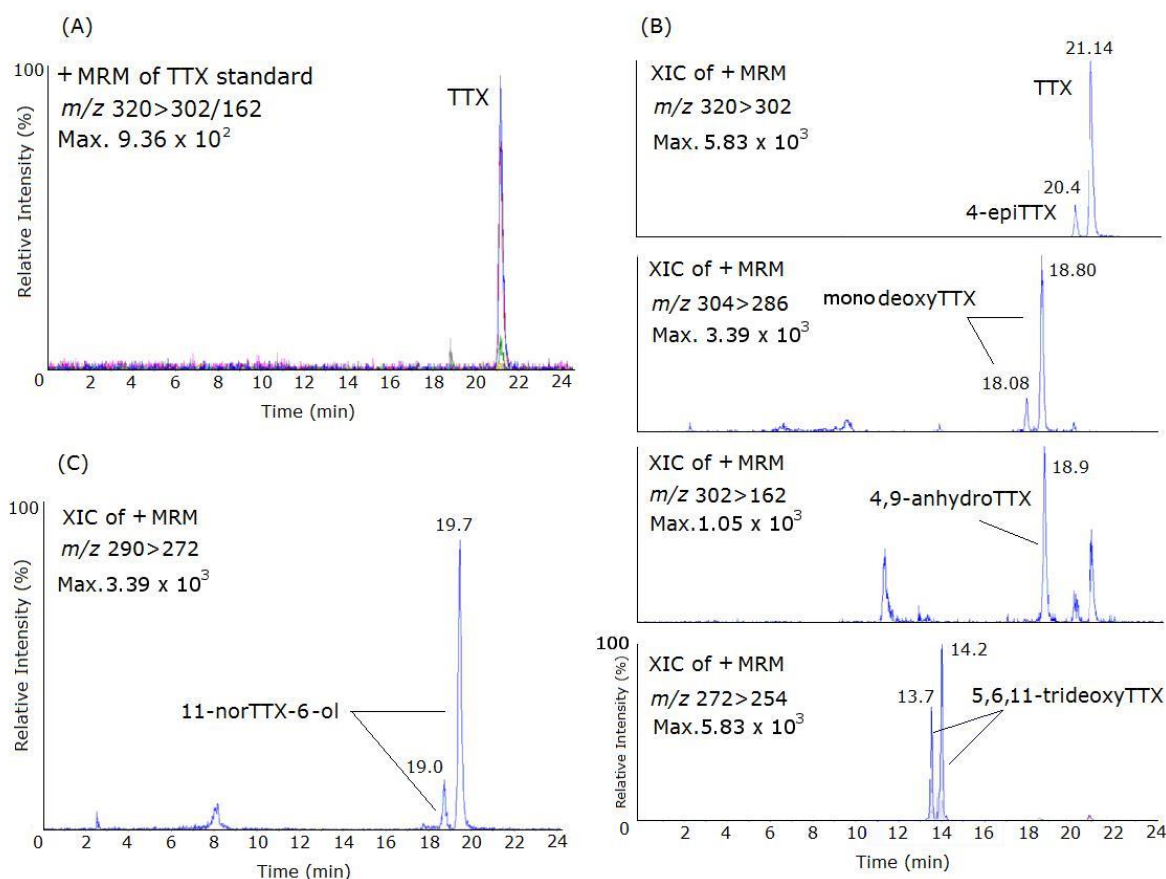


Figure 2.6 - Mass chromatograms of the LC-ESI-CID-MS/MS obtained under MRM operation of the TTX standard and naturally-contaminated samples of *Charonia lampas* and *Lagocephalus sceleratus*. (A) MRM of TTX standard (1500 ng/mL), m/z 320 > 302/162; (B) Extracted Ion Chrom

Five protonated molecules $[M + H]^+$ at m/z 320, 302, 304, 290 and 272 corresponding to TTX plus its derivatives were detected. The MRM transitions selected were: TTX and 4-*epi*TTX: 320 > 302/162; 4,9-anhydroTTX: 302 > 256/162; monodeoxyTTX: 304 > 286/176; 11-norTTX-6-ol: 290 > 272/162 and 5,6,11-trideoxyTTX: 272 > 254/162. Quantification was done with the most abundant ion in the fragment spectra: 302 (TTX and 4-*epi*TTX), 162 (4,9-anhydroTTX), 286 (monodeoxyTTX), 272 (11-norTTX-6-ol) and 254 (5,6,11-trideoxyTTX) (Figure 2.6).

The limits of detection and quantification (LOD/LOQ) of the LC-ESI-CID-MS/MS for TTX were 16 ng/mL ($S/N > 3$) and 63 ng/mL ($S/N > 10$), respectively.

For the UPLC-MS/MS the LOD ($S/N > 3$) was 1.7 ng/mL, and the LOQ ($S/N > 10$) was 5 ng/mL. TTX and analogue contents were identified and calculated against TTX standard (Figure 2.7), presuming that the toxin and its derivatives had the same molar response factor in each apparatus. A sample was considered positive when the toxin levels detected were above the LOQ.

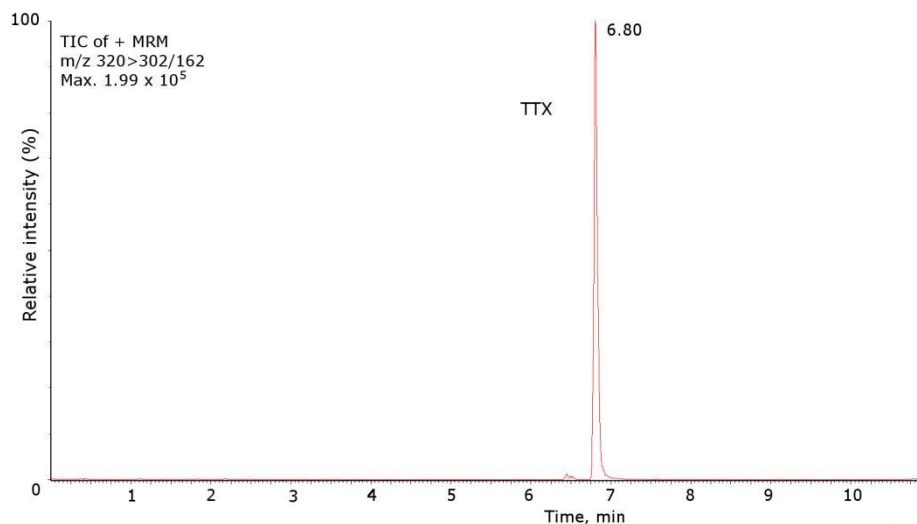


Figure 2.7 - Mass chromatograms of the UPLC-MS/MS obtained under MRM operation of the TTX standard. TIC (total ion chromatogram). TTX standard 1000 ng/mL, m/z 320 > 302/162.

The analysis of the gastropod collected in our waters allowed us to detect some positive samples, including *G. umbilicalis*, collected on Memória beach in July 2009. The peaks corresponded to monodeoxyTTX, with an amount of 63.81 ng/g (Figure 2.8).

Two other gastropod species had positive results: *M. lineata* collected in April 2010 in Vila Nova de Milfontes and *C. lampas* collected in September 2010 in Angeiras (Figure 2.9). While the first revealed the co-occurrence of TTX (90.50 ng/g) and 4-*epi*TTX (21.48 ng/g), in *C. lampas* we detected low levels of 5,6,11-trideoxyTTX (6.22 ng/g). This last variant was detected previously in the same species, together with TTX [21]. Nevertheless, this is the first report on the occurrence of TTX and variants in the two small gastropods *M. lineata* and *G. umbilicalis*. These two species, regardless of their small size, are harvested and consumed by locals, being rarely found in markets. Thus, the exposure of TTX and analogues via these small gastropods is neither regulated nor controlled.

The levels of these toxins found in our samples are relatively low when compared to other species that had caused human intoxications, or even with the *C. lampas* that caused the intoxication episode in Malaga [21]. In Table 2.3 the toxin levels detected in the present work and examples of other works for comparison are displayed. Nevertheless, one should be careful when comparing data, since the levels we report are due to the toxin content in the whole animal (edible part), while the data on the *C. lampas* reported by Rodriguez *et al.* 2008 refers to levels in the digestive gland only [21].

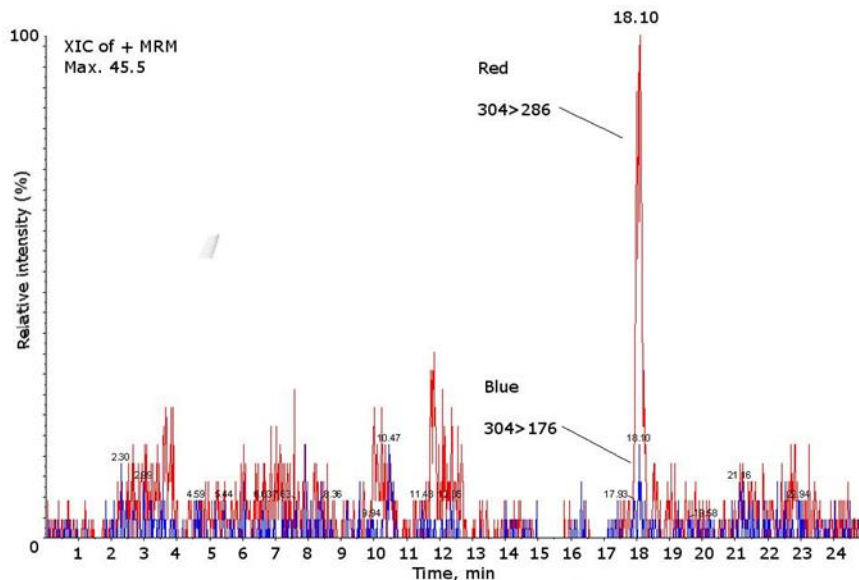


Figure 2.8 - Mass chromatograms of the LC-ESI-CID-MS/MS obtained under MRM operation of the positive sample of *Gibbula umbilicalis* for the analogue monodeoxyTTX (m/z 304 > 286/176). XIC extracted ions chromatogram.

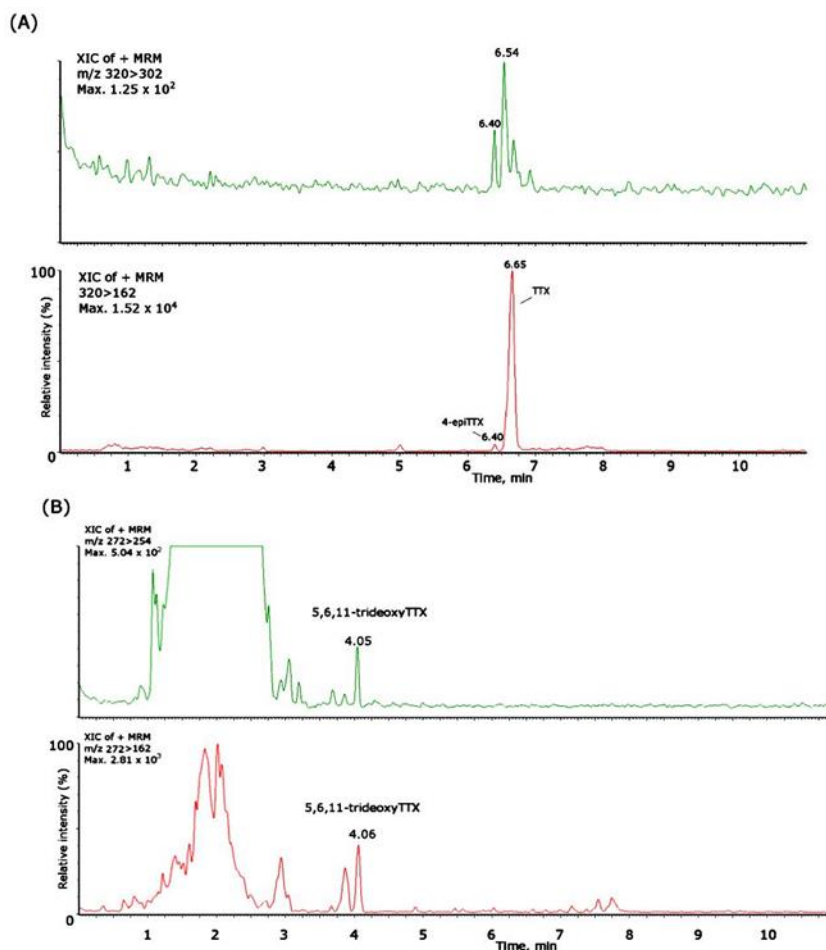


Figure 2.9 - Mass chromatograms of the UPLC-MS/MS obtained under MRM operation of the positive samples of *Monodonta lineata* and *C. lampas* for (A): TTX (m/z 320 > 162/302), 4-epiTTX (m/z 320 > 162/302); and (B): 5,6,11-trideoxyTTX (m/z 272 > 254/162).

Table 2.3 - TTX and analogues levels ($\mu\text{g/g}$) in marine gastropods from Portugal (pw-present work and [21]), China and Taiwan.

Species	Location	TTX	4-ep/TTX	MonodeoxyTTX	5,6,11-trideoxyTTX	Ref.
<i>G. umbilicalis</i>	Memória			0.063		pw
<i>M. lineata</i>	Vila Nova de Milfontes	0.090	0.021			pw
<i>C. lampas</i>	Angeiras				0.006	pw
	Algarve	315.00 *			1004.00 *	[21]
<i>N. nitidus</i>	China	1350				[25]
<i>N. semiplicatus</i>		26.10	3.37			[12]
<i>N. papillosus</i>	Taiwan	42–60				[26]

* Data obtained from digestive gland only.

The low levels we have found so far may also be due to the fact that TTX synthesis in warmer waters is higher than in the cold North Atlantic ones. The Lessepsian migration phenomenon may play an important role in the migration of TTX-bearers to more temperate waters, such as the elongated puffer *Lagocephalus sceleratus*, since 1902 more than 62 Red Sea fish species have migrated via the Suez Canal to the Mediterranean [22]. Global warming may also have influenced the migration and settling of TTX-bearing species to more temperate waters, once the rise in water temperature facilitates the migration of Red Sea exotic species to the Mediterranean Sea. Since 5.7% of the Mediterranean fauna is composed of Red Sea fish species, the risk of future invasion by these alien species is potentially very high [22]. There is not yet enough epidemiological and toxicological data concerning human intoxications by TTX in the temperate waters of the Atlantic Ocean. Nevertheless, a study done in the South Pacific area concerning the possible association of ciguatera and climate change revealed interesting assumptions that may give us some hints for future studies [27]. The study projected the idea that a rise in temperature is expected to increase the incidence of ciguatera poisoning of 35–70 per thousand people in 1990 to 160–430 per thousand people in 2050 in Papua New Guinea [27]. Taking into account that we need to be cautious when using simplistic models such as these, the rise in temperature may alter the growth rate of the toxic organisms [28] and also the rates of toxin production [29]. Nevertheless, temperature may also alter accumulation, metabolism and detoxication kinetics in fish vectors as it can for TTX [30]. In fact, another work showed that the *in vitro* uptake of TTX into liver tissue slices of *Takifugo rubripes* is temperature dependent, being significantly higher at 20 °C compared to 5 °C [30]. Cellular and molecular studies on the TTX kinetics are also needed in order to better predict the potential effects of global warming on TTX levels in vector species. Taking into account that the microflora of many puffer fish may be the origin of TTX, studies on the TTX production by the main bacterial species are important. A study on the diversity of bacteria isolated from the skin, gill and intestine of *Fugu niphobles* showed that temperature may have an effect on the

diversity and density of some bacteria [31]. The identification of *Vibrio* sp. species, and in particular of *V. alginolyticus*, was achieved when fish were exposed at 20 and 29 °C but not when reared at 10 °C. In laboratory culture, all strains of this species grew at 20 to 37 °C but very few grew at 10 °C, suggesting a preference for higher temperatures [31]. So, water temperature may have an impact on the growth rate of TTX producing bacteria, being responsible for higher bacteria counts in fish exposed to higher temperatures. More laboratory experiments are needed to support this hypothesis. All these circumstances, together with the fact that TTX travels along the food-chain [5–8], may favor the establishment of TTX in Atlantic temperate waters.

In this work, TTX plus its derivatives were detected in the Portuguese coast, not only in species already reported as TTX-bearers (*Charonia lampas*) but also in indigenous species not yet assigned, this being the first report of TTX presence in *Gibbula umbilicalis* and *Monodonta lineata*. Apart from the quantities detected in these animals, there is an imminent danger to the human population, since the toxin travels in the food-chain and it is unknown whether or not there is biomagnification of TTX [5–8]. This increases the potential danger, because all the species reported are edible and the toxin is water soluble and thermostable [32,33].

The species *Gibbula umbilicalis* and *Monodonta lineata* belong to the same family, Trochidae [34], and in this study we confirm their ability to accumulate TTX. Possibly there is a potential adaptation that is common for both. In addition, TTX was not detected in all specimens belonging to these two species, which could be due to different strains having different capabilities of adaptation. We can make no inferences about the seasonal intake of the toxin, due to the fact that we only had three positives (2.24% of the total sampling), although all of them were in the warmer months.

TTX is present in Portuguese waters, Angeiras being the most northern point of a TTX report in Atlantic temperate waters. Nevertheless, the low concentrations detected are not sufficient to cause a fatal outcome, since the Minimum Lethal Dose for humans is 2 mg [35]. Surveillance is advisable to avoid poisoning incidents and to understand the progress of this emergent phenomenon.

Experimental Section

3.1. Sampling Points and Selected Species

TTX was reported in gastropods and sea-stars in many parts of the world [5–8,21,36–43]. Due to this fact and also because we were searching for potential new vectors, fourteen benthic species were selected, belonging to gastropods (*Monodonta lineata*, *Monodonta turbinata*, *Gibbula umbilicalis*, *Gibbula magus*, *Littorina littorea*, *Littorina saxatilis*, *Nucella lapillus*, *Ocenebra erinacea*, *Calliostoma zizyphinum*, *Patella intermedia*, *Charonia lampas*), bivalves (*Mytilus galloprovincialis*), sea-urchins (*Paracentrotus lividus*) and sea-stars (*Marthasterias glacialis*). Samples were collected monthly at various sampling sites distributed along the coast of continental Portugal (Figure 1): Almogrove (37°39'11.52"N; 8°48'09.18"W), Vila Nova de Milfontes (37°43'02.19"N; 8°47'34.40"W), Monte Clérigos (37°20'06.92"N; 8°50'48.09"W), Porto Côvo (37°53'33.19"N; 8°47'38.25"W), São Torpes (37°58'53.56"N; 8°47'45.58"W), São Martinho do Porto (39°30'18.29"N; 9°08'18.07"W), Aguda (41°02'52.13"N; 8°39'13.19"W), Valadares (41°5'29.76"N; 8°39'27.05"W), Memória (41°13'50.96"N; 8°43'18.09"W), Angeiras (41°15'50.01"N; 8°43'37.14"W), Póvoa do Varzim (41°22'41.61"N; 8°46'7.39"W), Esposende (41°29'5.19"N; 8°46'45.76"W) and Viana do Castelo (41°41'35.38"N; 8°50'56.70"W). *Charonia lampas* were purchased at local fish markets, being caught along the Angeiras coast (41°15'49.06"N; 8°43'48.43"W). Organisms were collected in the intertidal area during low tide and were transported to the laboratory and refrigerated as soon as possible. Whenever they were not processed immediately, they were frozen at –20 °C. The number of samples collected and the average number of specimens needed to set a composed sample are displayed in Table 2.4.

Table 2.4 - Average number of specimens to set a composed sample and number of samples collected since July 2009 until the end of 2010. Availability of animals is dependent on their geographical distribution and ecology.

Species	Average Number of Animals	
	Number of Samples Collected from July 2009 till End 2010	Collected to Set a Composed Sample
<i>Gibbula umbilicalis</i>	34	100
<i>Gibbula magus</i>	1	90
<i>Monodonta lineata</i>	20	86
<i>Monodonta turbinata</i>	21	86
<i>Nucella lapillus</i>	13	15
<i>Littorina littorea</i>	2	10
<i>Littorina saxatilis</i>	4	15

<i>Ocenebra erinacea</i>	4	4
<i>Calliostoma zizyphinum</i>	1	1
<i>Patella intermedia</i>	4	15
<i>Charonia lampas</i>	5	1
<i>Mytilus galloprovincialis</i>	7	30
<i>Paracentrotus lividus</i>	10	10
<i>Marthasterias glacialis</i>	8	1

3.2. Sample Treatment

Due to the small size of some species, and in order to have enough biomass for the extraction procedure (1 g), all animals were treated in groups with the exception of *Charonia lampas*, *Calliostoma zizyphinum* and *Marthasterias glacialis*. In Table 2 the number of animals needed to set a composed extractable sample is displayed. Samples were extracted based on the methods of Shoji *et al.* and Ito *et al.* with appropriate amendments to the type of sample [44,45] as follows. Gastropods were boiled in water for 30 min due to their hard consistency. All samples were dissolved in acetic acid (1%), then homogenized with a mechanical crusher (1000 rpm, 5 min), (Silentcrusher M, Heidolph, VWR, Carnaxide, Portugal), and ultrasonication (70 Hz, 3 min), (Vibra Cell, Sonic & Materials, Reagent 5, Porto, Portugal). The extracts were then centrifuged at 4495 g for 20 min (Centrifugal-Legend RT, Sorvall). This procedure was repeated twice, and the two supernatants were defatted with dichloromethane (v/v). Both layers were collected and concentrated under reduced pressure (40 °C) in a rotary evaporator (Büchi) and stored at -20 °C [44–48]. This method was used in the first 50 samples, and then the extraction method was optimized in order to obtain cleaner extracts, based on the protocols of Jen *et al.* and Tsai *et al.* [49,50]: 1 g of sample tissue was homogenized with a blender (A320R1, 700W, Moulinex) then extracted in 3 mL of acetic acid (1%)/methanol with the help of a vortex mixer for 5 min (Top Mix 1118, Fisher Bioblock Scientific) and ultrasonic bath, (5 min, 100 Hz) (RK100H, Bandelin SONOREX). A double extraction was performed, extracts were centrifuged at 4495 g for 15 min at 4 °C (Centrifugal-Legend RT, Sorvall), supernatants were combined and adjusted to a final volume of 7 mL. Then 1 mL of the extract was cleaned through a C18 solid-phase extraction (SPE) cartridge (500 mg/3 mL volume from Supelco, Bellefonte, PA, USA). The cartridges were previously conditioned with 6 mL of methanol, followed by 6 mL of water (milliQ). The sample was eluted with 10 mL of 100% methanol and diluted with the same solvent to a final volume of 12 mL. Finally, each sample was concentrated by drying and re-suspended in 1 mL of methanol, and 100 µL were filtered through 0.45 µm filters (UltraFree-MC centrifugal devices, Millipore, Spain) before LC-MS/MS analysis [49,50]. All reagents used were paranalysis grade from Merck®.

3.3. LC-MS/MS Analysis

The LC-MS conditions were the same as reported by Rodriguez *et al.* [24]. Briefly, the analyses were performed in high-performance liquid chromatography (LC) equipment consisting of a binary system of LC-10ADVP pumps, an autoinjector (SIL-10ADVP) with degasser (DGU-14A), refrigerated rack, column oven (CTO-10ACvp) and a system controller (SCL-10Avp) from Shimadzu (Kyoto, Japan). The LC system was coupled to a 2000 QTRAPLC/MS/MS instrument from Applied Biosystems (Calrsbad, CA, USA), formed by a hybrid quadrupole-linear ion trap mass spectrometer (MS), equipped with an atmospheric pressured ionisation (API) unit, fitted with an electrospray ionisation source (ESI), operating in the conventional mode of low energy of collision dissociation induced (CID) of MS/MS. Nitrogen was produced by a Nitrocraft NC_{LC/MS} generator from Air Liquide (Madrid, Spain).

The LC system operated with the ESI interface in positive ion mode using the following parameters: curtain gas, 15 psi; collision-activated dissociation gas, 6 psi; IonSpray voltage, 4000 V; temperature, 450 °C; gas 1, 50 psi; gas 2, 50 psi; these parameters had been previously optimized using the TTX standard (Calbiochem Corporation). For the equipment control, data processing and analysis, Analyst software was used. Eluent (A) of the mobile phase was composed by formic acid (Merck, Madrid, Spain) and ammonium formate (Sigma Aldrich, Madrid, Spain), both with a concentration of 10 mM in water, and eluent (B) consisted of acetonitrile (Panreac Quimica, Barcelona, Spain) in water (95:5) with ammonium formate (5 mM) and formic acid (2 mM). The gradient used started with 100% of mobile phase (B), decreasing to 65% at minute 15, rising to 100% at minute 18 until the end of the run (25 min). An XBridge™ Amide column (i.d. 2.1 × 150 mm; 3.5 μm) with a guard cartridge (i.d. 2.1 × 10 mm) from Waters (Cerdanyola del Vallès, Spain) was used to achieve the separation of TTX, and analogues present in the samples. Column oven temperature was set at 25 °C and injection volume was 5 μL. The MS was operated in multiple reactions monitoring (MRM) mode, analysing two product ions per compound: one for quantification and the other for confirmation.

The mass spectrometer parameters were adjusted to obtain a signal of maximum intensity and stability. For the MS optimization, the sample solution was directly infused in the electrospray source at a 0.2 mL/min flow rate with a syringe pump. The MS was operated in the positive ion mode using the product ion scan with a cone gas, 40 V; capillary voltage, 2.8 kV; source temperature, 120 °C; desolvation temperature, 350 °C; collision energy, 45 eV. Helium and nitrogen were used as collision and drying gases, respectively [24].

To overcome the challenge of the lack of standards for TTX analogues [44,51–55] a sample of a naturally-contaminated *Charonia lampas* and *Lagocephalus sceleratus* used in former works [21,24] was injected in the LC-MS/MS, this way determining the respective retention times (RT) (Figure 2). The following variants were aimed for: TTX, 4-*epi*TTX, 5,6,11-trideoxyTTX, monodeoxyTTX, 11-norTTX-6-ol and 4-anhydroTTX. For the calibration curve, several dilutions from the TTX standard were performed, from 50 ng/mL concentration to 2000 ng/mL. TTX and its derivatives were quantified, using their peak areas to calculate amounts and using the curve obtained from TTX standard [56].

3.4. UPLC-MS/MS Analysis

Samples were analysed in Ultra High Performance Liquid Chromatography equipment ACQUITY UPLC system, coupled to a Xevo TQ MS mass spectrometer from Waters (Manchester, UK). The apparatus is equipped with a multimode source ESI/APCI/ESCI, a vacuum system composed of two air-cooled Edwards Vacuum turbo molecular pumps evacuating the source and analyzer, one Varian rotary backing pump. The nitrogen generator was a Nitrocraft NC_{LC/MS} from Air Liquide (Madrid, Spain). Chromatographic separation and detection of TTX and its derivatives was achieved with a Waters Acquity UPLC BEH Amide column (100 mm × 2.1, 1.7 μm), equipped with a 0.2 μm Acquity UPLC in-line filter and column oven at 35 °C. The LC operated with eluent (A), consisting of 10 mM ammonium formate (Sigma Aldrich, Madrid, Spain) and 10 mM formic acid (Merck, Madrid, Spain) in water. Eluent (B) contained acetonitrile (Panreac Quimica, Barcelona, Spain) in water (95:5) with a final concentration of 5 mM ammonium formate and 2 mM formic acid. The gradient programme used to elute the toxins was 100% mobile phase (B) at the beginning, decreasing to 65% (B) after 7 min, then kept for 2 min and back to 100% (B) over the next 0.5 min and finally kept 100% (B) for 1.5 min before the next injection. Flow rate was 0.4 mL/min and injection volume was 5 μL.

The Xevo TQ MS mass spectrometer operated with the following optimized source-dependent parameters (ESI source): capillary potential 2.7 kV, cone voltage 40 V, desolvation temperature 350 °C, desolvation gas flow 850 L/h N₂, cone gas flow 50 L/h N₂, source temperature 150 °C, collision gas flow 20 V. Argon was used as the collision gas at 4.5×10^{-3} mbar.

The mass spectrometer operated in MRM, detecting in positive mode, analysing two product ions per compound: one for quantification and another for confirmation. The transitions employed were: TTX and 4-*epi*TTX (m/z 320 > 302/162) and 5,6,11-trideoxyTTX (272 > 254/162), with retention times: TTX (6,80 min), 4-*epi*TTX (6.50 min) and 5,6,11-trideoxyTTX (4.06 min). Quantification was undertaken with the most abundant ion in the fragment spectra: 162 for TTX, 4-*epi*TTX and 5,6,11-trideoxyTTX. TTX analogues in sample solutions were identified according to the daughter ion spectra of the analogues reported in the literature [44]. For the calibration curve, several dilutions from the TTX standard (Calbiochem Corporation) were performed, from 31.25 ng/mL concentration to 3000 ng/mL. TTX and its derivatives were quantified using their peak areas to calculate amounts and using the curve obtained from TTX standard [56].

Conclusions

In this work we used LC-MS/MS and UPLC-MS/MS to detect TTX and several analogues in three autochthonous gastropod species of the Atlantic Portuguese continental coast. TTX, 4-*epi*TTX, monodeoxyTTX and 5,6,11-trideoxyTTX were detected in *Monodonta lineata*, *Gibbula umbilicalis* and *Charonia lampas*, being the most northern point of the Atlantic Ocean where these toxins were reported. All these species are edible, raising the probability of human health hazards. Despite the low

concentrations detected, ranging from 6.22 to 90.50 ng/g, it was clearly shown that TTX and analogues should be monitored in the species reported positive, and in others that can potentially accumulate the toxins and can be used as human food.

Acknowledgments

We acknowledge the INTERREG IV projects Atlantox and Pharamatlantic for partially funding this research, UP through IJUP projects and Ministerio de Ciencia y Tecnología, Spain: AGL2009-13581-C02-01. MS acknowledges FCT (SFRH/BD/73269/2010).

References

- Tahara, Y.; Hirata, Y. Studies on the puffer fish toxin. *J. Pharm. Soc. Jpn.***1909**, *29*, 587–625.
- Miyazawa, K.; Noguchi, T. Distribution and origin of tetrodotoxin. *J. Toxicol. Toxin Rev.***2001**, *20*, 11–33.
- Matsumura, K. Production of tetrodotoxin in puffer fish embryos. *Environ. Toxicol. Pharmacol.***1998**, *6*, 217–219.
- Lehman, E.M.; Brodie, E.D.; Brodie, E.D. No evidence for an endosymbiotic bacterial origin of tetrodotoxin in the newt *Taricha granulosa*. *Toxicon***2004**, *44*, 243–249.
- Lin, S.J.; Hwang, D.F. Possible source of tetrodotoxin in the starfish *Astropecten scoparius*. *Toxicon* **2001**, *39*, 573–579.
- Yasumoto, T.; Yotsu-Yamashita, M. Chemical and etiological studies on tetrodotoxin and its analogs. *J. Toxicol. Toxin Rev.***1996**, *15*, 81–90.
- Kono, M.; Matsui, T.; Furukawa, K.; Yotsu-Yamashita, M.; Yamamori, K. Accumulation of tetrodotoxin and 4,9-anhydrotetrodotoxin in cultured juvenile kusafugu *Fugu niphobles* by dietary administration of natural toxic komonfugu *Fugu poecilonotus* liver. *Toxicon***2008**, *51*, 1269–1273.
- Noguchi, T.; Arakawa, O.; Takatani, T. Toxicity of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. *Comp. Biochem. Physiol. Part D: Genomics Proteomics***2006**, *1*, 153–157.

Hwang, D.F.; Arakawa, O.; Saito, T.; Noguchi, T.; Simidu, U.; Tsukamoto, K.; Shida, Y.; Hashimoto, K. Tetrodotoxin-producing bacteria from the blue-ringed octopus *Octopus maculosus*.

Mar. Biol. **1989**, *100*, 327–332.

Noguchi, T.; Hwang, D.F.; Arakawa, O.; Sugita, H.; Deguchi, Y.; Shida, Y.; Hashimoto, K. *Vibrio alginolyticus*, a tetrodotoxin-producing bacterium, in the intestines of the fish *Fugu vermicularis vermicularis*. *Mar. Biol.* **1987**, *94*, 625–630.

Yotsu, M.; Yamazaki, T.; Meguro, Y.; Endo, A.; Murata, M.; Naoki, H.; Yasumoto, T. Production of tetrodotoxin and its derivatives by *Pseudomonas* sp. isolated from the skin of a pufferfish.

Toxicon **1987**, *25*, 225–228.

Wang, X.J.; Yu, R.C.; Luo, X.; Zhou, M.J.; Lin, X.T. Toxin-screening and identification of bacteria isolated from highly toxic marine gastropod *Nassarius semiplicatus*. *Toxicon* **2008**, *52*, 55–61.

Cestele, S.; Catterall, W.A. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* **2000**, *82*, 883–892.

Choudhary, G.; Yotsu-Yamashita, M.; Shang, L.; Yasumoto, T.; Dudley, S.C., Jr. Interactions of the C-11 hydroxyl of tetrodotoxin with the sodium channel outer vestibule. *Biophys. J.* **2003**, *84*, 287–294.

Hille, B. The receptor for tetrodotoxin and saxitoxin: A structural hypothesis. *Biophys. J.* **1975**, *15*, 615–619.

Scheib, H.; McLay, I.; Guex, N.; Clare, J.; Blaney, F.; Dale, T.; Tate, S.; Robertson, G. Modeling the pore structure of voltage-gated sodium channels in closed, open, and fast-inactivated conformation reveals details of site 1 toxin and local anesthetic binding. *J. Mol. Model.* **2006**, *12*, 813–822.

Kaku, N.; Meier, J. Clinical Toxicology of Fugu Poisoning. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*; Meier, J., White, J., Eds.; CRC Press: Boca Raton, FL, USA, 1995; pp. 75–83.

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

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

Zaki, M.A.; Mossa, A.E.W. Red Sea puffer fish poisoning: Emergency diagnosis and management of human intoxication. *Egypt. J. Aquat. Res.***2005**, *31*, 370–378.

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

Lasram, F.B.; Mouillot, D. Increasing southern invasion enhances congruence between endemic and exotic Mediterranean fish fauna. *Biol. Invasions***2009**, *11*, 697–711.

Akyol, O.; Unal, V.; Ceyhan, T.; Bilecenoglu, M. First confirmed record of *Lagocephalus sceleratus* (Gmelin, 1789) in the Mediterranean Sea. *J. Fish Biol.* **2005**, *66*, 1183–1186.

Rodriguez, P.; Alfonso, A.; Otero, P.; Katikou, P.; Georgantelis, D.; Botana, L.M. Liquid chromatography-mass spectrometry method to detect tetrodotoxin and its analogues in puffer fish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters. *Food Chem.* **2012**, *132*, 1103–1111.

Huang, H.N.; Lin, J.; Lin, H.L. Identification and quantification of tetrodotoxin in the marine gastropod *Nassarius* by LC-MS. *Toxicon***2008**, *51*, 774–779.

Jen, H.C.; Lin, S.J.; Lin, S.Y.; Huang, Y.W.; Liao, I.C.; Arakawa, O.; Hwang, D.F. Occurrence of tetrodotoxin and paralytic shellfish poisons in a gastropod implicated in food poisoning in southern Taiwan. *Food Addit. Contam.***2007**, *24*, 902–909.

Llewellyn, L.E. Revisiting the association between sea surface temperature and the epidemiology of fish poisoning in the south Pacific: Reassessing the link between ciguatera and climate change.

Toxicon **2010**, *56*, 691–697.

Chinain, M.; Germain, M.; Deparis, X.; Pauillac, S.; Legrand, A.M. Seasonal abundance and toxicity of the dinoflagellate *Gambierdiscus* spp. (Dinophyceae), the causative agent of ciguatera in Tahiti, French Polynesia. *Mar. Biol.***1999**, *135*, 259–267.

Ashton, M.; Tosteson, T.; Tosteson, C. The effect of elevated temperature on the toxicity of the laboratory cultured dinoflagellate *Ostreopsis lenticularis* (Dinophyceae). *Rev. Biol. Trop.***2003**, *51*, 1–6.

Matsumoto, T.; Nagashima, Y.; Kusuvara, H.; Sugiyama, Y.; Ishizaki, S.; Shimakura, K.; Shiomi, K. Involvement of carrier-mediated transport system in uptake of tetrodotoxin into liver tissue slices of puffer fish *Takifugu rubripes*. *Toxicon***2007**, *50*, 173–179.

Sugita, H.; Iwata, J.; Miyajima, C.; Kubo, T.; Noguchi, T.; Hashimoto, K.; Deguchi, Y. Changes in microflora of a puffer fish *Fugu niphobles*, with different water temperatures. *Mar. Biol.***1989**, *101*, 299–304.

Kao, C.Y. Structure-activity relations of tetrodotoxin, saxitoxin and analogues. *Ann. N.Y. Acad. Sci.***1986**, *479*, 52–67.

Hommel, D.; Hulin, A.; Saignavong, S.; Desbordes, J.M. Intoxication par poisson-coffre (tetrodotoxine). A propos d'une intoxication familiale. *Méd. Afr. Noire***1992**, *39*, 146–148.

Bouchet, P.; Rocroi, J.-P. Classification and nomenclator of gastropod families. *Malacologia***2005**, *47*, 1–397.

Noguchi, T.; Ebesu, J.S.M. Puffer poisoning: Epidemiology and treatment. *J. Toxicol. Toxin Rev.***2001**, *20*, 1–10.

Hwang, D.F.; Lu, S.C.; Jeng, S.S. Occurrence of tetrodotoxin in the gastropods *Rapanarapiformis* and *R. venosa venosa*. *Mar. Biol.* **1991**, *111*, 65–69.

Hwang, P.A.; Tsai, Y.H.; Deng, J.F.; Cheng, C.A.; Ho, P.H.; Hwang, D.F. Identification of tetrodotoxin in a marine gastropod (*Nassarius glans*) responsible for human morbidity and mortality in Taiwan. *J. Food Protect.***2005**, *68*, 1696–1701.

Maruyama, J.; Noguchi, T.; Narita, H.; Nara, M.; Jeon, J.K.; Otsuka, M.; Hashimoto, K. Occurrence of tetrodotoxin in a starfish, *Astropecten scoparius*. *Agric. Biol. Chem. Tokyo***1985**,

49, 3069–3070.

Narita, H.; Noguchi, T.; Maruyama, J.; Nara, M.; Hashimoto, K. Occurrence of a tetrodotoxin-associated substance in a gastropod, Hanamushirogai *Zeuxis siquijorensis*. *Bull. Jpn. Soc. Sci. Fish.* **1984**, *50*, 85–88.

Noguchi, T.; Narita, H.; Maruyama, J.; Hashimoto, K. Tetrodotoxin in the starfish *Astropectenpolyacanthus*, in association with toxification of a trumpet shell, Boshubora *Charonia sauliae*.

Bull. Japan. Soc. Sci. Fish. **1982**, *48*, 1173–1177.

Hwang, P.A.; Tsai, Y.H.; Lu, Y.H.; Hwang, D.F. Paralytic toxins in three new gastropod (Olividae) species implicated in food poisoning in southern Taiwan. *Toxicon* **2003**, *41*, 529–533.

Narita, H.; Noguchi, T.; Maruyama, J.; Ueda, Y.; Hashimoto, K.; Watanabe, Y.; Hida, K. Occurrence of tetrodotoxin in a trumpet shell, Boshubora *Charonia sauliae*. *Bull. Jpn. Soc. Sci. Fish.* **1981**, *47*, 935–941.

Noguchi, T.; Maruyama, J.; Ueda, Y.; Hashimoto, K.; Harada, T. Occurrence of tetrodotoxin in the Japanese ivory shell *Babylonia japonica*. *Bull. Jpn. Soc. Sci. Fish.* **1981**, *47*, 909–914.

Shoji, Y.; Yotsu-Yamashita, M.; Miyazawa, T.; Yasumoto, T. Electrospray ionization mass spectrometry of tetrodotoxin and its analogs: Liquid chromatography/mass spectrometry, tandem mass spectrometry, and liquid chromatography/tandem mass spectrometry. *Anal. Biochem.* **2001**, *290*, 10–17.

Ito, K.; Okabe, S.; Asakawa, M.; Bessho, K.; Taniyama, S.; Shida, Y.; Ohtsuka, S. Detection of tetrodotoxin (TTX) from two copepods infecting the grass puffer *Takifugu niphobles*: TTX attracting the parasites? *Toxicon* **2006**, *48*, 620–626.

Lee, M.J.; Jeong, D.Y.; Kim, W.S.; Kim, H.D.; Kim, C.H.; Park, W.W.; Park, Y.H.; Kim, K.S.; Kim, H.M.; Kim, D.S. A tetrodotoxin-producing *Vibrio* strain, LM-1, from the puffer fish *Fugovermicularis radiatus*. *Appl. Environ. Microbiol.* **2000**, *66*, 1698–1701.

Mahmud, Y.; Yamamori, K.; Noguchi, T. Occurrence of TTX in a brackish water puffer

—Midorifugull, *Tetraodon nigroviridis*, collected from Thailand. *J. Food Hyg. Soc. Jpn.* **1999**, *40*, 363–367.

Maruyama, J.; Noguchi, T.; Jeon, J.K.; Harada, T.; Hashimoto, K. Occurrence of tetrodotoxin in the starfish *Astropecten latespinosus*. *Experientia***1984**, *40*, 1395–1396.

Jen, H.C.; Lin, S.J.; Tsai, Y.H.; Chen, C.H.; Lin, Z.C.; Hwang, D.F. Tetrodotoxin poisoning evidenced by solid-phase extraction combining with liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.***2008**, *871*, 95–100.

Tsai, Y.H.; Ho, P.H.; Hwang, C.C.; Hwang, P.A.; Cheng, C.A.; Hwang, D.F. Tetrodotoxin in several species of xanthid crabs in southern Taiwan. *Food Chem.***2006**, *95*, 205–212.

Yotsu-Yamashita, M. Chemistry of puffer fish toxin. *J. Toxicol. Toxin Rev.***2001**, *20*, 51–66.

Jang, J.H.; Lee, J.S.; Yotsu-Yamashita, M. LC/MS analysis of tetrodotoxin and its deoxy analogs in the marine puffer fish *Fugu niphobles* from the southern coast of Korea, and in the brackishwater puffer fishes *Tetraodon nigroviridis* and *Tetraodon biocellatus* from southeast Asia. *Mar. Drugs***2010**, *8*, 1049–1058.

Diener, M.; Christian, B.; Ahmed, M.S.; Luckas, B. Determination of tetrodotoxin and its analogs in the puffer fish *Takifugu oblongus* from Bangladesh by hydrophilic interaction chromatography and mass-spectrometric detection. *Anal. Bioanal. Chem.***2007**, *389*, 1997–2002.

Pires, O.R.; Sebben, A.; Schwartz, E.F.; Bloch, C.; Morales, R.A.V.; Schwartz, C.A. The occurrence of 11-oxotetrodotoxin, a rare tetrodotoxin analogue, in the brachycephalidae frog

Brachycephalus ephippium. *Toxicon* **2003**, *42*, 563–566.

Nakagawa, T.; Jang, J.; Yotsu-Yamashita, M. Hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry of tetrodotoxin and its analogs. *Anal. Biochem.* **2006**, *352*, 142–144.

Yotsu-Yamashita, M.; Jang, J.H.; Cho, Y.K.; Konoki, K. Optimization of simultaneous analysis of tetrodotoxin, 4-epitetrodotoxin, 4,9-anhydrotetrodotoxin, and 5,6,11-trideoxytetrodotoxin by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Forensic Toxicol.***2011**, *29*, 61–64.

Marine Drugs 2013, 11, 1936-1960

New Invertebrate Vectors for PST, Spirolides and Okadaic Acid in the North Atlantic

Marisa Silva ^{1,2}, Aldo Barreiro ^{1,2}, Paula Rodriguez ³, Paz Otero ³, Joana Azevedo ^{2,4}, Amparo Alfonso ³, Luis M. Botana ³ and Vitor Vasconcelos ^{1,2,*}

Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, Porto 4619-007, Portugal; E-Mails: marisasilva17@gmail.com (M.S.); aldo.barreiro@gmail.com (A.B.)

Center of Marine and Environmental Research—CIMAR/CIIMAR, University of Porto, Rua dos Bragas 289, Porto 4050-123, Portugal; E-Mail: joana_passo@hotmail.com

Department of Pharmacology, Faculty of Veterinary, University of Santiago of Compostela, Lugo 27002, Spain; E-Mails: paula.rodriguez17@rai.usc.es (P.R.); mariapaz.otero@rai.usc.es (P.O.); amparo.alfonso@usc.es (A.A.); Luis.Botana@usc.es (L.M.B.)

Department of Chemical and Biomolecular Sciences, School of Health and Technology of Porto, Vila Nova de Gaia 4400-330, Portugal

Abstract: The prevalence of poisoning events due to harmful algal blooms (HABs) has declined during the last two decades through monitoring programs and legislation, implemented mainly for bivalves. However, new toxin vectors and emergent toxins pose a challenge to public health. Several locations on the Portuguese coast were surveyed between 2009 and 2010 for three distinct biotoxin groups [saxitoxin (PST), spirolide (SPX) and okadaic acid (OA)], in 14 benthic species of mollusks and echinoderms. Our main goals were to detect new vectors and unravel the seasonal and geographical patterns of these toxins. PSTs were analyzed by the Lawrence method, SPXs by LC-MS/MS, and OA by LC-MS/MS and UPLC-MS/MS. We report 16 new vectors for these toxins in the North Atlantic. There were differences in toxin contents among species, but no significant geographical or seasonal patterns were found. Our results suggest that legislation should be adjusted to extend the monitoring of marine toxins to a wider range of species besides edible bivalves.

Keywords: New vectors; PST; okadaic acid; spirolides; North Atlantic

Introduction

The occurrence of harmful algal blooms (HABs) has been increasing globally throughout the 20th century [1]. Rising water temperature and eutrophication were pointed out as the most significant factors in this increase [2]. HABs have a severe impact on the economy and human health, because phycotoxins may travel along the food chain, contaminating edible shellfish, resulting in intoxication incidents [3–7]. These phycotoxins can be classified as being either hydrophilic or lipophilic, with typical molecular weights (MW) of 500 Da and 600 Da, respectively [8]. Besides commercially important bivalves, other organisms, such as crustaceans, gastropods and fish, have been reported as phycotoxin vectors [9–11]. HAB poisoning events have been sporadic worldwide during the past two decades. The establishment of legislation, mostly for edible bivalves and monitoring programs are of extreme importance for poisoning prevention [7,12,13].

Paralytic shellfish toxins (PSTs) are a group of alkaloids that cause Paralytic Shellfish Poisoning (PSP). Intoxication events caused by these toxins result from the ingestion of contaminated shellfish [4,5]. These alkaloids are comprised of saxitoxin (STX) plus 57 analogs that bind specifically to site 1 of voltage-gated Na⁺ channels (Nav), causing paralysis. Currently, there is no antidote available. Ventilation support plus fluid therapy are the only treatments available [14]. After the first report in the USA in 1920, PSTs have been reported all over the globe [4,5]. The main producers of these neurotoxins are several species of dinoflagellates (*Alexandrium* sp., *Gymnodinium catenatum* and *Pyrodinium bahamense* var. *compressum*), as well as certain brackish and freshwater cyanobacteria (*Anabaena circinalis*, *A. lemmermannii*, *Aphanizomenon gracile*, *A. issatschenkoi*, *Rivularia* sp., *Lynbya wolleii*, *Planktothrix* sp. and *Cylindrospermopsis raciborskii*) [15–19]. The European Food Safety Authority (EFSA), proposes the use of toxicity equivalency factors (TEFs) as a unit that integrates all PST analogues for their conversion to SXT equivalents [14]. Fourteen TEF values have been calculated based on acute intra peritoneal (i.p.) toxicity in mice: NEO = 1; GTX₁ = 1; dc-STX = 1; GTX₄ = 0.7; GTX₃ = 0.6; GTX₂ = 0.4; dc-NeoSTX = 0.4; dc-GTX₃ = 0.4; 11-hydroxy-STX = 0.3; dc-GTX₂ = 0.2; GTX₅ = 0.1; GTX₆ = 0.1; C₂ = 0.1; C₄ = 0.1 [14]. There are only 15 standards available as certified material. In 2009, EFSA established the acute reference dose (ARfD) for PSTs as 0.5 µg STX equivalents/kg body weight, due to the lack of repeated oral intake data of these toxins in humans and animals [14]. The guideline limit value for PSTs is 800 ng SXT equivalents/g shellfish meat [20].

Spirolides (SPXs) belong to the group of the cyclic imines that includes other compounds, like gymnodimines, pinnatoxins, pteriatoxins, symbioimines, prorocentrolides and spiro-prorocetrolides [21,22]. These marine toxins have a cyclic imine group that is responsible for their neurotoxicity. SPXs bind specifically to both the muscarinic and nicotinic acetylcholine receptors (mAChR and nAChR, respectively) in the central and peripheral nervous system [23]. These biotoxins have acute toxicity by i.p. and oral administration in mouse bioassays (MBAs); however, there have been no reports of poisoning incidents [23,24]. SPXs have already been reported in microalgae and shellfish in Canada [25–27], Scotland [28], USA [29], Norway [30], Italy [31], Denmark [21], France [32], Spain [33] and

Chile [34]. Other cyclic imines seem to be confined to warmer waters of the Pacific Ocean, with reports from Japan, China and New Zealand [23,35]. SPXs are produced by dinoflagellate species, such as *Alexandrium ostenfeldii* and *A. peruvianum* [36]. The C group is the most toxic among SPXs and the only one with certified material available [37]. There are no legislated limits for cyclic imines, due to the lack of toxicological data [38].

Okadaic acid (OA) and its derivatives, dinophysistoxin-1 and 2 (DTX-1, DTX-2), are responsible for Diarrhetic Shellfish Poisoning (DSP). This group of neurotoxins is composed of polyethers that inhibit type 1 and 2A serine/threonine phosphatases [39]. OA and its analog, DTX-1, are also tumor promoters [40]. DSP incidents have been reported all over the globe [41–44]. Several species of phytoplankton have already been reported as causing DSP, particularly from the genera *Phalacroma*, *Prorocentrum* and *Dinophysis* [45–49]. The accepted limit established in Europe is 160 ng OAEquivalents/g shellfish meat [20].

The occurrence of emergent toxins and new vectors in the Atlantic Ocean brings new challenges for monitoring programs. It is necessary to fill the lack of knowledge and improve monitoring programs in order to minimize risks to human health. Close monitoring, in conjunction with reliable detection methods, helps to reduce the number of poisonings. Benthic organisms are generally poorly studied regarding their role as potential vectors for marine toxins, with the exception of bivalves [3–5,7,13,26,37]. Several benthic species were surveyed in this study, including gastropods (sea-snails, sea-slugs and limpets), bivalves (mussels) and echinoderms (starfishes and sea-urchins). We have chosen these particular species, because most of them are edible and we strongly believe that the human health risk is underestimated. In addition, all of them play an important role in benthic food-chains. Our aims were to unravel new vectors of PST, OA and SPX, to study the existence of seasonal, geographical and interspecific patterns of toxin accumulation. Table 2.5 shows the species sampled in this work and their trophic level, edibility and monitoring status.

Table 2.5 - Average number of specimens to set a composed sample and number of samples collected since July 2009 until the end of 2010. Availability of animals is dependent on their geographical distribution and ecology.

Species	Trophic level	Edibility	Monitored	References
<i>Gibbula</i> sp.	Grazer	Yes	No	[20,50]
<i>Monodonta</i> sp.	Grazer	Yes	No	[20,50]
<i>Littorina</i> sp.	Grazer	Yes	No	[20,50]
<i>Patella intermedia</i>	Grazer	Yes	No	[20,51]
<i>Paracentrotus lividus</i>	Grazer	Yes	No	[20,52]
<i>Echinus esculentus</i>	Grazer	Yes	No	[20,53]
<i>Aplysia depilans</i>	Grazer	No	No	[20,54]
<i>Mytilus galloprovincialis</i>	Filter feeder	Yes	Yes	[20,51]
<i>Nucella lapillus</i>	1st level Predator	Yes	No	[20,51]
<i>Marthasterias glacialis</i>	2nd level Predator	No	No	[20,51]
<i>Charonia lampas</i>	3rd level Predator	Yes	No	[20,55]

Results and Discussion

2.1. PSTs

In 104 samples analyzed, we obtained 51% positive results. A sample was considered positive when the toxin levels detected were above the limit of detection (LOD); however, 30.2% of these samples were below the limit of quantification (LOQ) for SXT and its analogs (Figure 2.10). Regarding gastropods, bivalves and echinoderms, most of the positive results occurred in the late spring-summer season. In the general linear model, significant differences were not detected for any of the fixed factors: sampling site ($F_{11,90} = 1.9$; $p = 0.08$) or species ($F_{9,92} = 1.8$; $p = 0.09$). These results can be explained by the high resemblance between species average values, although there were differences in their dispersion. It should be noted that the average PST concentration was in general very low (Figure 2.11). The percentage of analogs in each group screened (Figure 2.12) shows that the most toxic groups, carbamate (STX, NEO, GTX1, GTX2, GTX3 and GTX4) and decarbamoyl (dcSTX, dcGTX1, dcGTX2, dcGTX3 and dcGTX4), were always present in all species [14]. The former group was the most common, since 67% of the positives have carbamate forms, constituting 50%–83% of the PSTs' total content. The decarbamoyl group contributes from 50% to 67% of the total toxin content in the majority of the positive samples. The less toxic *N*-sulfo-carbamoyl group (C1, C2, C3, C4, GTX5 and GTX6) was only detected in 33% of the positive samples, reaching a maximum of 20% of the total PST content.

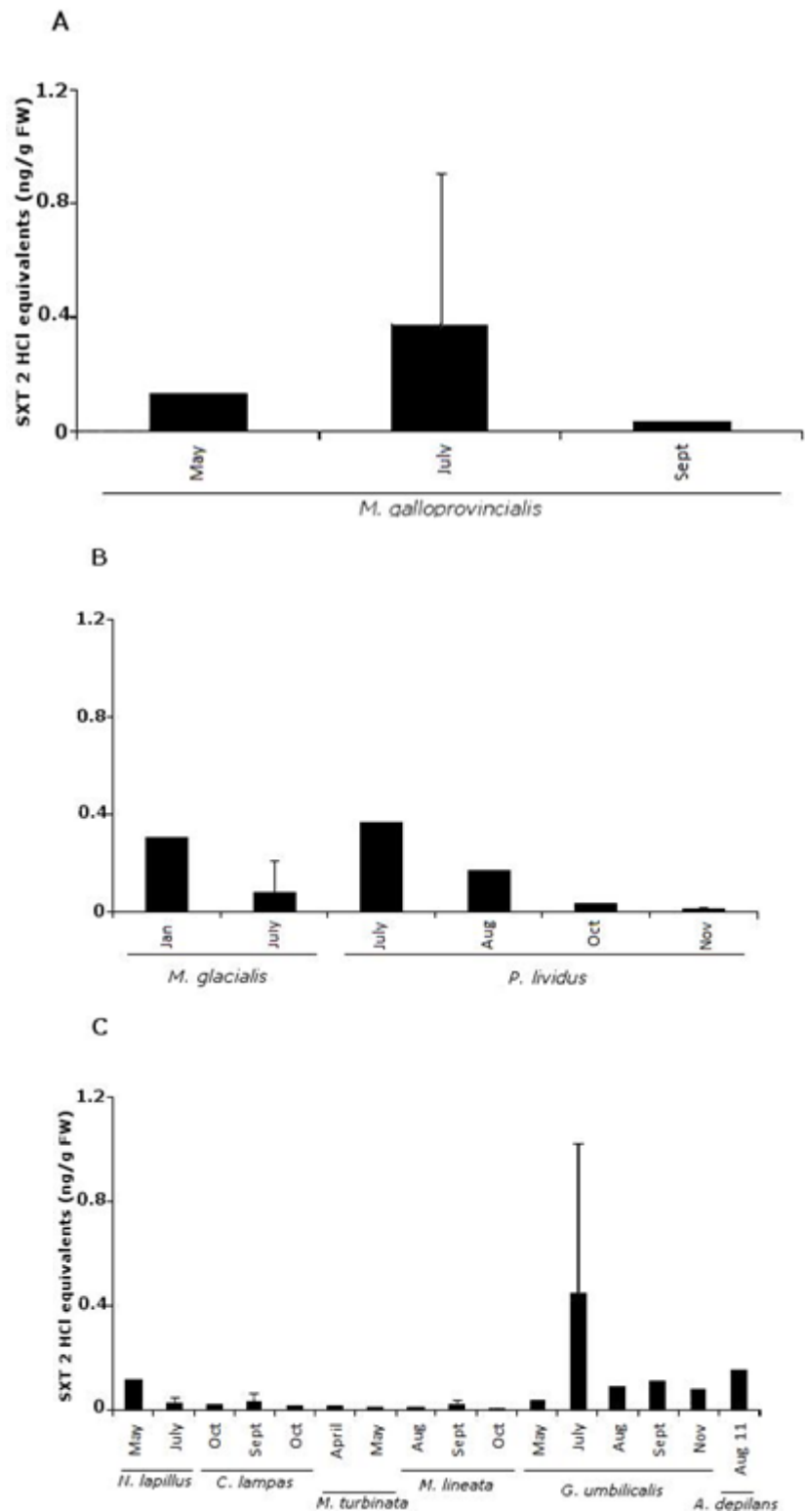


Figure 2.10 - Paralytic shellfish toxin (PST) content in saxitoxin (STX) 2 HCl equivalent ng/g fresh weight (fw) in all groups: (A) bivalves—*Mytilus galloprovincialis*; (B) echinoderms—*Marthasterias glacialis* and *Paracentrotus lividus*; (C) gastropods—*Nucella lapillus*

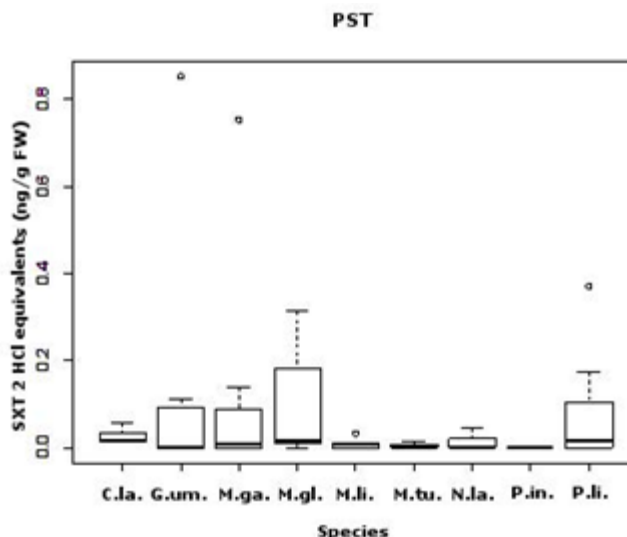


Figure 2.11 - Box and whisker plots for PST concentrations [SXT HCl 2 equivalents (ng/g fw)] found in each species (C.la.- C. lampas; G. um.- G. umbilicalis; M.ga.- M. galloprovincialis; M. gl.- M. glacialis; M. li.- M. lineata; M. tu.- M. turbinata;

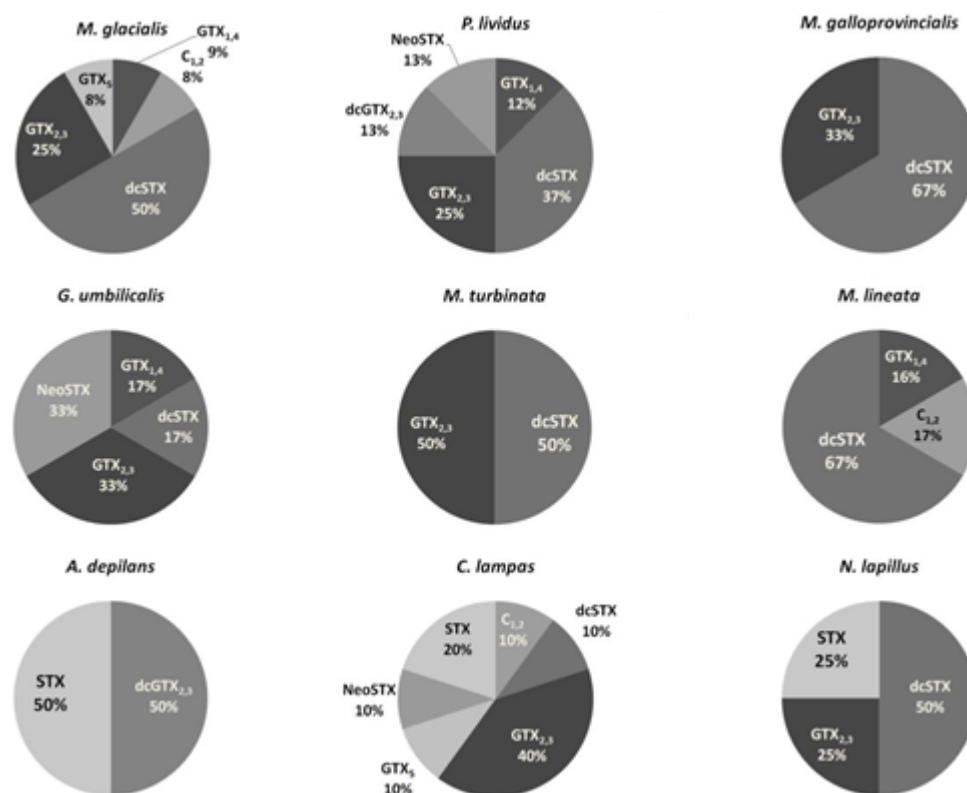


Figure 2.12 - PST profiles in each group screened.

In all the species, positives were below the limit implemented in Europe [20]. In comparison with recent work performed on the Portuguese coast [4], the present toxin profile detected was wider, showing a higher prevalence of the most potent analogs. However, the detected concentrations were lower. There was no previous data available regarding PSTs on the Portuguese coast in taxa other than bivalves [13,56]. PST metabolism in bivalves is very complex, since some species show the ability to convert

low toxic forms to high toxic ones, therefore presenting different toxin profile than the producers [57–59]. Gastropods are known for their ability to accumulate PSTs and having low depuration rates and, thus, being quite dangerous to humans, since they remain toxic for long periods of time. Most poisoning events due to gastropod ingestion have been reported in Eastern Asia [9,11]. Regarding bivalves, *M. galloprovincialis* has 67% of its PSTs as dcSTX, with the remainder being GTX_{2,3} (Figure 3). This profile could be due either to a high carbamoylase activity or feeding on more toxic dinoflagellate species [9], in contrast to a previous work [13], which showed mussels with a toxin profile enriched in the less toxic groups. There is evidence that GTX analogs can be converted into SXT in shellfish, due to the action of bacteria [60], and mediated by glutathione (GSH) [61]. This could be the reason why the mussel predator, *N. lapillus*, has 25% of SXT. Nevertheless, grazer gastropods can also acquire PSTs directly [62]. This is the case with *Monodonta* sp., *G. umbilicalis* and *A. depilans*, with the latter showing a higher toxin content (50% STX). *Gibbula umbilicalis* had 30% of NEO, and in *Monodonta* species, the carbamate group was absent (Figure 3). *Charonia lampas* is a scavenger and a predator, so it most probably acquired PSTs indirectly. In comparison with other works, the levels found in this species were low [63]. Although PSTs have already been detected in echinoderms, namely in starfish [64,65], the report of PSTs in sea-urchins is a novelty. Comparing the toxin content of both species, *M. glacialis* had 84% of its toxin content as carbamate analogs, while *P. lividus* had 50%, which could be due to a dietary effect. Asakawa *et al.* (1997) [64] found higher concentrations of PSTs in the starfish *Asterias amurensis*; however, in this work, we detected a higher diversity and concentration of the carbamate group. Our data show six new PST vectors among the species screened: four gastropods (*G. umbilicalis*, *N. lapillus*, *Monodonta* sp. and *A. depilans*) and two echinoderms (*P. lividus*, *M. glacialis*).

2.2. OA

A total of 51% of 55 samples were positive for OA (>LOD), with 14.3% of these below LOQ. No analogs were detected. Most of the positives (61.5%), as well as the higher concentrations detected in the late spring-summer season (Figure 2.13). The average concentration found for each species ranged between 0.58 ng/g fw (in *C. lampas*) and 175.71 ng/g fw (*N. lapillus*). Statistically significant differences for sampling site were detected, although they were close to the limit of statistical significance ($F_{8,43} = 2.4$; $p = 0.049$). This was possibly due to the fact that OA had a greater proportion of positive results and higher toxin concentration. It is possible that a greater sampling effort for PSTs and SPX could result in significant p -values for the sampling site as well. For species, clear significant differences were found ($F_{8,43} = 2.4$; $p < 0.001$).

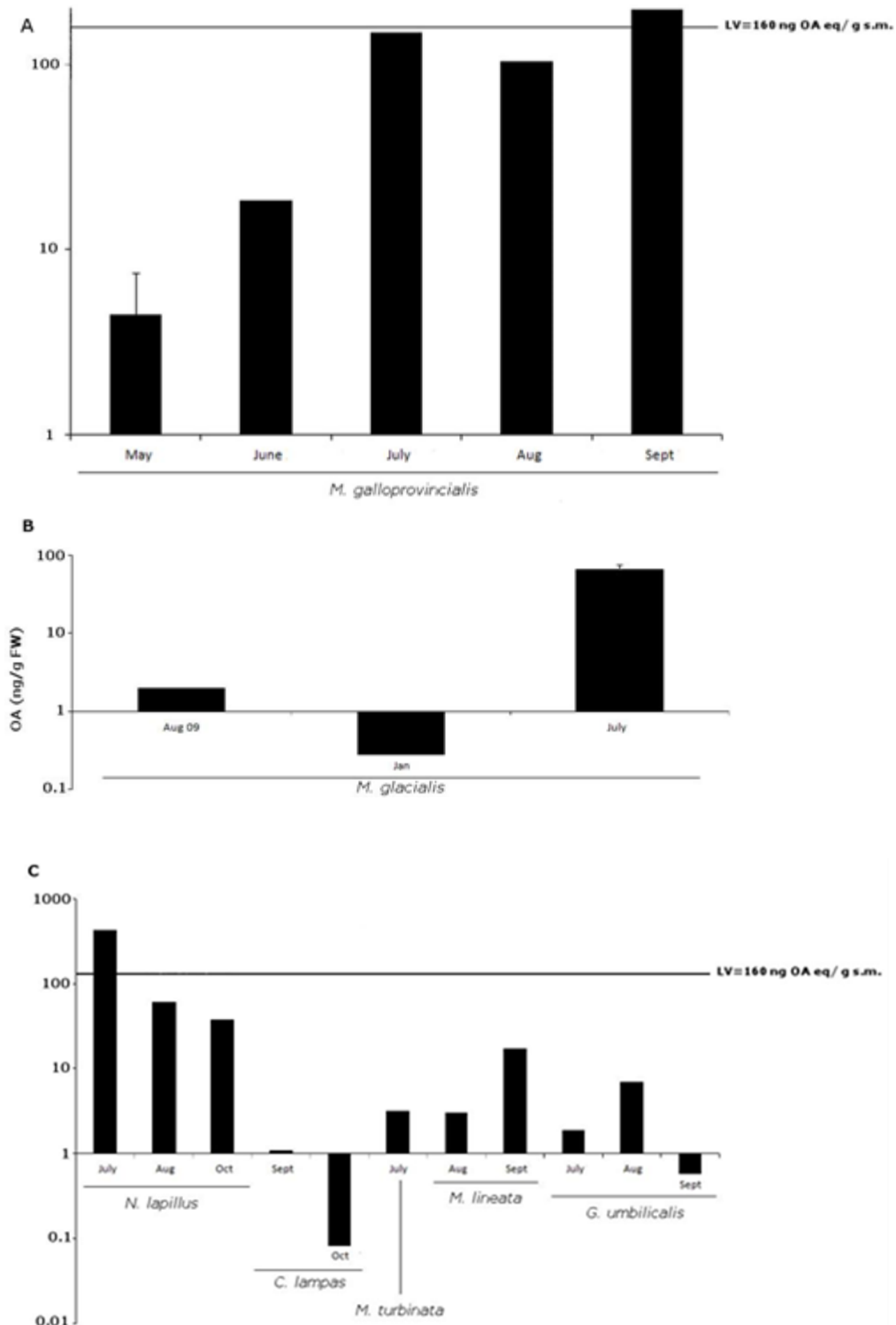


Figure 2.13 - Okadaic acid (OA) positive results (ng/g fw) for all sampled groups of organisms: (A) bivalves; (B) echinoderms; (C) gastropods. Y-axis in logarithmic scale. Limit value (LV) established in Europe is 160 ng OA equivalents (eq)/g shellfish meat (s.m.).

The majority of significant differences in pairwise comparisons between species appeared in this group of toxins. Differences were due to two major groups; those that have the lowest average OA values (six species—*C. lampas*, *G. umbilicalis*, *M. lineata*, *M. turbinata*, *P. lividus*, *P. intermedia*) versus those that have the highest average values (three species—*M. glacialis*, *M. galloprovincialis*, *N. lapillus*) (see Figure 2.14 and Table 2.6). Interestingly, the species with highest average OA values are linked through

the food chain, with *M. galloprovincialis* being predated by *N. lapillus* and both by *M. glacialis* (Figure 2.15) [51]. We show five first reports of OA in *G. umbilicalis*, *N. lapillus*, *Monodonta* sp., *P. lividus* and *M. glacialis*. These results suggest the need for a revision of marinetoxin monitoring policies in order to consider the inclusion of groups other than bivalves. In addition, the highest concentration detected did not occur in bivalves, but in the edible gastropod, *N. lapillus*.

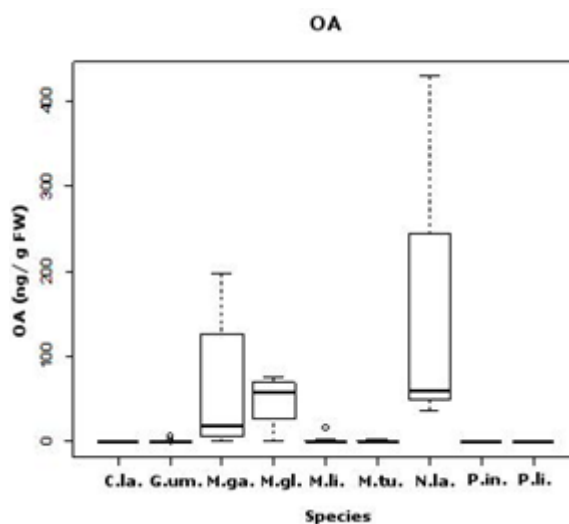


Figure 2.14 - Box and whisker plots of OA concentrations (ng/g fw) found in each species (C. la.- C. lampas; G. um.- G. umbilicalis; M. ga.- M. galloprovincialis; M. gl.- M. glacialis; M. li.- M. lineata; M. tu.- M. turbinata; N. la.- N. lapillus; P. in.- P. interm)

Table2.6 - Species' OA concentration pairwise comparisons using post hoc Tukey test.

Species pairwise comparisons	z	p
<i>N. lapillus</i> – <i>C. lampas</i>	3.3	0.03
<i>M. glacialis</i> – <i>G. umbilicalis</i>	3.8	<0.01
<i>M. galloprovincialis</i> – <i>G. umbilicalis</i>	5.2	<0.001
<i>N. lapillus</i> – <i>G. umbilicalis</i>	4.7	<0.001
<i>M. lineata</i> – <i>M. glacialis</i>	–3.6	<0.01
<i>M. turbinata</i> – <i>M. glacialis</i>	–3.4	0.02
<i>P. lividus</i> – <i>M. glacialis</i>	–5.6	<0.001
<i>P. intermedia</i> – <i>M. glacialis</i>	–5.5	<0.001
<i>M. galloprovincialis</i> – <i>M. lineata</i>	4.8	<0.001
<i>N. lapillus</i> – <i>M. lineata</i>	4.6	<0.001
<i>M. galloprovincialis</i> – <i>M. turbinata</i>	4.1	<0.01
<i>N. lapillus</i> – <i>M. turbinata</i>	4.0	<0.01
<i>P. lividus</i> – <i>M. galloprovincialis</i>	–7.1	<0.001
<i>P. intermedia</i> – <i>M. galloprovincialis</i>	–6.4	<0.001
<i>P. lividus</i> – <i>N. lapillus</i>	–6.4	<0.001
<i>P. intermedia</i> – <i>N. lapillus</i>	–5.9	<0.001
<i>P. intermedia</i> – <i>P. lividus</i>	–0.6	<0.001

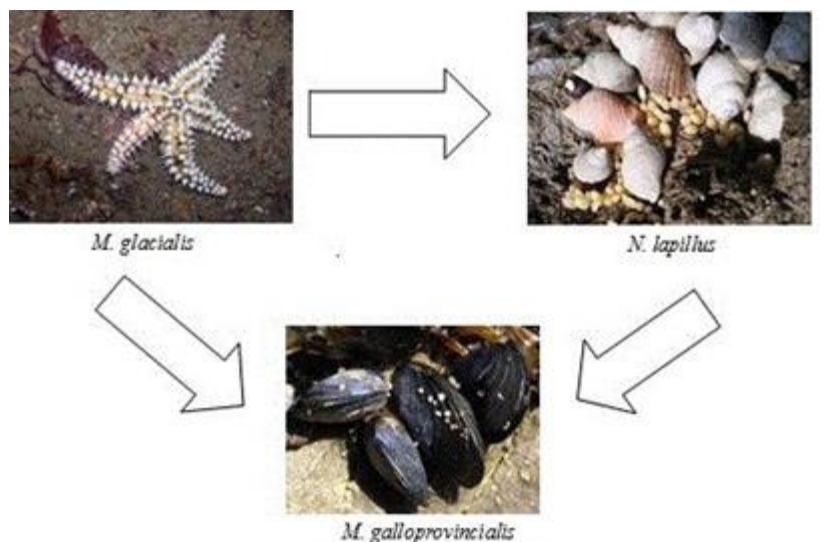


Figure 2.15 - Trophic relation between *M. galloprovincialis*, *N. lapillus* and *M. glacialis*.

Two samples exceeded the 160 ng OA equivalents/g limit [20]: *N. lapillus* collected in July 2010 in Memória with 429.41 ng/g fw and *M. galloprovincialis* collected in September 2010 from the same site with 198.17 ng/g fw. Our average OA levels detected were similar to other reports on the Portuguese coast [13]. We detected OA in January, which seemed to be unusual [13], but this could be explained by our wider range of species screened.

2.3. 13-Desmethyl Spirolide C

In 55 samples analyzed, 38.2% of the results were positive for 13-desmethyl Spirolide C (Figure 2.16). A sample was considered positive when the toxin levels detected were above the LOD. Concentrations ranged between 0.49 ng/g fw and 3.86 ng/g fw. Approximately half (48%) of the positive results occurred in the late spring-summer season. Concentrations were also higher in this period (Figure 2.16).

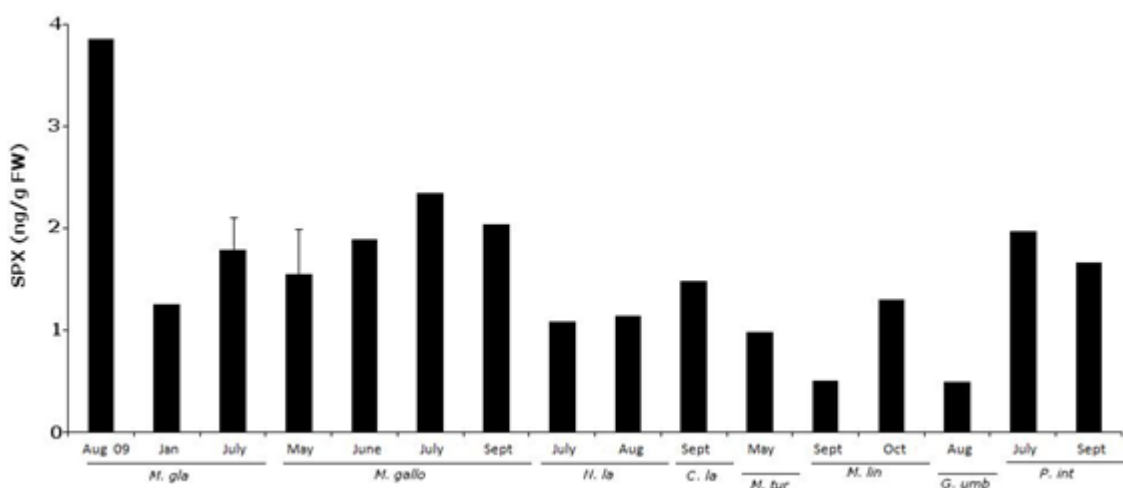


Figure 2.16 - SPX positive results (ng/g fw) for all sampled groups of organisms.

Regarding SPX toxin concentrations, significant differences were found for between species, but not for sampling sites. Here, as for OA, differences in pairwise comparisons were due to the same differentiated groups, with the exception of the species *C. lampas* and *P. intermedia*, as shown in Figure 2.17. Both species showed a large dispersion (see Table 2.7). We have shown five first reports for this biotoxin in *G. umbilicalis*, *N. lapillus*, *Monodonta* sp., *M. glacialis* and *P. intermedia*. It is important to point out that this is not only the first report of SPX in these species, but also on the Portuguese coast. SPX showed a wide range of dispersion, not only geographical, but also in terms of vectors.

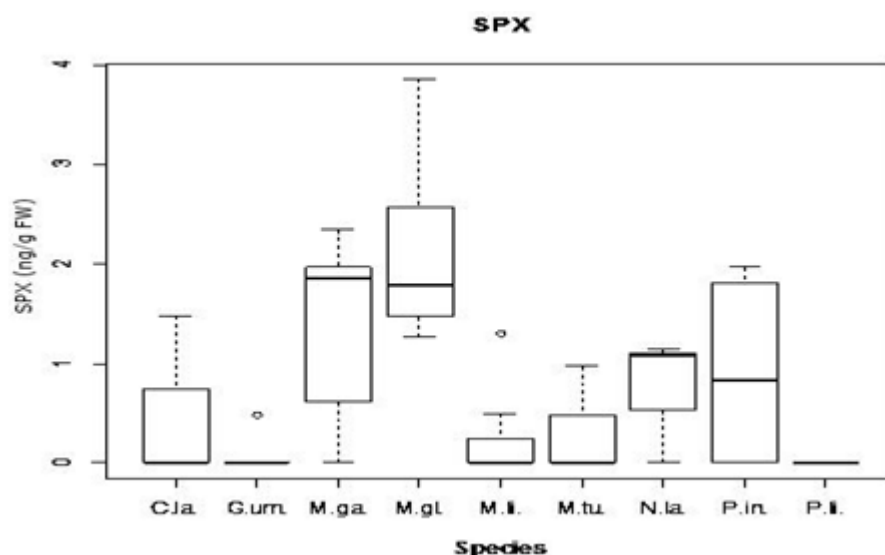


Figure 2.17 - Box and whisker plots of SPX concentration (ng/g fw) found in each species (C. la.- *C. lampas*; G. um.- *G. umbilicalis*; M. ga.- *M. galloprovincialis*; M. gl.- *M. glacialis*; M. li.- *M. lineata*; M. tu.- *M. turbinata*; N. la.- *N. lapillus*; P. in.- *P. interm*)

Table 2.7 - Species' SPX concentration pairwise comparisons using post hoc Tukey test.

Species pairwise comparisons	z	p
<i>M. glacialis</i> – <i>G. umbilicalis</i>	4.3	<0.001
<i>M. galloprovincialis</i> – <i>G. umbilicalis</i>	3.2	0.02
<i>M. lineata</i> – <i>M. glacialis</i>	-3.9	<0.01
<i>M. turbinata</i> – <i>M. glacialis</i>	-3.5	0.01
<i>P. lividus</i> – <i>M. glacialis</i>	-4.2	<0.001
<i>P. lividus</i> – <i>M. galloprovincialis</i>	-3.1	0.04

2.4. Toxins Concentrations Found: Comparison between Species and Sampling Sites

The results of the MANOVA test show that there were clear significant differences between species ($F_{8,42} = 2.4$; $p < 0.001$) and small differences between sampling sites ($F_{8,42} = 1.7$; $p = 0.04$). These were, in general, the same results as those from the analysis for each individual toxin. In the pairwise multivariate comparisons performed with the Hotelling test, generally, the species that showed more significant differences regarding toxin concentrations were *M. glacialis*, *M. galloprovincialis* and *N. lapillus*. The previous Tukey tests showed a similar pattern, *i.e.*, the same pairs of species showed the most significant differences (see Table 2.8). With respect to sampling site, differences between sites were, as expected, from the test for individual toxins, mainly due to OA (Table 2.8). However, if the

sampling effort was more balanced, differences between sampling sites could also be detected for PSTs and SPX. Memória was the most intensively sampled site and showed more differences in this test. It should be noted that the species with the highest toxin concentrations are linked by the food-chain. *Mytilus galloprovincialis* is predated by both *N. lapillus* and *M. glacialis*, while the latter also preys on *N. lapillus* [51,66]. The advantage of this multivariate test is that our data support the possibility of bioaccumulation of toxins along the food chain, taking into account all toxins together.

Table 2.8 - Pairwise comparisons between factor levels of sampling site and species, using the three groups of toxins as dependent factors.

Pairwise Comparison	<i>p</i>
<i>C. lampas</i> – <i>P. intermedia</i>	0.01
<i>G. umbilicalis</i> – <i>M. glacialis</i>	<0.001
<i>G. umbilicalis</i> – <i>M. galloprovincialis</i>	0.03
<i>G. umbilicalis</i> – <i>N. lapillus</i>	<0.01
<i>P. intermedia</i> – <i>M. glacialis</i>	<0.001
<i>P. intermedia</i> – <i>M. galloprovincialis</i>	0.02
<i>P. intermedia</i> – <i>N. lapillus</i>	<0.001
<i>M. lineata</i> – <i>M. glacialis</i>	0.01
<i>M. turbinata</i> – <i>M. glacialis</i>	<0.01
<i>M. galloprovincialis</i> – <i>P. lividus</i>	<0.001
<i>P. lividus</i> – <i>M. glacialis</i>	0
<i>P. lividus</i> – <i>N. lapillus</i>	
Angeiras–Memória	0.03
Memória–Porto Côvo	0.01
Memória–Viana do Castelo	0.03
Memória–Almograve	0.01

Experimental Section

3.1. Selected Species and Sampling Points

In this study, we were searching for unconventional vectors of marine toxins, so we surveyed several benthic species. We focused our sampling on edible species, but also other species that play an important role in the food-chain: gastropods (*Monodonta lineata*, *Monodonta turbinata*, *Gibbula umbilicalis*, *Gibbula magus*, *Littorina littorea*, *Littorina saxatilis*, *Nucella lapillus*, *Patella intermedia*, *Aplysia depilans*, *Charonia lampas*), bivalves (*Mytilus galloprovincialis*), sea-urchins (*Paracentrotus lividus*, *Echinus esculentus*) and starfish (*Marthasterias glacialis*).

Animals were collected monthly from several locations along the northern and southern Portuguese coast (Figure 2.18) from July 2009 till the end of 2010. Samples of *Charonia lampas* were purchased in local markets from the same areas. Organisms were collected from the intertidal area during low tides and were transported to the laboratory in refrigerated containers. If they were not processed immediately, they were frozen at $-20\text{ }^{\circ}\text{C}$. The number of samples collected and average number of specimens needed to set a pooled sample are detailed in Table 2.9.

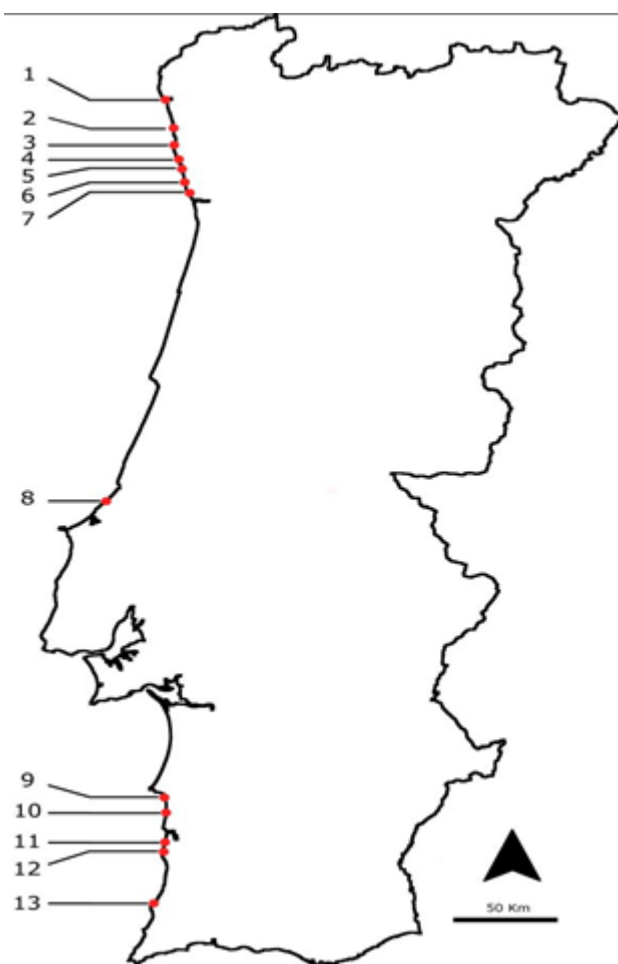


Figure 2.18 - Location of the sampling points on the Atlantic Portuguese coast: 1: Viana do Castelo; 2: Esposende; 3: Póvoa do Varzim; 4: Angeiras; 5: Memória; 6: Valadares; 7: Aguda; 8: São Martinho do Porto; 9: São Torpes; 10: Porto Côvo; 11: Monte Clérigos; 12:

Table 2.9 - Average number of specimens comprising a pooled sample and number of samples collected since July 2009 till 2010. Availability of animals is dependent on their geographical distribution and ecology.

Species	Number of pooled samples collected	Average number of animals collected to set a pooled sample
	from July 2009 till end 2010	
<i>Gibbula umbilicalis</i>	34	100
<i>Gibbula magus</i>	1	90

<i>Monodonta lineata</i>	20	86
<i>Monodonta turbinata</i>	21	86
<i>Nucella lapillus</i>	13	15
<i>Littorina littorea</i>	2	10
<i>Littorina saxatilis</i>	4	15
<i>Patella intermedia</i>	4	15
<i>Charonia lampas</i>	5	1
<i>Mytilus galloprovincialis</i>	7	30
<i>Paracentrotus lividus</i>	10	10
<i>Marthasterias glacialis</i>	8	1
<i>Echinus esculentus</i>	2	1
<i>Aplysia depilans</i>	1	1

3.2. Sample Preparation

3.2.1. Paralytic Shellfish Toxin (PST) Extraction

Samples were extracted following the Lawrence *et al.* method [67]. Animals were dissected and homogenized with a blender (A320R1, 700 W, Moulinex) in pooled groups in order to obtain 1 g of extractable tissue, with the exception of *Charonia lampas*, *Marthasterias glacialis*, *Echinus esculentus* and *Aplysia depilans*, which were treated individually. The homogenized tissue was extracted with 3 mL 1% acetic acid (Merck, Portugal) using a vortex mixer for 5 min (Top Mix 1118, Fisher Scientific, Portugal), then heated in a boiling water bath for 10 min, remixed on a vortex mixer and placed in ice for 5 min. The resulting mixture was sonicated (1 min, 70 Hz, Vibra Cell, Sonic & Materials, USA) and subsequently centrifuged at 4495×g for 10 min at 4 °C (Centrifugal-Legend RT, Sorvall). Then, 2 mL of 1% acetic acid was added to the centrifuge tube containing the solid residue, mixed well on a vortex mixer and centrifuged again (4495 g/10 min/4 °C). The supernatant solution was collected pooled into the same tube that contained the first portion of extract and finally diluted to 5 mL with 1% acetic acid. An aliquot (1 mL) of the crude extract was passed through a C18 solid-phase extraction (SPE) cartridge (500 mg/3 mL volume from Supelco, Bellefonte, PA, USA), previously conditioned with 10 mL methanol (Fisher Scientific, Leics, UK), followed by 10 mL water (MilliQ). Toxin was absorbed onto the column and eluted at a flux of 0.4 mL/min using 2 mL of ultrapure water. The pH was adjusted to 6.5 using 0.2 M NaOH (Sigma Aldrich, Portugal). The eluents were evaporated to dryness (Acid-resistant Centrивap Concentrator, Labconco, MO, USA) and dissolved in 0.5 mL of 0.03 M acetic acid. The extracts were passed through 0.45 µm filters (Ultrafree-MC centrifugal filter devices from Millipore, Spain) and pre-oxidized before HPCL-FLD analysis. The extracts were oxidized with hydrogen peroxide and periodate (Panreac Quimica, Spain). Peroxide oxidation consisted of mixing 25 µL of 10% hydrogen peroxide in water (v/v) with 250 µL 1 M NaOH and 100 µL of sample. The solution was then mixed on a vortex mixer and rested at room temperature for 2 min. Then 20 µL of acetic acid was added. At this point, the solution was homogenized and 25 µL was injected into the HPLC system. Peroxide oxidant solution

was prepared daily. Periodate oxidation consisted of mixing 100 μ L sample with 100 μ L of deionized water and 500 μ L periodate oxidant. The solution reacted for 1 min at room temperature before the addition of 5 μ L of acetic acid. Periodate oxidant was prepared daily by mixing 5 mL of 0.03 M periodic acid with 5 mL of 0.3 M ammonium formate and 5 mL of 0.3 M sodium phosphate dibasic. The pH of the final solution was adjusted to 8.2 with 1 M NaOH.

3.2.2. Lipophilic Toxins Extraction

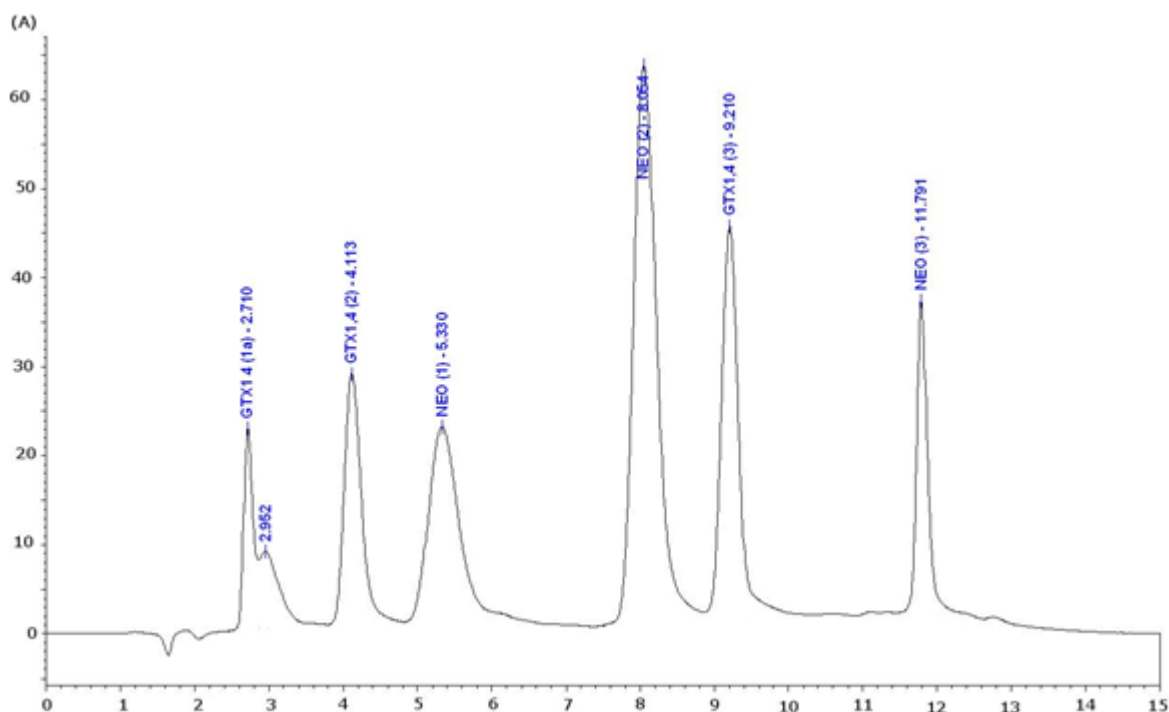
The protocol from Otero *et al.* (2010) [68] was followed for the extraction of okadaic acid (OA), dinophysistoxin (DTX-1 and DTX-2) and spirolides (13,19-didesmethyl SPX C; 13-desmethyl SPX C). Animals were dissected and homogenized with the help of a blender (A320R1, 700 W, Moulinex) in pooled groups in order to obtain 1 g of extractable tissue, with the exception of *Charonia lampas*, *Marthasterias glacialis*, *Echinus esculentus* and *Aplysia depilans*. In these cases, each animal was treated separately. The 1 g of homogenized tissue was extracted with 3 mL of methanol (Fisher Scientific), then centrifuged during 10 min at 2932 g at 4 °C (Centrifugal-Legend RT). This procedure was repeated twice, and the supernatants combined and concentrated to dryness (Acid-resistant Centrivap Concentrator, Labconco). Residues were then re-suspended in 10 mL of water (MilliQ) and partitioned twice against dichloromethane (Merck). The organic layers (20 mL) were reserved and concentrated by drying and re-suspended in 1 mL of methanol. Then 500 μ L was concentrated to dryness, re-suspended in 100 μ L of methanol and filtered through a 0.45 μ m filter (UltraFree-MC centrifugal devices, Millipore) before LC-MS/MS analysis.

3.3. Sample Analysis

3.3.1. PSP HPLC-FLD Conditions

The conditions were the same as reported by Rodriguez *et al.* (2010) [69]. Briefly, the analyses were performed using high-performance liquid chromatography with fluorescence detection (HPLC-FLD) equipment (Waters 2695), consisting of a pump (Waters 515) and a column (SupelcosilTM LC-18, 5 μ m, 15 \times 4.6 mm, Sigma Aldrich) kept at 35 °C. Empower software (Waters, Manchester, UK) was used to control the process. Toxins were detected with a Waters 2475 fluorescence detector, with excitation set to 340 nm and emission to 395 nm. Injection volume was 25 μ L. Eluent (A) of the mobile phase was composed by 0.1 M of ammonium formate (Sigma Aldrich). Eluent (B) was composed by 0.1 M of ammonium formate in 5% of acetonitrile (Panreac Quimica). Both eluents had their pH adjusted to 6 with 1 M acetic acid. The gradient started with 5% of mobile phase (B) for the first 5 min, then increasing to 70% up to min 9, then decreasing to 0% in min 11 and stayed in 0% till the end of the run (15 min). The flow rate was 1 mL/min. For toxin detection and determination, standards of SXT, decarbamoyl gonyautoxin 2 and 3 (dcGTX₂ and dcGTX₃) and decarbamoylsaxitoxin (dcSXT) combined (Mix I), gonyautoxin 2 and 3 (GTX₂ and GTX₃), gonyautoxin 5 (GTX₅) and sulfo-carbamoyl saxitoxin 1 and 2 (C₁ and C₂) combined (Mix II) were pre-oxidized with hydrogen peroxide and neosaxitoxin (NEO),

gonyautoxin 1 and 4 (GTX₁ and GTX₄) combined (Mix III) were oxidized with periodate. Standards were obtained from NRC Certified Reference Material Program (Institute for Marine Biosciences, Halifax, Canada). The three mixtures of standards were diluted 10-fold in water (MilliQ), a calibration curve was made with subsequent dilutions in water with five points for each mixture. Concentrations ranging from 6 ng/mL to 257 ng/mL for Mix I, from 8 ng/mL to 354 ng/mL for Mix II and from 6 ng/mL to 290 ng/mL for Mix III. Every calibration solution was made daily for each set of analysis. PSTs were identified by comparison of oxidation products of the standards retention times. PSTs were quantified by direct comparison of peak areas with the calibration curves. Quantified levels for each analog must be converted in STX 2HCl equivalents ng/g, using applied TEFs for each analog [14]. Retention times were: dcGTX_{2,3} (3.75 and 4.05 min), C_{1,2} (5.2 min), dcSTX (7.06 and 7.92 min), GTX_{2,3} (9.17 min), GTX₅ (10.6 min), SXT (11.8 min) and NEO (8.05 min) (Figure 2.19). Due to the complexity of the samples and to overcome the matrix effect, non-oxidized samples of each species were injected in the HPLC. Blanks were prepared daily. The limits of detection (LOD) and quantification (LOQ) for the HPLC-FLD are displayed in Table 2.10.



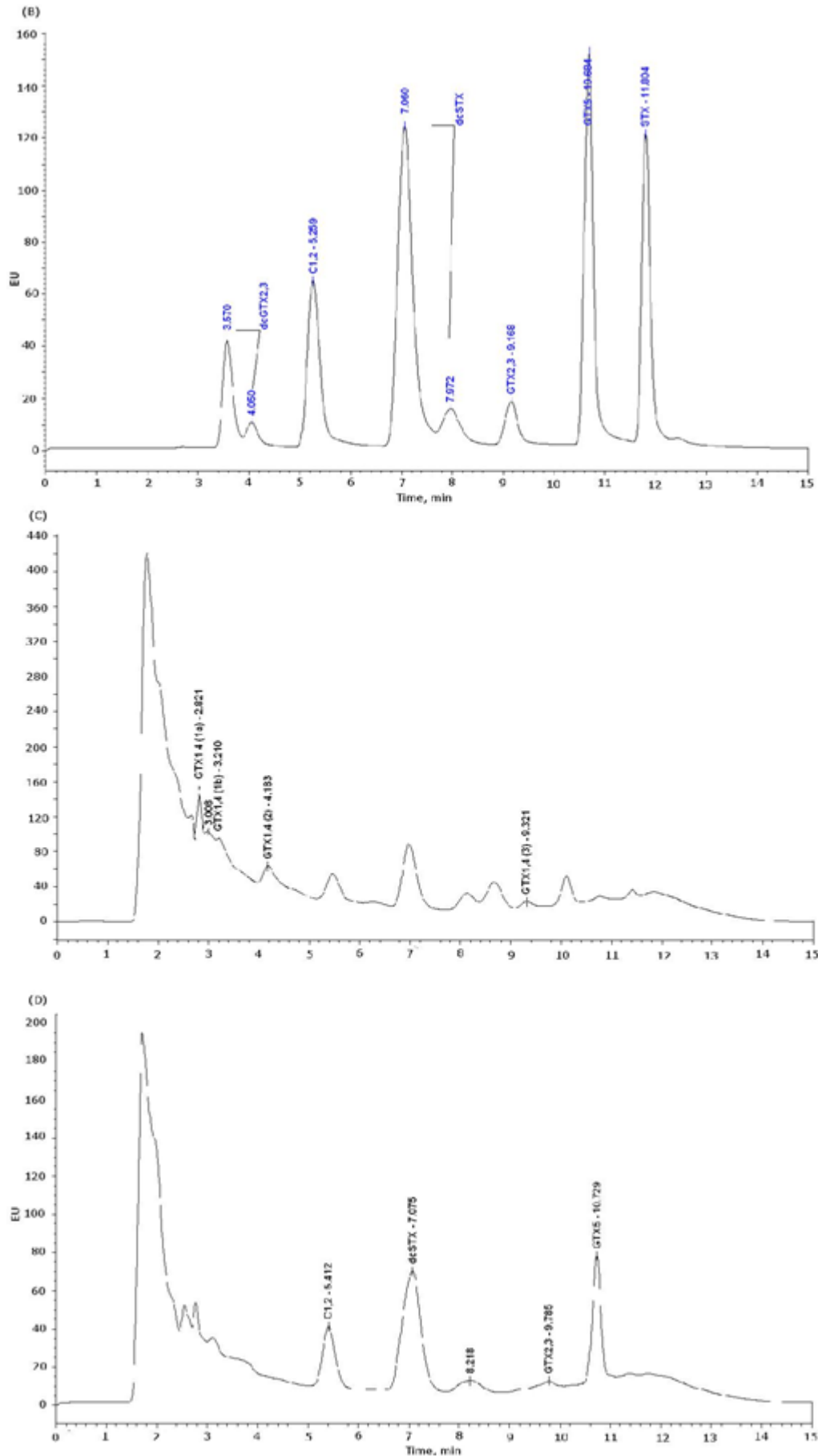


Figure 2.19 - Chromatograms of the PSTs standards and a positive sample for *M. glacialis*, hydroxylated toxins are oxidized with periodate and non-hydroxylated toxins with peroxide. (A) Standards injected with periodate oxidation, peak GTX_{1,4}(1) and GTX_{1,4}(3) are secondary oxidation products of GTX_{1,4}. Peaks NEO(1) and NEO(3) are secondary oxidation products of NEO. (B) Standards injected with peroxide

oxidation. (C) Chromatogram of a positive sample in *M. glacialis* injected with periodate oxidation. (D) Chromatogram of a positive sample in *M. glacialis* injected with peroxide oxidation.

Table 2.10 - Limits of detection (LODs) and quantification (LOQs) of the method for each toxin (expressed in ng/mL).

Toxin	LOD	LOQ
dcGTX _{2,3}	2.7	8
C1,2	3	11
dcSTX	0.2	6
GTX _{2,3}	1.5	9
GTX ₅	0.4	8
SXT	0.4	7
GTX _{1,4}	3	9
NEO	1.2	8

3.3.2. Lipophilic Toxins LC-MS/MS Conditions

The LC-MS/MS conditions were the same as reported in Otero *et al.* (2011) [70]. Briefly, the analyses were performed with high-performance liquid chromatography (LC) equipment (Shimadzu, Kyoto, Japan) consisting of a binary system of LC-10ADVP pumps, an autoinjector (SIL-10ADVP) with degasser (DGU-14A), refrigerated rack, column oven (CTO-10ACvp) and a system controller (SCL-10Avp). The LC system was coupled to a hybrid quadrupole-linear ion trap mass spectrometer (MS) (2000 QTRAPLC/MS/MS, Applied Biosystems, Carlsbad, CA, USA), equipped with an atmospheric pressure ionization unit (API) and fitted with an electrospray ionization source (ESI). The equipment operated in the conventional mode of low energy of collision induced dissociation (CID) of MS/MS. Nitrogen was produced by a Nitrocraft NC_{LC/MS} generator from Air Liquide (Madrid, Spain). The LC system operated with the ESI interface using the following parameters: curtain gas, 15 psi; collision-activated dissociation gas (CAD), 6 psi; IonSpray voltage, 4000 V; temperature, 450 °C; gas 1, 50 psi; gas 2, 50 psi. Analyst software was used to control the whole process. Toxins separation was performed with a BDS-Hypersil-C8 column (i.d. 2 × 50 mm; 3 μm) and a guard cartridge (i.d. 10 × 2.1 mm) from Thermo (Waltham, MA, USA). Column oven temperature was set at 25 °C. Injection volume of 5 μL. Eluent A of the mobile phase was composed of 50 mM formic acid (Merck, Madrid, Spain) and 2 mM ammonium formate (Sigma Aldrich, Madrid, Spain) in water. Eluent B consisted of acetonitrile (Panreac Quimica, Barcelona, Spain) in water (95:5) with ammonium formate (2 mM) and formic acid (50 mM). The gradient started with 30%–90% of mobile phase (B) for 8 min, then maintained at 90% until min 11, decreasing to 30% over 0.5 min and maintained during 5.5 min until the end of the run. Flow rate was 0.2 mL/min. The mass spectrometer was operated in multiple reaction monitoring (MRM), detecting in positive and negative modes. Two product ions were analyzed per compound, one for quantification and another for confirmation. The transitions employed were: OA and DTX-2 (m/z 803.5 > 255.5/113.5), DTX-1 (m/z 817.5 > 255.5/113.5), 13,19-didesmethyl SPX C (m/z 678.5 > 660.5/430.5), 13-desmethyl

SPX C (m/z 692.5 > 674.4/444.4). Retention times were: OA (8.2 min), DTX-1 (9.7 min), DTX-2(8.7 min), 13,19-didesmethyl SPX C (4 min) and 13-desmethyl SPX C (5.6 min). For the calibration curve, eight different concentrations of the standard (Laboratorios Cifga, Spain) were injected in duplicate: OA/DTX-1/DTX-2 from 1 ng/mL to 200 ng/mL; SPX from 0.5 ng/mL to 200 ng/mL (Figure 2.20). All toxins were quantified, using their peak areas to calculate amounts and using the curve obtained from each standard. The LOD and LOQ of the LC-ESI-CID-MS/MS for each toxin were: OA/DTX-1/DTX-2 (4/10 ng/mL) and SPX (0.1/0.5 ng/mL). Due to technical reasons, the LC-MS/MS device was not available, so the analyses for DSP's proceeded in a UPLC-MS/MS (50 samples of 55).

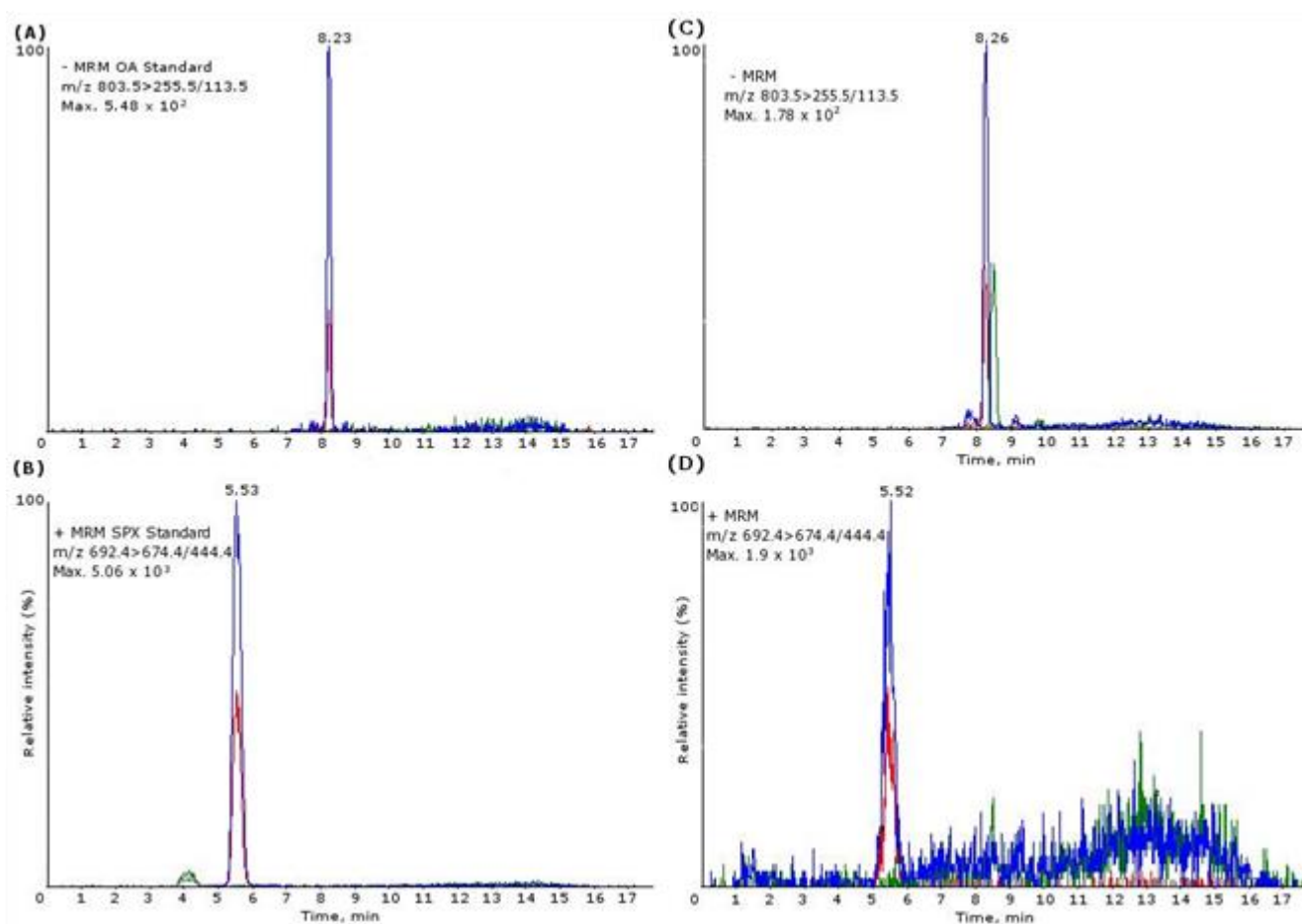


Figure 2.20 -Mass chromatograms of the LC-ESI-CID-MS/MS obtained under multiplexed reaction monitoring (MRM): (A) extracted ion chromatogram (XIC) of OA standard (200 ng/mL), m/z 803.5 > 255.5/113.5; (B) XIC of 13-desmethyl SPX C standard (200 ng/mL), m/z 692.4 > 674.4/444.4; (C) XIC of a positive sample for OA in *N. lapillus* (m/z 803.5 > 255.5/113.5); (D) XIC of a positive sample for 13-desmethyl SPX C in *N. lapillus* (m/z 692.4 > 674.4/444.4).

3.3.3. Lipophilic Toxin UPLC-MS/MS Conditions

Samples were analyzed according to Otero *et al.* (2011) [70] with the ultra-high performance liquid chromatography equipment, ACQUITY UPLC system, coupled to a Xevo TQ MS mass spectrometer from Waters (Manchester, UK). The apparatus was equipped with a multimode source ESI/APCI/ESCI, a vacuum system with two air-cooled Edwards Vacuum turbo molecular pumps and one Varian rotary backing pump. The nitrogen generator was a Nitrocraft NCLC/MS from Air Liquide (Madrid, Spain). Chromatographic separation and detection of OA and its derivatives was performed with a Waters Acquity UPLC BEH C₁₈ column (100 mm × 2.1, 1.7 μm) with an in-line 0.2 μm Acquity UPLC filter. The

column oven was set at 30 °C. Eluent A consisted of water, and eluent B contained acetonitrile (Panreac Quimica, Spain) and water (95:5). Both eluents contained 2 mM ammonium formate (Sigma Aldrich, Spain) and 50 mM formic acid (Merck, Spain). The gradient program used to elute the toxins started with 30% mobile phase B for 3 min, increasing to 90% B over 1.5 min, then kept stable for 1 min and reducing to 30% of B over the next 0.1 min and finally kept for 2 min before the next injection. The flow rate was 0.4 mL/min, and the injection volume was 5 µL. The Xevo TQ MS mass spectrometer operated with the following optimized source-dependent parameters (ESI source): capillary potential 2.5 kV, cone voltage 20 V, desolvation temperature 350 °C, desolvation gas flow 850 L/h N₂, cone gas flow 50 L/h N₂, source temperature 120 °C and collision gas flow 20 V. Argon was used as the collision gas at 4.5 × 10⁻³ mbar. The mass spectrometer operated in MRM, detecting in negative mode, analyzing two product ions per compound, one for quantification another for confirmation. The transitions employed were the same used in the LC-MS/MS device; retention times were 2.91 min for OA, 3.08 min for DTX-2 and 3.52 min for DTX-1. For the calibration curve, several dilutions of the standards (Laboratorios Cifga) from 1 ng/mL to 200 ng/mL were set (Figure 2.21). OA and its derivatives were quantified using their peak areas to calculate amounts and using the curve obtained from each standard. The LOD and LOQ of the method were: 3.7/6.4 ng/mL (OA), 6/10 ng/mL (DTX-1) and 1.6/5.4 ng/mL (DTX-2).

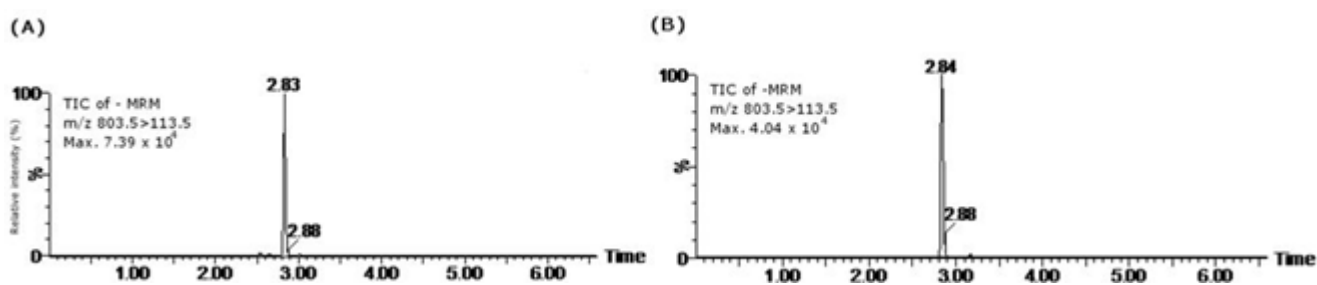


Figure 2.21 - Mass chromatograms of the UPLC-MS/MS obtained under MRM operation: (A) total ion chromatogram (TIC) of OA standard 200 ng/mL, m/z 803.5 > 255.5/113.5; (B) TIC of a positive sample for OA in *M. glacialis*, m/z 803.5 > 255.5/113.5

3.4. Statistical Analyses

Analyses were performed using R (version 2.14.1) software. In order to test for differences in toxin content for each group of toxins, a general linear mixed model was fitted using toxin concentration as the dependent variable and species and sampling site as additive fixed factors. Seasonal patterns were visually observed in our data distribution (see results), but because the selected dates did not represent the whole possible seasonal effects, the sampling date was included in the model as a random factor. For those fixed factors that showed significant differences, pairwise comparisons between levels were made with the *post hoc* Tukey test. Normality was tested on model residuals with the Shapiro test. When needed, data were normalized by transformation with the Box-cox function. In order to test for differences in toxin content for all toxins together, MANOVA tests were performed using toxin concentration as the dependent variable. Species and sampling site were analyzed as fixed factors in separate models. Hotelling tests were performed for pairwise comparisons between the levels of factors

that showed significant differences. Data were tested for normality with a multivariate Shapiro test and normalized with the Box-cox function when needed [71]. Some of the datasets analyzed here contain unbalanced numbers of samples per factor level (see Table 5). This might compromise the accuracy of linear models with complicated factor structures and should be taken into account when interpreting the significance values reported in the tables.

Conclusions

In this work, we surveyed the Atlantic continental coast of Portugal for unconventional vectors of three groups of biotoxins—PSTs, OA and SPX. Using HPLC-FLD, LC-MS/MS and UPLC-MS/MS techniques, we were able to detect SPX for the first time on the Portuguese coast and also new vectors for this group of biotoxins. We report 16 new vectors for these toxins in the North Atlantic. The values obtained for some species, such as starfish, *M. glacialis*, and the gastropod, *N. lapillus*, suggest that toxin transfer along the food chain probably occurred via mussels. There was no significant evidence of geographical patterns in terms of toxin content among the selected species. However, these differences might be found with a stronger sampling effort. The detection of new vectors, particularly those that are potentially used as food resources, suggests that monitoring of marine toxins should be extended to species other than bivalves in order to limit human health risks. The same could be considered for regulated toxins limits, which are usually calculated for edible bivalves, since our study showed that these toxins are bioaccumulated in upper levels of the food chain. We hope that this work contributes towards the establishment of new legislation, especially for cyclic imines.

Acknowledgments

This work was partially funded by the Project MARBIOTECH (reference NORTE-07-0124-FEDER-000047) within the SR & TD Integrated Program MARVALOR—Building research and innovation capacity for improved management and valorization of marine resources, supported by the Programa Operacional Regional do Norte (ON.2—O Novo Norte) and by the European Regional Development Fund, by the INTERREG IV projects Atlantox, Pharmatlantic, FCT project Pest-C/MAR/LA0015/2011, UP through IJUP projects and FEDER cofunded-grant: From Ministerio de Ciencia y Tecnología, Spain: AGL2012-40485-CO2-01. MS acknowledges FCT, FSE and MCTES for co-funding SFRH/BD/73269/2010, Vitor Ramos, João Paulo Machado and Jonathan Wilson.

References

1. Hallegraef, G.M. Algal blooms are not a simple toxic broth. *Search* **1993**, *24*, 179.

2. Glibert, P.M.; Anderson, D.; Gentien, P.; Granéli, E.; Sellner, K. The global, complex phenomena of harmful algal blooms. *Oceanography***2005**, *18*, 136–147.
3. Kat, M. The Occurrence of *Prorocentrum* Species and Coincidental Gastrointestinal Illness of Mussel Consumers. In *Toxic Dinoflagellate Blooms*; Taylor, D.L., Seliger, H.H., Eds.; Elsevier: New York, NY, USA, 1979; pp. 215–220.
4. Rodrigues, S.M.; de Carvalho, M.; Mestre, T.; Ferreira, J.J.; Coelho, M.; Peralta, R.; Vale, P. Paralytic shellfish poisoning due to ingestion of *Gymnodinium catenatum* contaminated cockles—Application of the AOAC HPLC official method. *Toxicon***2012**, *59*, 558–566.
5. Meyer, K.F.; Sommer, H.; Schoenholz, P. Mussel poisoning. *Am. J. Prev. Med.***1928**, *2*, 365–394.
6. Steward, I.; Seawright, A.A.; Shaw, G.R. Cyanobacterial Poisoning in Livestock, Wild Mammals and Birds—An Overview. In *Cyanobacterial Harmful Algal Blooms: State of Science and Research Needs*; Hudnell, H.K., Ed.; Springer: New York, NY, USA, 2008; pp. 613–637.
7. Vale, P.; Antonia, M.; Sampayo, M. Esters of okadaic acid and dinophysistoxin-2 in Portuguese bivalves related to human poisonings. *Toxicon***1999**, *37*, 1109–1121.
8. Gerssen, A.; Pol-Hofstad, I.E.; Poelman, M.; Mulder, P.P.; van den Top, H.J.; de Boer, J. Marine toxins: Chemistry, toxicity, occurrence and detection, with special reference to the Dutch situation. *Toxins***2010**, *2*, 878–904.
9. Deeds, J.R.; Landsberg, J.H.; Etheridge, S.M.; Pitcher, G.C.; Longan, S.W. Non-traditional vectors for paralytic shellfish poisoning. *Mar. Drugs***2008**, *6*, 308–348.
10. Gainey, L.S.J.; Shumway, S. A compendium of the responses of bivalve molluscs to toxic dinoflagellates. *J. Shellfish Res.***1988**, *7*, 623–628.
11. Shumway, S.E. Phycotoxin-related shellfish poisoning: Bivalve molluscs are not the only vectors. *Rev. Fish. Sci.* **1995**, *3*, 1–31.
12. Correia, F.S. Um caso raro de intoxicação alimentar colectiva. *Bol. Inst. Super. Hig. Doutor Ricardo Jorge* **1946**, *3*, 216–221.

13. Vale, P.; Botelho, M.J.; Rodrigues, S.M.; Gomes, S.S.; Sampayo, M.A.D. Two decades of marine biotoxin monitoring in bivalves from Portugal (1986–2006): A review of exposure assessment.
14. *Harmful Algae* **2008**, 7, 11–25.
15. Alexander, J.; Benford, D.; Cockburn, A.; Cravedi, J.P.; Dogliotti, E.; Di Domenico, A.; Fernández-Cruz, M.L.; Fink-Gremmels, J.; Fürst, P.; Galli, C.; *et al.* Scientific opinion of the panel on contaminants in the food chain on a request from the European commission on marine biotoxins in shellfish—Saxitoxin Group. *EFSA J.* **2009**, 1019, 1–76.
16. Codd, G.A. Cyanobacterial toxins: Occurrence, properties and biological significance. *Water Sci. Technol.* **1995**, 32, 149–156.
17. Clemente, Z.; Busato, R.H.; Oliveira Ribeiro, C.A.; Cestari, M.M.; Ramsdorf, W.A.; Magalhaes, V.F.; Wosiack, A.C.; Silva de Assis, H.C. Analyses of paralytic shellfish toxins and biomarkers in a southern Brazilian reservoir. *Toxicon* **2010**, 55, 396–406.
18. Carmichael, W.W.; Evans, W.R.; Yin, Q.Q.; Bell, P.; Moczydlowski, E. Evidence for paralytic shellfish poisons in the freshwater cyanobacterium *Lyngbya wollei* (Farlow ex Gomont) comb. nov.
19. *Appl. Environ. Microbiol.* **1997**, 63, 3104–3110.
20. Ballot, A.; Fastner, J.; Wiedner, C. Paralytic shellfish poisoning toxin-producing cyanobacterium *Aphanizomenon gracile* in northeast Germany. *Appl. Environ. Microbiol.* **2010**, 76, 1173–1180.
21. Sivonen, K.; Jones, G. Cyanobacterial Toxins. In *Toxin Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management*, Chorus, I., Bartram, J., Eds.; WHO E& FN Spon: London, UK, 1999; pp. 41–111.
22. European Commission. Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Available online: <http://eur-lex.europa.eu> (27 December 2010).
23. Mackinnon, S.L.; Cembella, A.D.; Burton, I.W.; Lewis, N.; LeBlanc, P.; Walter, J.A. Biosynthesis of 13-desmethyl spirolid C by the dinoflagellate *Alexandrium ostenfeldii*. *J. Org. Chem.* **2006**, 71, 8724–8731.

24. MacKinnon, S.L.; Walter, J.A.; Quilliam, M.A.; Cembella, A.D.; LeBlanc, P.; Burton, I.W.; Hardstaff, W.R.; Lewis, N.I. Spirolides isolated of the danish strains of the toxigenic dinoflagellate *Alexandrium ostenfeldii*. *J. Nat. Prod.***2006**, *69*, 983–987.
25. Munday, R. Toxicology of Cyclic Imines: Gymnodimine, Spirolides, Pinnatoxins, Pteriatoxins, Prorocentrolide, Spiro-Prorocentrimine, and Symbioimines. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, 2nd ed.; Botana, L.M., Ed.; CRC Press (Taylor andFrancys Group): Boca Raton, FL, USA, 2008; pp. 581–594.
26. Rhodes, L.; Smith, K.; Selwood, A.; McNabb, P.; van Ginkel, R.; Holland, P.; Munday, R. Production of pinnatoxins by a peridinoïd dinoflagellate isolated from Northland, New Zealand. *Harmful Algae* **2010**, *9*, 384–389.
27. Hu, T.; Curtis, J.M.; Walter, J.A.; Wright, J.L.C. Characterization of biologically inactive spirolides E and F: Identification of the spirolide pharmacophore. *Tetrahedron Lett.***1996**, *37*, 7671–7674.
28. Hu, T.; Curtis, J.M.; Oshima, Y.; Quilliam, M.A.; Walter, J.A.; Watson-Wright, W.M.; Wright, J.L.C. Spirolides B and D, two novel macrocycles isolated from the digestive glands of shellfish. *J. Chem. Soc. Chem. Commun.***1995**, *20*, 2159–2161.
29. Hu, T.; Burton, I.W.; Cembella, A.D.; Curtis, J.M.; Quilliam, M.A.; Walter, J.A.; Wright, J.L. Characterization of spirolides a, c, and 13-desmethyl c, new marine toxins isolated from toxic plankton and contaminated shellfish. *J. Nat. Prod.***2001**, *64*, 308–312.
30. John, U.; Cembella, A.D.; Hummert, C.; Elbrächter, M.; Groben, R.; Medlin, L.K. Discrimination of the toxigenic dinoflagellate species *Alexandrium tamarense* and *Alexandrium ostenfeldii* in co-occurring natural populations from Scottish coastal waters. *Eur. J. Phycol.***2003**, *38*, 25–40.
31. Anderson, D.M.; Kulis, D.M.; Keafer, B.A.; Gribble, K.E.; Marin, R.; Scholin, C.A. Identification and enumeration of *Alexandrium* spp. from the Gulf of Maine using molecular probes. *Deep SeaRes. II* **2005**, *54*, 2467–2490.
32. Aasen, J.A.B.; MacKinnon, S.L.; LeBlanc, P.; Walter, J.A.; Hovgaard, P.; Aune, T.; Quilliam, M.A. Detection and identification of spirolides in Norwegian shellfish and plankton. *Chem. Res.Toxicol.* **2005**, *18*, 509–515.

33. Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Magno, S.; Tartaglione, L.; Cangini, M.; Pompei, M.; Guerrini, F.; Boni, L.; Pistocchi, R. Toxin profile of *Alexandrium ostenfeldii* (Dinophyceae) from the Northern Adriatic Sea revealed by liquid chromatography-mass spectrometry. *Toxicon***2006**, *47*, 597–604.
34. Amzil, Z.; Sibat, M.; Royer, F.; Masson, N.; Abadie, E. Report on the first detection of pectenotoxin-2, spirolide a and their derivatives in French shellfish. *Mar. Drugs***2007**, *5*, 168–179.
35. Villar Gonzalez, A.; Rodriguez-Velasco, M.L.; Ben-Gigirey, B.; Botana, L.M. First evidence of spirolides in Spanish shellfish. *Toxicon***2010**, *48*, 1068–1074.
36. Álvarez, G.; Uribe, E.; Ávalos, P.; Mariño, C.; Blanco, J. First identification of azaspiracid and spirolides in *Mesodesma donacium* and *Mulinia edulis* from Northern Chile. *Toxicon***2010**, *55*, 638–641.
37. Seki, T.; Satake, M.; Mackenzie, A.L.; Kaspar, H.F.; Yasumoto, T. Gimnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium* sp. *Tetrahedron Lett.* **1995**, *36*, 7093–7096.
38. Touzet, N.; Franco, J.M.; Raine, R. Morphogenetic diversity and biotoxin composition of *Alexandrium* (Dinophyceae) in Irish coastal waters. *Harmful Algae* **2008**, *7*, 782–797.
39. Otero, P.; Alfonso, A.; Alfonso, C.; Araoz, R.; Molgo, J.; Vieytes, M.R.; Botana, L.M. First direct fluorescence polarization assay for the detection and quantification of spirolides in mussel samples. *Anal. Chim. Acta***2011**, *701*, 200–208.
40. Alexander, J.; Benford, D.; Boobis, A.; Ceccatelli, S.; Cravedi, J.P.; di Domenico, A.; Doerge, D.; Dogliotti, E.; Edler, L.; Farmer, P.; *et al.* Scientific Opinion on marine biotoxins in shellfish—Cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins). *EFSA J.***2010**, *8*, 1628–1887.
41. Bialojan, C.; Takai, A. Inhibitory effect of a marine sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.***1988**, *256*, 283–290.
42. Maneiro, E.; Rodas, V.L.; Costas, E.; Hernandez, J.M. Shellfish consumption: A major risk factor for colorectal cancer. *Med. Hypotheses***2008**, *70*, 409–412.

43. Elgarch, A.; Vale, P.; Rifai, S.; Fassouane, A. Detection of diarrhetic shellfish poisoning and azaspiracid toxins in moroccan mussels: Comparison of the LC-MS method with the commercial immunoassay kit. *Mar. Drugs***2008**, *6*, 587–594.
44. Garcia, C.; Truan, D.; Lagos, M.; Santelices, J.P.; Diaz, J.C.; Lagos, N. Metabolic transformation of dinophysistoxin-3 into dinophysistoxin-1 causes human intoxication by consumption of O-acyl-derivatives dinophysistoxins contaminated shellfish. *J. Toxicol. Sci.* **2005**, *30*, 287–296.
45. Scoging, A.; Bahl, M. Diarrhetic shellfish poisoning in the UK. *Lancet***1998**, *352*, 117.
46. Yasumoto, T.; Oshima, Y.; Yamaguchi, M. Occurrence of a new type of shellfish poisoning in tohoku district. *Bull. Jpn. Soc. Sci. Fish.***1978**, *44*, 1249–1255.
47. MacKenzie, L.; Beuzenberg, V.; Holland, P.; McNabb, P.; Suzuki, T.; Selwood, A. Pectenotoxin and okadaic acid-based toxin profiles in *Dinophysis acuta* and *Dinophysis acuminata* from New Zealand. *Harmful Algae***2005**, *4*, 75–85.
48. Hu, T.M.; Curtis, J.M.; Walter, J.A.; Wright, J.L.C. Identification of DTX-4, a new water-soluble phosphatase inhibitor from the toxic dinoflagellate *Prorocentrum lima*. *J. Chem. Soc. Chem. Comm.* **1995**, *5*, 597–599.
49. Caroppo, C.; Congestri, R.; Bruno, M. On the presence of *Phalacrocoma rotundatum* in the southern Adriatic Sea (Italy). *Aquat. Microb. Ecol.***1999**, *17*, 301–310.
50. Jorgensen, K.; Andersen, P. Relation between the concentration of *Dinophysis acuminata* and diarrhetic shellfish poisoning toxins in blue mussels (*Mytilus edulis*) during a toxic episode in the Limfjord (Denmark), 2006. *J. Shellfish Res.***2007**, *26*, 1081–1087.
51. Spatharis, S.; Dolapsakis, N.P.; Economou-Amilli, A.; Tsirtsis, G.; Danielidis, D.B. Dynamics of potentially harmful microalgae in a confined Mediterranean Gulf—Assessing the risk of bloom formation. *Harmful Algae***2009**, *8*, 736–743.
52. Crothers, J.H. Common topshells: An introduction to the biology of *Osilinus lineatus* with notes on other species in the genus. *Field Stud.***2001**, *10*, 115–160.
53. Knox, G.A. Hard Shores. In *The Ecology of Seashores*; Kennish, M.J., Ed.; CRC Press: Boca Raton, FL, USA, 2001; pp. 20–86.

54. Lemée, R.; Boudouresque, C.F.; Gobert, J.; Malestroit, P.; Mari, X.; Meinesz, A.; Menager, V.; Ruitton, S. Feeding behaviour of *Paracentrotus lividus* in the presence of *Caulerpa taxifolia* introduced in the Mediterranean Sea. *Oceanol. Acta* **1995**, *19*, 245–253.
55. Forster, G.R. The ecology of *Echinus esculentus* L. Quantitative distribution and rate of feeding. *J. Mar. Biol. Assoc. UK* **1959**, *38*, 361–367.
56. Carefoot, T.H. Aplysia: Its biology and ecology. *Oceanogr. Mar. Biol. Annu. Rev.* **1987**, *25*, 167–284.
57. Lin, S.J.; Hwang, D.F. Possible source of tetrodotoxin in the starfish *Astropecten scoparius*. *Toxicon* **2001**, *39*, 573–579.
58. Vale, P. Hydrolysis of hydroxybenzoate saxitoxin analogues originating from *Gymnodiumcatenatum*. *Food Chem.* **2011**, *125*, 1160–1165.
59. Ogata, T.; Kodama, M.; Fukuyo, Y.; Inoue, T.; Kamiya, H.; Matsuura, F.; Sekiguchi, K.; Watanabe, S. Studies on paralytic shellfish poisoning in ofunato bay. 1. The occurrence of protogonyaulax spp in Ofunato Bay, in association with the toxification of the scallop *Patinopecten yessoensis*. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48*, 563–566.
60. Montojo, U.M.; Sakamoto, S.; Cayme, M.F.; Gatdula, N.C.; Furio, E.F.; Relox, J.R.; Sato, S.; Fukuyo, Y.; Kodama, M. Remarkable difference in accumulation of paralytic shellfish poisoning toxins among bivalve species exposed to *Pyrodinium bahamense* var. compressum bloom in Masinloc bay, Philippines. *Toxicon* **2006**, *48*, 85–92.
61. Sekiguchi, K.; Sato, S.; Kaga, S.; Ogata, T.; Kodama, M. Accumulation of paralytic shellfish poisoning toxins in bivalves and an ascidian fed on *Alexandrium tamarense* cells. *Fish. Sci.* **2001**, *67*, 301–305.
62. Asakawa, M.; Takagi, M.; Iida, A.; Oishi, K. Studies on the conversion of paralytic shellfish poison (Psp) components by biochemical reducing agents. *Eisei Kagaku* **1987**, *33*, 50–55.
63. Oshima, Y. Chemical and Enzymatic Transformation of Paralytic Shellfish Toxins in Marine Organisms. In *Harmful Marine Algal Blooms*; Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou, C., Eds.; Lavoisier Intercept Ltd.: Paris, France, 1995; p. 475.
64. Noguchi, T. Marine toxins. *Nippon Suisan Gakkaishi* **2003**, *69*, 895–909.

65. Nagashima, Y.; Arakawa, O.; Shiomi, K.; Noguchi, T. Paralytic Shellfish Toxins in a Trumpet Shell. *Charonia lampas* from Spain. In *Harmful and Toxic Algal Blooms*; Yasumoto, T., Oshima, Y., Fukuyo, Y., Eds.; Intergovernmental Oceanic Commission of UNESCO: Sendai, Japan, 1996; pp. 425–427.
66. Asakawa, M.; Nishimura, F.; Miyazawa, K.; Noguchi, T. Occurrence of paralytic shellfish poison in the starfish *Asterias amurensis* in Kure Bay, Hiroshima Prefecture, Japan. *Toxicon* **1997**, *35*, 1081–1087.
67. Lin, S.J.; Tsai, Y.H.; Lin, H.P.; Hwang, D.F. Paralytic toxins in Taiwanese starfish *Astropectenscoparius*. *Toxicon* **1998**, *36*, 799–803.
68. Brusca, R.C.; Brusca, G.J. *Invertebrates*, 2nd ed.; Sinauer Associates, Inc.: Sunderland, MA, USA, 2002.
69. Lawrence, J.F.; Niedzwiadek, B.; Menard, C. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: Collaborative study. *J. AOAC Int.* **2005**, *88*, 1714–1732.
70. Otero, P.; Alfonso, A.; Alfonso, C.; Vieytes, M.R.; Louzao, M.C.; Botana, A.M.; Botana, L.M. New protocol to obtain spirolides from *Alexandrium ostenfeldii* cultures with high recovery and purity. *Biomed. Chromatogr.* **2010**, *24*, 878–886.
71. Rodriguez, P.; Alfonso, A.; Botana, A.M.; Vieytes, M.R.; Botana, L.M. Comparative analysis of pre- and post-column oxidation methods for detection of paralytic shellfish toxins. *Toxicon* **2010**, *56*, 448–457.
72. Otero, P.; Alfonso, A.; Alfonso, C.; Rodriguez, P.; Vieytes, M.R.; Botana, L.M. Effect of uncontrolled factors in a validated liquid chromatography-tandem mass spectrometry method question its use as a reference method for marine toxins: Major causes for concern. *Anal. Chem.* **2011**, *83*, 5903–5911.
73. Guisande, C.G.; Vaamonde, A.L.; Barreiro, A. *Tratamiento de datos con R, Statistica y SPSS*; Díaz de Santos: Madrid, Spain, 2011; p. 978.

New Invertebrate Vectors of Okadaic Acid from the North Atlantic Waters - Portugal (Azores and Madeira) and Morocco

Marisa Silva^{1,2}, **Inés Rodríguez**³, **Aldo Barreiro**^{1,2}, **Manfred Kaufmann**^{2,4,6}, **Ana Isabel Neto**^{2,5}, **Meryem Hassouani**⁷, **Brahim Sabour**⁷, **Amparo Alfonso**³, **Luis M. Botana**³, **Vitor Vasconcelos**^{1,2*}.

- ¹ Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, Porto 4619-007, Portugal; E-Mails: marisasilva17@gmail.com (M.S.); aldo.barreiro@gmail.com (A.B.)
- ² Interdisciplinary Center of Marine and Environmental Research–CIMAR/CIIMAR, University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal
- ³ Department of Pharmacology, Faculty of Veterinary, University of Santiago of Compostela, 27002 Lugo, Spain; E-Mails: ines.rodriguez.filgueiras@usc.es (I.R.); amparo.alfonso@usc.es (A.A.); Luis.Botana@usc.es (L.M.B.)
- ⁴ University of Madeira, Marine Biology Station of Funchal, 9000-107 Funchal, Madeira Island, Portugal; E-Mails: mkaufmann@ciimar.up.pt(M.K.)
- ⁵ Department of Marine Biology, University of Azores, 9501-801 Ponta Delgada, Azores, Portugal; E-Mails: aneto@uac.pt (A.I.N.)
- ⁶ Center of Interdisciplinary Marine and Environmental Research of Madeira - CIIMAR-Madeira, Edif. Madeira Tecnopolo, Caminho da Penteada, 9020 – 105 Funchal, Madeira, Portugal
- ⁷Phycology Research Unit – Biotechnology, Ecosystems Ecology and Valorization Laboratory. Faculty of Sciences El Jadida, University Chouaib Doukkali, BP20 El Jadida, Morocco; E-Mails: hassouani@hotmail.com (M.H.); sabour.b@ucd.ac.ma (B.S.)

Abstract: Okadaic acid and its analogues are potent neurotoxic polyethers that cause the diarrhetic shellfish poisoning syndrome through the ingestion of contaminated shellfish by humans. This group of toxins is worldwide disseminated but the frequency of poisoning incidents has declined in the last 20 years due to legislation and monitoring programs implemented for bivalves. During the summers of 2012 and 2013 we collected a total of 101 samples of 23 different species of benthic and subtidal organisms such echinoderms, crustaceans, bivalves and gastropods from the Madeira Island, São Miguel Island (Azores archipelago) and the Moroccan northwestern coast. Samples were analyzed by UPLC-MS/MS. Our main objective was to detect new vectors for these biotoxins. We report 9 new vectors for these toxins in the North Atlantic: *Astropecten aranciacus*, *Arbacia lixula*, *Echinaster sepositus*, *Holothuria sanctori*, *Ophidiaster ophidianus*, *Onchidella celtica*, *Aplysia depilans*, *Patella* spp., *Stramonita haemostoma*. There were differences in toxin

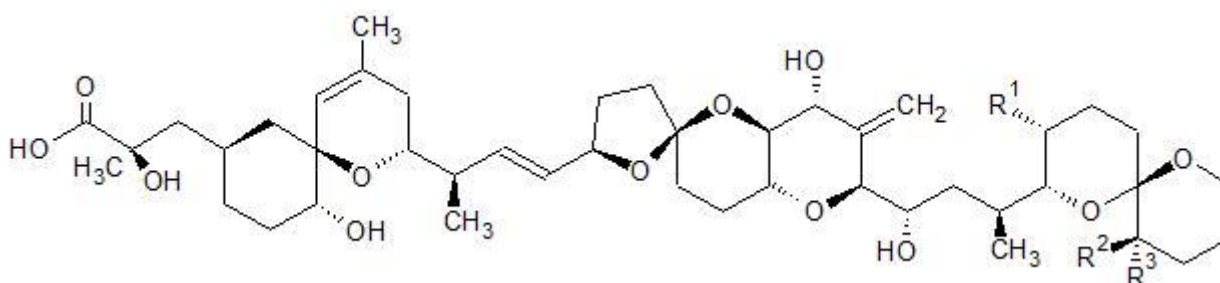
contents among species. Despite the low concentrations detected, the levels of toxins found in especial in edible species indicate the importance of this kind of studies. Monitoring routines should be extended to comprise a wider number of vectors other than bivalves for of okadaic acid and its analogues.

Keywords: Okadaic Acid; New vectors; Madeira Island; São Miguel Island; Morocco.

Introduction

Diarrhetic shellfish poisoning (DSP) is a syndrome caused by the ingestion of organisms contaminated with the neurotoxic polyether group of Okadaic acid (OA) and its analogs, dynophysistoxin 1 and 2 (DTX1, DTX2) (Figure 2.22). These diarrhetic shellfish toxins (DST) were first isolated from two sponge species: *Halichondria okadae* and *H. melanodocia* [1,2], and are mainly produced by dinoflagellates of the genera *Dynophysis*, *Phalacroma* and *Prorocentrum* [3-5]. Regarding the mechanism of action, these toxins are strong inhibitors of serine/threonine phosphatases, especially type 1 and 2A with particular high affinity to 2A [6,7]. This inhibition results in the increase of the phosphorylation of a number of proteins leading to significant cell alterations, being OA and DTX1 also reported as tumor promoters [8].

Albeit being first reported in Japan, poisoning incidents occur all over the world, and the most common route of intoxication is via ingestion of contaminated fish and shellfish [9-12]. These phycotoxins are heat and frost resistant, and taste and odor of the contaminated organisms remains unchanged [13-15]. DSP is known for its serious gastrointestinal symptoms, from chills to diarrhea, and the severity of the intoxication depends on the amount of toxin that the patient was exposed to [16].



Toxin	R ¹	R ²	R ³
OA	CH ₃	H	H
DTX1	CH ₃	CH ₃	H
DTX2	H	H	CH ₃

Figure 2.22 - Chemical structures of OA, DTX1&2.

Given their global incidence, DSTs are regulated worldwide and mice studies together with epidemiologic ones led to the establishment of the Lowest Observed Effect Level (LOEL) of 50 µg OA equivalents/person [17]. Currently the toxic equivalent factors (TEF) have been established, (Table 2.11), and the limit value for the European Union is 160 µg OA equivalents/ kg shellfish meat (SM) [17,18]. Regarding detection methods, the European Food Safety Agency (EFSA) recommends the use of analytical techniques such as LC-MS/MS [17]. Also, latter in 2011, this recommendation was reinforced by the European Commission (EC), suggesting that this technique should be established as reference method for DST detection until 31 December 2014 [19]. Analytical procedures avoid ethical issues comparatively to the use of mice, also they are able to identify several toxins in a mixture with high degree of sensitivity.

Table 2.11 - Toxic equivalent factors for OA and its analogs.

Toxin	TEF	Ref
OA	1	[17]
DTX1	1	[17]
DTX2	0,6	[17]

Prior investigation demonstrated that DSTs can be found in unusual vectors along the food-chain, considering that monitoring for this group of toxins exclusively in bivalves is simplistic and underestimates the risk to public health [20]. This argument is reinforced by the evidence that OA is bioaccumulated through the food-web [20]. In this work we surveyed the Portuguese islands of Madeira (Madeira archipelago) and São Miguel (Azores archipelago), and the northwestern Moroccan coast for new vectors, through intertidal and SCUBA diving harvesting. We collected 23 species of benthic organisms including gastropods (sea-snails, sea-slugs and limpets), bivalves (mussels), crustaceans (barnacles) and echinoderms (starfishes, sea-urchins and sea-cucumbers). The decision of the selection of these species is related to the fact that most of them are edible and are commercially important species (Table 2.12).). Inedible species were also sampled for their importance in the food chain. We believe that our data contribute to the development and updating of legislation regarding the monitoring procedures of these toxins in order to better protect public health.

Table2.12 - Species sampled and their trophic level, average number of specimens comprising a pooled sample (AvNr) and number of samples collected (NrPSamples) on Madeira Island in September 2012, São Miguel Island, Azores, in June 2013 and Morocco in July 2013,

Species	Trophic level	Sampling Site(s)	Nr P Samples	AvNr	Edible	M. Status	Ref
<i>Astropecten aranciacus</i>	2 nd level predator	Madeira	1	2	No	No	[21]
<i>Aplysia depilans</i>	Grazer	Morocco	3	1	No	No	[22]
<i>Arbacia lixula</i>	Grazer	Madeira/Azores/ Morocco	9	4	No	No	[23]
<i>Charonia lampas</i>	3 rd level predator	Madeira/Morocco	3	1	Yes	No	[24]
<i>Cerithium vulgatum</i>	Grazer	Morocco	1	40	Yes	No	[25]
<i>Diadema africanum</i>	Grazer	Madeira	2	1	No	No	[26]
<i>Echinaster sepositus</i>	2 nd level predator	Madeira	1	3	No	No	[27]
<i>Gibbula umbilicalis</i>	Grazer	Morocco	3	100	Yes	No	[28]
<i>Holothuria(Platyrhina)sanctori</i>	Deposit feeder	Morocco	4	1	Yes	No	[29,30]
<i>Marthasterias glacialis</i>	2 nd level predator	Madeira/Azores/ Morocco	8	1	No	No	[31]
<i>Monodonta lineata</i>	Grazer	Morocco	5	86	Yes	No	[28]
<i>Mytilus</i> spp.	Filter feeder	Morocco	4	30	Yes	Yes	[32]
<i>Onchidella celtica</i>	Grazer	Morocco	1	50	No	No	[33]
<i>Ophidiaster ophidianus</i>	Detritivorous	Madeira/Azores	5	1	No	No	[27]
<i>Pattela aspera</i>	Grazer	Madeira	2	15	Yes	No	[31]
<i>Patella</i> spp.	Grazer	Morocco	4	12	Yes	No	[31]
<i>Pattela candei</i>	Grazer	Azores	3	10	Yes	No	[31]
<i>Paracentrotus lividus</i>	Grazer	Madeira/Azores/ Morocco	7	1	Yes	No	[34]
<i>Pollicipes pollicipes</i>	Filter feeder	Morocco	3	35	Yes	No	[31]
<i>Sphaerechinus granularis</i>	Grazer	Azores	4	1	Yes	No	[35]
<i>Umbraculum umbraculum</i>	Grazer	Madeira	1	1	No	No	[36]
<i>Stramonita haemostoma</i>	2 nd level predator	Madeira/Azores/ Morocco	5	15	No	No	[37]

Results and Discussion

In this work, a total of 101 samples were collected from three different sampling sites: Madeira Island (25 samples) in September 2012, São Miguel Island, Azores (37 samples), in June 2013 and the northwestern Moroccan coast (39 samples) in July 2013 (Figure 2.23).

Several species belonging to distinct taxa were collected, comprising starfish (*A. aranciacus*, *E. sepositus*, *M. glacialis*, *O. ophidianus*), sea-urchins (*A. lixula*, *D. africanum*, *P. lividus*, *S. granularis*), sea-cucumber (*H. sanctori*), crustaceans (*P. pollicipes*), bivalves (*Mytilus* spp.) and gastropods (*A. depilans*, *C. vulgatum*, *C. lampas*, *G. umbilicalis*, *M. lineata*, *O. celtica*, *Patella* spp., *P. candeji*, *P. tenuistenuis*, *P. aspera*, *S. haemostoma*, *U. umbraculum*).

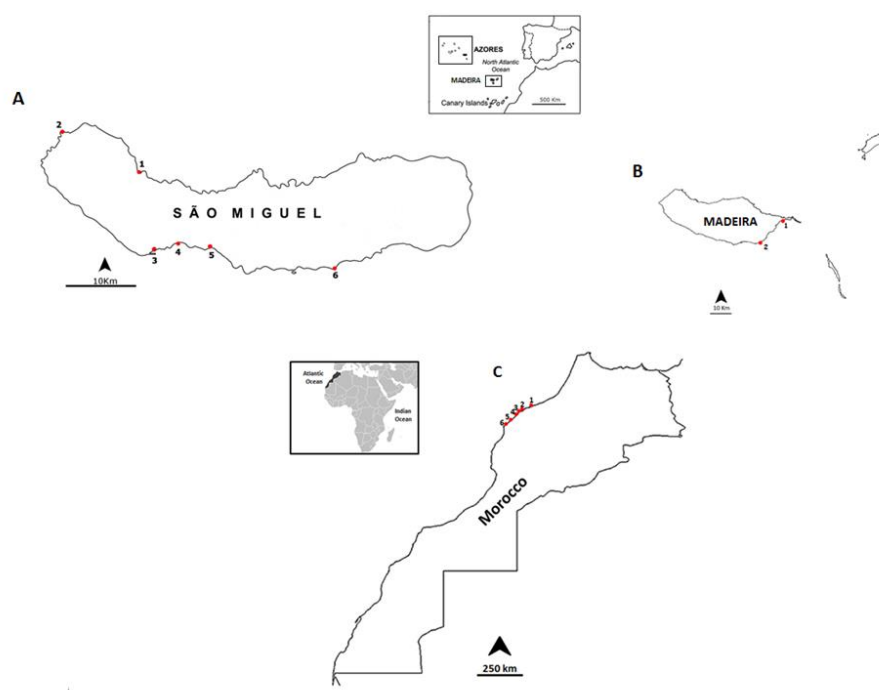


Figure 2.23 - Location of the sampling points: (A) São Miguel island coast, Azores archipelago: 1- Cruzeiro; 2- Mosteiros; 3- Étar; 4- São Roque; 5- Lagoa; 6- Caloura; (B) Madeira island coast: 1- Reis Magos; 2- Caniçal; (C) Northwestern Moroccan coast: 1- Casablanca Corniche; 2- El Jadida Haras; 3- El Jadida Sâada; 4- Sidi Bouzid; 5- Mrizika; 6- Qualidia.

OA and its analogues were screened in the three sampling sites, being a total of 19% of samples with quantifiable OA contents for OA (>LOQ), but neither DTX1, DTX2 were detected (Figure 2.24). DTX3 was also screened but was below the limit of detection of the equipment. The majority of them (73.7%), as well as the highest concentrations detected were in the Moroccan coast, followed by Madeira Island (21.1%). This might be due to the fact that both sampling sites are at the same latitude. For São Miguel Island (Azores) only one measurable sample was detected in the starfish *O. ophidianus*.

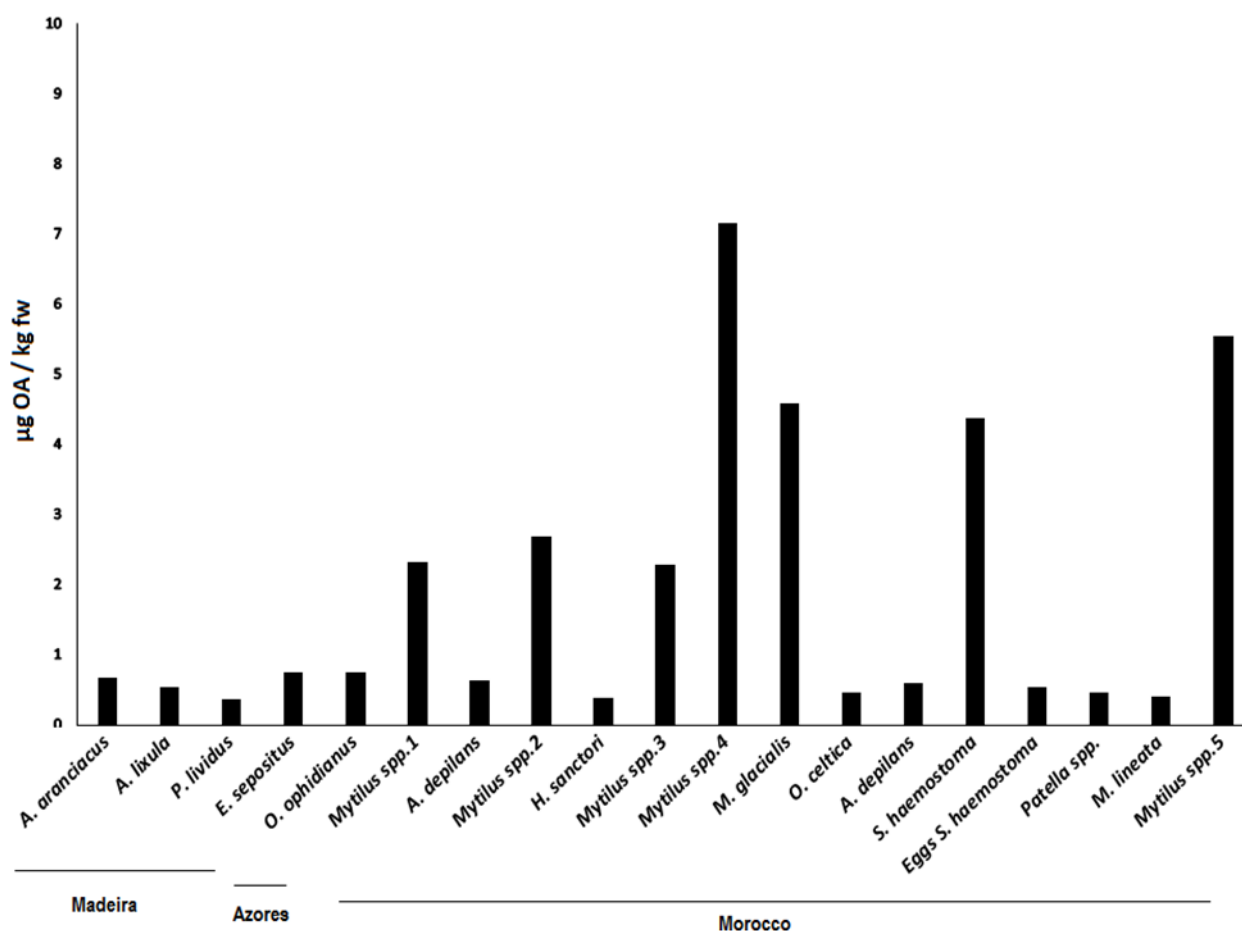


Figure 2.24 - OA ($\mu\text{g}/\text{Kg}$ fw) for all sampled groups of organisms in Azores, Madeira and Morocco. Numbers correspond to different sampling locations in Morocco: 1- Casablanca Corniche; 2- Sidi Bouzid; 3- El Jadida Saâda; 4- Mrizika; 5- Oualidia.

Average concentrations detected were all below the current limit implemented in the European legislation – $160 \mu\text{g}$ OA equivalents/ kg Shellfish meat [17], and ranged from $0.368 \mu\text{g}/\text{kg}$ fresh weigh (fw), in *P. lividus*, to $7.157 \mu\text{g}/\text{kg}$ fw in *Mytilus spp.*

Regarding statistics, the first step of the gamma hurdle model was a Generalized Linear Model (GLZ) performed with the data of presence/absence of OA, using Binomial distribution error.

This model was applied separately to each geographical location. The results of the model's analysis of deviance as well as the coefficients rescaled to a logistic probability [0,1] are shown in Table 2.13. "Organism" did not result to be a significant factor, probably due to the low number of samples with quantifiable OA contents, except in Morocco. In Morocco, the highest probability corresponded to the bivalve, whereas all other organisms had very low probabilities of containing OA. The second part of the model, with the gamma error distribution, analyzes the variation in OA concentration, among those samples showing quantifiable results. This analysis was not possible to be performed in Azores because there was one single sample containing OA. The factor "Organism" was significant both in Madeira and Morocco (Table 2.14). In Madeira, were OA appeared only in sea urchin and star fish, the latter contained in average, around three times more OA (1.58 versus 0.45 , Table 2.14). In Morocco,

the bivalves contained much more OA than all the other organisms, being the closest one the sea start, with in average approximately 1 forth of bivalve OA content (4 versus 1.15, Table 2.14).

Due to the oligotrophic waters of Madeira and Azores archipelagos bivalves are not common being gastropods and echinoderms a good alternative for the monitoring of OA and its derivatives in both archipelagos.

Table2.13 - Results of the binomial regression model for OA occurrence with “organism” as factor.

Analysis of deviance				
Location	factor	χ^2	df	p
Madeira	Organism	3.9	3	0.28
	Rescaled model coefficients: sea urchin = 0.16; star fish = 0.71; gastropod = 1.6×10^{-8} ; limpet = 1.6×10^{-8}			
Azores	Organism	2.1	3	0.56
	Rescaled model coefficients: sea urchin = 4.3×10^{-10} ; star fish = 1; gastropod = 0.5; limpet = 0.5			
Morocco	Organism	19.8	7	<0.01
	Rescaled model coefficients: bivalve = 1; crustacean = 1×10^{-17} ; sea urchin = 1×10^{-17} ; star fish = 3.2×10^{-9} ; gastropod = 1.1×10^{-9} ; limpet = 1.1×10^{-9} ; sea snail = 6.4×10^{-9} ; sea cucumber = 1.1×10^{-9}			

Table2.14 - Results of the gamma regression model for OA occurrence with organism as factor.

Analysis of deviance				
Location	factor	χ^2	df	p
Madeira	Organism	5.4	1	<0.05
	Rescaled model coefficients: sea urchin = 0.45; star fish = 1.58			
Morocco	Organism	12.8	5	<0.05
	Rescaled model coefficients: bivalve = 4; star fish = 1.15; gastropod = 0.44; limpet = 0.12; sea snail = 0.15; sea cucumber = 0.1			

We report 9 new vectors for OA in the species *A. aranciacus*, *A. lixula*, *A. depilans*, *E. sepositus*, *H. sanctori*, *O. celtica*, *O. ophidianus*, *Patella spp.*, *S. haemostoma*. Also, importantly we detected OA in the eggs of *S. haemostoma* suggesting that this toxin can be transferred to the offspring. These kind of

cases have already been reported, as an example in *Takifugo rubripes* larvae that are protected by maternal Tetrodotoxin [38].

Experimental Section

Selected Species and Sampling Sites

The coasts of the Portuguese islands Madeira (Madeira archipelago), São Miguel (Azores archipelago) and the northwestern coast of Morocco, where surveyed for non-traditional vector species for Okadaic Acid and its analogs. Several edible and non-edible species were selected (n=23), to search for potential new vectors and also the prevalence of the screened biotoxins in the food web: gastropods (*Patella tenuis tenuis*, *Patella aspera*, *Stramonita haemostoma*, *Umbraculum umbraculum*, *Charonia lampas*, *Patella candei*, *Patella spp.*, *Aplysia depilans*, *Monodonta lineata*, *Cerithium vulgatum*, *Gibbula umbilicalis*, *Onchidellaceltica*), crustaceans (*Pollicipes pollicipes*), bivalves (*Mytilus spp.*), starfish (*Astropecten aranciacus*, *Ophidiaster ophidianus*, *Marthasterias glacialis*, *Echinaster sepositus*), sea-cucumber (*Holothuria (Platyperona) sanctori*), sea-urchins (*Paracentrotus lividus*, *Arbacia lixula*, *Sphaerechinus granularis*, *Diadema africanum*). Benthic organisms were harvested from the intertidal areas during the low tide and by scuba diving expeditions: the Madeira Island was surveyed in September 2012 São Miguel Island, Azores, and the Moroccan coast were sampled in June and July 2013 respectively, with the sampling sites: Reis Magos (32°39'16.21"N; 16°49'05.29"W) and Caniçal (32°44'20.08"N; 16°44'17.55"W)(Figure 2). São Miguel Island, Azores, and the Moroccan coast were sampled in June and July 2013 respectively. Sampling sites for Azores are shown in Figure 3: Cruzeiro (37° 50'31.19"N; 25° 41'33.61"W), Mosteiros (37°53'25.57"N; 25°49'14.72"W), and Etar (37°44'19.31" N; 25°39'38.84" W), São Roque (37°45'15.35"N; 25°38'31.60"W), Lagoa (37°44'42.38"N; 25° 19'47"W), Caloura (37°42'49.34"N; 25°29'54.54"W). In Figure 4 shows the Moroccan sampling sites: Casablanca corniche (33°36'01.2"N; 7°39'57.5"W), El Jadida Haras (33°14'42.0"N; 8°28'37.5"W), El Jadida Sâada (33°14'42.4"N; 8°32'26.9"W), Sidi Bouzid (33°13'57.1"N; 8°33'20.9"W), Mrizika (32°57'21.8"N; 8°46'53.2"W), Oualidia (32°43'55.8"N; 9°02'57.6"W). Two samples of *Patella tenuis* and *P. aspera* were purchased on local markets in Madeira, being caught in the northern coast of the island (32° 51'17.02"N; 17° 01'54.02"W). Organisms were transported to the laboratory in refrigerated containers. Samples were frozen at -20 °C, if they were not processed immediately.

Sample extraction and hydrolysis procedure

The Otero *et al.* (2010) extraction protocol was followed [39]. The recuperation rate of the method was calculated with a 95% recovery of OA. Briefly, shells were removed when necessary and then animals were homogenized with a blender (A320R1, 700 W, Moulinex) in pooled groups in order to obtain 1 g of tissue, with the exception of *Ophidiaster ophidianus*, *Paracentrotus lividus*, *Sphaerechinus*

granularis, *Umbraculum umbraculum*, *Diadema africanum*, *Holoturia (Platyperona) sanctori*, *Charonialampas*, *Marthasterias glacialis*, *Aplysia depilans*. In these cases, each animal was treated separately since they had enough extractable biomass. Homogenized tissue – 1 g - was processed with 3 mL of methanol (Fisher Scientific), then centrifuged during 10 min at 2932 g at 4 °C (Centrifugal-Legend RT). This procedure was repeated twice, and the supernatants combined and concentrated to dryness (Acid-resistant Centrivap Concentrator, Labconco). Afterwards, residues were re-suspended in 10 mL of water (MilliQ) and doubly partitioned with dichloromethane (Merck). Aqueous layer was discarded and the organic layers (20 mL) were concentrated by drying and re-suspended in 1 mL of methanol. After that 500 µL was concentrated to dryness, re-suspended in 100 µL of methanol and filtered through a 0.45 µm filter (UltraFree-MC centrifugal devices, Millipore). In order to detect and quantify the total content of OA and DTXs, 20 µL of methanolic extract were brought to a final volume of 1 mL. This dilution was hydrolysed with 125 µL 2.5 M NaOH, the mixture was homogenized and heated at 76°C for 40 min. Then cooled to room temperature and neutralised with 125 µL 2.5 M HCl and homogenised in the vortex [40]. The extract was filtered with 0.45 µm filter and injected 5 µL in the LC column.

Sample Analysis

Analyses were performed using a 1290 Infinity ultra-high-performance liquid chromatography system coupled to a 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany). Toxin separation was performed with an AQUITY UPLC BEH C18 column (2.1x100mm, 1.7 µm, Waters). Column oven was set at 40 °C, samples in the autosampler were cooled to 4 °C and injection volume was 5 µL. Eluent A consisted in 100% water and B acetonitrile (Panreac Quimica, Barcelona, Spain) in water (95:5), both containing 50 mM formic acid (Merck, Madrid, Spain) and 2mM ammonium formate (Sigma Aldrich, Madrid, Spain). The gradient started with 30%-70% of mobile phase (B) until 3 min, then maintained in 70% B until 4.5 min and decreasing to 30% over 0.1 min and maintained during 1.99 min until the end of the run. Flow rate was 0.4 mL/min.

MS detection was performed using an Agilent G6460C triple quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany). Source conditions were optimized to achieve the best sensitivity for all compounds. A drying gas temperature of 350 °C and a flow of 8 L/min, a nebulizer gas pressure of 45 psi (Nitrocrafter NCLC/MS from Air Liquid), a sheath gas temperature of 400 °C and a flow of 11 L/min were used. The capillary voltage was set to 4000 V in negative mode with a nozzle voltage of 0 V and 3500 V. The fragmentor was 260 V and the cell accelerator voltage was 4 for each toxin in this method. The collision energy, optimized using MassHunter Optimizer software, was 52 and 60 eV for OA and DTX2 and 53 and 66 eV for DTX1. The mass spectrometer was operated in multiple reaction monitoring (MRM), detecting in negative mode. Two product ions were analyzed per compound, one for quantification and another for confirmation.

The transitions employed were: OA and DTX2 (m/z 803.5 > 255.2/113.2), DTX1 (m/z 817.5 > 255.2/113). Retention times were: OA (3.94 min), DTX1 (4.46 min), DTX2 (4.09 min). For the calibration curve, eight different concentrations of the standard (Laboratorios Cifga, Spain) were injected in triplicate: OA/DTX1/DTX2 from 1.56 ng/mL to 100 ng/mL (Figure 2.25). All toxins were quantified, using their peak areas to calculate amounts and using the curve obtained from each standard. The LOD and LOQ for OA/DTX1/DTX2 were 0.0936 and 0.312 $\mu\text{g/Kg}$ respectively.

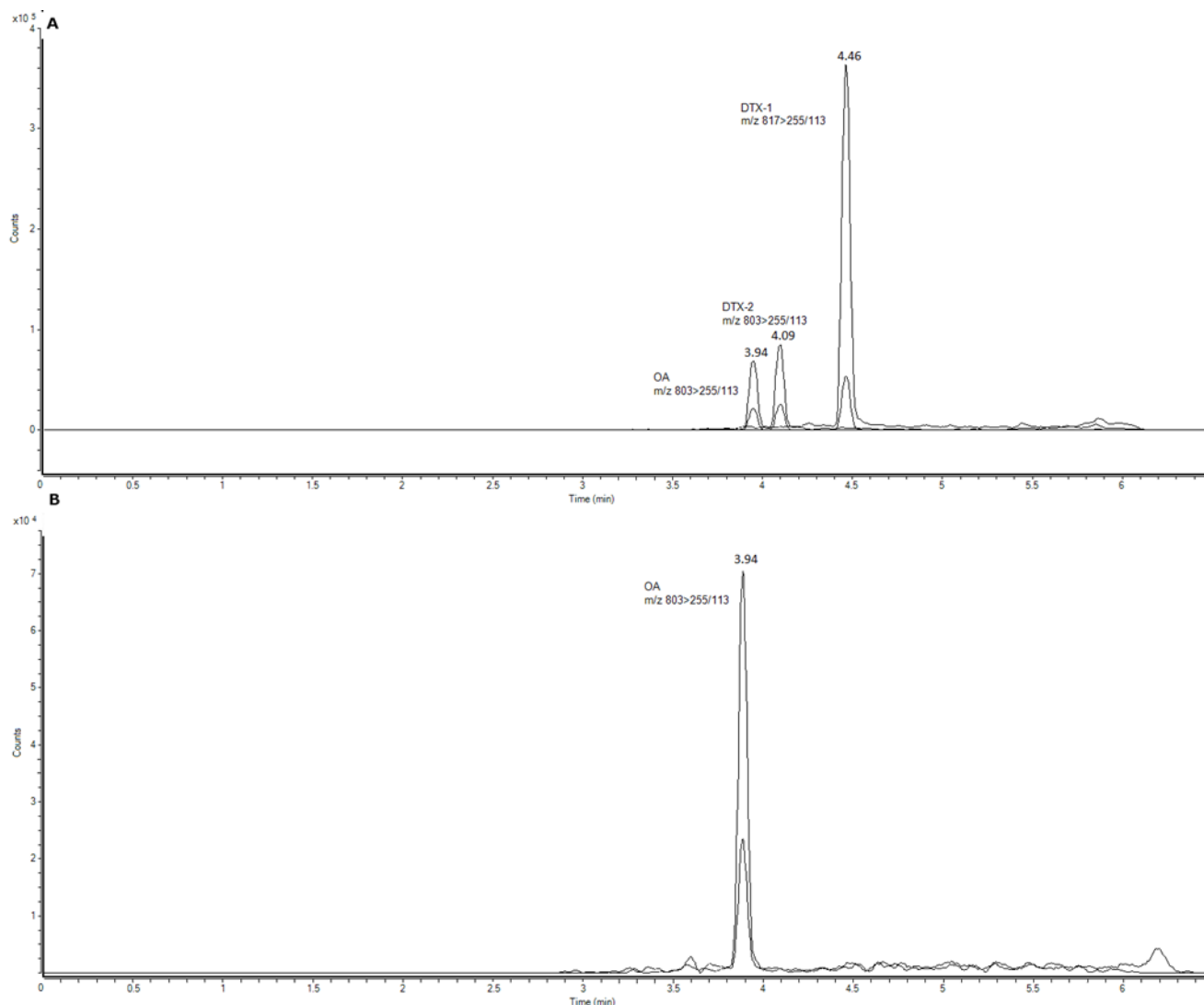


Figure 2.25-Mass chromatograms of the UPLC- MS/MS obtained under multiple reaction monitoring (MRM) in negative mode. (A) Total ion chromatogram (TIC) of OA standard (100 ng/mL), m/z 803.5 > 255.2/113.2, DTX2 standard (100 ng/mL), m/z 803.5 > 255.5/113.5 and DTX1 standard (100 ng/mL), m/z 817.5 > 255.2/113. (B) TIC of a sample with quantifiable OA contents in *Mytilus* spp. (m/z 803.5 > 255.5/113.5)

Statistical Analyzes

The influence of the factors sampling site (Morocco, Madeira and Azores islands) and organism type in the OA occurrence was analyzed using Generalized Linear Models (GLZ). The dependent variable was the concentration of OA in the organisms flesh ($\mu\text{g kg}^{-1}$). The organisms were grouped according to their most distinctive taxonomical degree, which was not necessarily species, since in some cases, very similar species were sampled at the same time. So the levels of the factor organism were: bivalve, sea cucumber, sea urchin, sea star, sea snail and limpet. The data set consists in OA concentrations found

in pooled samples from each organism. There is one single data per pooled sample, being the pools constituted by similar numbers of organisms, for each organism level (see methods). The data set could be considered as a zero – inflated data set, with variance larger than the mean. These models are usually handled with Poisson or negative binomial distributions [41]. However, we can use neither of these distributions because our data are continuous. Instead, we used the approach of gamma hurdle models [42], which performs the analysis in two steps: first, the analysis of presence/absence of the toxin (managed with a binomial or negative binomial distribution) and second, on these data showing measurable concentrations of OA, a GLZ with gamma distribution. Besides the analysis including “sampling site” and “organism” as factors, another analysis considering only organism as factor was performed, for which new organism levels that only occurred in a single sampling site were included: mussel, barnacle, sea cucumber and sea hare.

All the models were performed with R software [43], package *stats* function *glm*.

Conclusions

In this work we used the UPLC-MS/MS technique for the screening of non-described vectors for Okadaic acid and its derivatives in the northwestern Moroccan coast, the Madeira and São Miguel Islands (Madeira and Azores archipelagos respectively). We detected OA in a total of 9 new vectors among the 23 screened species: echinoderms (*A. aranciacus*, *A. lixula*, *E. sepositus*, *H. sanctori*, *O. ophidianus*) and gastropods (*O. celtica*, *A. depilans*, *Patella spp.*, *S. haemostoma*). We also detected OA in the progeny of *S. haemostoma*, suggesting that this biotoxin could be transferred to the offspring. Regarding species uptake, mussels were the organisms with higher uptake tendency, followed by gastropods and echinoderms. Since the scarcity of mussels in Madeira and Azores archipelagos, gastropods and echinoderms can be a good alternative for the monitoring of this group of toxin. Although the detected values are below the limit value currently implemented in the European Union, it is important to extend the monitored organisms beyond bivalves to learn more about the trophic transfer of these toxins and OA seasonal dynamics to better calculate human health risk in these poorly studied areas.

Acknowledgments

This research was partially funded by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Program - and national funds through FCT - Foundation for Science and Technology - under the project PEst-C/MAR/LA0015/2013 and by the projects MARBIOTECH (reference NORTE-07-0124-FEDER-000047) within the SR&TD Integrated Program MARVALOR - Building research and innovation capacity for improved management and valorization of marine resources, supported by the Programa Operacional Regional do Norte (ON.2-O Novo Norte) and NOVOMAR. MS acknowledge also FCT for the grant SFRH/BD/73269/2010 and Ana Regueiras, Isadora Diniz, Afonso Prestes and Manuela Maranhão. This research was also partially funded by the

FCT-Portugal / CNRST-Morocco Cooperation Convention under the project 1006/13 CNR “Marine emergent toxins in the north east Atlantic (Portugal-Morocco) produced by microalgae and bacteria”. The research leading to USC results has received funding from the following FEDER cofunded-grants, from CDTI and Technological Funds. Supported by Ministerio de Economía y Competitividad, AGL2012-40185-CO2-01 and Consellería de Cultura, Educación e Ordenación Universitaria, GRC2013-016. Inés Rodriguez is supported by a fellowship from Subprograma de Formación de Personal Investigador (AGL2012-40185-CO2-01), Spain.

Author Contributions

M.S. and V.V. conceived the idea, M.S. performed the sampling, sample analyzes and paper writing.

I.R. and A.B. collaborated in sample analyzes.

A.A. contributed on experimental design.

M.H. collaborated in sample collection.

V.V. and L.M. contributed in funding, materials and analyses tools.

V.V., B.S., A.I.N., M.K.: Collaborated in the collections and provided sampling and laboratory facilities

All: proof reading of the manuscript

Conflict of Interest

The authors declare no conflict of interest.

References and Notes

1. Schmitz, F.J.; Prasad, R.S.; Gopichand, Y.; Hossain, H.B.; Van Der Helm, D.; Schmidt, P., Acanthifolicin, a new episulfide-containing polyether carboxylic acid from extracts of the marine sponge *pandaros acanthifolium*. *Journal of the American Chemical Society* 1981, 103, 2467–2469.
2. Tachibana, K.; Scheurrer, P.J.; Tsukitani, Y.; Kikuchi, H.; Engen, D.V.; Clardy, J.; Gopichand, Y.; Schimitz, F.J., Okadaic acid, a cytotoxicity polyether from two marine sponges of the genus *halichondria*. *Journal of the American Chemical Society* 1981, 103, 2469–2471.
3. MacKenzie, L.; Beuzenberg, V.; Holland, P.; McNabb, P.; Suzuki, T.; Selwood, A., Pectenotoxin and okadaic acid-based toxin profiles in *dinophysis acuta* and *dinophysis acuminata* from new zealand. *Harmful Algae* 2005, 4, 75-85.
4. Hu, T.M.; Curtis, J.M.; Walter, J.A.; Wright, J.L.C., Identification of dtx-4, a new water-soluble phosphatase inhibitor from the toxic dinoflagellate *prorocentrum lima*. *Journal of the Chemical Society, Chemical Communications* 1995, 5, 597–599.
5. Caroppo, C.; Congestri, R.; Bruno, M., On the presence of *phalacrocoma rotundatum* in the southern adriatic sea (italy). *Aquatic Microbial Ecology* 1999, 17, 301-310.

6. Bialojan, C.; Takai, A., Inhibitory effect of a marine sponge toxin, okadaic acid, on protein phosphatases. *Biochemistry Journal* 1988, 256, 283–290.
7. Takai, A.; Murata, M.; Torigoe, K.; Isobe, M.; Mieskes, G.; Yasumoto, T., Inhibitory effect of okadaic acid derivatives on protein phosphatases. A study on structure-affinity relationship. *Biochemistry Journal* 1992, 284, 539–544.
8. Maneiro, E.; Rodas, V.L.; Costas, E.; Hernandez, J.M., Shellfish consumption: A major risk factor for colorectal cancer. *Medical Hypotheses* 2008, 70, 409–412.
9. Yasumoto, T.; Oshima, Y.; Yamaguchi, M., Occurrence of a new type of shellfish poisoning in tohoku district. *Bulletin of the Japanese Society of Scientific Fisheries* 1978, 44, 1249–1255.
10. Elgarch, A.; Vale, P.; Rifai, S.; Fassouane, A., Detection of diarrhetic shellfish poisoning and azaspiracid toxins in moroccan mussels: Comparison of the lc-ms method with the commercial immunoassay kit. *Marine Drugs* 2008, 6, 587-594.
11. Garcia, C.; Truan, D.; Lagos, M.; Santelices, J.P.; Diaz, J.C.; Lagos, N., Metabolic transformation of dinophysistoxin-3 into dinophysistoxin-1 causes human intoxication by consumption of o-acyl-derivatives dinophysistoxins contaminated shellfish. *Journal of Toxicological Sciences* 2005, 30, 287-296.
12. Scoging, A.; Bahl, M., Diarrhetic shellfish poisoning in the uk. *Lancet* 1998, 352.
13. Reboreda, A.; Lago, J.; Chapela, M.J.; Vieites, J.M.; Botana, L.M.; Alfonso, A.; Cabado, A.G., Decrease of marine toxin content in bivalves by industrial processes. *Toxicon* 2010, 55, 235-243.
14. McCarron, P.; Kilcoyne, J.; Hess, P., Effects of cooking and heat treatment on concentration and tissue distribution of okadaic acid and dinophysistoxin-2 in mussels (*mytilus edulis*). *Toxicon* 2008, 51, 1081-1089.
15. Lawrence, J.; Loreal, H.; Toyofuku, H.; Hess, P.; Karunasagar, I.; Ababouch, L. Assessment and management of biotoxin risks in bivalve mollusks; 551; FAO: Rome, 2011; p 337.
16. Hamano, Y.; Kinoshita, Y.; Yasumoto, T., Enteropathogenicity of diarrhetic shellfish toxins in intestinal models. *Journal of the Food Hygienic Society of Japan* 1985, 27, 375–379.
17. Alexander, J.; Auðunsson, G.A.; Benford, D.; Cockburn, A.; Cravedi, J.P.; Dogliotti, E.; Di Domenico, A.; Fernández-Cruz, M.L.; Fink-Gremmels, J.; Fürst, P., et al., Opinion of the scientific panel on contaminants in the food chain on a request from the european commission on marine biotoxins in shellfish – okadaic acid and analogues. *The EFSA Journal* 2008, 589, 1-62.
18. Aune, T.; Larsen, S.; Aasen, J.A.; Rehmann, N.; Satake, M.; Hess, P., Relative toxicity of dinophysistoxin-2 (dtx-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* 2007, 49, 1-7.
19. Ec (2011). Official journal of the european union. Commission regulation (eu) no 15/2011 amending regulation (ec) no 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve mollusks. 2074/2005 15/2011.

20. Silva, M.; Barreiro, A.; Rodriguez, P.; Otero, P.; Azevedo, J.; Alfonso, A.; Botana, L.M.; Vasconcelos, V., New invertebrate vectors for pst, spirolides and okadaic acid in the north atlantic. *Marine Drugs* 2013, 11, 1936-1960.
21. Burla, H.; Ferlin, V.; Pabst, B.; Ribi, G., Notes on the ecology of *astropecten aranciacus*. *Marine Biology* 1972, 14, 235-241.
22. Carefoot, T.H., *Aplysia*: Its biology and ecology. *Oceanography and Marine Biology Annual Review* 1987, 25, 167-284.
23. Bulleri, F.; Benedetti-Cecchi, L.; Cinelli, F., Grazing by the sea urchins *arbacia lixula* I. And *paracentrotus lividus* lam. In the northwest mediterranean. *Journal of Experimental Marine Biology and Ecology* 1999, 241, 81-85.
24. Lin, S.J.; Hwang, D.F., Possible source of tetrodotoxin in the starfish *astropecten scoparius*. *Toxicon* 2001, 39, 573-579.
25. Nicolaidou, A.; Nott, J.A., The role of the marine gastropod *cerithium vulgatum* in the biogeochemical cycling of metals. *Biogeochemical Cycling and Sediment Ecology* 1999, 59, 137-146.
26. Rodríguez, A.; Hernández, J.C.; Clemente, S.; Coppard, S.E., A new species of *diadema* (echinodermata: Echinoidea: Diadematidae) from the eastern atlantic ocean and a neotype designation of *diadema antillarum* (philippi, 1845). *Zootaxa* 2013, 3636, 144-170.
27. Ferguson, J.C., Feeding activity in *echinaster* and its induction with dissolved nutrients. *Biology Bulletin* 1969, 136, 374-384.
28. Crothers, J.H., Common topshells: An introduction to the biology of *osilinus lineatus* with notes on other species in the genus. *Field Studies* 2001, 10, 115-160.
29. Navarro, P.G.; García-Sanz, S.; Barrio, J.M.; Tuya, F., Feeding and movement patterns of the sea cucumber *holothuria sanctori*. *Marine Biology* 2013, 160, 2957–2966.
30. Toral-Granda, V.; Lovatelli, A.; Vasconcellos, M. In Scientific committee composed of conand c., hamel j.F., mercier a., purcell s. And uthicke s. - international workshop on the sustainable use and management of sea cucumber fisheries, SPC Beche-deMer Information Bulletin, Puerto Ayora, Galapagos Islands, Ecuador, 19-23 November, 2008; Puerto Ayora, Galapagos Islands, Ecuador.
31. Knox, G.A., Hard shores. In *The ecology of seashores*, Kennish, M.J., Ed. CRC Press: Boca Raton, Florida, USA, 2001; pp 20-86.
32. Buschbaum, C.; Dittmann, S.; Hong, J.-S.; Hwang, I.; Strasser, M.; Thiel, M.; Valdivia, N.; Yoon, S.; Reise, K., Mytilid mussels: Global habitat engineers in coastal sediments. *Helgoland Marine Research* 2008, 63, 47-58.
33. Dayrat, B., Review of the current knowledge of the systematics of *onchidiidae* (mollusca: Gastropoda: Pulmonata) with a checklist of nominal species. *Zootaxa* 2009, 2068, 1-26.
34. Lemée, R.; Boudouresque, C.F.; Gobert, J.; Malestroit, P.; Mari, X.; Meinesz, A.; Menager, V.; Ruitton, S., Feeding behaviour of *paracentrotus lividus* in the presence of *caulerpa taxifolia* introduced in the mediterranean sea. *Oceanologica Acta* 1995, 19, 245-253.

35. Martínez-Pita, I.; Sanches-Espana, A.; Garcia, F.J., Gonadal growth and reproduction in the sea urchin *sphaerechinus granularis* (Lamarck 1816) in southern Spain. *Scientia Marina* 2008, 72, 603-611.
36. Valdes, A., On the publication data, authorship, and type species of *Umbraculum* and *Tylodina* (Gastropoda: Opisthobranchia: Tylodinoidea). *The Nautilus* 2001, 115, 29-34.
37. Ramírez, R.; Tuya, F.; J., H.R., Spatial patterns in the population structure of the whelk *Stramonita haemastoma* (Linnaeus, 1766) (Gastropoda: Muricidae) in the Canarian archipelago (Eastern Atlantic). *Scientia Marina* 2009, 73, 431-437.
38. Itoi, S.; Yoshikawa, S.; Asahina, K.; Suzuki, M.; Ishizuka, K.; Takimoto, N.; Mitsuoka, R.; Yokoyama, N.; Detake, A.; Takayanagi, C., et al., Larval pufferfish protected by maternal tetrodotoxin. *Toxicon* 2014, 78, 35-40.
39. Otero, P.; Alfonso, A.; Alfonso, C.; Vieytes, M.R.; Louzao, M.C.; Botana, A.M.; Botana, L.M., New protocol to obtain spirochetes from *Alexandrium ostenfeldii* cultures with high recovery and purity. *Biomed Chromatogr* 2010, 24, 878-886.
40. European Union Reference Laboratory for Marine Biotoxins (2015). Eu-harmonised standard operating procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS. Pp. 1-33. European Union Reference Laboratory for Marine Biotoxins. Available online: http://aesan.msssi.gob.es/en/crlmb/web/procedimientos_crlmb/crlmb_standard_operating_procedures.shtml (3 February 2015).
41. Zuur, A.F.; Ieno, E.N.; Walker, N.J.; Saveliev, A.A.; Smith, G.M., *Mixed effects models and extensions in ecology with R*. Springer Science+Business Media: 2009.
42. Frees, E.W., *Regression modeling with actuarial and financial applications*. Cambridge University Press: 2011.
43. R Core Team (2014). *A language and environment for statistical computing*. R Foundation for Statistical Computing. <http://www.R-project.org/>

Toxins 2015, 7, 3740-3757

First Report of CIGUATOXINS in two starfish species: *Ophidiaster ophidianus* and
Marthasterias glacialis

Marisa Silva ^{1,2,†}, **Inés Rodríguez** ^{3,†}, **Aldo Barreiro** ^{1,2}, **Manfred Kaufmann** ^{2,4,5}, **Ana Isabel Neto** ^{2,6},
Meryem Hassouani ⁷, **Brahim Sabour** ⁷, **Amparo Alfonso** ³, **Luis M. Botana** ³ and **Vitor Vasconcelos** ^{1,2,*}

¹ Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, Porto 4619-007, Portugal; E-Mails: marisasilva17@gmail.com (M.S.); aldo.barreiro@gmail.com (A.B.)

² Interdisciplinary Center of Marine and Environmental Research–CIMAR/CIIMAR, University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

³ Department of Pharmacology, Faculty of Veterinary, University of Santiago of Compostela, 27002 Lugo, Spain; E-Mails: ines.rodriguez.filgueiras@usc.es (I.R.); amparo.alfonso@usc.es (A.A.); Luis.Botana@usc.es (L.M.B.)

⁴ University of Madeira, Marine Biology Station of Funchal, 9000-107 Funchal, Madeira Island, Portugal; E-Mails: mkaufmann@ciimar.up.pt (M.K.)

⁵ Center of Interdisciplinary Marine and Environmental Research of Madeira - CIIMAR-Madeira, Edif. Madeira Tecnopolo, Caminho da Penteada, 9020 – 105 Funchal, Madeira, Portugal

⁶ Department of Marine Biology, University of Azores, 9501-801 Ponta Delgada, Azores, Portugal; E-Mails: aneto@uac.pt (A.I.N.)

⁷ Phycology Research Unit – Biotechnology, Ecosystems Ecology and Valorization Laboratory, Faculty of Sciences El Jadida, University Chouaib Doukkali, BP20 El Jadida, Morocco; E-Mails: hassouani@hotmail.com (M.H.); sabour.b@ucd.ac.ma (B.S.)

† These authors contributed equally to this work.

Abstract: Ciguatera fish poisoning (CFP) is a syndrome caused by the ingestion of fish contaminated with Ciguatoxins (CTXs). These phycotoxins are produced mainly by dinoflagellates that belong to the genus *Gambierdiscus* that are transformed in more toxic forms in predatory fish guts, and are more present in the Indo-Pacific and Caribbean areas. It is estimated that CFP causes per year more than 10,000 intoxications worldwide. With the rise of water temperature and anthropogenic intervention, it is important to study the prevalence of CFP in more temperate waters. Through inter- and subtidal sampling, 22 species of organisms were collected, in Madeira and Azores archipelagos and in the northwestern Moroccan coast, during September of 2012 and June and July of 2013. A total of 94 samples of 22 different species of bivalves, gastropods, echinoderms and crustaceans were analyzed by Ultra Performance Liquid Chromatography-Mass Spectrometry-Ion Trap-Time of Flight (UPLC-MS-IT-TOF) and Ultra Performance Chromatography- Mass Spectrometry (UPLC-MS). Our main aim was to

detect new vectors and ascertain if there were some geographical differences. We detected for the first time putative CTXs in echinoderms, in two starfish species—*M. glacialis* and *O. ophidianus*. We detected differences regarding uptake values by organisms and geographical location. Toxin amounts were significant, showing the importance and the need for continuity of these studies to gain more knowledge about the prevalence of these toxins, in order to better assess human health risk.

In addition, we suggest monitoring of these toxins should be extended to other vectors, starfish being a good alternative for protecting and assessing human health risk.

Keywords: Ciguatera; New vectors; Madeira Island; São Miguel Island; Morocco.

Introduction

Ciguatoxins (CTXs) are lipophilic compounds that result from the biotransformation in finfish of precursor gambiertoxins produced by dinoflagellates from the genus *Gambierdiscus* [1–3].

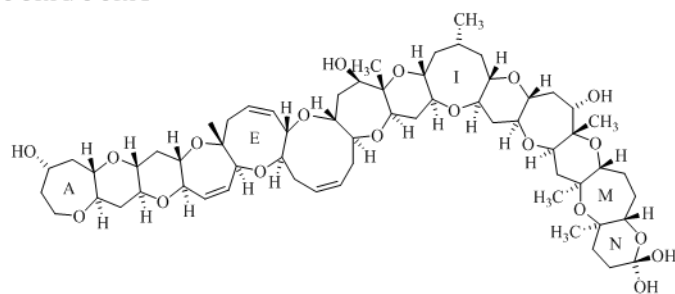
Geographically, CTXs are typical for the tropical and sub-tropical regions of the Atlantic (C-CTX), Indian (I-CTX) and Pacific (P-CTX) Oceans [1,4–8]. Both the structure and potency of CTXs appear to be geographically determined, in resemblance to Maitoxin that shares the same producer, and to date more than 30 CTX analogues have been described [3–5,7,9–13].

CTXs are polyethers with rigid structure, formed by rings 13–14 fused by ether linkages (Figure 2.26) [2]. These potent biotoxins are odorless, tasteless, heat resistant and lipid soluble, so they are not destroyed by cooking processes [7]. Regarding action mechanism, CTXs bind to non-selective, non-voltage activated ion channels, causing their opening, leading to the increase of intracellular calcium levels to toxic concentrations [14]. In terms of symptomatology, during the acute period in the first 24 h gastrointestinal problems such as diarrhea, nausea, abdominal pain and vomiting appear. In addition, cardiovascular complications can occur. Neurologic symptoms that can emerge from within a few hours to two weeks after exposure, like paresthesias, dysesthesias, and hyperesthesias [15].

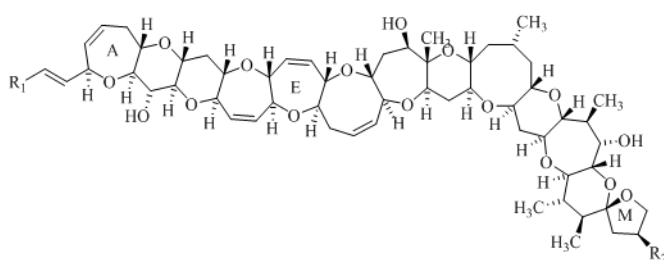
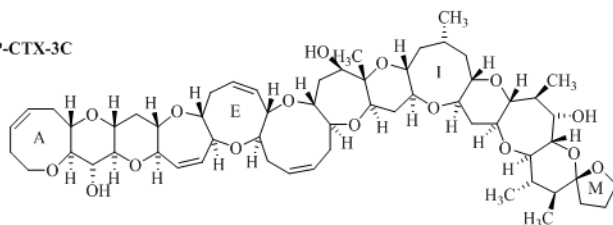
Ciguatera fish poisoning (CFP) is the most common type of intoxication syndrome, even in non-endemic areas, due to the large quantity of fish exports, and this makes it a worldwide concern [16].

The most common route of intoxication is through ingestion of contaminated fish, being estimated 10,000 to 50,000 intoxications per year [4,17]. Recent reports document the presence of CTXs as far north as the Mediterranean [18]. In addition, high abundance of *Gambierdiscus* cells are not visible as blooms, as in other harmful algal bloom species, and this hinders the process of monitoring and managing of these blooms [19].

C-CTX-1/ C-CTX-2



P-CTX-3C



	R ₁	R ₂
P-CTX-1	¹ CH ₂ OHCHOH	OH
P-CTX-3 / P-CTX-2	¹ CH ₂ OHCHOH	H
P-CTX-4B/ P-CTX-4A	¹ CH ₂ CH	H

Figure 2.26 - Structures Caribbean (C) and Pacific (P) CTX-group toxin. The epimers, P-CTX-2 (52-epi P-CTX-3), P-CTX-4A (52-epi P-CTX-4B) and C-CTX-2 (56-epi C-CTX-1) are indicated in parenthesis.

The common vectors for these phycotoxins are finfish, some mollusks (e.g., turban snail, *Lunella cinerea*, and in giant clams, *Tridacna gigas*), achieving higher concentrations in top predatory fish like groupers (Family: Serranidae), barracuda (Family: Sphyraenidae) and snapper (Family: Lutjanidae) [20]. Regarding the regulatory status in the European Union (EU), EFSA proposes the use of toxicity equivalency factors (TEFs), based on their acute intraperitoneal LD50 in mice, determining eleven values: P-CTX-1 = 1, P-CTX-2 = 0.3, P-CTX-3 = 0.3, C-CTX-2 = 0.3, P-CTX-3C = 0.2, 2,3-dihydroxy PCTX-3C = 0.1, 51-hydroxy P-CTX-3C = 1, P-CTX-4A = 0.1, C-CTX-1 = 0.1 and P-CTX-4B = 0.05. In the same scientific opinion on CTXs, EFSA claims that a concentration of 0.01 µg P-CTX-1 equivalents/kg of fish as expectable not to exert effects in sensitive individuals. In addition, due to very few reported cases for CTX occurrences European markets no limits nor ARfD (acute reference dosage) have been established until now, nor advisement regarding analytical methodologies to use [1]. Nevertheless, Commission Regulation (EC) nr 854/2004 stated mandates that checks have to be made to ensure that fishery products containing CTX are forbidden to enter the market [21]. Other countries like the United States of America, have safety levels (<0.1 µg/kg C-CTX-1 equivalents and <0.01 µg/kg P-CTX-1 equivalents), other countries opted for more radical measures, like Japan that banned the importation of some fish species reported as CFP vectors (e.g., barracuda) [1,22]. The aim of our work was to search for new vectors, to better access the human health risk.

We screened the Madeira Island (Madeira archipelago), São Miguel Island (Azores archipelago), and the Moroccan coast by scuba diving expeditions and intertidal harvesting of 22 edible and

inedible species. The inedible species were collected due to their importance in the food chain. We hope that our work contributes to the development and establishment of monitoring procedures as well as legislation in the EU to better protect consumers and public health.

Results and Discussion

In this work, a total of 94 samples were analyzed for CTX plus its analogues from 14 sampling points distributed in three different locations: Madeira Island, São Miguel Island and along the northwestern Moroccan Coast (Figure 2.27). Twenty-two species of benthic organisms, including bivalves (mussels), gastropods (sea-snails, sea-slugs and limpets), echinoderms (starfishes, sea-urchins and sea-cucumbers) and crustaceans (barnacles) were collected. The decision to sample these particular species was linked to the fact we wanted to screen edible and commercially important species and inedible species for their importance in the trophic chain. The number of samples collected and average number of specimens needed to set a pooled sample are detailed (Table 2.15).

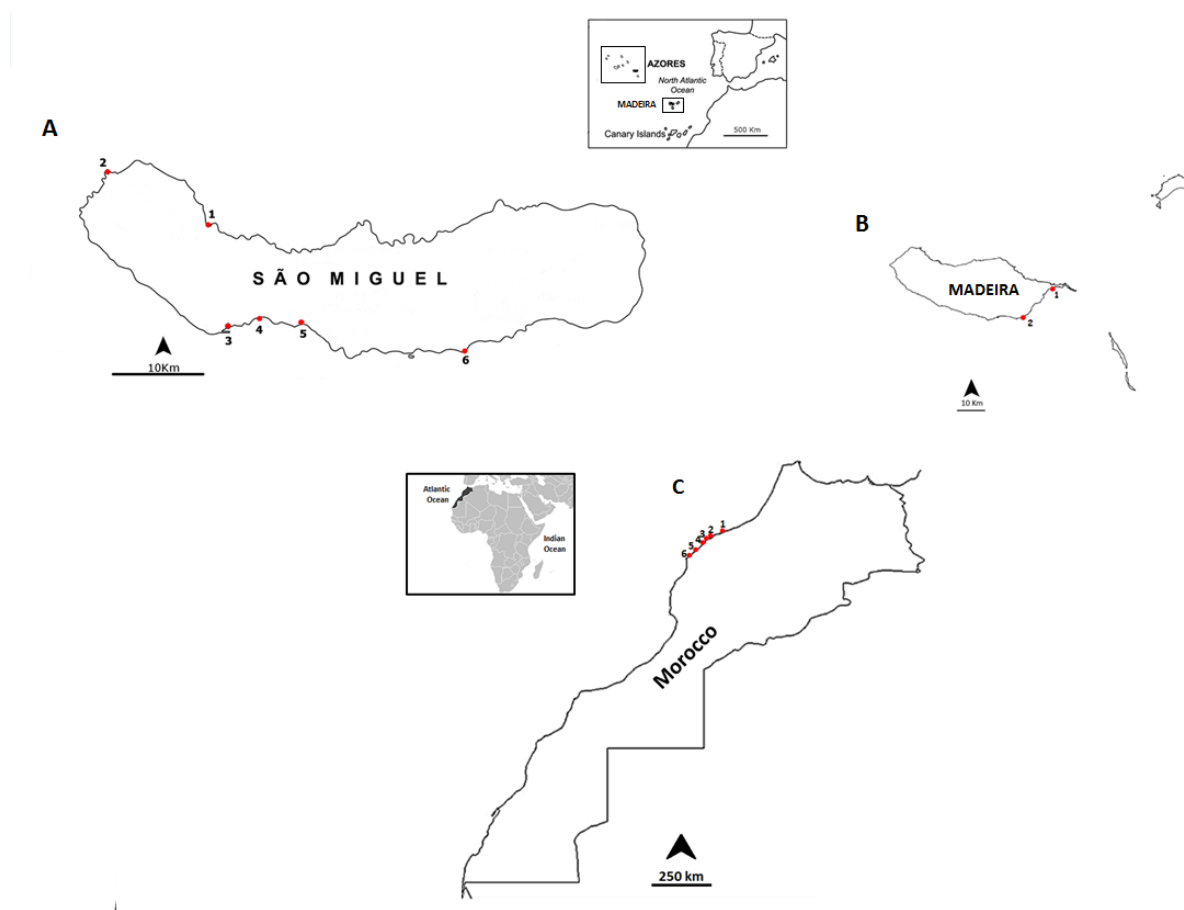


Figure 2.27 - Location of the sampling points: (A) São Miguel island coast, Azores archipelago: 1, Cruzeiro; 2, Mosteiros; 3, Étar; 4, São Roque; 5, Lagoa; and 6, Caloura. (B) Madeira island coast: 1, Reis Magos and 2, Caniçal. (C) Northwestern Moroccan coast: 1, Casablanca Corniche; 2, El Jadida Haras; 3, El Jadida Sâada; 4, Sidi Bouzid; 5, Mrizika; and 6, Oualidia..

Table2.15 - Species sampled and their trophic level, average number of specimens comprising a pooled sample (AvNr) and number of samples collected (NrP Samples) on Madeira Island in September 2012, São Miguel Island, Azores, in June 2013 and Morocco in July 2013 and their edibility. Availability of animals is dependent on their geographical distribution and ecology.

Species	Trophic Level	Sampling Site(s)	NrP Samples	AvNr	Edible	Ref.
<i>Aplysia depilans</i>	Grazer	Morocco	3	1	No	[23]
<i>Arbacia lixula</i>	Grazer	Madeira/Azores/Morocco	9	4	No	[24]
<i>Charonia lampas</i>	3rd level predator	Madeira/Morocco	3	1	Yes	[25]
<i>Cerithium vulgatum</i>	Grazer	Morocco	1	40	Yes	[26]
<i>Diadema africanum</i>	Grazer	Madeira	2	1	No	[27]
<i>Echinaster sepositus</i>	2nd level predator	Madeira	1	3	No	[28]
<i>Gibbula umbilicalis</i>	Grazer	Morocco	3	100	Yes	[29]
<i>Holothuria(Platyperona)sanctori</i>	Deposit feeder	Morocco	4	1	Yes	[30,31]
<i>Marthasterias glacialis</i>	2nd level predator	Madeira/Azores/Morocco	8	1	No	[32]
<i>Monodonta lineata</i>	Grazer	Morocco	5	86	Yes	[29]
<i>Mytilus spp.</i>	Filter feeder	Morocco	4	30	Yes	[33]
<i>Onchidella celtica</i>	Grazer	Morocco	1	50	No	[34]
<i>Ophidiaster ophidianus</i>	Detritivorous	Madeira/Azores	5	1	No	[28]
<i>Pattela aspera</i>	Grazer	Madeira	2	15	Yes	[32]
<i>Patella spp.</i>	Grazer	Morocco	4	12	Yes	[32]
<i>Pattela candei</i>	Grazer	Azores	3	10	Yes	[32]
<i>Paracentrotus lividus</i>	Grazer	Madeira/Azores/Morocco	7	1	Yes	[35]
<i>Pollicipes pollicipes</i>	Filter feeder	Morocco	3	35	Yes	[32]
<i>Sphaerechinus granularis</i>	Grazer	Azores	4	1	Yes	[36]
<i>Umbraculum umbraculum</i>	Grazer	Madeira	1	1	No	[37]
<i>Stramonita haemostoma</i>	2nd level predator	Madeira/Azores/Morocco	5	15	No	[38]

After sample extraction, the CTXs profile was determined in each sample by Ultra Performance Liquid Chromatography- Mass Spectrometry (UPLC-MS) method. Initially, samples were screened by UPLC-MS in Selected Ion Monitoring (SIM) mode for 20 CTXs analogues described in the literature (Table 2.16) and in seven samples some suspicious peak was detected, all of them from starfish.

Table2.16 - Screened CTX's UPLC-MS in Positive Mode.

Compound Name	Mass	Polarity
I-CTX-3 and I-CTX-4	1157.6	Positive
Unknown CTX	1143.6	Positive
Caribbean-CTX	1141.7	Positive
C-CTX-1127	1127.6	Positive
CTX-1B	1111.6	Positive

Compound Name	Mass	Polarity
54-deoxy-CTX-1B	1095.6	Positive
52-epi-54-deoxy-CTX-1B		
M-CTX-4A and M-CTX-4B	1079.6	Positive
MTX small	1060	Positive
2,3-OH-CTX-3C	1055.6	Positive
M-CTX-3C methylacetal		
2-OH-CTX-3C and M-CTX-3C	1041.6	Positive
Analogs CTX	1040.6	Positive
51-OH-CTX-3C	1039.6	Positive
49-epo-CTX-3C and CTX-3C	1023.6	Positive
Unknown CTX	1159.6	Positive

Afterwards, the scan of each sample was carefully analyzed, checking the characteristic mass pattern fragmentation of CTX; that is, the formation of sodium and ammonium adducts and losses of water. In this sense, Figure 2.28 shows the SIM and the spectrum of CTX-3C standard. The mass spectrum from Figure 2.28B showed the ion CTX3C $[M+H]^+ m/z$ 1023.5, and the water losses m/z 1005.5 associated with $[M+H-H_2O]^+$ and m/z 987.5 due to second water lost $[M+H-2H_2O]^+$. In addition, the sodium adduct $[M+Na]^+ m/z$ 1045.7 is also detected.

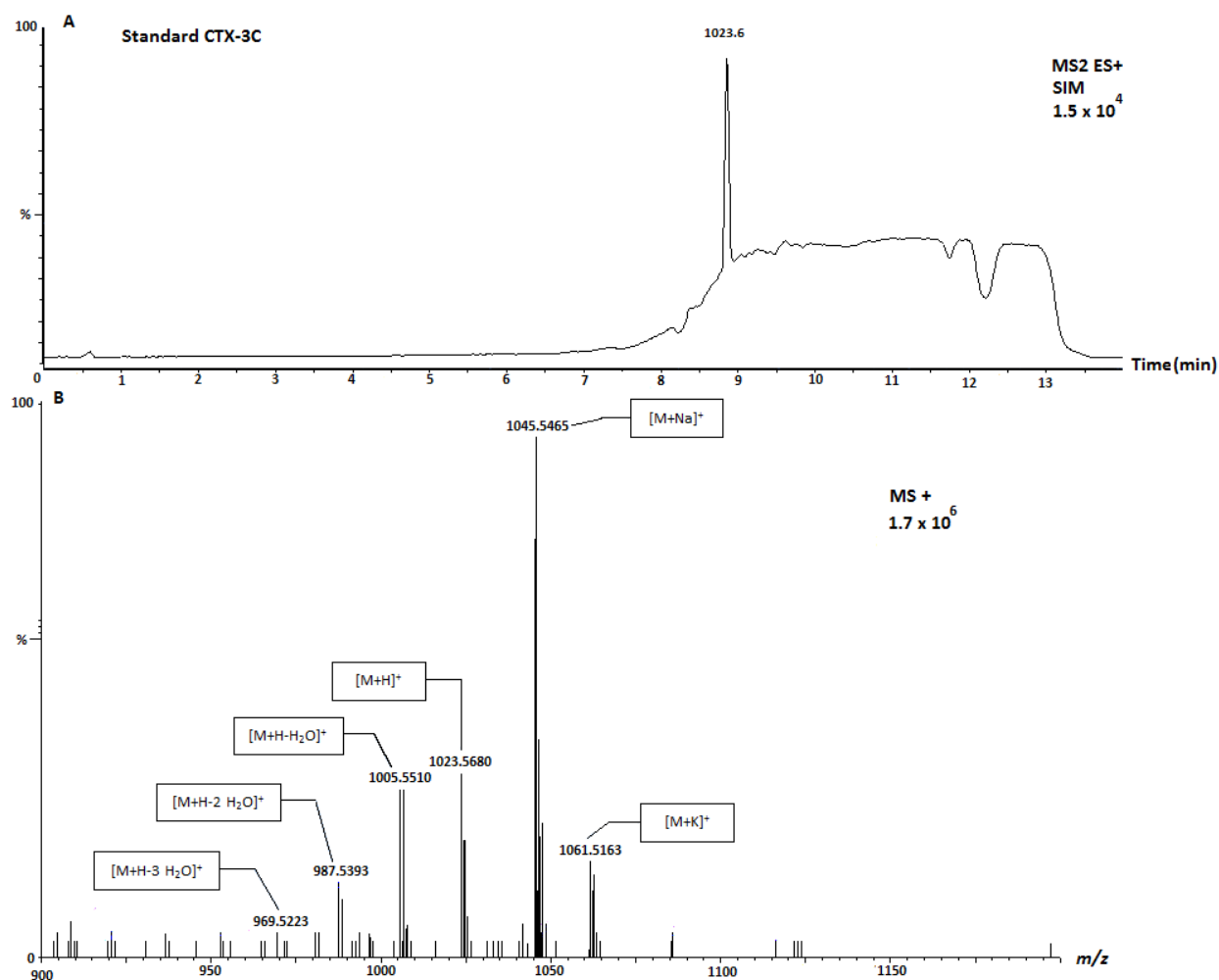
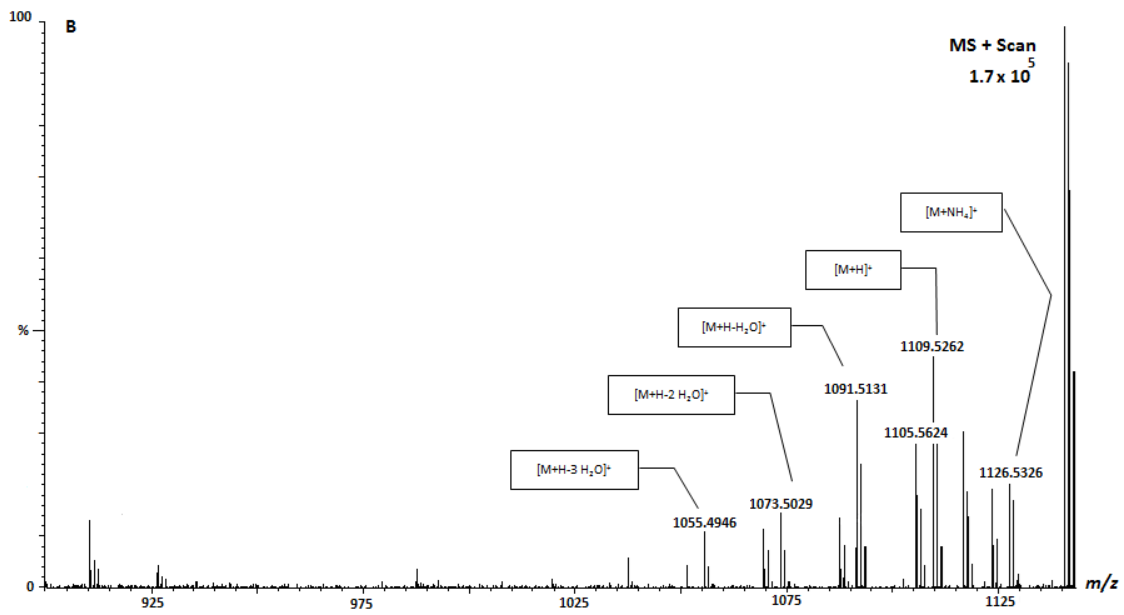
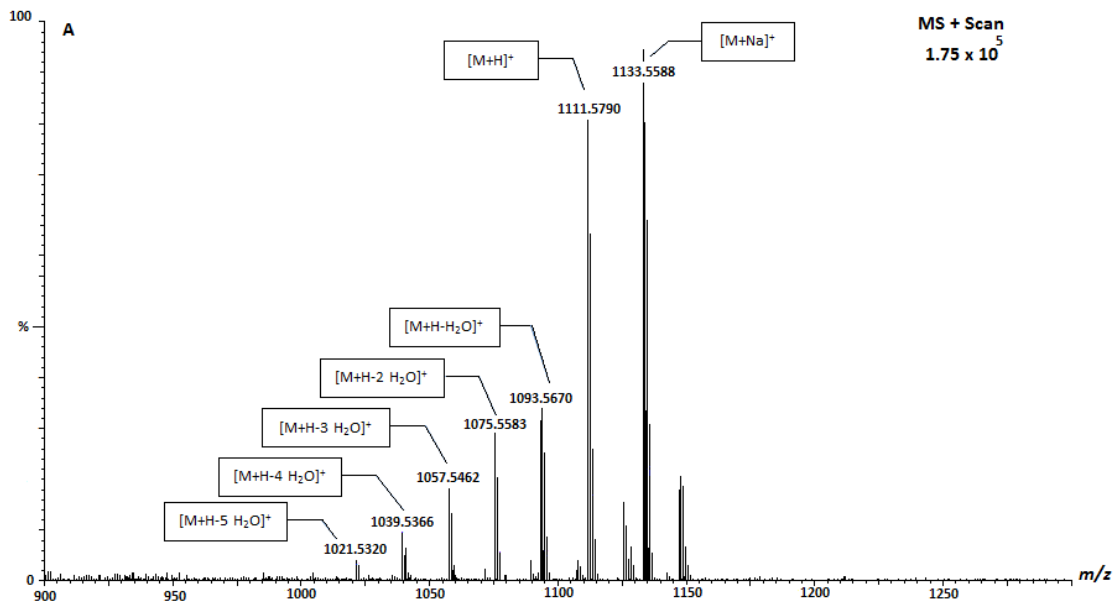


Figure 2.28 - Selected Ion Monitoring (SIM) chromatogram (A) and mass spectrum (B) of standard CTX-3C. SIM obtained by UPLC-MS/MS and spectrum obtained by UPLC-MS-IT-TOF.

Through this analysis, three CTX analogues were detected. From SIM data of samples, m/z 1111.5 was found and from scan mass mode (m/z 800–1300) of samples, two molecules with m/z 1109.5 and m/z 1123.5 were found. The mass spectrum of these molecules (Figure 2.29) shows the typical fragmentation pattern of CTX-like compounds, with several losses of water and adducts of ammonium and sodium. The mass spectrum in Figure 2.29A shows two intense masses, one ($[M+H]^+$) at m/z 1111.5 and other ($[M+Na]^+$) at m/z 1133.5. Moreover, five losses of water were observed while the ammonium adduct did not appear, probably due to its low intensity. Therefore CTX-1B, m/z 1111.5, could be proposed, although not confirmed due to the absence of a standard. The mass spectrum in Figure 2.29B shows an intense mass ($[M+NH_4]^+$) at m/z 1116.6 associated with ammonium adduct from m/z 1109.5. The other three masses shown in the spectrum correspond to one, two and three losses of water at m/z 1099.6 and 1073.5 and 1055.5, respectively, from m/z 1109.5 molecule. The mass spectrum in Figure 2.29C shows an ion at $[M+H]^+$ m/z 1123.5 and two intense masses, $[M+NH_4]^+$ at m/z 1140.5 and $[M+Na]^+$ at m/z 1145.5. In addition, the four losses of water and the potassium adduct of m/z 1123.5 were also detected in the spectrum.



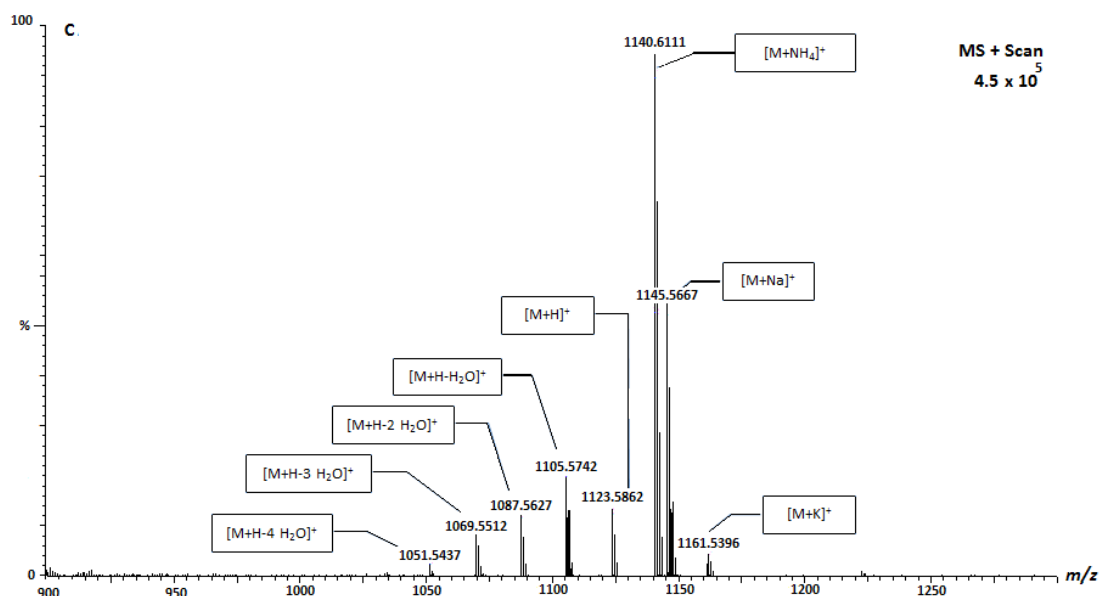


Figure 2.29 - MS2 spectrum of CTX analogue at m/z 1111.5 $[M+H]^+$ (A); CTX analogue at m/z 1109.5 $[M+H]^+$ (B); and CTX analogue m/z 1123.5 $[M+H]^+$ (C). Spectra obtained by Ultra Performance Liquid Chromatography-Mass Spectrometry-Ion Trap-Time of Flight UPLC-MS-IT-TOF.

Once the presence of CTX analogues was identified, the toxin amount was quantified (Table 2.17). In order to calculate concentrations of CTX analogues, it was assumed that related analogues would give a similar response to that of CTX-3C, because of this, and since there is an absence of correspondence with EFSA, TEFs results will be expressed in μg CTX-3C equivalents/Kg. Therefore, the calibration curve was done with CTX-3C standard. The quantification of these toxins was carried out using SIM acquisition in positive mode. For CTX-3C standard, a good seven-point calibration curve with range between 18.75–600 ng/mL was obtained ($R^2 = 0.999$). The limit of detection (LOD) was 1.125 $\mu\text{g}/\text{Kg}$ and the limit of quantification (LOQ) was 3.75 $\mu\text{g}/\text{Kg}$.

Table 2.17 - Presumed CTX detected in UPLC-MS (FW-Fresh weight).

Sample	Location	Species	1111 (μg CTX/Kg Fw)	1123 (μg CTX/Kg Fw)	1109 (μg CTX/Kg Fw)
341 #1	Madeira	<i>O. ophidianus</i>	21.55	7.24	
341 #2	Madeira	<i>O. ophidianus</i>	46.49	29.80	
411 #2	Azores	<i>M. glacialis</i>	<LOQ	<LOQ	
412	Azores	<i>O. ophidianus</i>	19.73		
424	Azores	<i>O. ophidianus</i>	124.04	30.90	
426 #1	Azores	<i>M. glacialis</i>	7.92		

435 Azores *O. ophidianus* 58.78 4.40

Only 7.45% of the 94 samples analyzed were quantifiable for CTX. All of the measurable samples were one of two starfish species, *O. ophidianus* and *M. glacialis*, from Portuguese territory. This is the first report of CTX in echinoderms. Average concentrations detected ranged from 4.40 µg CTX-3C equivalents/Kg fresh weigh (fw), to 124.04 µg CTX-3C equivalents/Kg fw in *O. ophidianus*, if it were possible to apply EFSA's, TEFs both would be above the U.S. American safety level (<0.1 µg/kg C-CTX-1 equivalents and <0.01 µg/kg P-CTX-1 equivalents) [22]. Regarding statistics, the first step of the gamma hurdle model was a GLZ performed with the data of presence/absence of CTX with Binomial distribution of the error. The results of the model's analysis of deviance are shown in Table 2.18, as well as the coefficients rescaled to a logistic probability (0,1). Sampling site had no significant effect. Among sampling sites, the coefficients showed the lowest probability of occurrence in Morocco, and the highest in Madeira. The second part of the model, which considers the variation in CTX concentration, analyzed with a Gamma distribution of the error among those samples showing positive results, did not show significance of sampling site as factor either, although its p value was closer to significance (Table 2.18). Looking at model coefficients, it is clear that the largest concentrations found occurred in Azores Islands, and lowest in Morocco (zero). This differs from the probability results of the presence/absence data.

The analysis performed with starfish species as factor (Table 2.19) showed significant differences in both the presence/absence and the analysis of the positives (differences in putative CTX concentrations), conclusively, *O. ophidianus* had both higher probability of showing a positive result, and significantly higher amounts of toxin per body mass. This can be explained by their distinct feeding habits, since *O. ophidianus* is a detritivorous acquiring CTX from the sediment bed, against the predatory ones from *M. glacialis*. In addition, Llewellyn (2010) suggested that the rise of water temperature, derived by climate change, can increase the incidence of CFP in Papua New Guinea from 35–70 per thousand people in 1990 to 160–430 per thousand people in 2050 [39]. In addition, it is known that the rise of water temperature can influence the growth rate of the producers [40], toxin production as well as the uptake rate of the vectors [41,42], and that could be the main reason of the differences between sampling sites, though Morocco and Madeira are at the same latitude. Nevertheless, the main species that we detected quantifiable amounts of CTX is absent in Morocco. Since Madeira and Azores archipelagos present oligotrophic waters, making these ecosystems poor in bivalves, the red starfish (*O. ophidianus*) presents a possible alternative as key species for CTX monitoring. We have to interpret the results from our models carefully, since the actual number of data is low. This may affect the non-significance of the models from sampling sites (Table 2.18) where the differences found might become significant by simply adding more data. However, the models using species as factor (Table 2.19) reveal a strong pattern that is not affected by the small number of data.

Table2.18 - Results of the gamma hurdle model for CTX occurrence with sampling site as factor.

Analysis of Deviance				
Model	Factor	χ^2	df	p
Binomial Error	Site	2.9	2	0.23
	Rescaled model coefficients for Site: Intercept (Azores) = 0.36; Madeira = 0.78; Morocco = 4×10^{-8}			
Gamma Error	Site	5.5	2	0.06
	Rescaled model coefficients for Site: Intercept (Azores) = 229; Madeira = 1.39; Morocco = 0.004			

Table2.19 - Results of the gamma hurdle model for CTX occurrence with starfish species as factor.

Analysis of Deviance				
Model	Factor	χ^2	df	p
Binomial Error	Species	8.5	1	<0.01
	Rescaled model coefficients: Intercept (<i>M. glacialis</i>) = 0.11; <i>O. ophidianus</i> = 0.98			
Gamma Error	Organism	9.7	1	<0.01
	Rescaled model coefficients: Intercept (<i>M. glacialis</i>) = 9.4; <i>O. ophidianus</i> = 57.7			

Our results add new information to this topic, we can say, to the best of our knowledge, that this is the first report of putative CTX in starfish. It is also noteworthy that significant amounts of this group of toxins were detected at the bottom of the trophic chain. We hope this contribute towards the establishment of legislation, as well as the promotion of the monitoring of these toxins in the EU.

Experimental Section

Selected Species and Sampling Sites

The coasts of the Portuguese islands Madeira (Madeira archipelago), São Miguel (Azores archipelago) and the northwestern coast of Morocco, were surveyed for non-traditional vector species for Ciguatoxins. Several edible and non-edible species were selected (n = 22) to search for potential new vectors and also the prevalence of the screened biotoxins in the food web: gastropods (*Aplysia depilans*, *Cerithium vulgatum*, *Charonia lampas*, *Gibbula umbilicalis*, *Monodonta lineata*, *Onchidella celtica*, *Patella tenuis tenuis*, *Patella aspera*, *Patella candei*, *Patella spp.*, *Stramonita haemostoma*, *Umbraculum umbraculum*), crustaceans (*Pollicipes pollicipes*), bivalves (*Mytilus spp.*), starfish (*Echinaster sepositus*, *Marthasterias glacialis*, *Ophidiaster ophidianus*), sea-cucumber (*Holothuria (Platyperona) sanctori*), sea-urchins (*Arbacia lixula*, *Diadema africanum*, *Paracentrotus lividus*, *Sphaerechinus granularis*). Benthic organisms were harvested from intertidal areas during low tide and by scuba diving expeditions: the Madeira Island was surveyed in September 2012, and São Miguel Island, Azores, and the Moroccan coast were sampled in June and July 2013, respectively. Sampling

sites are displayed in Table 2.20. Two samples of *Patella tenuis tenuis* and *P. aspera* were purchased in local markets in Madeira, being caught in the northern coast of the island (32°51'17.02" N; 17°01'54.02" W). Organisms were transported to the laboratory in refrigerated containers. Samples were frozen at -20 °C, if they were not processed immediately.

Table 2.20 - Sampling Sites and respective geographical coordinates, surveyed during September of 2012 and June and July of 2013.

Date	Location	Sampling Site	Geographic Coordinates
September 2012	Madeira Island	Reis Magos	32°39'16.21" N; 16°49'05.29" W
		Canical	32°44'20.08" N; 16°44'17.55" W
June 2013	São Miguel Island	Cruzeiro	37° 50'31.19" N; 25° 41'33.61" W
		Étar	37°44'19.31" N; 25°39'38.84" W
		São Roque	37°45'15.35" N; 25°38'31.60" W
		Mosteiros	37°53'25.57" N; 25°49'14.72" W
		Lagoa	37°44'42.38" N; 25°19'47" W
		Caloura	37°42'49.34" N; 25°29'54.54" W
July 2013	Morocco Coast	Casablanca corniche	33°36'01.2" N; 7°39'57.5" W
		El Jadida Haras	33°14'42.0" N; 8°28'37.5" W
		El Jadida Sâada	33°14'42.4" N; 8°32'26.9" W
		Sidi Bouzid	33°13'57.1" N; 8°33'20.9" W
		Mrizika	32°57'21.8" N; 8°46'53.2" W
		Oualidia	32°43'55.8" N; 9°02'57.6" W

Reagents

Acetonitrile and methanol were supplied by Panreac (Barcelona, Spain). All solvents employed in this work were high performance liquid chromatography or analytical grade and the water was distilled and passed through a water purification system (Milli-Q, Millipore, Madrid, Spain). Formic acid was purchased from Merck (Darmstadt, Germany). Ammonium formate was from Fluka (Sigma-Aldrich, Madrid, Spain).

A synthetic standard of CTX-3C was provided by Dr. Masahiro Hiram. The methodology applied for the synthesis was described for the first time in 2001 and improved in 2004 [43,44].

Sample extraction

The Otero *et al.* (2010) extraction protocol was followed [45]. The efficiency of the method was studied by analyzing the extracts discarded in each stage of the protocol. Data showed no loss of toxin in each step. The results agree with the efficiency achieved in the method previously described (>95% for P-CTX-1B) [46]. Animals were dissected and homogenized with a blender (A320R1, 700 W, Moulinex, Lisbon, Portugal) in pooled groups in order to obtain 2 g of tissue, with the exception of *Aplysia depilans*, *Charonia lampas*, *Diadema africanum*, *Holothuria (Platyperona) sanctori*, *Marthasterias glacialis*, *Ophidiaster ophidianus*, *Paracentrotus lividus*, *Sphaerechinus granularis*, and *Umbraculum umbraculum*. In these cases, each animal was treated separately since they had enough extractable

biomass. The homogenized tissue was cooked for 20 min at 70 °C, then homogenized with 8 mL of Methanol/Hexane (3:1), sonicated (1 min, 70 Hz, Vibra Cell, Sonic & Materials, Newtown, CT, USA), and subsequently centrifuged at 4000 rpm for 20 min. The upper hexane layer was discarded, and the lower methanol phase was filtered through a 0.45- μ m filter (Millipore Ultrafree-MC centrifugal filter units, Bedford, MA, USA). The resulting filtered was diluted into methanol water (50:50). Thereafter Solid Phase Extraction (SPE) was performed using C18 SPE cartridges (500 mg/3 mL volume from Supelco, Bellefonte, PA, USA). Cartridges were previously conditioned with 4 mL of milliQ water, then samples were loaded and washed with 65% Methanol, and finally samples were eluted in 80% Methanol. Thereafter, samples were mixed with 4.2 mL of 1 M NaCl and 6.7 mL of Chloroform and centrifuged for 4 min at 2000 rpm (Centrifugal-Legend RT, Sorvall, Waltham, MA, USA). The upper methanolic layer was discarded and the lower organic layer was evaporated to dryness in a rotary evaporator (Büchi, Flawil, Switzerland) and dissolved in 4 mL chloroform. For reducing matrix interference, another cleanup procedure was done with Silica Sep-Pak cartridges (Waters, Milford, CT, USA). After loading the sample cartridges were conditioned with chloroform, samples were washed with chloroform and eluted 90% of chloroform. Extract was concentrated to dryness and then re-suspended in methanol. In Figure 2.30 is displayed the totality of the purification procedure. Before UPLC-MS analysis, positive samples were confirmed and the exact mass was obtained by UPLC-MS-IT-TOF.

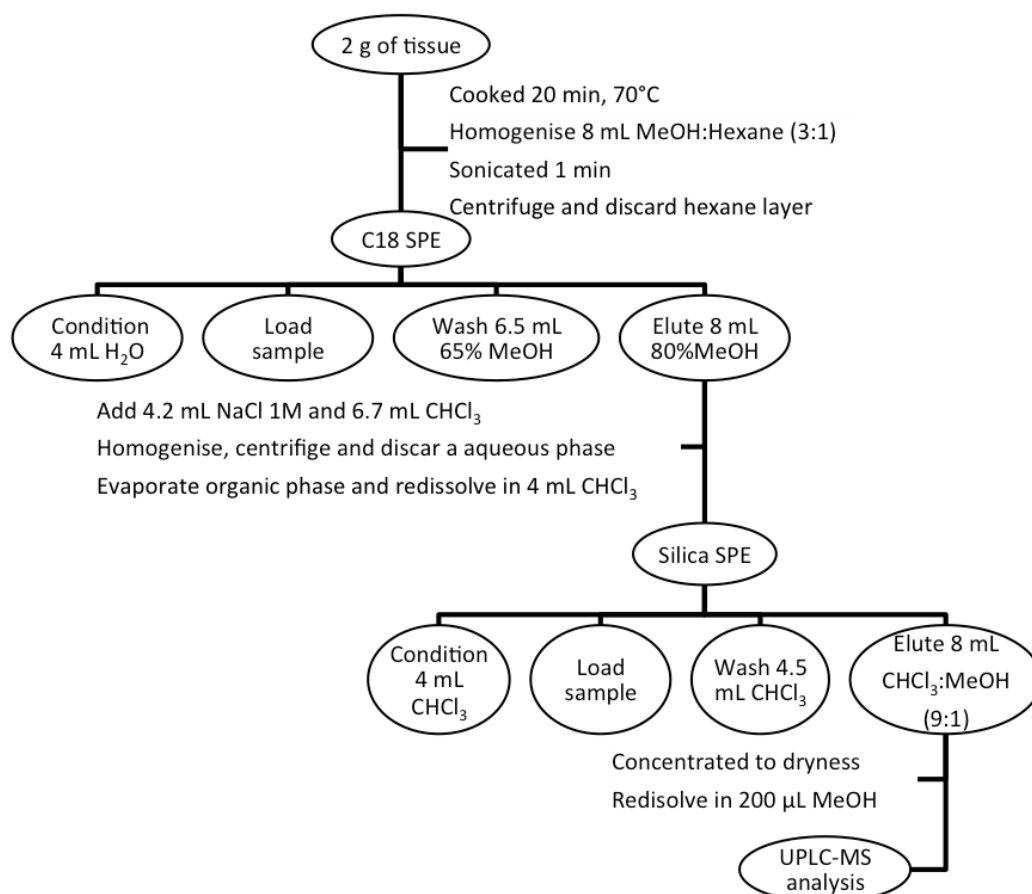


Figure 2.30- CTX purification scheme.

UPLC-MS conditions

For the analysis, a 1290 Infinity ultra-high-performance liquid chromatography (UHPLC) system coupled to a 6460 Triple Quadrupole mass spectrometer (both Agilent Technologies, Waldbronn, Germany) was used. Chromatographic separation was performed at 35 °C, the injection volume was 5 µL and flow rate of 0.4 mL/min using a column ACQUITY UPLC BEH C18 (2.1 × 100 mm, 1.7 µm, Waters, Manchester, UK). The nitrogen generator is a Nitrocraft NCLC/MS from Air Liquid (Madrid, Spain). Mobile phases A and B were water and acetonitrile:water (95:5), respectively, both acidified with 50 mM formic acid and 2mM ammonium formate. Chromatographic separation was performed by gradient elution starting with 50% B for 2.5 min, then increasing to 100% B for 4.5 min., this condition was hold for 4.5 min and reducing afterward to 50% B over 0.1 min. This proportion was maintained for 2.4 min, until the next injection to equilibrate the system. The electrospray (ESI) source of 6460 mass spectrometer was operated with the following values of source-dependent parameters: gas temperature, 350 °C; gas flow, 8 L/min; sheath gas temperature, 400 °C; sheath gas flow, 11 L/min, nebulizer, 45 psi; capillary voltage, and 4000 V; and nozzle voltage 0 V. All analyses were performed in MS scan and selected ion monitoring (SIM).

For CTX-3C standard, a six-point calibration curve among the range 37.5-600 ng/mL was done. The limit of detection (LOD) was 1.125 µg/Kg and a limit of quantification (LOQ) was 3.75 µg/Kg.

UPLC-MS-IT-TOF conditions

The UPLC system, from Shimadzu (Kyoto, Japan) consists of two pumps (LC-30AD), autoinjector (SIL-10AC) with refrigerated rack, degasser (DGU-20A), column oven (CTO-10AS) and a system controller (SCL-10Avp). The system is coupled to an IT-TOF-MS system with an electrospray ionization (ESI) interface (Shimadzu, Kyoto, Japan). The nitrogen generator is a Nitrocraft NCLC/MS from Air Liquid (Spain). The separation was performed with an ACQUITY UPLC Phenyl-Hexyl column (2.1 × 100 mm, 1.7 µm particle size, Waters, Spain). Mobile phases A and B were water and acetonitrile:water (95:5), respectively, both acidified with 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation was performed by gradient elution starting with 50% B for 2.5 min, then increasing to 100% B for 4.5 min., this condition was hold for 4.5 min and reduced afterward to 50% B over 0.1 min. This proportion was maintained 2.4 min. until the next injection to equilibrate the system. The mobile phase flow rate was 0.4 mL/min, the injection volume was 5 µL and the temperature was maintained at 35 °C. The MS method was operated in positive mode with the following ESI source conditions: nebulizing gas flow, 1.5 L/min, heat block temperature and CDL temperature, 200 °C and detector voltage, and 1.65 kV. The molecules were analyzed using an ion accumulation time of 10 ms.

Statistical Analyzes

The influence of the factors Sampling site (Morocco, Madeira and Azores islands) and organism type in the CTX occurrence was analyzed using Generalized Linear Models (GLZ). The dependent variable was the concentration of CTX in the organisms flesh ($\mu\text{g CTX-3C equivalents/Kg}$). Because only the starfish yielded positive results, primarily, we performed an analysis considering only the starfish data, using sampling site as factor. The dataset consists of CTX concentrations found in individual samples from each organism. There is one single data per pooled sample, being the pools constituted by similar numbers of organisms. The data set could be considered as a zero—inflated dataset, with variance larger than the mean. These models are usually handled with Poisson or negative binomial distributions [47]; however, we can use neither of these distribution because our data are continuous. Instead, we used the approach of gamma hurdle models [48], which performs the analysis in two steps: first, the analysis of presence/absence of the toxin (managed with a binomial or negative binomial distribution) and second, on these data showing positive concentrations of CTX, a GLZ with gamma distribution. Besides the analysis including sampling site as factor, we performed another analysis considering starfish species as factor. All the models were performed with R software [49], package stats (), function glm.

Conclusions

The primary aim of this work was to search for new vectors for CTX in the Portuguese islands and the northwestern coast of Morocco using UPLC-IT-TOF-MS and UPLC-MS/MS techniques. From 22 surveyed species, we detected CTX in two species of starfish: *M. glacialis* and *O. ophidianus*. In addition, the quantifiable results were all in Portuguese territory, being São Miguel Island (Azores), the location with greater propensity to find these biotoxin groups. This is an important finding since it is, to the best of our knowledge, the first report of these toxins in echinoderms ever. We detected three analogues, CTX-1B and two unnamed derivatives, in concentrations that ranged from 4.40 to 124.04 $\mu\text{g CTX-3C equivalents/Kg fw}$, especially in the red starfish, *O. ophidianus*. Since this species has detritivorous feeding habits, this could be the main explanation for considerable difference with regard to accumulated amounts, comparing to the predatory spiny starfish (*M. glacialis*). Regarding Ciguatera monitoring, starfish present themselves as a good alternative, though more studies should be done in order to understand correlation of CTX uptake between echinoderms and predatory fish, to better evaluate human health risk.

Acknowledgments

This research was partially funded by the FCT project UID/Multi/04423/2013 and by the projects MARBIOTECH (reference NORTE-07-0124-FEDER-000047) within the SR&TD Integrated Program

MARVALOR—Building research and innovation capacity for improved management and valorization of marine resources, supported by the Programa Operacional Regional do Norte (ON.2-O Novo Norte) and NOVOMAR (reference 0687-NOVOMAR-1-P), supported by the European Regional Development Fund. M.S. also acknowledges FCT for the grant SFRH/BD/73269/2010 and Ana Regueiras, Isadora Diniz, Afonso Prestes and Manuela Maranhão. This research was also partially funded by the FCT-Portugal/CNRST-Morocco Cooperation Convention under the project 1006/13 CNR “Marine emergent toxins in the north east Atlantic (Portugal-Morocco) produced by microalgae and bacteria”. I. R. was supported by a fellowship from Subprograma de Formación de Personal Investigador (AGL2012-40185-CO2-01), Spain. The research leading to these results has received funding from the following FEDER cofunded-grants. From CDTI and Technological Funds, supported by Spanish Ministerio de Economía y Competitividad, AGL2012-40185-CO2-01, AGL2014-58210-R, and Consellería de Cultura, Educación e Ordenación Universitaria, GRC2013-016 (Galicia) Spain.

Author Contributions

M.S. and V.V. conceived the idea, M.S. performed the sampling, sample analyzes and paper writing. I.R. performed the LC-MS/MS and IT-TOF analysis. A.B. collaborated in sample analyzes. A.A. contributed on experimental design. M.H. collaborated in sample collection. V.V. and L.B. contributed in funding, materials and analyses tools. V.V., B.S., A.I.N., and M.K. collaborated in the collections and provided sampling and laboratory facilities. All authors participated in proof reading of the manuscript.

Conflicts of Interest

State any potential conflicts of interest here or “The authors declare no conflict of interest”.

References and Notes

1. Alexander, J.; Benford, D.; Boobis, A.; Ceccatelli, S.; Cravedi, J.P.; di Domenico, A.; Doerge, D.; Dogliotti, E.; Edler, L.; Farmer, P.; *et al.* Efsa panel on contaminants in the food chain; scientific opinion on marine biotoxins in shellfish—Emerging toxins: Ciguatoxin group. *EFSA J.* **2010**, *1627*, 1–38.
2. Murata, M.; Legrand, A.M.; Ishibashi, Y.; Yasumoto, T. Structures of ciguatoxin and its congener. *J. Am. Chem. Soc.* **1989**, *111*, 8929–8931.

3. Murata, M.; Legrand, A.M.; Ishibashi, Y.; Fukui, M.; Yasumoto, T. Structures and configurations of ciguatoxin from the *Moray eel Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. *J. Am. Chem. Soc.* **1990**, *112*, 4380–4386.
4. Lehane, L.; Lewis, R.J. Ciguatera: Recent advances but the risk remains. *Int. J. Food Microbiol.* **2000**, *61*, 91–125.
5. Lewis, R.J.; Sellin, M.; Poli, M.A.; Norton, R.S.; MacLeod, J.K.; Sheil, M.M. Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, muraenidae). *Toxicon* **1991**, *29*, 1115–1127.
6. Lewis, R.J.; Vernoux, J.P.; Brereton, I.M. Structure of caribbean ciguatoxin isolated from *Caranx latus*. *J. Am. Chem. Soc.* **1998**, *120*, 5914–5920.
7. Pottier, I.; Vernoux, J.P.; Jones, A.; Lewis, R.J. Characterization of multiple caribbean ciguatoxins and congeners in individual specimens of horse-eye jack (*Caranx latus*) by high performance liquid chromatography/mass spectrometry. *Toxicon* **2002**, *40*, 929–939.
8. Vernoux, J.P.; Lewis, R.J. Isolation and characterisation of caribbean ciguatoxins from the horse-eye jack (*Caranx latus*). *Toxicon* **1997**, *35*, 889–900.
9. Satake, M.; Fukui, M.; Legrand, A.M.; Cruchet, P.; Yasumoto, T. Isolation and structures of new ciguatoxin analogs, 2,3-dihydroxyctx3c and 51-hydroxyctx3c, accumulated in tropical reef fish. *Tetrahedron Lett.* **1998**, *39*, 1197–1198.
10. Satake, M.; Ishibashi, Y.; Legrand, A.M.; Yasumoto, T. Isolation and structure of ciguatoxin-4a, a new ciguatoxin precursor, from cultures of dinoflagellate *Gambierdiscus toxicus* and parrotfish *Scarus gibbus*. *Biosci. Biotechnol. Biochem.* **1997**, *60*, 2103–2105.
11. Satake, M.; Murata, M.; Yasumoto, T. Gambierol: A new toxic polyether compound isolated from the marine dinoflagellate *Gambierdiscus toxicus*. *J. Am. Chem. Soc.* **1993**, *115*, 361–362.
12. Satake, M.; Murata, M.; Yasumoto, T. The structure of CTX3C, a ciguatoxin congener isolated from cultured *Gambierdiscus toxicus*. *Tetrahedron Lett.* **1993**, *34*, 1975–1978.
13. Holland, W.C.; Litaker, R.W.; Tomas, C.R.; Kibler, S.R.; Place, A.R.; Davenport, E.D.; Tester, P.A. Differences in the toxicity of six gambierdiscus (dinophyceae) species measured using an *in vitro*

human erythrocyte lysis assay. *Toxicon* **2013**, *65*, 15–33.

14. Lu, X.Z.; Deckey, R.; Jiao, G.L.; Ren, H.F.; Li, M. Caribbean maitotoxin elevates $[Ca^{2+}]_i$ and

activates non-selective cation channels in HIT-T15 cells. *World J. Diabetes* **2013**, *4*, 70–75.

Toxins **2015**, *7* 3755

15. Pottier, I.; Vernoux, J.P.; Jones, A.; Lewis, R.J., Analysis of toxin profiles in three different fish

species causing ciguatera fish poisoning in guadalupe, french west indies. *Food Addit. Contam.* **2002**, *19*, 1034–1042.

16. Friedman, M.A.; Fleming, L.E.; Fernandez, M.; Bienfang, P.; Schrank, K.; Dickey, R.; Bottein, M.Y.;

Backer, L.; Ayyar, R.; Weisman, R.; *et al.* Ciguatera fish poisoning: Treatment, prevention and management. *Mar. Drugs* **2008**, *6*, 456–479.

17. De Fouw, J.C.; van Egmond, H.P.; Speijers, G.J.A. Ciguatera Fish Poisoning: A Review. Available

online: <http://www.rivm.nl/bibliotheek/rapporten/388802021.pdf> (accessed on 17 December 2010).

18. Bentur, Y.; Spanier, E. Ciguatoxin-like substances in edible fish on the eastern mediterranean.

Clin. Toxicol. **2007**, *45*, 695–700.

19. Hamilton, B.; Hurbungs, M.; Jones, A.; Lewis, R.J. Multiple ciguatoxins present in indian ocean

reef fish. *Toxicon* **2002**, *40*, 1347–1353.

20. Fda. Fish and Fishery Products Hazards and Controls Guidance. 2011. Available online: [http://www.Fda.Gov/downloads/food/guidancecomplianceregulatoryinformation/guidancedocuments/](http://www.Fda.Gov/downloads/food/guidancecomplianceregulatoryinformation/guidancedocuments/seafood/ucm251970.Pdf)

[seafood/ucm251970.Pdf](http://www.Fda.Gov/downloads/food/guidancecomplianceregulatoryinformation/guidancedocuments/seafood/ucm251970.Pdf) (accessed on 12 March 2013).

21. Regulation (ec) No 854/2004 of the european parliament and of the council of 29 April 2004 laying

down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Oj L 139, 30.4.2004, pp. 206–320. EURO-LEX; Access to European Law.

Available online: <http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32007R1246> (accessed on 18 June 2015).

22. Cdc (centers for disease control and prevention), 2009. Cluster of ciguatera fish poisoning-north

carolina, 2007. Morbidity and mortality weekly report (mmwr), *58*, 283–285. Available from

- <http://www.Cdc.Gov/mmwr/pdf/wk/mm5811.Pdf>. Centers of Disease Control and Prevention. Available online: <http://www.cdc.gov/> (accessed on 18 June 2015).
23. Carefoot, T.H. Aplysia: Its biology and ecology. *Oceanogr. Mar. Biol. Ann. Rev.* **1987**, *25*, 167–284.
24. Bulleri, F.; Benedetti-Cecchi, L.; Cinelli, F. Grazing by the sea urchins *Arbacia lixula* L. and *Paracentrotus lividus* Lam. in the northwest Mediterranean. *J. Exp. Mar. Biol. Ecol.* **1999**, *241*, 81–85.
25. Lin, S.J.; Hwang, D.F. Possible source of tetrodotoxin in the starfish *Astropecten scoparius*. *Toxicon* **2001**, *39*, 573–579.
26. Nicolaidou, A.; Nott, J.A. The role of the marine gastropod *Cerithium vulgatum* in the biogeochemical cycling of metals. *Biogeochem. Cycl. Sediment Ecol.* **1999**, *59*, 137–146.
27. Rodríguez, A.; Hernández, J.C.; Clemente, S.; Coppard, S.E. A new species of diadema (Echinodermata: Echinoidea: Diadematidae) from the eastern Atlantic Ocean and a neotype designation of *Diadema antillarum* (Philippi, 1845). *Zootaxa* **2013**, *3636*, 144–170.
28. Ferguson, J.C. Feeding activity in *Echinaster* and its induction with dissolved nutrients. *Biol. Bull.* **1969**, *136*, 374–384.
29. Crothers, J.H. Common topshells: An introduction to the biology of *Osilinus lineatus* with notes on other species in the genus. *Field Stud.* **2001**, *10*, 115–160.
30. Navarro, P.G.; García-Sanz, S.; Barrio, J.M.; Tuya, F. Feeding and movement patterns of the sea cucumber *Holothuria sanctori*. *Mar. Biol.* **2013**, *160*, 2957–2966. *Toxins* **2015**, *7*, 3756
31. Toral-Granda, V.; Lovatelli, A.; Vasconcellos, M. Scientific committee composed of Conand C., Hamel J.F., Mercier A., Purcell S. and Uthicke S. In Proceedings of the International Workshop on the Sustainable Use and Management of Sea Cucumber Fisheries, SPC Beche-deMer Information Bulletin, Puerto Ayora, Galapagos Islands, Ecuador, 19–23 November 2008.
32. Knox, G.A. Hard shores. In *The Ecology of Seashores*; Kennish, M.J., Ed.; CRC Press: Boca Raton, FL, USA, 2001; pp. 20–86.

33. Buschbaum, C.; Dittmann, S.; Hong, J.S.; Hwang, I.; Strasser, M.; Thiel, M.; Valdivia, N.; Yoon, S.;
Reise, K. Mytilid mussels: Global habitat engineers in coastal sediments. *Helgol. Mar. Res.* **2008**,
63, 47–58.
34. Dayrat, B. Review of the current knowledge of the systematics of onchidiidae
(mollusca:Gastropoda:Pulmonata) with a checklist of nominal species. *Zootaxa* **2009**, *2068*, 1–
26.
35. Lemée, R.; Boudouresque, C.F.; Gobert, J.; Malestroit, P.; Mari, X.; Meinesz, A.; Menager,
V.;
Ruitton, S. Feeding behaviour of *paracentrotus lividus* in the presence of *caulerpa taxifolia*
introduced in the mediterranean sea. *Oceanol. Acta* **1995**, *19*, 245–253.
36. Martinez-Pita, I.; Sanches-Espana, A.; Garcia, F.J. Gonadal growth and reproduction in the
sea
urchin *Sphaerechinus granularis* (lamarck 1816) in southern spain. *Sci. Mar.* **2008**, *72*, 603–611.
37. Valdes, A. On the publication data, authorship, and type species of *Umbraculum* and
Tylodina
gastropoda; opisthobranchia; tylodinoidea. *Nautilus* **2001**, *115*, 29–34.
38. Ramírez, R.; Tuya, F.; Haroun, R.J. Spatial patterns in the population structure of the whelk
Stramonita haemastoma (linnaeus, 1766) (gastropoda: Muricidae) in the canarian archipelago
(eastern atlantic). *Sci. Mar.* **2009**, *73*, 431–437.
39. Llewellyn, L.E. Revisiting the association between sea surface temperature and the
epidemiology
of fish poisoning in the south pacific: Reassessing the link between ciguatera and climate
change.
Toxicon **2010**, *56*, 691–697.
40. Chinain, M.; Germain, M.; Deparis, X.; Pauillac, S.; Legrand, A.M. Seasonal abundance and
toxicity of the dinoflagellate *Gambierdiscus* spp. (dinophyceae), the causative agent of ciguatera
in
tahiti, french polynesia. *Mar. Biol.* **1999**, *135*, 259–267.
41. Ashton, M.; Tosteson, T.; Tosteson, C. The effect of elevated temperature on the toxicity of
the laboratory cultured dinoflagellate *Ostreopsis lenticularis* (dinophyceae). *J. Trop. Biol.* **2003**,
51,
1–6.
42. Matsumoto, T.; Nagashima, Y.; Kusuhara, H.; Sugiyama, Y.; Ishizaki, S.; Shimakura, K.;
Shiomi, K.
Involvement of carrier-mediated transport system in uptake of tetrodotoxin into liver tissue slices
of puffer fish *Takifugu rubripes*. *Toxicon* **2007**, *50*, 173–179.

43. Hirama, M.; Oishi, T.; Uehara, H.; Inoue, M.; Maruyama, M.; Oguri, H.; Satake, M. Total synthesis of ciguatoxin CTX3C. *Science* **2001**, *294*, 1904–1907.
44. Inoue, M.; Hirama, M., Evolution of a practical total synthesis of ciguatoxin CTX3C. *Account. Chem. Res.* **2004**, *37*, 961–968.
45. Otero, P.; Lopez, S.P.; Alfonso, A.; Vale, C.; Rodríguez, P.; Gouveia, N.N.; Gouveia, N.; Delgado, J.; Vale, P.; Hirama, M.; *et al.* First toxin profile of ciguateric fish in madeira arquipelago (europe). *Anal. Chem.* **2010**, *82*, 6032–6039.
46. Lewis, R.J.; Yang, A.; Jones, A. Rapid extraction combined with lc-tandem mass spectrometry (crem-lc/ms/ms) for the determination of ciguatoxins in ciguateric fish flesh. *Toxicon* **2009**, *54*, 62–66.
Toxins **2015**, *7* 3757
47. Zuur, A.F.; Ieno, E.N.; Walker, N.J.; Saveliev, A.A.; Smith, G.M. *Mixed Effects Models and Extensions in Ecology with R*; Springer Science: New York, NY, USA, 2009.
48. Frees, E.W. *Regression Modeling with Actuarial and Financial Applications*; Cambridge University Press: New York, NY, USA, 2011.
49. R Core Team. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2014. Available online: <http://www.R-project.org/> (accessed on 2 June 2015).

Third Section

Chapter 4. General Discussion

General discussion

In this thesis were screened several groups of legislated and emergent toxins, analyzing a total of 33 different species of benthic organisms in the Portuguese coast, islands and northwestern Moroccan coast (Table 3.1). Our prime aim was to search for new vectors of these toxins, but also to try to unravel geographical patterns and the prevalence of emergent toxins in temperate waters.

Table 3.1. Tested species versus detected toxins (TTX- Tetrodotoxin; PSTs – Paralytic Shellfish toxins; OA- Okadaic Acid; CI – Cyclic Imines; CTX- Ciguatoxins).

Toxin / Species	TTX	PSTs	OA	CI	CTX
<i>Monodonta lineata</i>	X	X	X	X	
<i>M. turbinata</i>		X	X	X	
<i>Gibbula umbilicalis</i>	X		X	X	
<i>Nucella lapillus</i>			X	X	
<i>Patella intermedia</i>				X	
<i>Patella spp.</i>			X		
<i>Onchidella celtica</i>			X		
<i>Charonia lampas</i>	X				
<i>Aplysia depilans</i>		X	X		
<i>Stramonita haemostoma</i>			X		
<i>Marthasterias glacialis</i>		X	X	X	X
<i>Ophidiaster ophidianus</i>			X		X
<i>Echinaster sepositus</i>			X		

<i>Astropecten</i>		X
<i>aranciacus</i>		
<i>Paracentrotus</i>	X	X
<i>lividus</i>		
<i>Arbacia lixula</i>		X
<i>Holothuria</i>		X
<i>sanctori</i>		

Emergent toxins pose a challenge to monitoring programs and legislation in temperate waters (Paper 1), for being a novelty in these ecosystems, therefore is pertinent that international collaboration occur to obtain reference material to assist in optimizing screening methods (analytical and molecular), so that the detection is fast and reliable. Collaboration with the Health sector is relevant not only to provide medical staff awareness and information, but also to implement epidemiologic studies, to understand the degree of impact of these biotoxins in coastal areas. It is also relevant, the adjustment of good monitoring practices and updated legislation for better protection of consumers, since these emergent toxins have higher potential for lethality in comparison with legislated ones, (with exception of the CI group who needs further research regarding this issue).

The optimization of detection methods is crucial regarding biotoxins monitoring, since they have huge impact in human economy and health. In this thesis, we worked with several different matrices which posed some analytical challenges (Paper 2), more precisely, due to overlapping phenomena between toxin peaks and matrix ones. The method, PCOX, is extremely sensitive, and toxin free matrix is used to prepare the calibration solution to overcome the matrix effect and also to facilitate data interpretation. Two matrices, gastropod and starfish, cloaked the GTX1,4 peak. This issue was surpassed by refinement of oxidation technique of conversion of these toxins into NEO to be able to quantify reliably the masked toxins. We present a faster and economical protocol that facilitates the interpretation and quantification o PSPs in new matrices.

In this thesis several monitoring surveys were performed, in order to screen emergent and legislated toxins:

- Tetrodotoxin (TTX) is potent marine neurotoxin, typical from warm ecosystems, has probable bacterial origin and was reported in several taxa not close related [1-3]. Since the opening of the Suez Canal the majority of poisoning incidents

occurred in Asia, were Fugo (puffer fish), a TTX-bearer, is considered a delicacy. The opening of this artificial corridor provided the migration of toxic alien species from the Red Sea to the Mediterranean, raising the number of poisoning incidents in that region, the majority of them caused by misidentification of the alien specimens like the elongated puffer (*Lagocephalus sceleratus*) [4,5]. TTX was reported for the first time in October 2008 in the temperate waters of the Atlantic, through a nonfatal poisoning incident, caused by ingestion of a trumped shell (*Charonia lampas*) harvested in the southern Portuguese Coast [6].

- In Paper 3, thirteen sampling sites along the Portuguese Coast were surveyed between the September 2009 and November of 2010, and 14 benthic species were screened for this group of toxins. As a result of this survey we detected TTX and its analogs in three species with concentrations ranging between 0.006 to 0.090 µg/g. Apart from the low concentration detected, compared with the values detected by Rodriguez et al. 2008 [6] (highest value detected 1004.00 µg/g fw) the major novelties of this work were:
- The report of this group of toxins in two new species of gastropods (*G. umbilicalis* and *M. lineata*), and also the most northern point of detection of TTX plus derivatives till date in the temperate waters of the Atlantic Ocean. . Also, since TTX has probable bacterial origin, bivalves could not be the most effective choice as indicator species for monitoring purposes, since the producers of this emergent toxin are not suspended in the water column. This is a major issue, since there's a lack of safety measures regarding this biotoxins, the only guiding value belongs to the Japanese Ministry of Health and Welfare [7]. This kind of studies should be continued and encouraged to gather more information for better protect human health.
- The Saxitoxin group (PSTs) as well as the Okadaic Acid (OA) group, are legislated biotoxins and due to established monitoring practices and legislation, poisoning incidents have been scarce in the last two decades [8-10]. In this work (Paper 4 and 5) data showed a total of 16 new vectors: for PSTs in *G. umbilicalis*, *N. lapillus*, *Monodonta sp.*, *A. depilans*, *P. lividus* and *M. glacialis*; for OA in *G. umbilicalis*, *N. lapillus*, *Monodonta sp.*, *P. lividus* and *M. glacialis*, *A. aranciacus*, *A. lixula*, *A. depilans*, *E. sepositus*, *H. sanctori*, *O. celtica*, *O. ophidianus*, *Patella spp.* and *S. haemostoma*; data also showed that OA can be a bioaccumulation phenomenon along the food-chain.

Though for PSTs concentrations detected were all below the limit value established by EFSA (800 ng SXT equivalents/g shellfish meat) [10], the toxin profile detected was wider with prevalence of more toxic analogs comparing to recent work published till that date [11].

Our work regarding legislated phycotoxins showed that monitoring these toxins based in bivalves alone can be reductive and misleading, since there are evidences of bioaccumulation along the food-chain. We hope that our work can encourage more studies to better understand the trophic chain transfer phenomenon to assess the human health risks.

The Cyclic Imine (CI) group was also surveyed in this thesis, composed by several phycotoxins characterized by the presence of a cyclic imine group responsible for its neurotoxicity [12]. Though these biotoxins have acute neurotoxicity by oral administration in MBA, poisoning incidents were not recorded till date [12-14]. Albeit all the efforts for the refinement and optimization of methods of detection regarding this group [15,16], there is a need of epidemiological studies to learn about the potential chronic toxicity of these phycotoxins, in order to establish good monitoring practices and legislation.

The major contribute of this work was the report of CIs for the first time in the Portuguese Coast in 5 benthic species: *G. umbilicalis*, *N. lapillus*, *Monodonta sp.*, *M. glacialis* and *P. intermedia*. Concentrations detected ranged from 0.49 ng/g fw and 3.86 ng/g fw for 13-desmethyl-SPX C, we hope that our data aids bridging the lack of toxicological data respecting this group.

Ciguatoxins (CTXs) are responsible for the Ciguatera fish poisoning syndrome, caused after the ingestion of top level predatory fish [17]. These toxins are common in warm ecosystems and the first report of them in the temperate waters of the Atlantic remarks the year of 2010 [18]. In our work (Paper 6) we surveyed two Portuguese Islands (Madeira and São Miguel (Azores archipelago)) and the Moroccan northwestern coast in a total of 14 sampling points for 22 benthic species. Our results show the detection of three CTXs analogs in two starfish species: *M. glacialis* and *O. ophidianus* in the Portuguese islands. Average concentrations detected ranged from: 4.40 µg CTX-3C equivalents/Kg fw, to 124.04 µg CTX-3C equivalents/Kg fw. This discovery represents a major advance towards CTXs knowledge, since so far was thought that this group of toxins had finfish as its only vectors. Also, echinoderms present themselves as fine candidates for CTXs monitoring programs, since bivalves are not common in the oligotrophic waters of the Portuguese archipelagos.

Our work show that there is much to be done to unravel the dynamics and human health risk assessment regarding emerging toxins. Also, the legislation should be updated with respect to monitored toxins, since new vectors have been reported and monitoring practices based on bivalve mollusks alone may underestimate the real risk to public health.

References

1. Miyazawa, K.; Noguchi, T., Distribution and origin of tetrodotoxin. *Journal of Toxicology - Toxin Reviews* **2001**, *20*, 11-33.
2. Hwang, D.F.; Arakawa, O.; Saito, T.; Noguchi, T.; Simidu, U.; Tsukamoto, K.; Shida, Y.; Hashimoto, K., Tetrodotoxin-producing bacteria from the blue-ringed octopus *octopus maculosus*. *Marine Biology* **1989**, *100*, 327-332.
3. Noguchi, T.; Hwang, D.F.; Arakawa, O.; Sugita, H.; Deguchi, Y.; Shida, Y.; Hashimoto, K., *Vibrio alginolyticus*, a tetrodotoxin-producing bacterium, in the intestines of the fish *fugu vermicularis vermicularis*. *Marine Biology* **1987**, *94*, 625–630.
4. Katikou, P.; Georgantelis, D.; Sinouris, N.; Petsi, A.; Fotaras, T., First report on toxicity assessment of the lessepsian migrant pufferfish *lagocephalus sceleratus* (gmelin, 1789) from european waters (aegean sea, greece). *Toxicon* **2009**, *54*, 50-55.
5. Bentur, Y.; Ashkar, J.; Lurie, Y.; Levy, Y.; Azzam, Z.S.; Litmanovich, M.; Golik, M.; Gurevych, B.; Golani, D.; Eisenman, A., Lessepsian migration and tetrodotoxin poisoning due to *lagocephalus sceleratus* in the eastern mediterranean. *Toxicon* **2008**, *52*, 964-968.
6. Rodriguez, P.; Alfonso, A.; Vale, C.; Alfonso, C.; Vale, P.; Tellez, A.; Botana, L.M., First toxicity report of tetrodotoxin and 5,6,11-trideoxyttx in the trumpet shell *charonia lampas lampas* in europe. *Analytical Chemistry* **2008**, *80*, 5622–5629.
7. Kaku, N.; Meier, J., Clinical toxicology of fugu poisoning. In *Handbook of clinical toxicology of animal venoms and poisons*, Meier, J.; White, J., Eds. CRC: Boca Raton, FL, USA, 1995; pp 75-83.
8. Regulation (ec) no 854/2004 of the european parliament and of the council of 29 april 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Oj L 139, 30.4.2004, p. 206–320 (18 june 2015) <http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32007R1246>
9. Alexander, J.; Auðunsson, G.A.; Benford, D.; Cockburn, A.; Cravedi, J.P.; Dogliotti, E.; Di Domenico, A.; Fernández-Cruz, M.L.; Fink-Gremmels, J.; Fürst, P., *et al.*, Opinion of the scientific panel on contaminants in the food chain on a request from the european commission on marine biotoxins in shellfish – okadaic acid and analogues. *The EFSA Journal* **2008**, *589*, 1-62.
10. Alexander, J.; Auðunsson, G.A.; Benford, D.; Cockburn, A.; Cravedi, J.P.; Dogliotti, E.; Di Domenico, A.; Fernández-Cruz, M.L.; Fink-Gremmels, J.; Fürst, P., *et al.*, Scientific opinion of the panel on contaminants in the food chain on a request from the european commission on marine biotoxins in shellfish – saxitoxin group. *The EFSA Journal* **2009**, *1019*, 1-76.

11. Rodrigues, S.M.; de Carvalho, M.; Mestre, T.; Ferreira, J.J.; Coelho, M.; Peralta, R.; Vale, P., Paralytic shellfish poisoning due to ingestion of gymnodinium catenatum contaminated cockles—application of the aoac hplc official method. *Toxicon* **2012**, *59*, 558-566.
12. Munday, R., Toxicology of cyclic imines: Gymnodimine, spirolides, pinnatoxins, pteriatoxins, prorocentrolide, spiro-prorocentrimine, and symbioimines. In *Seafood and freshwater toxins: Pharmacology, physiology and detection*, 2 ed.; Botana, L.M., Ed. CRC Press (Taylor and Francys Group): Boca Raton, FL, USA., 2008; Vol. 173, pp 581–594.
13. Munday, R.; Selwood, A.I.; Rhodes, L., Acute toxicity of pinnatoxins e, f and g to mice. *Toxicon* **2012**, *60*, 995-999.
14. Rhodes, L.; Smith, K.; Selwood, A.; McNabb, P.; van Ginkel, R.; Holland, P.; Munday, R., Production of pinnatoxins by a peridinoid dinoflagellate isolated from northland, new zealand. *Harmful Algae* **2010**, *9*, 384–389.
15. Otero, P.; Alfonso, A.; Alfonso, C.; Vieytes, M.R.; Louzao, M.C.; Botana, A.M.; Botana, L.M., New protocol to obtain spirolides from *alexandrium ostenfeldii* cultures with high recovery and purity. *Biomedical Chromatography* **2010**.
16. Otero, P.; Alfonso, A.; Alfonso, C.; Araoz, R.; Molgo, J.; Vieytes, M.R.; M., B.L., First direct fluorescence polarization assay for the detection and quantification of spirolides in mussel samples. *Analytica Chimica Acta* **2011**, *701*, 200-208.
17. Pottier, I.; Vernoux, J.P.; Jones, A.; Lewis, R.J., Analysis of toxin profiles in three different fish species causing ciguatera fish poisoning in guadalupe, french west indies. *Food Additives & Contaminants* **2002**, *19*, 1034-1042.
18. Otero, P.; Pérez, S.; Alfonso, A.; Vale, C.; Rodríguez, P.; Gouveia, N.N.; Gouveia, N., Delgado, J., Vale, P., Hiram, M., Ishihara, Y., Molgó, J., Botana, L.M., First toxin profile of ciguateric fish in madeira archipelago (europe). *Analytical Chemistry* **2010**, *82*, 6032-6039.

General Conclusions

The data showed in this work indicates that regarding monitoring procedures when it comes to an indicator species, the evaluation of human health risk based only in bivalves is reductive. Of a total of 33 screened vectors, the major conclusions are:

- TTX and its derivatives were detected for the first time in two endemic gastropod species from the Portuguese Coast: *Gibbulla umbilicalis* and *Monodonta lineata*.
- The most northern report of TTX in the Atlantic ocean till that date was Angeiras and concentrations ranged between 6.22 to 90.50 ng/g.
- Six new vectors were reported for the Saxitoxin group, four in gastropods (*G. umbilicalis*, *N. lapillus*, *Monodonta sp.* and *A. depilans*) and two echinoderms (*P. lividus*, *M. glacialis*), all detected values were below the limit value recommended by EFSA.
- We optimized the protocol for PSTs detection, overcoming the challenge of overlapping toxin peaks with natural fluorescent matrices.
- We reported fourteen new vectors for OA in: *G. umbilicalis*, *N. lapillus*, *Monodonta sp.*, *Patella spp.*, *S. haemostoma*, *O. celtica*, *P. lividus*, *M. glacialis*, *A. aranciacus*, *A. lixula*, *A. depilans*, *E. sepositus*, *H. sanctori*, *O. ophidianus*.
- Detected values ranged from 0.37 ng/g to 429.41 ng/g, being the highest concentration detected in *N. lapillus* a gastropod predator of mussels.
- OA results suggest that the biomagnification phenomenon along the trophic chain occurs and legislation and monitoring practices should be revised and updated.
- In this work we report the first detection of CIs in the Portuguese Coast, concentrations ranged between 0.49 ng/g fw and 3.86 ng/g fw, and five new vectors were detected: *G. umbilicalis*, *N. lapillus*, *Monodonta sp.*, *M. glacialis* and *P. intermedia*.
- Ciguatoxins were detected for the first time in echinoderms, in two species of starfish (*O. ophidianus* and *M. glacialis*) with concentrations ranging from: 4.40 ng/g fw and 124.04 ng/g fw.
- Starfish represent themselves as a potential indicator species for CTXs monitoring though more studies have to be made in order to understand the correlation between echinoderms and predatory fish to better assess human health risk.

A total of 29 new vectors were detected, for emergent toxins and legislated ones in several species of benthic organisms. This results show the importance of these kind of

studies, data show changes in the ecosystems. New toxins are entering the food-chain, giving extra defenses to benthic organisms, and still we don't know the consequences for the ecosystems and human health. New toxin routes, especially edible ones show that monitoring routines based only in bivalves are inefficient and don't calculate the real risk for human health.

We hope that our contribution is the beginning to develop tighter monitoring routines and most importantly to help in drafting new legislation for the protection of public health.