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Marine toxins in Mozambique: The first approach to public health risk assessment

Isidro José Tamele

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INSTITUTO CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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MARINE TOXINS IN MOZAMBIQUE: THE FIRST APPROACH TO PUBLIC HEALTH RISK ASSESSMENT

Thesis of Candidacy for the Ph.D. degree in Toxicology and Environmental Contamination. Doutoral Program of the University of Porto (Institute of Biomedical Science Abel Salazar)

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Dedication

I dedicate this work to my parents José Tamele and Cândida Banze who were my first school, to my children José, Xinave Tamele and Francília and finally to my brothers Alfina, Carla, Cândida and Ricardo.

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Previous note

This thesis was prepared according to 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 the chapters of this thesis was made. This 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) and IPMA (Portuguese Institute of the Sea and Atmosphere)

Publications

Publications listed in this thesis include all reviews, articles, oral communications, and posters published during doctoral program (2018 – 2022)

Thesis Publications

Published papers

1. **Tamele, Isidro**; Silva, Marisa, and Vasconcelos, Vitor. "The incidence of marine toxins and the associated seafood poisoning episodes in the African countries of the Indian Ocean and the Red Sea." Toxins 11.1 (2019): 58. https://doi.org/10.3390/toxins11010058
2. **Tamele, Isidro**; Silva, Marisa, and Vasconcelos, Vitor. "The incidence of tetrodotoxin and its analogs in the Indian Ocean and the Red Sea." Marine drugs 17.1 (2019): 28. https://doi.org/10.3390/md17010028
3. **Tamele, Isidro**; Timba, Ilário; Costa, Pedro and Vasconcelos, Vitor. "Tetrodotoxin and analogs in two local pufferfish species from Inhaca Island–South of Mozambique: First report in the Mozambican coast." Toxicon (2022). https://doi.org/10.1016/j.toxicon.2022.06.011
4. **Tamele, Isidro**; Timba, Ilário; Costa, Pedro and Vasconcelos, Vitor "First report of Pinnatoxins in bivalve molluscs *Atrina vexillum, Pintacta imbricata,* and *Anadara antiquata* from Inhaca Island (South of Mozambique) – South of the Indian Ocean" Journal of Marine Science and Engineering. 2022. https://doi.org/10.3390/jmse10091215
5. **Tamele, Isidro**; Garrine, Natércia; Costa, Pedro and Vasconcelos, Vitor "Management of marine toxins risk in Mozambique – A monitoring program is needed: An opinion" Mozambican Journal of Applied Science. 2023. https://doi.org/10.53224/mjas/ispg/2022v1n5

Oral Communication

1. **Tamele, Isidro**; Timba, Ilário; Costa, Pedro and Vasconcelos, Vitor. Screening of marine toxins in seafood species from the Inhaca Island: First report of Tetrodotoxins and Pinnatoxins in pufferfishes and bivalves species

from the Mozambique coast – South Indian Ocean, in XIV Reunião Ibérica sobre Microalgas Nocivas e Biotoxinas Marinhas. 2022: Lisbon, Portugal.

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Resumo

As costas africanas do Oceano Índico e do Mar Vermelho possuem um clima subtropical e tropical considerado ótimo para o desenvolvimento e proliferação de muitos microrganismos incluindo algas nocivas produtoras de toxinas marinhas (TM). Paradoxalmente, estudos relacionados com a ocorrência e incidência destas algas nocivas e TM são muito limitados, desde África do Sul ao Egipto. Dos poucos estudos disponíveis nesta área, as TM mais relatadas incluem ciguatoxinas (CTXs), toxinas paralisantes de marisco (TPM) e tetrodotoxinas (TTXs). As TM no pescado constituem uma grande ameaça à saúde pública mundial principalmente em países do Oceano Índico onde não há um plano de monitorização. Em Moçambique, os dados sobre ocorrência de TM são escassos embora haja relatos de intoxicações humanas, que em situações extremas conduziram à morte. A presente tese, organizada em 5 capítulos, foi desenvolvida com o objetivo de avaliar o risco de TM na costa moçambicana através da triagem no pescado de Moçambique das TM legisladas na UE e de outras consideradas recentemente como toxinas emergentes.

Foi detetada TTX e os seguintes análogos 4-epiTTX, 4,9-anhydroTTX, 11-deoxyTTX, e 11-norTTX-6-(R/S)-ol em espécies de peixe-balão (*Arothron hispidus* e *Diodon hystrix*). *A. hispidus* apresentou uma concentração mais elevada de TTX (9522.0 µg TTX kg^{-1}) do que *D. hystrix* (350.9 µg TTX kg^{-1}). A distribuição de TTX e análogos foi estudada em *A. hispidus*, tendo sido encontrada a seguinte ordem decrescente de TTX nos vários tecidos analisados: intestino > fígado > pele ≫ músculo > gónadas. Outras toxinas emergentes como pinnatoxinas G, F and E foram encontradas em bivalves (*Atrina vexillum, Pintacta imbricata* e *Anadara antiquata*). A quantificação de PnTX G revelou os seguintes valores: 7.7 e 14.3 $\mu g \cdot kg^{-1}$ em *A. vexillum*; 1.6 e 2.4 $\mu g \cdot kg^{-1}$ em *P. imbricata*, e 4.5 e 5.9 $\mu g \cdot kg^{-1}$ em *A. antiquata* em extratos hidrolisados e não hidrolisados respectivamente.

Estes resultados sugerem que os moçambicanos podem estar expostos a TM a partir do pescado. Não foram detetadas TM legisladas na UE nestas espécies de bivalves, nomeadamente toxinas lipofílicas, nem toxinas PSP ou ASP. Os

resultados encontrados nesta tese são os primeiros dados sobre TM no pescado de Moçambique e podem evidenciar a existência de uma das grandes ameaças à saúde pública. Apesar de os resultados serem ainda limitados, abrem uma importante discussão e reflecção sobre implementação dum programa de monitorização de TM em Moçambique. As TM mais relevantes que devem ser monitorizadas são descritas nesta tese. Técnicas analíticas como LC-MS/MS são recomendadas como métodos de determinação e quantificação devido à sua maior reprodutibilidade, especificidade, sensibilidade e capacidade de discriminar análogos de determinadas toxinas na amostra.

A monitorização de TM em Moçambique poderá ser atribuída às instituições responsáveis pela investigação pesqueira (Instituto Nacional de Investigação Pesqueira e Instituto Nacional de Inspeção de Pescado) envolvendo todas as delegações provinciais. Numa primeira fase, o laboratório de análise de TM pode estar localizado na cidade de Maputo, devido à disponibilidade de equipamentos de análises químicas de TM (LC – MS/MS) em comparação com outras delegações provinciais e à facilidade logística e troca de experiências com centros universitários de investigação como a Estação de Biologia Marinha da Universidade Eduardo Mondlane e Laboratório Nacional de Higiene de Águas e Alimentos (Ministério da Saúde). O processo de amostragem pode ser realizado sazonalmente em locais selecionados, uma no verão (outubro a março) e outra no inverno (abril a setembro) para avaliar uma possível sazonalidade da ocorrência de TM.

O limite máximo de toxinas em mariscos pode ser adotado a partir de outros países que Moçambique tem comércio de marisco, como a região da UE, EUA, Japão, Austrália, Nova Zelândia e África do Sul. Nesta tese, foi proposto o seguinte limite para cada grupo de TM: ácido ocadáico (AO) - 0,16mg (AO) kg^{-1}; CTX (0,01 µg (P-CTX-1) kg^{-1}); iminas cíclicas - 400 µg [espirolide (SPXs) kg^{-1}]; brevetoxina (PbTX) - 0,8 mg (PbTX-2) kg^{-1}; yessotoxina (YTX) - 3,75 mg (YTX) kg^{-1}; azaspiracido (AZA) - 0,16 mg (AZA) kg^{-1}; ácido domóico (DA) - 20 mg (DA) kg^{-1}; PST - 0,8 mg [saxitoxina (STX) kg^{-1}], TTX - 44 µg (TTX) kg^{-1}; palitoxina (PlTX) - 250 µg (PlTX) kg^{-1}.

Para o sucesso da monitorização de TM, é crucial a integração e intercolaboração de autoridades ambientais, de saúde pública e universidades de todos os países africanos do Oceano Índico e do Mar Vermelho.

Abstract

The African coasts of the Indian Ocean and the Red Sea have a subtropical and tropical climate considered optimal for the development and proliferation of microorganisms, including harmful algae bloom (HABs) that may produce marine toxins (MT) as secondary metabolites. Paradoxically, studies related to the occurrence and incidence of HABs and their MT are limited, from South Africa to Egypt. The few studies in this area describe ciguatoxins (CTXs), paralytic shellfish toxins (PSTs) and tetrodotoxins (TTXs) as the most reported MT. Accumulation of MT in shellfish and fish represents one of the greatest threats to public health worldwide, especially in Indian Ocean countries where there is no monitoring programs. In Mozambique, despite of cases of human intoxications including deaths involving marine fish, data on the occurrence of MT are very scarce. Thus, the present thesis, organized in five chapters, was developed with the objective of evaluating the risk of MT on the Mozambican coast by screening the EU-legislated and emerging MT in the local shellfish and fish of Mozambique.

In this thesis, TTX e analogues 4-epiTTX, 4,9-anhydroTTX, 11-deoxyTTX, e 11-norTTX-6-(R/S)-ol were detected in species of pufferfishes (*Arothron hispidus* and *Diodon hystrix*). *A. Hispidus* (9522.0 µg TTX kg^{-1}) presented high level of TTX than *D. hystrix* (350.9 µg TTX kg^{-1}). The distribution of TTX and analogues in A. *hispidus* was intestine > liver >skin ≫ muscle > gonads. Emergent toxins such as pinnatoxins G, F and E were found in bivalves (*Atrina vexillum, Pintacta imbricata* and *Anadara antiquata*). Only PnTX G was quantified and the level found was: 7.7 and14.3 $\mu g \cdot kg^{-1}$ in *A. Vexillum*; 1.6 e 2.4 $\mu g \cdot kg^{-1}$ in *P. imbricata*, and.5 e 5.9 $\mu g \cdot kg^{-1}$ in *A. antiquata* in hydrolyzed and non-hydrolyzed extracts respectively. These results suggest that Mozambicans may be exposed to MT from seafood. No EU legislated lipophilic MT were found in these species of bivalves. The data found in this thesis are the first data regarding MT in seafood from Mozambique and they may evidence the existing of one the great threats to public health. These results, although very preliminary due to several aspects such as the reduced number of individuals and species analyzed, collection in one point and one period, may be an indicative for

implementation of monitoring program in Mozambique. In Mozambique, the relevant MTs that must be monitored in shellfish are described in this thesis. Analytical techniques such as LC-MS/MS are recommended as determination and quantification methods due to their higher reproducibility, specificity, sensitivity and capacity to discriminate analogs of given toxins in the sample.

The monitoring of MT in Mozambique can be attributed to institutions responsible for fishery research (*Instituto Nacional de Investigação Pesqueira* and *Instituto Nacional de Inspeção de Pescado*) involving all provincial delegations. In the first phase, the laboratory of MT analysis may be in Maputo city, due to the availability of the chemical analysis equipaments for MT (LC – MS/MS) compared to other provincial delegation and the easy logistic and experience changes with university research centers such as *Estação de biologia marinha da Universidade Eduardo Mondlane, Laboratório Nacional de Higiene de Águas e Alimentos* (Ministry of Health). The sampling process must carry out seasonally in selected sites, one in the summer (October to March) and another in the winter (April to September) in order to assess a possible seasonality of the MT. The permitted limit of toxins in shellfish can be adopted from other countries which Mozambique has seafood trading such as the EU region, USA, Japan, Australia, New Zealand, and South Africa. The proposal of permitted limit for each group of MT to be adopted is: okadaic acid (AO) - 0.16mg (AO)Kg^{-1}; CTX (0.01 µg (P-CTX-1)kg^{-1}); cyclic imines - 400 µg [spirolide (SPXs)kg^{-1}]; brevetoxin (PbTX)- 0.8 mg (PbTX-2)Kg^{-1}; pectenotoxin (PTX) - 0.16mg (AO)Kg^{-1}; yessotoxin (YTX) - 1 mg(YTX)kg^{-1}; azaspiracid (AZA) - 0.16 mg(YTX)kg^{-1}; domoic acid (DA) - (20 mg(DA)kg^{-1}; PST - 0.8 mg[saxitoxin (STX)kg^{-1}], TTX - 44 µg(TTX)Kg^{-1}; palytoxin (PlTX) - 250 µg (PlTX)kg^{-1}.

For the success of the MT monitoring programs, the integration and intercollaboration of environmental and public health authorities including universities of all African Countries of the Indian Ocean and the Red Sea is crucial.

Contents of the thesis

Table index

Figure index

Abbreviations and symbols

AD/DA - ácido domoic/domoic acid

ANTX - anatoxin-a

AO/OA - ácido ocadáico/okadaic acid

ASP -amnesic shellfish poisoning

ASTs - amnesic shellfish toxins

AT - aplysiatoxin

ATX - antillatoxins

AZAs - azaspiracidos/azaspiracids

AZP - azaspiracid shellfish poisoning

BA - bioassay

C/P/I- CTXs –caribbean/pacific-Indian- ciguatoxinas/ciguatoxins

CAS - chemical abstract numbers

CFP - Ciguatera Shellfish Poisoning

CI - iminas cíclicas/cyclic imines

CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental

CTA - cytotoxicity assay

CYN - cylindrospermopsins

DAT - debromoaplysiatoxin

DSP - diarrheic shellfish poisoning

DSTs - diarrheic shellfish toxins

DTXs– dinophysistoxins

EFSA - European Food Safety Authority

EIA - enzyme-immuno assay

ELISA - enzyme-linked immunosorbent assay

EU - European Union

FCG – Fundação Calouste Gulbenkian

FCUP – Faculdade de Ciências da Universidade

FL - Fluorescence

FLD - fluorescence detection

FPA - fluorescence polarization assay

GC - gas chromatography

GYMs - gymnodimines

HABs - Harmful Algal Blooms

HANTX - homoanatoxin-a

HPLC - high-performance liquid chromatography

IPMA – Instituto Português do Mar e Atmosfera

JCD - jamaicadimes

KTX - kalkitoxins

LA/B/C - lyngbyatoxins A, B and C

LC - liquid chromatography

LMT – lipophilic marine toxins

LOD - limit of detection

LOQ - limit of quantification

MBA - mouse bioassay

MRM - muiltreation monitoring

MS - mass spectrometry

MTMP – marine toxins monitoring program

MTX - maitotoxin

NACOSTI - National Commission for Science, Technology, and Innovation

NPI - no poisoning incidents

NSP - neurologic shellfish poisoning

NZ - New Zealand

PbTXs – brevetoxinas/brevetoxins

PDAD - photo diode array detection

PltXs – palitoxinas/palytoxins

PnTXs – pinnatoxinas/pinnatoxins

PST - paralytic shellfish toxins

PtTXs - pteriatoxins

PTXs – pectenotoxinas/pectenotoxins

RBA - receptor-based assay

SA - South Africa

SBA - saxitoxin binding assay

SPR - surface plasmon resonance

STXs - saxitoxinas/saxitoxins

TEF - toxicity equivalency factors

TFDA - Tanzania Food and Drugs Authority

TLC - thin-layer chromatography

TM – Toxinas Marinhas

TPM – toxinas paralisantes de moluscos

TTXs – tetrodotoxinas/tetrodotoxins

TX – toxin

USA - United States of America

UVD - ultraviolet detection

YTXs – yessotoxinas/yessotoxins

I. INTRODUCTION OF THE THESIS

Highlights of the chapter

- Three parts of the thesis: state of the art, screening, and monitoring proposal of marine toxins in Mozambique
- The thesis is organized in 5 chapters composed by two review, one manuscript and two research articles

Marine toxins (MT) in seafood constitute one of the great threats to public health worldwide and more specifically in countries of the Indian Ocean where there is no monitoring program. In Mozambique, occurrence data survey studies of MT are very limited [1,2] although cases of human intoxications with some fatalities involving marine fishes are reported [3-8]. So, this thesis was developed in order to perform a risk assessment of MT in the Mozambican coast by screening the EU legislated and emerging MT in the most consumed seafood.

Objectives of the thesis

For the present thesis, three parts were outlined:

1. State of the art of marine toxins and their producers in the Indian Ocean and the Red Sea.

2. Screening of marine toxins in seafood (fishes and shellfishes) from Mozambican coast; and

3. Proposal for the implantation of the marine toxins monitoring plan in Mozambique.

For preparation of this thesis, the candidate participated on the acquisition and analysis of the samples, results discussion, and preparation of the works for publication. The sample collections works were done by the candidate in the Mozambican coast with collaboration of Marine Biology Station of Inhaca of the Faculty of Sciences (Eduardo Mondlane University, Mozambique). MT analyses were carried out in Center of Marine and Environmental Research - University of Porto (CIIMAR - UP) and Portuguese Institute of the Sea and Atmosphere (IPMA).

Structure of the thesis

This thesis is organized in 5 chapters. The Chapter I describes a general introduction of the thesis focusing on the thesis structure, research lines as well as the content of other chapters. Chapter II contains information used to understand the state of the art of the MT in the Indian Ocean and the Red Sea as a response to research line 1. This chapter is composed by two reviews published in *Toxins* and *Marine Drugs*. The occurrence of MT and their producers along the African Indian and the Red Sea coasts (from coast of Egypt to South Africa) and associated human poisoning episodes were discussed as a contribution to public health and monitoring programs are discussed in this chapter. The existence of monitoring programs of MT was highlighted and suggestions for the control and prevention of marine toxins in this area were added. Chapter III describes the screening of MT in pufferfish (*Diodon hystrix* and *Arothron hispidus*) and bivalves (*Atrina vexillum, Pinctada imbricata, Anadara antiquata,* and *Saccostrea cucculata*) from Mozambique. Determination of MT was carried out via liquid chromatography with tandem mass spectrometry detection following the method proposed by EULRMB 2017 and 2015 for TTXs and PnTXs respectively. The chapter III was developed as response of the research line 2. Two research papers were published on this subject, one communication related to TTXs and another to PnTXs. The papers were published in *Toxicon* and *Journal of Marine Science and Engineering* respectively. Chapter IV evaluates the risk of MT in Mozambique basing on the experiences of other African countries of the Indian Ocean and the Red Sea and unclarified human intoxication cases reported in the coastal area of Mozambique. In this chapter, detailed suggestions are present to authorities of Mozambique for implementation of MT monitoring program. For that, a review article was accept in the *Mozambican journal of Applied Science*. The structure of the manuscripts and research articles used as chapters in this thesis are according to the journals guidelines in which they were published or submitted. All submitted manuscripts and published paper were written by the candidate with the contribution of other authors that are described in each paper. Finally, the chapter

V describes general discussion, conclusions and final considerations of the thesis and perspectives for further works regarding MT in Mozambican coast.

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II. STATE OF THE ART OF MARINE TOXINS AND THEIR PRODUCERS IN THE INDIAN OCEAN AND THE RED SEA

Highlights of the chapter

- Data regarding MT are limited in the Indian Ocean and the few available data report cases of human intoxications (including deaths) involving CTXs, PSTs and TTXs.
- To date, in African countries of the Indian Ocean and the Red Sea, to date, only South Africa has a specific monitoring program for marine toxins. And some other countries count only with centers of seafood poisoning control.
- In Mozambique, there is no monitoring program neither research regarding MT.
- The specific monitoring program and further studies regarding MT are strongly needed in the African countries of the Indian Ocean.

REVIEW ARTICLE - Toxins 2019, 11, 58: The Incidence of Marine Toxins and the Associated Seafood Poisoning Episodes in the African Countries of the Indian Ocean and the Red Sea.

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Abstract

The occurrence of Harmful Algal Blooms (HABs) and bacteria can be one of the great threats to public health due to their ability to produce marine toxins (MTs). The most reported MTs include paralytic shellfish toxins (PSTs), amnesic shellfish toxins (ASTs), diarrheic shellfish toxins (DSTs), cyclic imines (CIs), ciguatoxins (CTXs), azaspiracids (AZAs), palytoxin (PlTXs), tetrodotoxins (TTXs) and their analogs, some of them leading to fatal outcomes. MTs have been reported in several marine organisms causing human poisoning incidents since these organisms constitute the food basis of coastal human populations. In African countries of the Indian Ocean and the Red Sea, to date, only South Africa has a specific monitoring program for MTs and some other countries count only with respect to centers of seafood poisoning control. Therefore, the aim of this review is to evaluate the occurrence of MTs and associated poisoning episodes as a contribution to public health and monitoring programs as an MT risk assessment tool for this geographic region.

Keywords: Indian Ocean; marine toxins; harmful algal bloom

Key Contribution: The scarcity of MT data along African countries of the Indian Ocean and the Red Sea suggests the need for further studies and the creation of specific monitoring programs of MTs, particularly for dinoflagellates and diatoms since these constitute the phytoplankton that produces fatal MTs.

Introduction

The occurrence of Harmful Algal Blooms (HABs) in marine ecosystems can be one of the great threats to public health due to their capacity to produce marine toxins (MTs) as secondary metabolites [1–14]. MTs can be accumulated by distinct marine organisms such as fish, mollusks and crustaceans [15–24] which are the basic diet of coastal human populations. Suspected or confirmed episodes of human poisoning caused by MTs have been reported worldwide in the last century [20,21,25–48]. The occurrence of episodes of human poisoning occurs via ingestion of contaminated marine food due to the lack of

monitoring programs in some countries or violations of national health authorities' regulations imposing the closure of harvesting areas and seafood commercialization [18,20,26,35,39,45,47,49]. Despite the ideal environmental conditions for the formation of blooms in this geographical area, there are insufficient data related to their occurrence and toxin production [50]. This review analyses the occurrence of MTs and their producers along the African Indian and the Red Sea coasts (from Egypt to South Africa) and associated human poisoning episodes. The existence of monitoring programs of MTs will be also highlighted and finally, some suggestions for the control and prevention of marine toxins in this area will be presented.

Marine Toxins and Their Producers

Chemically, toxins can be grouped according to their polarity, lipophilic and hydrophilic. Concerning MT monitoring, analysis and quantification methods in seafood are described in Table II.1, including bioassays, immunoassays, and analytical chemistry methods. The bioassay methods (Mouse Bioassay (MBA), Rat Bioassay (RBA)) are no longer in use due to ethical reasons according to Directive 86/609/EEC [51] and procedural variation [52] (e.g., use of different extraction solvents and consequently shortcomings). Chemical methods, mainly liquid chromatography coupled to mass spectrometry, are considered as the most promising since they are fully validated and standardized to replace bioassays in many organizations worldwide. Further information related to each toxin group such as syndromes, producers, common vectors, symptoms, detections methods in seafood, limit of detection (LOD) and quantification (LOQ) and permitted limit used in some parts of the world is also described in Table II.1

Table II.1. Marine toxins and their symptoms, producers, permitted limit, detection methods, limit of detection/limit of quantification [LOD/LOQ] and toxicity equivalency factors [TEF] according to the European Food Safety Authority [EFSA].

Toxin (Syndrome)	Symptoms	Detection			Permitted Limit	Toxin (TEF)	Producer
		Methods	LOD, ε g K^{-1}g	LOQ, ε g K^{-1}g			
OA and analogs (DSP)	diarrhea, nausea, vomiting, abdominal pain and tumor formation in the digestive system [50]	BA [180,181]	160		0.16mg OA equivalents /Kg shellfish meat in EU region [182]	OA [**1.0**]	Dinoflagellates*: Prorocentrum* spp. [8], *Dinophysis* spp. [2,6,9,10,15,53,54] and *Phalacroma rotundatum*[55]
		EIA [183–186]	10–26	3–41		DTX1 [**1.0**]	
		LC-MS [183], -UVD [187]	15–30	1–50		DTX2 [**0.6**]	
						DTX3 [**1.0; 1; 0.6**]	
CTXs and analogs (CFP)	vomiting, diarrhea, nausea, tingling, itching, hypotension, bradycardia. In extreme cases, death through respiratory failure in 30 min and 48 h after fish consumption [50]	BA [188,189]	0.16–0.560 P-CTX [190]		0.01 µg P-CTX-1 equivalents/kg of fish in USA [191]	P-CTX-1[**1.0**]	Dinoflagellates: *Gambierdiscus toxicus, Ostreopsis siamensis* and *Prorocentrum lima* [59]
		CTA [192–194]	~10^6 - 0.039 C-CTX			P-CTX-2[**0.3**]	
		EIA [72,189,195–199]	-0.032 P-CTX			2,3-dihydroxy P-CTX-3C[**1.0**]	
		LC-MS/MS				C-CTX-1[**0.1**]	

		[67,70,71,74, 200], -UVD [62,201,202]				
CIs	non-specific symptoms such as gastric distress and tachycardia in humans[82]	BA	5.6–77 PnTXE	Not regulated	13-desmethyl SPX C[**1.0**]	Dinoflagellates: SPXs: *Alexandrium* spp. [351,76], GYMs: *Gymnodium* spp.[77], PnTXs: *Vulcanodinium rugosum* [78]and PtTXs: biotransformation from PnTXs via metabolic and hydrolytic transformation in shellfish [5,77–79,351]
		FPA [203]	80–85 13-SPXC			
		LC-MS/MS [79,204], -UVD [205]	0.8–20 13-SPXC/GYMA			
PbTxs **and analogs (NSP)**	nausea, vomiting, diarrhea, paresthesia, cramps, bronchoconstriction, paralysis, seizures in 30 min to 3 h [87]	BA [206]		800 µg BTX-2 equivalents/kg shellfish in USA[98], New Zealand, and Australia [99,100]	BTX-2, BTX-3, BTX2-B2 and S-deoxy-BTX-B2 [**same TEF**]	Dinoflagellate*: Karenia* spp.[4,16,87]
		CTA [192]	250 BTX-1			
		RB [108]	30BTX-3			
		EIA [207,208]	1 BTXs and 25 BTXs			
		LC – MS/MS [209]	0.2 – 2 BTXs			
PTX and analogs	No specific symptoms	MBA	-	160 µg OA equivalents./kg	PTX [1,2,3,4,6 and 11][**1.0**]	
		EIA[207]	-			

		LC – MS/MS [211,212]	1	shellfish meat in EU region [210]	PTX [7,8,9 and 2SA] and 7-*epi*PTX2 SA [**<<10**]	Dinoflagellate: *Dinophysis acuta* [101]
YTX and analogs	No specific symptoms	BA		3.75 mg YTX equivalents/Kg shellfish meat in EU region [124]	YTX[**1.0**]	Dinoflagellate: *Protoceratium reticuatum* [4,109], *Lingulodinium polyedrum [4]*and *Gonyaulax polyhedral [4]*
		EIA [213]			1a-homoYTX[**1.0**]	
		LC-MS/MS [111]	0.017		45-hydroxyYTX[**1.0**]	
					45-hydroxy-1a-homoYTX[**0.5**]	
AZA and analogs (AZP)	nausea, vomiting, diarrhea and decreased reaction to stomach cramps, deep pain, dizziness, hallucinations, confusion, short-term memory loss, seizure[214]	BA [181]		0.16 mg AZA1equivalents/Kg shellfish in EU region [210]	AZA1[**1.0**]	Dinoflagellates: *Azadinium spinosum* [117]and *Protoperidinum crassipes* [118]
		LC-MS/MS	0.05		AZA2[**1.8**]	
					AZA3[**1.4**]	
					AZA4[**0.4**]	
					AZA5[**0.2**]	
STX and analogs (PSP)	Numbness in the face and neck; headache, dizziness, nausea, vomiting, diarrhea,	BA [216,217]		0.8 mg STX equivalent/Kg shellfish in EU region [210]	STX[**1.0**]	Dinoflagellates: *Alexandrium* spp.[2,3,7], *Gymnodinium catenatum*[3], *Pyrodinium bahamense* [3]
		SBA [218]			NSTX[**1.0**]	
					GTX1[**1.0**]	
					GTX2[**0.4**]	
					GTX3[**0.6**]	
		CTA [192,219]			GTX4[**0.7**]	
					GTX5[**0.1**]	

	muscular paralysis; pronounced respiratory difficulty; death through respiratory paralysis [215]	Antibodies Assay [220–224]			GTX[**0.1**]	andcyanobacteria*Tric hodesmium erythraeum* [131]
					C2[**0.1**]	
		Eletrophoresis [225]			C4[**0.1**]	
					de-STX[**1.0**]	
					de-GTX3[**0.2**]	
		LC-MS/MS [226–229]	23–42 STX		de-NSTX2[**0.2**]	
					de-GTX3[**0.4**]	
					11-hydroxy-STX[**0.3**]	
DA and analogs (ASP)	nausea, vomiting, diarrhea or abdominal cramps] within 24 h of consuming DA contaminated shellfish and/or neurological symptoms or signs [confusion, loss of memory or other serious signs such as seizure or coma] occurring within 48 h	BA [230]	40		20 mg DA equivalents/Kg shellfish in EU region [210]	Diatoms*: Pseudo-nitzschia* spp. [126] and red algae: *Chondria armata* [127].
		(a) ASP-EIA[184,231]	0.003	0.01		
		SPR[232]	20			
		RB[233–235]	20			
		Capillary electrophoresis [236–238]	0.15 -1			
		LC -MS/MS [211,239,240], UVD [241,242]	0.015			
		TLC [243]	10			

TTX and analogs	Vomiting, strong headache, muscle weakness, respiratory failure, hypotension and even death in hours[244]	BA [144,245–247]	1.1[247]	2 mg TTX equivalents/Kg shellfish in Japan [248]	S/R 11-norTTX-[6]-ol[0.19/0.17]	Bacteria: *Serratia marcescens, Vibrio* spp. [83]*, V. Aeromonas sp.* [138], *Microbacterium, arabinogalactanolyticum* [139], *Pseudomonas* sp.[140], *Shewanella putrefaciens* [141], *Alteromonas* sp.[142], *Pseudoalteromonas* sp.[143], and *Nocardiopsis dassonvillei* [144]
		RB [249]	$2–4.10^{-3}$TTX		4-*epi*TTX[**0.16**]	
		EIA [245–247,250–256]	0.002/mL [255], 0.0001/mL [253]		4,9-anhydroTTX[**0.02**]	
		TLC [139,257]	2 [257]		5,6,11-deoxyTTX[**0.01**]	
		GC-MS [28,258,259]	500	1000 [258]		
		LC-MS/MS [260–264] – FLD [265]	0.00009?-24.5 [260–264]	40 [265] – 100 [265]		
PlTX	Vasoconstriction, hemorrhage, myalgia, ataxia, muscle weakness, ventricular fibrillation, ischemia and death [266,267] and rhabdomyolysis[268]	BA		Not regulated toxin but proposed value is 0.25mg PlTX equivalent/Kg shellfish in EU region [269]	PlTX[**1.0**]	Zoanthids: *Palythoa* spp.anddinoflagellates: *Ostreopsis ovata*. [153–155]and possibly cyanobacteria: *Trichodesmium* sp. [156]
		Hemolysis assay [270]	1.6			
		CTA [107]	50		streocin-D[**0.4ï 1.0**]	
		EIA [254]	1/mL			
		LC-MS/MS [204,271]–	$2,5.10^{-5}$–0, 50.10^{-5}			

		FLD and–UVD [272]		
MC	liver hemorrhage within a few hours of an acute dose and death [273]	LC-MS [167,274–276] and EIA [277]	Tolerable daily intake: 0.04 µg/kg of MC body weight/day [278]	Cyanobacteriaof genus: *Pseudoanabaena, Phormidium, Spirilia* [164], *Leptolyngbya, Oscillatoria, Geitlerinema* [165], *Trichodesmium*[166] and *Synechococcus* [167]
ANTX and HANTX	Hypersalivation, diarrhea, shaking and nasal mucus discharge [279], respiratory arrest and death [280]	RB and GC/MS [281,282]		Cyanobacteria: *Hydrocoleum lyngbyaceum*[177]
AT and DAT	Contact dermal: dermatitis initiating with erythema and burning sensations, appearing a few hours after exposure, gave way to blister formation and	LC-MS/MS [286]		Algae *Gracilaria coronopifolia* [172] and cyanobacteria *Lyngbya majuscula* [171]
LA, LB, and LC				Cyanobacteria *Lyngbya majuscule* [174]

	deep desquamation, lasting up to several days [283,284] and consumption of contaminated seafood; burning sensation in the mouth and throat, vomiting and diarrhea [285]			
ATX and analogs	No specific symptoms	LC [287]		Cyanobacteria: *Lyngbya majuscula* [179]
JCD and analogs	No specific symptoms	LC, TLC and [288]		Cyanobacteria: *Lyngbya majuscula* [176]
KTX and analogs	No specific symptoms	LC [173]		Cyanobacteria: *Lyngbya majuscula* [173]
CYN and analogs	Gastroenteritis [289]	LC-MS/MS [290],–PDAD [291] EIA[294]	1[292]–200 [293]	Cyanobacteria: *Cylindrospermopsis raciborskii* [175]

Toxins: DA—domoic acid, DTX, CTX -ciuatoxin, AZA—azaspiracid, CI—cyclic imines, PTX—pectenotoxin, YTX—yessotoxin, STX—saxitoxin, OA—okadaic acid, BTX—revetoxin, PlTX—palytoxin, TTX -tetrodotoxin, MC—microcystin, ANTX—anatoxin, HANTX—homoanatoxin, LA, LB and LC—lyngbyatoxins A, B and C respectively. ATX—antillatoxin, KTX—kalkitoxin, CYN—cylindrospermopsins AT—aplysiatoxin, DAT—debromoaplysiatoxin, JCD—jamaicamides, **Syndrome**: PSP—Paralyc Poisoning, DSP—Diarrheic Shellfish

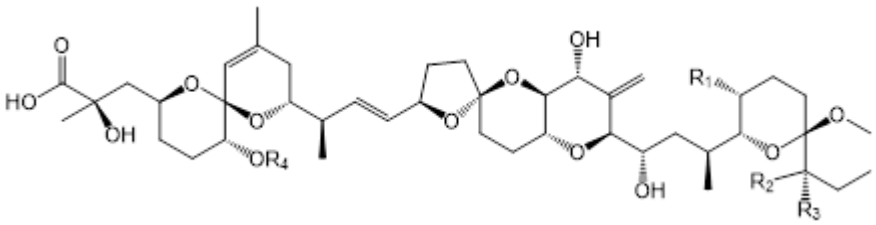
Poisoning, ASP—Amnesic Shellfish Poisoning, AZP—Azaspiracid Shellfish Poisoning, CFP—Ciguatera Shellfish Poisoning, NSP—Neurologic Shellfish Poisoning, **Detection methods**: CTA—Cytotoxicity assay, EIA—Enzyme-ImmunoAssay, SPR—Surface Plasmon Resonance, RB—Receptor-based, GC—Gas Chromatography, BA—Bioassay; UVD—Ultra Violet Detection; LC—Liquid Chromatography and MS—Mass Spectroscopy, FPA—Fluorescence Polarization Assay, TLC—Thin Layer Chromatography, SBA—Saxitoxin Binding Assay, PDAD—photo diode array detection.

Lipophilic Toxins

Lipophilic toxins are lipid soluble toxins and this group comprises okadaic acid (OA), ciguatoxins (CTX), cyclic imines (CIs) [spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs)], brevetoxins (PbTxs), pectenotoxins (PTXs), yessotoxins (YTXs) and azaspiracids [AZAs], Table II.1.

Okadaic Acid and Analogs

Okadaic acid (OA)and their analogs, dinophysistoxins-1, -2 and -3 (DTXs) (Figure II.1), are polyethers produced by dinoflagellates: *Prorocentrum* spp. [8], *Dinophysis* spp. [2,6,9,10,15,53,54] and *Phalacroma rotundatum* [55] (Table II.1).These polyethers are frost-resistant and heat-stable and consequently, their toxicity is not affected by the cooking procedures in water (they are stable at <150 ˚C) [56]. The OA group is responsible for the diarrheic shellfish poisoning syndrome (DSP), with OA being the main representative of DSP toxins. Okadaic acid (OA) and its analogs act as inhibitors of the serine/threonine phosphoprotein phosphatases 1,22B,4,5 types that are involved in modeling the functions of certain proteins crucial for synaptic transmission, transport and neutromissors release [57,58].



Analog	R_1	R_2	R_3	R_4
OA	CH_3	H	H	H
DTX1	CH_3	CH_3	H	H
DTX2	H	H	CH_3	H
DTX3	H/CH_3	H/CH_3	H/CH_3	Acyl

Figure II.1 Chemical structure of OA and main derivatives [DTX1, DTX2, and DTX3].

Ciguatoxins

Ciguatoxins (CTXs) (Figure II.2A) are a group of toxins produced by tropical and subtropical dinoflagellates species: *Gambierdiscus toxicus* and *Fukuyoa* spp. [59,60] (Table 1) mainly found in the Pacific, Caribbean and the Indian Ocean regions [P-CTX, C-CTX and I-CTX, respectively]. CTXs are lipid-soluble polyethers with 13-14 rings fused by ether linkages into a rigid ladder-like structure [60]. To date, the structures of20 P-CTXs, 10 C-CTXSand 4 I-CTXs analogs have been fully identified and the most reported include P-CTX-1, P-CTX-2, P-CTX-3, P-CTX-3C [61–67], gambiertoxin [GbTXs, namely, P-CTX-4A and P-CTX-4B] [68], C-CTX-1, C-CTX-2 [67,69], I-CTX-1, I-CTX-2, I-CTX-3 and I-CTX-4 [70,71] mostly in predatory fish and gastropods [20,21,23,66,69,72–74]. The major analog of each group of CTXsis P-CTX-1. C-CTX-1, C-CTX-2, I-CTX1, and I-CTX-2. The chemical structure of the last two (I-CTXs) have the same molecular weight and similar structures as C-CTX-1 [62,67,70,71]. CTXs are odorless and tasteless heat-stable molecules and are not affected when subjected to water cooking, freezing and acid or basic conditions, though they suffer structural alterations by oxidation [60]. CTXs and Maitotoxin (MTX) (Figure II.2B) (produced by *Gambierdiscus* spp. [68]) were the first group of toxins reported to be responsible for ciguatera shellfish poisoning (CFP) [23]. The mechanism of action of CTX and analogs is to elevate calcium ion concentration and activate non-selective cation channels in cells causing neurologic effects in humans [75].

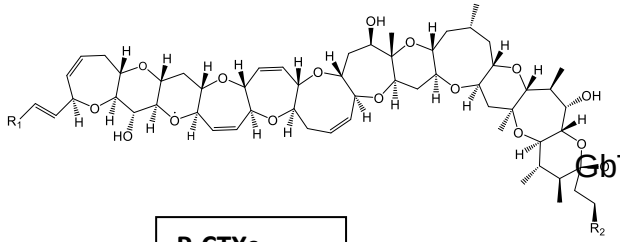
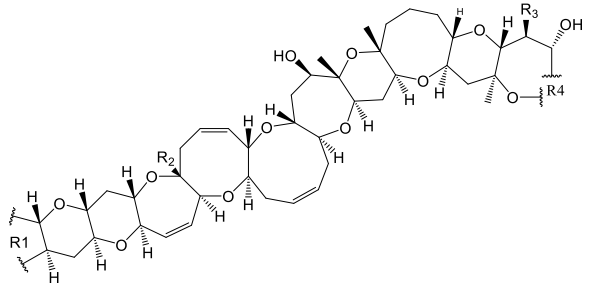
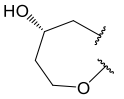
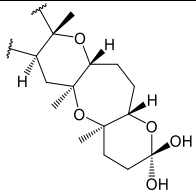
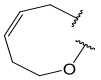
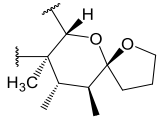
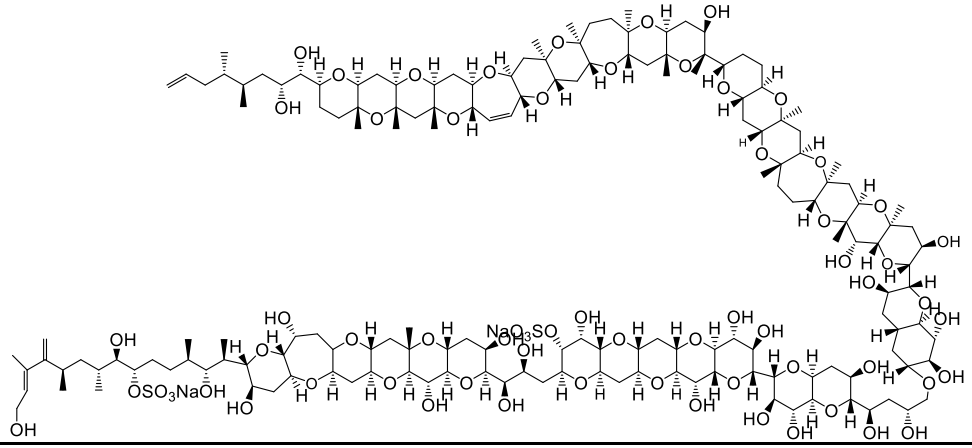
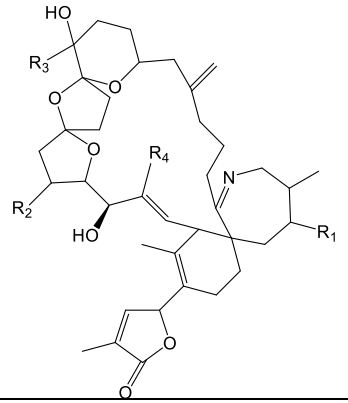
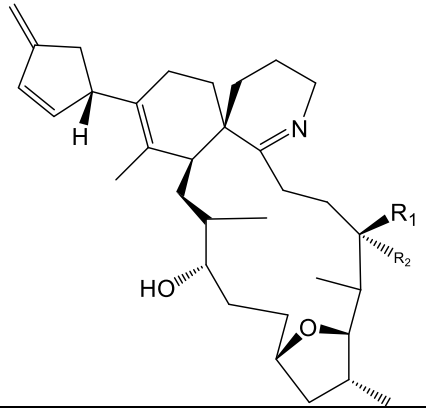
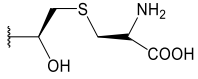
Order	Structure	Toxin	R_1	R_2	R_3	R_4
(a)	 P-CTXs	P-CTX-1	$CH_2OHCHOH$	OH		
		P-CTX-2 and P-CTX-3	$CH_2OHCHOH$	H		
		GbTX [P-CTX-4A and P-CTX-4B]	CH_2CH_3	H		
(b)		C-CTX-1 and C-CTX-2		CH_3	H	
		P-CTX-3C		H	CH_3	
(c)						

Figure II.2. Chemical structure of major CTXs analogs from Pacific (P-CTXs) (**a**) and Caribbean (C-CTXs) (**b**) regions. The major CTXs from Indian region (I-CTXs) have a similar structure with C-CTX-1. (**c**) Chemical structure of maitotoxin (MTX).

Cyclic Imines

Cyclic imines (CI) (Figure II.3) are toxins produced by dinoflagellates: SPXs: *Alexandrium* spp. [1,76], GYMs: *Gymnodium* spp. [77], PnTXs: *Vulcanodinium rugosum* [78] and PtTXs: biotransformation from PnTXs via metabolic and hydrolytic transformation in shellfish [1,5,77–79] (Table II.1). CIs are a heterogenous group composed ofspirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs) and more than 24 structural analogs have been described to date [80].

Regarding chemical properties, these toxins are a group of macrocyclic compounds that have in common an imine functional group and spiro-linked ether moieties in their structure [80]. They are colorless amorphous solid macrocyclic compounds with imine and spiro-linked ether moieties [80], considerably soluble in organic solvents such as methanol, acetone, chloroform and ethyl acetate [5,80]. CIs are neurotoxins and actby inhibiting the nicotinic and muscarinic acetylcholine receptors (mAChR and nAChR, respectively) in the nervous system and at the neuromuscular junction [81]. CI bioactivity seems to depend on the imine functional group since the hydrolysis of spirolides A–D produce spirolide E and F with a keto-amine structure that is fully inactive [81]. To date, there are no regulations for CIs and no common symptoms can be recognized as specific for CI [82].

Order	Structure	Toxin	R_1	R_2	R_3	R_4
		Spirolides				
(**a**)		SPX A	H	CH_3	CH_3	H
		SPX B	H	CH_3	CH_3	H
		SPX C	CH_3	CH_3	CH_3	H
		SPX D	CH_3	CH_3	CH_3	H
		13-desmethyl SPX C	CH_3	H	CH_3	H
		13, 19-desmethyl SPX C	CH_3	H	H	H
		13-desmethyl SPX D	CH_3	H	CH_3	H
		27-Hydroyx-13-didesmethyl SPX C	CH_3	H	H	CH_3
		Gymnodimines				
(**b**)		GYM A	H	H		
		GYM B	H	OH		
		GYM C	H	H		
		Pteriatoxins				
(**c**)		PtTX A 	OH	H	H	

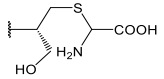
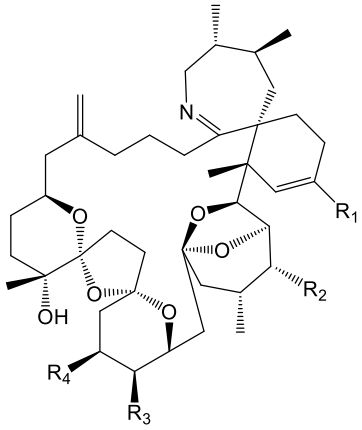
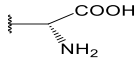
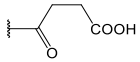
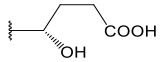
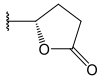
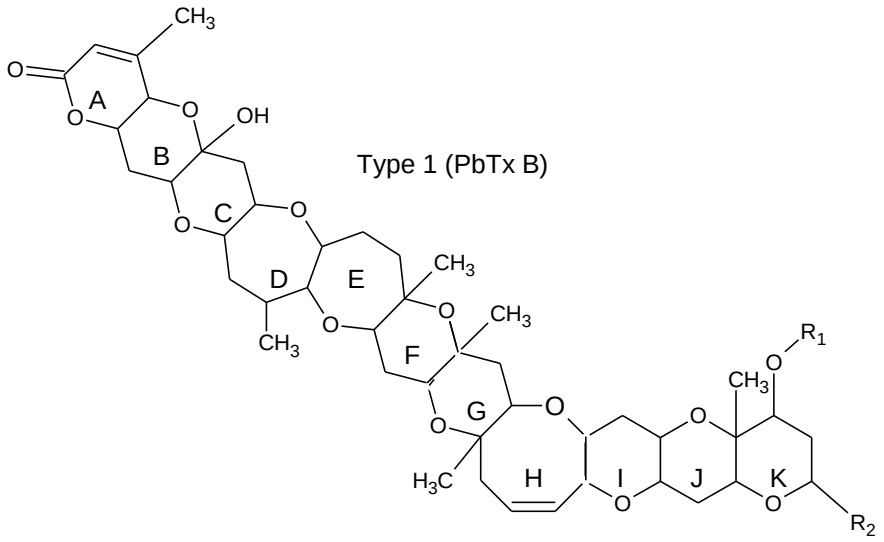
Order	Structure	Toxin	R_1	R_2	R_3	R_4
		PtTX B		OH	H	H
		Pinnatoxins				
		PnTX A		OH	H	H
		PnTX B and C		OH	H	H
		PnTX D		H	OH	CH_3
		PnTX E		H	OH	CH_3
		PnTX F		H	OH	CH_3
		PnTX G		OH	H	H

Figure II.3. Chemical structures of CI (SPXs (**a**), GYMs (**b)**, PnTXs (**c**), and PtTXs (**c**),) and Silva et al. [79,83–86].

Brevetoxins

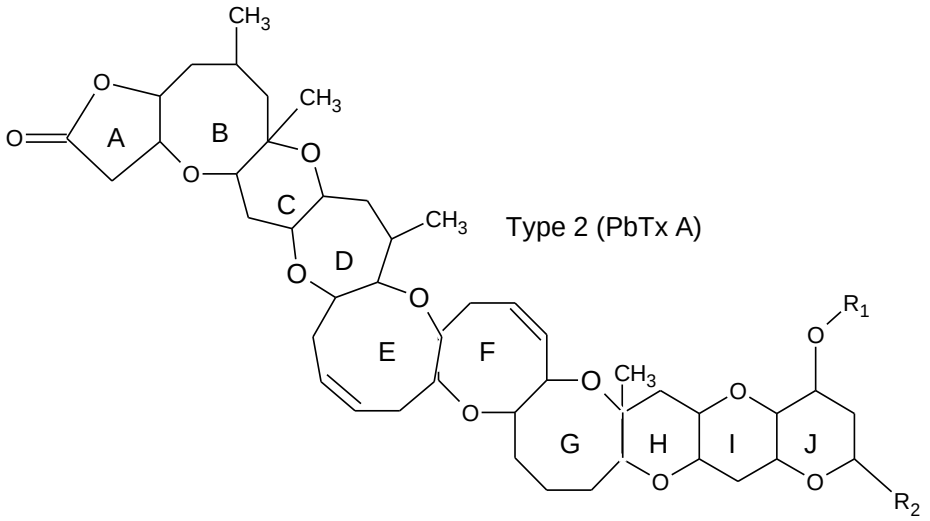
Brevetoxins (PbTxs) (Figure II.4) are cyclic polyethers produced by dinoflagellates: *Karenia* spp. [4,16,87] (Table II.1). There are two known types of BTXs, named type A and type B (also called type 1(PbTx-1) and type 2 (PbTx-2), respectively). The difference between two types of PbTxs consists in a few transfused rings that are ten for PbTx-1 and eleven for PbTx-2. The main analogs include PbTx-3, PbTx-6, PbTx-9, PbTx-B1, PbTx-B2, S-desoxy-PbTx-B2, PbTx-B3, PbTx-B4, and PbTx-B5 [44,88–94]. PbTxs are lipid-soluble cyclic polyether consisting of 10 to 11 transfused rings [95], stable and resistant to heat and steam autoclaving [96]. PbTxs cause neurotoxic shellfish poisoning (NSP) and actby binding with high affinity to receptor site 5 of the voltage-gated sodium channels (Na_V) in cell membranes, and lactone is important for the toxin activity [97]. PbTxs are regulated in USA [98], New Zealand, and Australia [99,100] (Table II.1).



Toxin	R_1	R_2
PbTx-2	H	$CH_2C[CH_2]CHO$
PbTx-3	H	$CH_2C[CH_2]CH_2OH$
PbTx-5	$COCH_3$	K-ring acetate PbTx-2
PbTx-6	H	H-ring epoxide PbTx-2
PbTx-8	H	CH_2COCH_3Cl
PbTx-9	H	$CH_2CH[CH_3]CH_2OH$

Figure II.4. Chemical structures of the main group of PbTxs (PbTxs-A and PbTxs-B). The capital letter A in first ring indicates type A and type B (also called type 1and type 2, respectively [4]). These rings contain lactone group that is most important for the toxin activity.

Figure II.4. continued.

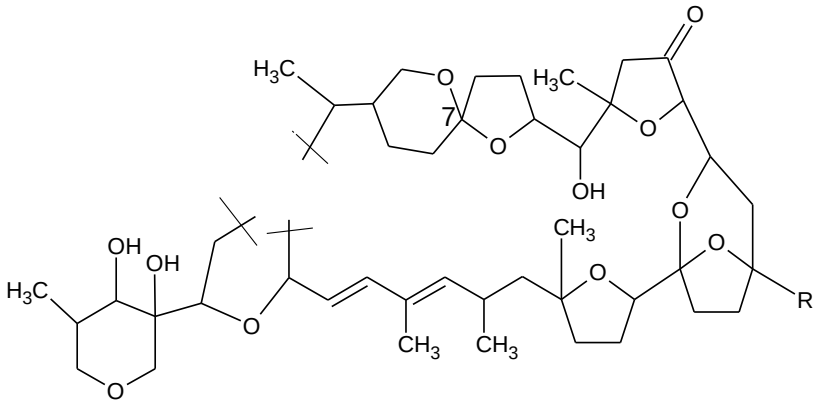


Toxin	Type	R_1	R_2
PbTx-1	2	H	$CH_2C[CH_2]CHO$
PbTx-7	2	H	$CH_2C[CH_3]CH_2OH$
PbTx-10	2	H	$CH_2CH[CH_3]CH_2OH$

Pectenotoxin Group

Pectenotoxins (PTXs) (Figure II.5) are lipophilic polyethers produced by several dinoflagellate species [101] (Table II.1). They contain spiroketal, bicyclic ketal, cyclic hemiketals, and oxolanes in their structure. To date, more than 15 PTX analogs have been documented and many are derived through biotransformation of PTX2 in marine organism metabolism such as bivalve mollusks [102]. The most reported analogs include PTX1, *epi*-PTX1, PTX2, PTX2 *seco* acid (PTX2 SA), 7-*epi*-PTX2 *seco* acid (7-*epi*-PTX2 SA), PTX3, PTX4, PTX6, *epi*-PTX6, PTX7, PTX11 (34S-hydroxy-PTX2) [6,101,103–105]. PTXs are heat-stable and unstable under alkaline conditions [103]. PTX and analogs alter actin-based structures [103,106] causing cell death and apoptosis [107]. PTXs co-occur with the OA—group and

contribute to DSP in humans [108].



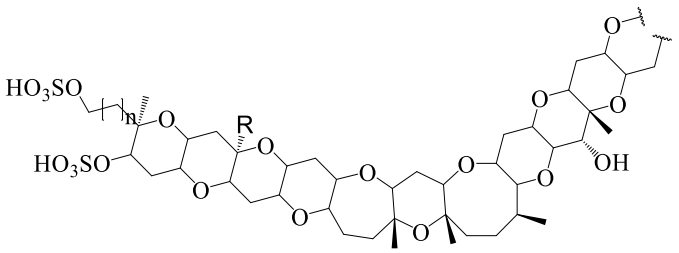
Toxin	Structure	R	Configuration at C7
PTX1		CH_2OH	R
PTX2		CH_3	R
PTX3		CHO	R
PTX4		CH_2OH	S
PTX6		COOH	R
PTX7		COOH	S
PTX11		OH	R
PTX2 SA		CH_3	R
7-epi-PTX2 SA		CH_3	S

Figure II.5. Chemical structures of main pectenotoxins.

Yessotoxins

Yessotoxins (YTXs) (Figure II.6) are produced by dinoflagellates species: *Protoceratium reticulatum* [4,109], *Lingulodinium polyhedral* [4] and *Gonyaulax polyhedra* [4] (Table II.1). They are a heat-stable polyether, with eleven transfused ether rings, an unsaturated side chain, and two sulfate esters [110]. To date, more than 90 YTX analogues have been isolated [102] and only YTX, 45-hydroxyYTX, carboxylic, 1a-homoYTX, 45,46,47-trinorYTX, ketoYTX, 40-epi-ketoYTX, 41a-homoYTX, 9Me-41a-homoYTX, 44,55-dihydroxyYTX, 45-hydroxy-1a-

homoYTX, carboxy-1a-homoYTX [111] have been fully identified [111]. The mechanism of action of YTX and their analogs is not fully understood; however, they are involved in phosphodiesterase activation [112] and modulation of calcium migration at several levels [113], alteration of protein disposal [114], cell change shape [115], apoptosis and cell death [116]. To date, there are no reports of human illness associated with YTXs [111]



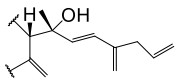
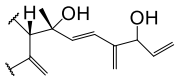
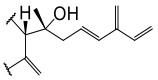
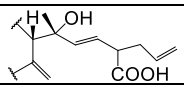
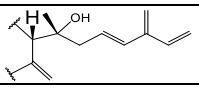
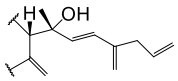
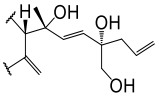
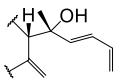
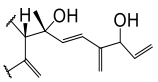
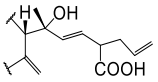
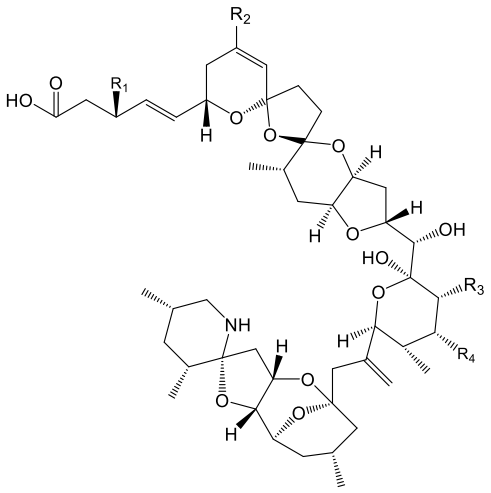
Toxin		R	n	Toxin		R	n
YTX		H	1	40-epi-ketoYTX		H	1
45-hydroxy YTX		H	1	41a-homoYTX		H	1
Carboxy YTX		H	1	9-Me-41a-homoYTX		CH_3	1
1a-homoYTX		H	2	44,45-dihydroxyYTX		H	2
45,46,47-trinorYTX		H	1	45-hydroxy-1a-homoYTX		H	1
KetoYTX		H	1	Carboxy-1a-homoYTX		H	2

Figure II.6. Chemical structures of YTXs n corresponds to the number of methyl groups in the molecule.

Azaspiracids

Azaspiracids (AZAs) (Figure II.7) are toxins produced by dinoflagellates: *Azadinium spinosum* [117] and *Protoperidinum crassipes* [118] (Table II.1). They are colorless, odorless and amorphous solids of toxins containing a heterocyclic

amine, a unique tri-spiro-assembly and an aliphatic carboxylic acid in their structures [117,119–124]. Around 21 compounds of AZAs are well known and documented [117,119–124] of which AZA, AZA2, AZA3, AZA4, and AZA5 are the most prevalent ones based on occurrence and toxicity in humans. AZAs are responsible for the AZP syndrome (Table II.1) and their mechanism of action is the inhibition of hERG voltage-gated potassium channels [125].



Toxin	R_1	R_2	R_3	R_4
AZA	H	H	CH_3	H
AZA2	H	CH_3	CH_3	H
AZA3	H	H	H	H
AZA4	OH	H	H	H
AZA5	H	H	H	OH

Figure II.7. Chemical structure of AZAs.

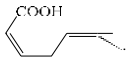
Hydrophilic Toxins

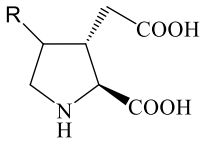
Hydrophilic Toxins are polar soluble compounds, and they include domoic acid (DA) and analogs, Paralytic Shellfish Toxins (PSTs), tetrodotoxins (TTXs) and palytoxins (PlTXs).

Domoic Acid and Analogs

Domoic acid (DA) (Figure II.8) and analogs are polar cyclic amino acid toxins of

diatom origin *Pseudo-nitzschia* spp. [126] and red algae: *Chondria armata* [127] (Table II.1). They present three carboxylic acid groups and the most reported DA analogs include *epi*-domoic acid (*epi*-DA), domoic acid C5′-diastereomer and isodomoic acids A, B, C, D, E, F, G and H [iso-DA A-H] [128,129]. DA is the representative molecule of the DA-group that is responsible for amnesic shellfish poisoning (ASP) syndrome [130]. The characteristic symptomology of ASP is detailed in Table II.1.





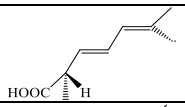
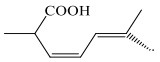
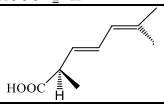
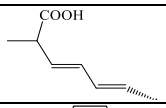
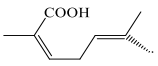
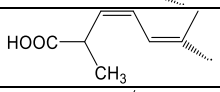
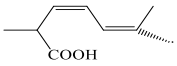
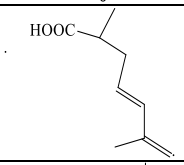
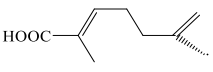
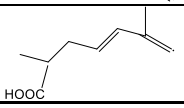
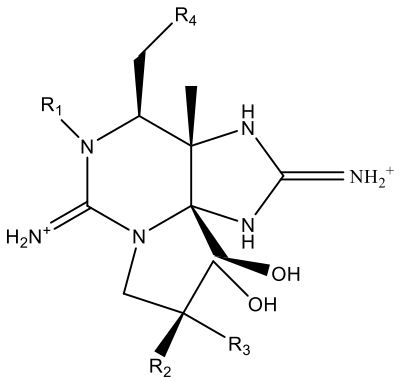
Toxin	R	Toxin	R
DA		Iso DA D	
5′-epiDA		Iso DA E	
Iso DA A		Iso DA F	
Iso DA B		Iso DA G	
Iso DA C		Iso DA H	

Figure II.8. Chemical structure of DA and analogs.

Paralytic Shellfish Toxins

Paralytic shellfish toxins (PSTs) (Figure II.9) are water-soluble tetrahydropurine toxins produced mainly by dinoflagellates *Alexandrium* spp. [2,3,7], *Gymnodinium catenatum* [3], *Pyrodinium bahamense* [3] and by cyanobacteria *Trichodesmium erythraeum* [131] except M (Figure II.9) toxins that are *Mytilus* spp. metabolism products [132]. This group is composed of several analogs and they are prone to

various conversions depending on pH (Figure II.9), being divided into several groups: carbamoyl (saxitoxin (STX), neosaxitoxin (NeoSTX) and gonyautoxins (GTX1-4)) decarbamoyl [dc-](dcSTX, dcNeoSTX, dcGTX1-4), Nsulfo-carbamoyl [GTX5-6, C1-4], hydroxylated saxitoxins [M1-4] [133–135] and benzoyl toxins (GC1-3) [135]. Their heat stability is pH dependent (except for Nsulfo-carbamoyl components) [136]. STX and analogs act by binding to Nav and consequently blocking ion conductance in nerves and muscles fibers leading to paralysis [137]. Symptoms resulting from PSTs poisoning are described in Table II.1.



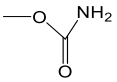
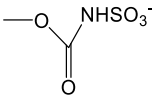
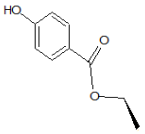
Toxin	R_1	R_2	R_3	R_4
STX	H	H	H	
GTX1	OH	H	OSO_3^-	
GTX2	H	H	OSO_3^-	
GTX3	H	OSO_3^-	H	
GTX4	OH	OSO_3^-	H	
NEO	OH	H	H	
dcSTX	H	H	H	-OH
dcGTX1	OH	H	OSO_3^-	
dcGTX2	H	H	OSO_3^-	
deGTX3	H	OSO_3^-	H	
dcGTX4	OH	OSO_3^-	H	
dcNEO	OH	H	H	
dcGTX5	H	H	H	
dcGTX6	OH	H	H	
C1	H	H	OSO_3^-	
C2	H	OSO_3^-	H	
C3	OH	H	OSO_3^-	
C4	OH	OSO_3^-	H	
GC1	H	H	OSO_3^-	
GC2	H	OSO_3^-	H	
GC3	H	H	H	

Figure II.9. Chemical structures of STX group.

Tetrodotoxins

Tetrodotoxins (TTXs) (Figure II.10) are toxins produced by bacteria in marine environments: *Serratia marcescens*, *Vibrio* spp. [83], *Aeromonas* sp. [138], *Microbacterium arabinogalactanolyticum* [139], *Pseudomonas* sp. [140], *Shewanella putrefaciens* [141], *Alteromonas* sp. [142], *Pseudoalteromonas* ssp. [143], and

Nocardiopsis dassonvillei [144] (Table II.1). They are colorless, crystalline-weak basic compounds with one positively charged guanidinium group and a pyrimidine ring [145,146]. TTX poisoning has been recognized since ancient Egyptian times [42]. To date, TTX is considered an extremely potent emergent toxin in the Atlantic Ocean [83] and acts by binding to Nav (neuron navigators – cytoskeletal associated proteins important for neuro migration, neurite growth, an axon guidance but they also function more widely in other tissues) on the surface of nerve cell membranes blocking the cellular communication and causing death by cardio-respiratory paralysis [147].

Several poisoning incidents have reported in Asia [Japan is the most affected country] [148], the Mediterranean Sea and the Indian Ocean [35]. TTX is usually concentrated in the ovaries, liver, intestines, and skin ofits principal vector [puffer fish] [42]. To date, the structures of 26 analogs of TTX have been fully elucidated but their relative toxicity and occurrence are not yet fully known [145,146] except for 12compounds, namely, TTX, 11-oxoTTX, 11-deoxyTTX, 11-norTTX-6[R]-ol, 11-norTTX-6[S]-ol, 4-epiTTX, 4,9-anhydroTTX, 5,6,11-trideoxyTTX. [131], 4-CysTTX, 5-deoxyTTX, 5,11-dideoxyTTX, and 6,11-dideoxyTTX [149–152].

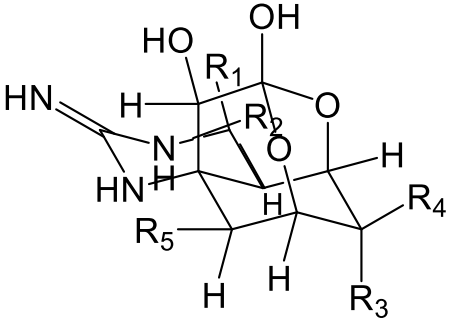
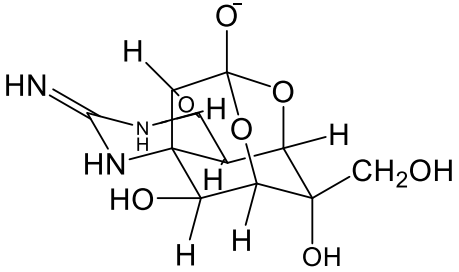
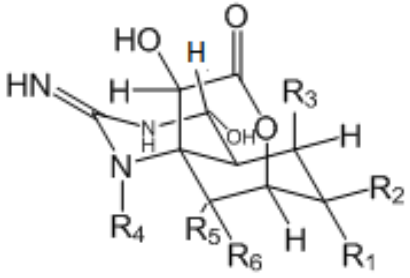
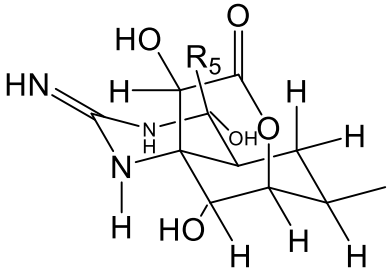
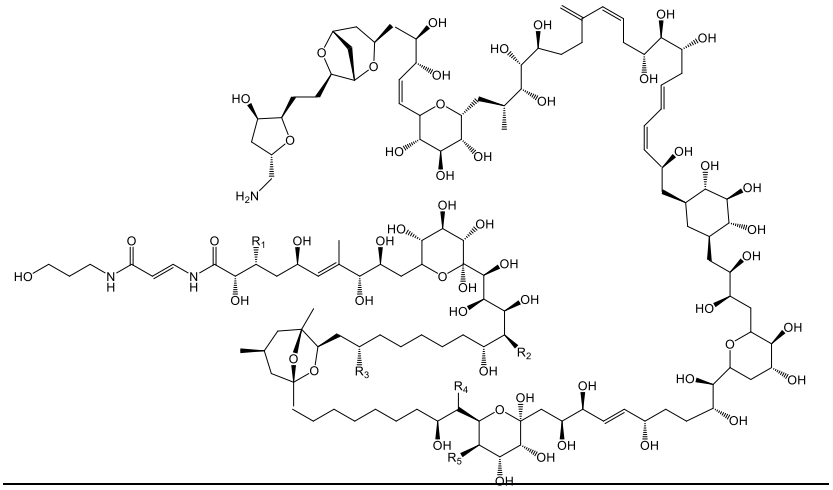
Structure	Toxin	R_1	R_2	R_3	R_4	R_5	Title
	TTX	H	OH	OH	CH_2OH	OH	
	4-*epi*TTX	OH	H	OH	CH_2OH	OH	
	11-deoxyTTX	H	OH	OH	CH3	OH	
	11-oxoTTX	H	OH	OH	$CH[OH]_2$	H	
	11-nor TTX-6[R]-ol	H	OH	H	OH	OH	
	11-norTTX-6[R]-ol	H	OH	H	OH	OH	
	11-norTTX-6[S]-ol	H	OH	OH	H	OH	
	5-deoxyTTX	OH	CH_2OH	H	H	OH	
	5,11-dideoxyTTX	OH	CH3	H	H	OH	
	5,6,11-trideoxyTTX	H	CH_3	H	H	OH	

Figure II.10. Chemical structure of TTX and their main analogues.

Palytoxin

Palytoxin (PlTX) and its derivatives (Figure II.11) are toxins produced by marine zoanthids *Palythoa* spp., dinoflagellates: *Ostreopsis ovata*. [153–155] and possibly by cyanobacteria: *Trichodesmium* sp. [156] (Table II.1). These polyhydroxylated toxins have both lipophilic and hydrophilic properties [157] with a partial unsaturated aliphatic backbone containing cyclic ethers, 64 chiral centers, 40–42 hydroxyl and 2 amide groups [157]. Among PlTX analogs, known are: isobaric PlTX, ostreocin-D, ovatoxin [a to f], mascarenotoxins, ostreotoxin-1 and 2, homopalytoxin, bishomopalytoxin, neopalytoxin, deopalytoxin and 42-hydroxypalytoxin and their molecular weights range from 2659 to 2680 DA [158–160]. PlTX and analogs act on Na^+, K^+ -ATPase pumps molecules in the cell membrane [161] and the loss of intracellular contents into the blood plasma and consequent injury causing rhabdomyolysis, among other signs, are the most reported as signs of PlTX poisoning [161].



Toxin	R_1	R_2	R_3	R_4	R_5
Palytoxin	CH_3	OH	CH_3	OH	OH
Ostreocin-D	H	H	H	OH	H

Figure II.11. Chemical Structure of PlTXs [PTX and Ostreocin-D].

Marine Cyanotoxins

Most marine toxins reported are produced mainly by microalgae (composed basically

by dinoflagellates, diatoms, and marine bacteria), while cyanobacteria are reported as toxin producers in fresh, brackish waters and terrestrial habitats. Recently, cyanotoxins typical from freshwater have been identified in the marine environment [162]. Thus, this section will be focused on the description of the most reported marine cyanotoxins involved in seafood poisoning, their producers and mode of action (Table II.1).

One of the most relevant groups of marine cyanotoxins is themicrocystin group (MCs) [163] (Figure II.12). MCs are produced by cyanobacteria of genus *Pseudoanabaena, Phormidium, Spirilia* [164], *Leptolyngbya, Oscillatoria, Geitlerinema* [165], *Trichodesmium* [166] and *Synechococcus* [167] and their occurrence have been reported in many parts of the world, namely: the central Atlantic coast of Portugal [168], Canary Islands Archipelago [166], Brazilian coast [169], Amvrakikos Gulf (Greece) [167] and Indian Ocean [170]. To date, MCs is regulated in freshwater habitats but should be extended to the marine environments since there are reports of these hepatotoxins in marine environments [162].

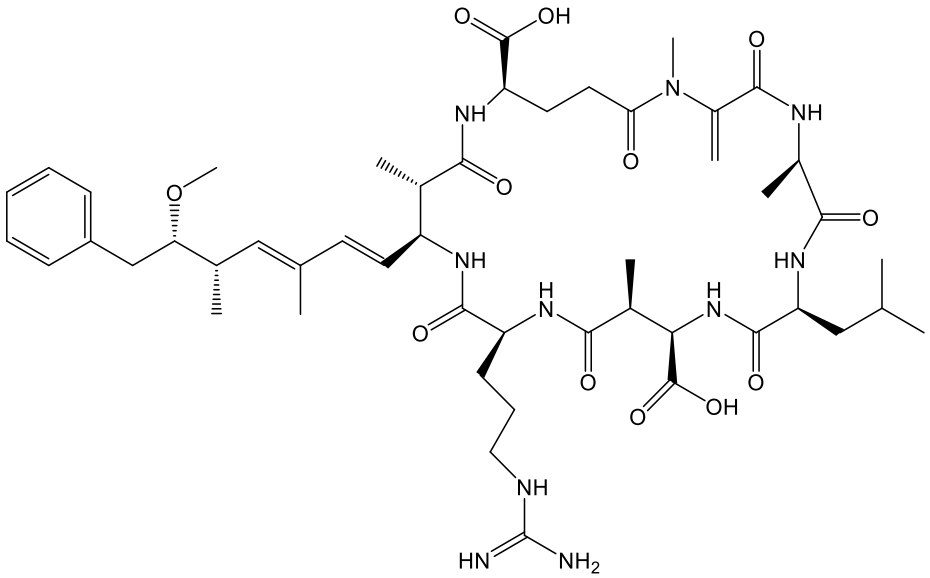
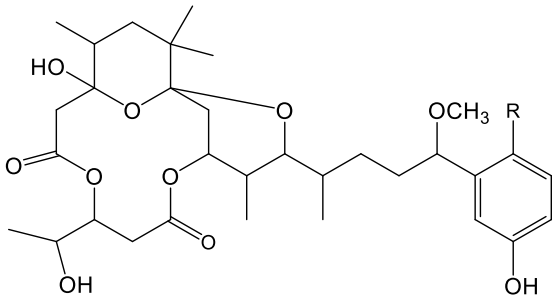
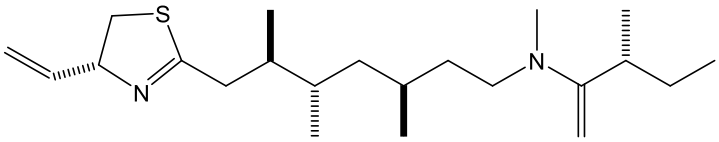
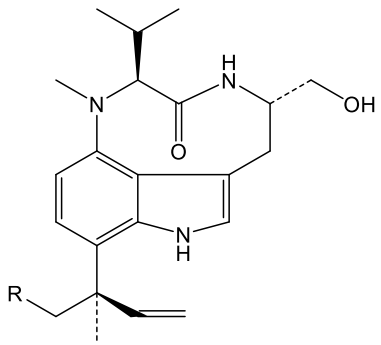
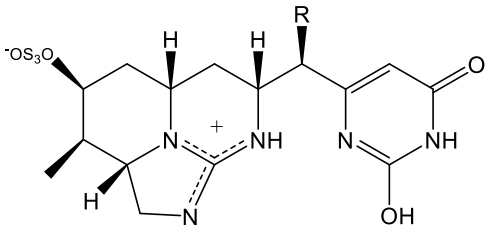
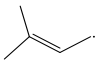
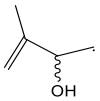
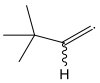
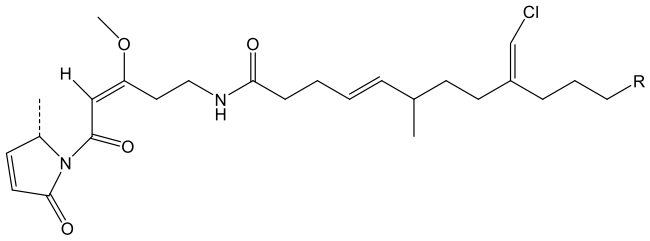
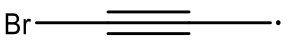
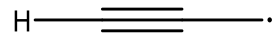
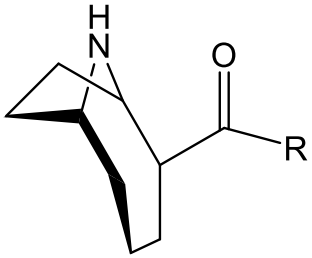


Figure II.12. Chemical structure of MC.

Other reported marine cyanotoxins [in parenthesis is indicated their producers] (Figure II.13) are aplysiatoxin (AT) [171] (Figure II.13a), debromoaplysiatoxin (DAT)

[171] (Figure II.13) (algae *Gracilaria coronopifolia* [172] and cyanobacteria *Lyngbya majuscule* [171]), kalkitoxin (KTX) (cyanobacteria *Lyngbyamajuscula* [173]) (Figure II.13b), lyngbyatoxins (LA, LB and LC) (cyanobacteria *Lyngbya majuscule* [174]) [Figure II.13c], cylindrospermopsins (CYNs) (cyanobacteria *Cylindrospermopsis raciborskii* [175]) (Figure II.13d), jamaicamides (JCDs) (Cyanobacteria *Lyngbya majuscule* [176]) (Figure 13e), anatoxins (ANTX) (cyanobacteria *Hydrocoleum lyngbyaceum* [177]) [178] (Figure II.13f) andantillatoxins (ATX) (cyanobacteria *Lyngbya majuscule* [179]) (Figure II.13g). The mechanism of action anddetection methods are presented in Table II.1.

Order	Structure	Toxin	R
(a)		AT	Br
		DAT	H
(b)			
(c)		7-*epi*CYN	OH, epimer at C7
		CYN]	OH
		do-CYN	H

Order	Structure	Toxin	R
(d)		LA	
		LB	
		LC	
(e)		JCD A	
		JCD B	
		JCD C	
(f)		ANTX-a	CH_3
		HANTX - a	CH_2CH_3
(g)		ATX A	

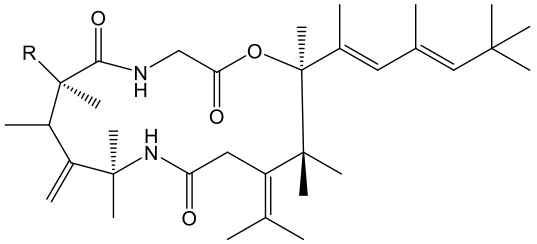
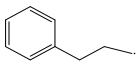
Order	Structure	Toxin	R
		ATX B	

Figure II.13. Chemical structures of Aplysiatoxin (AT) and Debromoaplysiatoxin (DAT) (**a**); kalkitoxins (KTX) (**b**); lyngbyatoxins A, B and C (LA, LB and LC) (**c**); cylindrospermopsins (CYN) (**d**); jamaicadimes (JCD) (**e**); anatoxin-a (ANTX) and homoanatoxin-a (HANTX) (**f**) and antillatoxins (ATX) (**g**).

Recent studies indicate Homoanatoxin-a (HANTX, a derivative of anatoxin-a) produced by the cyanobacteria *Hydrocoleum* sp. and *Trichodesmium* sp. which co-occur with *G. toxicus*, may be the causative toxin of CFP [43] (rather than CTXs). This evidence suggests further studies to clarify marine cyanotoxins responsible for CFP and their mechanism of action [178]. The reports of seafood poisoning involving marine cyanotoxins are very scarce and consequently, there is no specific symptomology that can be related to marine cyanotoxin human poisoning.

Incidence of Harmful Algal Blooms Marine Toxins and Consequent Poisoning Incidents along African Indian and the Red Sea Coasts

The main geographical focus of this review is the African Indian and the Red Sea coasts, including surrounding islands (Figure II.14). The marine environment of this area is understudied due to a lack of monitoring infrastructure. There is a high rate of poverty in local communities, and the local population is vulnerable to natural disasters [including HABs, tropical storms]. The exponential increase in population accompanied by industrialization and climate change contributes to eutrophication in coastal areas and it is of the main causes of the HABs proliferation in the marine in environment [295,296]. This study area is characterized as

subtropical to tropical climate with a water temperature above 20 °C [297]. Eutrophication and the transportation of cysts [through maritime traffic] are considered the main factors contributing to large phytoplankton blooms, including those comprised of HAB species and/or pathogenic bacteria [295,296]. Countries with monitoring programs of marine environments related to control of seafood poisoning are listed in Table II.2. A few of these programs have noted the presence of MTs (Figure II.14) and HAB species [dinoflagellates, cyanobacteria, diatoms], some of which [HAB species] were detected/confirmed by microscopic techniques and some confirmed by partial 16 S rRNA genes analysis [12,13,298–323].

Table II.2. MT monitoring scenario of the African countries of the Indian Ocean and the Red Sea.

Country	Monitored MT	Permitted Limit, mgKg^{-1} Shellfish	Detection	Laboratories for Toxin Analysis	Reference
South Africa	PST	0.8 STX		Research centers and Universities	[324]
	OA, DTX1-2, PTX1-2	0.16 mg OA	LC-MS/MS		
	YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX	8 mg YTX	LC-MS/MS		
	AST	20 mg DA			
	AZA1-3	0.16 mg OA	LC-MS/MS		
Mozambique	N.D.	N.D:	N.D.	N.D.	N.D.
Tanzania	CTX, TTX, AST	N.D.	Symptomology and vectors	N.D.	[325]
Kenya	MT producers [HAB]	N.D.	N.D.	Mombasa Research Center	[326]

Country	Monitored MT	Permitted Limit, $mgKg^{-1}$ Shellfish	Detection	Laboratories for Toxin Analysis	Reference
Madagascar	N.D.	N.D.	Educational programmes	Research centers and Universities	[327]
French Islands	N.D.	N.D.	N.D.	Researches centers	[35,328]
Mauritius	N.D.	N.D.	N.D.		333]
Comoros	N.D.	N.D.	N.D.	N.D.	
Somalia and Seychelles	N.D.	N.D.	N.D.	N.D.	
Eygpt	N.D.	N.D.	N.D.	Poison Control Center, Ain Shams University	[329,330]
Djibouti	N.D.	N.D.	N.D.	N.D.	
Eritrea	N.D.	N.D.	N.D.	N.D.	
Sudan	N.D.	N.D.	N.D.	N.D.	

N.D - No data

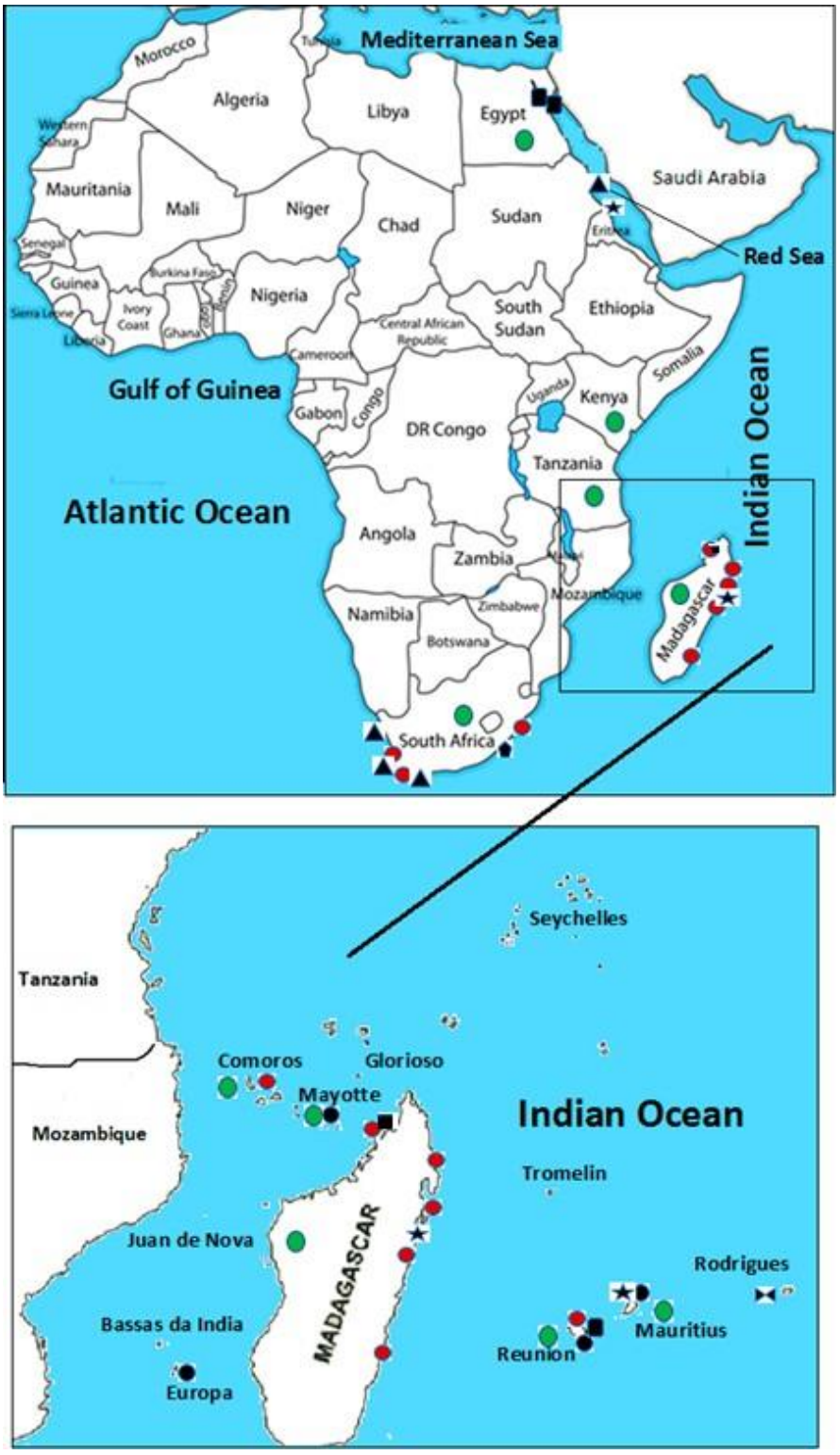


Figure II.14. Map of the incidence of marine toxins (MT) along African countries of the Indian Ocean and the Red Sea, from EgypttoSouth Africa and nearby islands. Red

circles [●]—confirmed or suspected seafood poisoning episodes caused by MT; green circles [●]—MT or Harmful Algal Blooms monitoring programmes or Centers of seafood poisonings; ▲—Saxitoxins group; ●—Okadaic Acid group; ★—Ciguatoxin group; ⋈—Palytoxin group; ⬟—Domoic Acid group and ■—Tetrodotoxin group.

South Africa

The occurrence of species of phytoplankton including MTs-producing HABs has been reported in coastal waters of South Africa through scientific reports and environmental monitoring programmes since 2011 [324]. Reported producer species include cyanobacteria (*Microcystisaeruginosa, Oscillatoria* sp., *Trichodesmium* sp.), dinoflagellates (*Dinophysisacuminata, D. rotundata*, *Alexandrium catenella, A. minutum*, *Gymnodinium* sp., *Prorocentrum* sp., *Gambierdiscustoxicus*, *Ostreopsis siamensis, O. ovata, P. lima, P. concavum*), diatoms (*Pseudo-nitzschia multiseries*) [19,305,309,315,331–333] and bacteria (*Vibrio parahaemolyticus*) [298]. Seafood poisoning cases were also reported in South Africa caused by PSTs, DSPs, PlTXs and GYM [19,216,309,334] (Table II.3) after the consumption of mussels (*Donax serra*, *Perna perna* and *Chloromytilus meridionalis*) (Table II.4) [37]. To minimize seafood poisoning by MTs, South Africa has implemented, through the Department of Agriculture, a program for MT monitoring in molluscan shellfish on all coasts (South African Molluscan Shellfish Monitoring and Control Programme) [324] (Table II.2). This program was created based on the regulations of the European Commission (EC) Regulation, namely: Commission Regulation (EC) No 2074/2005, No 853/2004 and No 15/2011 where limit values are described for MTs and analytical techniques are advised to monitor shellfish [324]. Due to the absence of legislation regarding CTXs, currently, there is an absence of monitoring programs regarding this group in South Africa since the Indian Ocean is considered an endemic site of CTXs, this is a matter of major importance.

Mozambique

Studies related to HAB occurrence in Mozambique are very scarce and the few published works indicate the occurrence of dinoflagellates of the genus *Alexandrium*

[313] and species of cyanobacteria (*Phormidium ambiguum, Lyngbya majuscula,* and *Lyngbya* cf. *putealis*) [307]. To date, due to the absence of a Monitoring Program and trained health staff to recognize specific symptoms of seafood poisoning in humans, there are no records of published data of MT occurrence or reports of seafood poisoning cases in this country.

Tanzania

Published studies indicate the occurrence of cyanobacteria, namely: *Pseudanabaena* sp., *Spirulina labyrinthiformis*, *Spirulina* sp., *Leptolyngbya* sp., *Phormidium* sp., *Oscillatoria* sp., *Lyngbyaaestuarii, Lyngbya* sp., *Lyngbya majuscula*, *Nodularia* sp., *Synechococcus* sp., *Microcystis* sp.; Dinoflagellates: *Gambierdiscus toxicus*, *Procentrum* sp. and diatoms: *Pseudo-nitzschia* sp., *Pseudo-nitzschia pungens*, *P. seriata* and *P. cuspidate* [335–341]. Data related to MTs and seafood poisoning episodes are very scarce in Tanzania. In 2003, the Tanzanian government created guidelines for investigation and control of foodborne diseases and the regulatory institution is the Tanzania Food and Drugs Authority (TFDA) (Table II.2) [325]. The main objective of TFDA is to regulate matters related to food quality and safety for consumers through the dissemination of the information related to causative agents, latency period [duration], principal symptoms, typical vectors, and prevention of poisoning as measures of public health protection [325]. Among several foodborne disease sources, MTs such as CTXs, TTXs, DA, and PSTs are described by TFDA. The creation of alert and monitoring programs is an effective way to prevent poisoning episodes caused by MT-contaminated seafood.

Kenya

In order to reduce the cases of seafood poisoning caused by MTs, the Kenya Marine and Fisheries Research has carried out projects funded by governmental and non-governmental institutions for monitoring levels of HABs and their toxins (Table II.2) in coastal waters and shellfish as well as the possible transfer in the trophic food web [326].Since October 2017, there is an ongoing project (BIOTOXINS Research Project) funded by National Commission for Science,

Technology and Innovation (NACOSTI) at Mombasa Research Center [326]. This project will cover a period of 2 years, which is not enough for long-termmonitoring. In these coastal waters were reported to occur several species of diatoms: *Nitzschia* sp., *N. closterium, N. longisigma, N. sigma, Pseudo-nitzschia* sp. *Guinardia* sp., *G. striata, G.delicatula, Skeletonema* sp, *Leptocylindrus* sp., *Rhizosolenia* sp., *Cerataulina* sp., *Coscinodiscus* sp., *Thalassiosira* sp., *Corethron* sp., *C. criopilum, C. cenofemus* and *Chaetoceros* sp.; dinoflagellates: *Alexandrium* sp., *Dinophysis* sp., *D. caudata, Gambierdiscus* sp., *G. toxicus, Gonyaulax* sp., *Gymnodinium* sp., *Gyrodinium* sp., *Ostreopsis* sp., *Peridinium* sp., *Prorocentrum* sp., *Ceratium* sp., *C. fusus, C. furca, Noctiluca* sp., *N. scintillans*, *Protoperidinium* sp., *Scrippsiella* sp. and *S. trochoidea* [301,310]. Cyanobacteria were also reported: *Lyngbya* sp., *Oscillatoria* sp., *Fischerella epiphytica*, *Anabaena* sp., *Nodularia spumigena*, *Umezakia natans*, *Aphanizomenon flos-aquae, Microcystis aeruginosa and Trichodesmium* sp. [342].

Madagascar

Madagascar is the country with more records of published data regarding MT occurrence (Figure II.14) and consequently, many reported cases of seafood poisoning [36,47,49,343]. The seafood poisoning cases in Madagascar have been registered since 1930 mainly after the consumption of fish of the family *Sphyrnidae, Cacharinidae, Clupeidae* (herrings, sardines), and marine turtles species (*Eretmochelys imbricata* and *Chelonia mydas*) [36,47,49,343]. The main marine poisoning causative agents reported are CTXs, TTXs, and PlTXs [18,344] (Table II.4). To reduce the number of seafood poisoning events, the MadagascarMinistry of Health has created a Seafood Poisoning National Control Program (Table II.2) based on the setting of an epidemiological surveillance network, prevention of the communities through educational programs and the development of research on marine eco-environment [327].

Indian Ocean French Islands

Mayotte, Europa, Banc du Geyser, Bassas da India, Glorioso, Juan de Nova, Reunion and Tromelin islands administratively make part in the French government

but since they are in the Indian Ocean, were considered for the present study. In these islands, there are reports of the occurrence of HABs and cases of seafood poisoning linked to MTs. The reported HAB forming species include: dinoflagellates (*Prorocentrum lima, P. convacum, Ostreopsis ovata, Gambierdiscus toxicus, Alexandrium* spp.), cyanobacteria (*Hydrocoleum* sp., *Lyngbya majuscula, Phormidium* sp., *Leptolyngbya* sp. and *Oscillatoria* sp.) [70,300,317,319,345]. The recorded human intoxications were due to DSTs and TTXs [35,328] (Table II.4). Centers of Disease for control and Preventing is the organization responsible for National Biomonitoring Program of toxins (PSTs) in these islands [35,328] (Table II.2).

Mauritius

In Mauritius there are registered cases of seafood poisoning caused mainly by CTXs [346] after the consumption of reeffish (*Lutjanus sebae*) [70,71,71] (Table II.4). The Ministry of Ocean Economy, Marine Resources, Fisheries and Shipping of Mauritius is the institute responsible for themonitoring of HABs (Table II.2) [347,348], developing several activities and reporting the principal vectors species involved in seafood poisoning, namely: fish (*Variola louti, Plectroponus maculatus, ceragidae, Vieille loutre, V. plate, V. cuisinier, Lutjanus gibbus, L. sebae, L. monostigmus, L. bohar, Anyperodon leucogramnicus, Harengula ovalis, Sphyraena barracuda, Synancela verrucose, Remora remora, Lactoria carnuta, Diodon hystrix*), turtles (*Eretmochelys imbricate*), crabs (*Carpillus maculatus*), sea-urchins (*Echinothrix* sp.) and bivalves (*Tridaena* sp.) [348].

HAB producers recorded in Mauritius include several dinoflagellates species (*Ostreopsis mascarenensis*, *Gambierdiscus toxicus* Adachi & Fukuyo, *Ostreopsis ovata* Fukuyo, *Ostreopsis siamensis*, *O. mascarenensis*, *Prorocentrum lima*, *P. concavum*, *P. hoffmanianum*, *Amphidinium* sp., *A. carterae*, *Coolia* sp., *Sinophysis* sp., *Gymnodinium* sp., *Gonyaulax* sp., and *Alexandrium* sp.), diatoms (*Pseudo-nitzschia* sp.) and cyanobacteria (*Phormidium* sp., *Oscillatoria* sp. and *Lyngbya* sp., *Phormidium* sp., *Oscillatoria* sp. and
Lyngbya sp.) [308].

The Archipelago of Comoros

Published data of the archipelago of Comoros indicate the occurrence of *Gambierdiscus toxicus*, *G. yasumotoi*, *G. belizeanus*, *Prorocentrum arenarium*, *P. maculosum*, *P. belizeanum*, *P. lima*, *P. mexicanum*, *P. hoffmanianum*, *P. concavum*, *P. emarginatum*, *P. elegans*, *P*. sp., *Ostreopsis caribbeanus*, *O. mascarenensis*, *O. ovata O. heptagona*, *O. labens*, *O. siamensis*, *O. lenticularis*, *O. marinus*, *Cooliamonotis*, *C. tropicalis*, *Sinophysis microcephalus*, *S. canaliculate* and *Amphidiniopsis* sp. [10,300]. Suspected seafood poisoning episodes linked to MTs were registered in the archipelago of Comoros after the consumption of turtle Eretmochelys imbricate with symptomatology similar to CFP [26], suggesting the presence of CTXs (Table II.4).

Somalia and Seychelles

There are no published studies related to the occurrence of HABs and MTs in Somalia and Seychelles. While there are no published reports of HABs or MTs in Somalia and Seychelles waters, the proximity to other countries with such reports and currents in the area suggest that investigations are necessary to avoid potential seafood poisoning events [62].

Table II.3. Geographic occurrence MT per country, MT producer, and MT vector along African countries of the Indian ocean and red sea coasts. TX - toxin.

Toxin	Date	Location	Toxin Producer	Determination Method	Toxin Vector	TX Concentration, (mg TX Equivalents per Kg Shellfish Meat)	Cell/Extract Toxicity	Reference
PSTs	1999	South Africa	*Alexandrium catenella*	AOAC mouse bioassay	*Haliotis midae*	0.01609 STX		[22]
	1998–2002	South Africa: Yzerfontein,	*Alexandrium catenella* / *Alexandrium tamiyavanichi*	HPLC-FLD	-	-	4.8 pg STX eq cell^{-1} / 0.14 pg STX eq cell^{-1}	[334]
	2003–2004	South Africa: Cape Town	*Alexandrium minutum*	LC-FD and HILIC-MS/MS	-	-	0.65 pg GTX cell^{-1}	[309]
	2012 –2014	Central Red Sea	*Pyrodinium bahamense,Ceratium* sp*, Alexandrium* sp. and *Protoperidinium* spp.	ELISA	-	-	>> 0.4 ng mL^{1}	[349]
DSTs	2000	Europa Island Mozambic channel, France]	*Prorocentrum arenarium*	FR3T3 fibroblast	-	-	IC_{50} = 0,1 µg OA ml^{-1} and 50 µg extract ml^{-1}	[11]

Toxin	Date	Location	Toxin Producer	Determination Method	Toxin Vector	TX Concentration, (mg TX Equivalents per Kg Shellfish Meat)	Cell/Extract Toxicity	Reference
				PPIA HPLC-FD HPLC-MS			22 ng OA/mg of extract	
	2001	Lagoons of La Reunion Mayotte and Mauritius Islands	*Prorocentrum lima*	PPIA	-	-	IC_{50} 1.3–25 mg/mL on on fibroblast; 6261.3 ± 156.5 − 128.3±17.2 ng eq OA/mg crude extract	[328]
	2002–2018	South Africa:Abalgold	-	-	*Haliotis asinina*	-	-	[324]
	2008		*Dinophysis acuminata*	LC-MS/MS	*Crassostrea gigas*	0.267 OA		

Toxin	Date	Location	Toxin Producer	Determination Method	Toxin Vector	TX Concentration, (mg TX Equivalents per Kg Shellfish Meat)	Cell/Extract Toxicity	Reference
		South Africa: Saldanha Bay and Lambert's Bay			*Choromytilus meridionalis*	0.012 OA		
CTXs	2001	Mauritius: Nazareth, Saya de Malha and Soudan	-	HPLC-MS/RLB, Mongoose feeding test, and MBA	*Lutjanus sebae* and *Lutjanus Lab*	Qualitative analysis	-	[71]
	2002	North of the Republic of Mauritius, Banks fishery	-	HPLC-MS/RLB	*Lutjanus sebae*		-	[70]
	2012–2013	Central Red Sea	*Gambierdiscus belizeanus* and *Ostreopsis* spp.	Mouse neuroblastoma cell-based assay	-	-	6,50–1,14.10 $^{-5}$ pg P-CTX^{-1} eq. cell^{-1}	[350]
	2013	Madagascar: district of Fenoarivo Atsinanana	*Gambierdiscus* spp.	CBA / MBA	*Carcharhinus leucas*	0.083 P-CTX-1 / 0.09272 P-CTX-1	-	[20]

Toxin	Date	Location	Toxin Producer	Determination Method	Toxin Vector	TX Concentration, (mg TX Equivalents per Kg Shellfish Meat)	Cell/Extract Toxicity	Reference
				LC-ESI-HRMS		0.01628 P-CTX-1		
				MBA		752 MU/g		
PlTXs	1994	Madagascar: Antalaha District	*Ostreopsis siamensis*	MBA	*Herklotsichthys quadrimaculatus*	0.00045 PTXs/ fish [head and esophagus]		[18]
				Hemolysis assays		0.00002 PTXs/fish [head and esophagus]		
				Cytotoxicity tests		0.00000005 /fish [head and esophagus]		
				MS				
	1996	Mauritius: Rodrigues Island	*Ostreopsis mascarenensis*	HPLC-diode array detector, Nanoelectrospray ionization quadrupole time-of-flight and	-	-		[14,160]

Toxin	Date	Location	Toxin Producer	Determination Method	Toxin Vector	TX Concentration, (mg TX Equivalents per Kg Shellfish Meat)	Cell/Extract Toxicity	Reference
				HPLC-ESI-MS/MS analysis				
				Hemolysis assays			8.00 ± 0.01 ng PTX mL^{-1}	
				Cytotoxicity Assay			IC50 = 10 µM against human H460 lung cancer cells	
	2008	South Africa: Saldanha Bay and Lambert's Bay	*Dinophysis acuminata*	LC-MS/MS	*Crassostrea gigas*	0.267 OA		
					Choromytilus meridionalis	0.012 OA		
DA cultures	2012	South Africa: Algoa Bay	*Pseudo-nitzschia multiseries*	ELISA	-	-	0.076 pg DA $cell^{-1}$– 0.098 pg DA $cell^{-1}$	[12]
				LC/MS–MS			0.086 pg DA $cell^{-1}$–	

Toxin	Date	Location	Toxin Producer	Determination Method	Toxin Vector	TX Concentration, (mg TX Equivalents per Kg Shellfish Meat)	Cell/Extract Toxicity	Reference
							0.086 pg DA cell^{-1}	
TTXs	1990–1991	Egypt: Suez City, in the northwestern part of the Red Sea		TLC, electrophoresis, UV, GC–MS MBA	*Pleuranacanthus sceleratus*	752 MU/g		[316]
	1998	Madagascar: Nosy Be Island -	-	MBA		16 MU/g		[41]
	2002–2003	Egypt: Gulf of Suez		MBA	*Lagocephalus sceleratus*	3950 MU/g		[351]
	2013	Reunion Island		MBA and LC-MS/MS	*Lagocephalus sceleratus*	17 TTX	-	[35]

The Red Sea (Djibouti, Eritrea, Sudan, Egypt)

Several research works related to MTs are carried out in the Red Sea but are very limited on the African coast. Saudi Arabia is the country with the most published studies related to the occurrence of HABs along the Red Sea [13,308,311,316,321,322,352,353]. The Dinoflagellates (*Alexandrium* sp., *Dinophysis* sp., *Prorocentrum* sp., *Pyrodinium* sp., *Gymnodinium* sp.), cyanobacteria (*Lyngbya* sp., *Oscillatoria* sp., *Trichodesmium* sp.) and diatoms (*Pseudonitzschia* spp.) are the most reported marine producer species [13,308,311,316,321,322,352,353]. The bacteria *Vibrio paraehemolyticus*, producer of TTX, was detected in shrimp (*Penaeus latisulcatus*) in the Suez Gulf [299]. MTs reported in the Red Sea, mainly the Egyptian coast, described in Tables II.3 and II.4, include CTXs, TTXs, PSTs detected in puffer fish such as *Pleuranacanthus sceleratus* and *Lagocephalus sceleratus* [13,316,349–353]. Cases of seafood poisoning caused by CTXs and TTXs were reported, and according to the Poison Control Center, affiliated with Ain Shams University (Cairo, Egypt), CTXs are the third most responsible agents that induce food poisoning in Egypt [354]. Puffer fish poisoning has been recorded since ancient Egyptian times [42]. In Egypt, there is monitoring of HABs in aquatic ecosystems since 1994 when Egypt became a member of the Convention on Biological Diversity although the Nature Conservation Sector, Egyptian Environment Affairs Agency and the Ministry of State for Environmental Affairs (Table II.2) are focal points [330]. There are no reports of HABs and MT occurrence in coastal areas of Djibouti, Eritrea, and Sudan.

Table II.4. Seafood poisoning episodes caused by MTs, observed effects/Symptoms, fish or shellfish consumed and victim number affected along African countries of the Indian Ocean and Red Sea coasts. TX – Toxin

Local	Date	Seafood	Observed Effects/Symptoms	TX	Detection Method	TX Concentration, (mg TX Equivalents/ Kg Shellfish Meat)	Victim Number	Reference
Comoros islands: Ndrondroni	24 December 2012	*Eretmochelys imbricata* (turtle)	Itching, Asthenia, Vomiting, Abdominal pain, Rash Myalgia Shortness of breath, Nausea Itching of the mouth/throat, Fever, Diarrhea Vertigo, Paresthesia, Dysphagia Mouth burn Sore throat, Erectile dysfunction	-	-	-	49 suspected cases and 8 probable cases, age range [0-40 years], 1 death	[26]
North-eastern coast of Madagascar	December 1994	Turtle	Nausea, vomiting, dysphagia, acute stomatitis	-	-	-	60 persons with poisoning attack rate were 48% with a lethality of 7.7%	[47]
Madagascar: district of	November 2013			CTXs	MBA	0.083 P-CTX-1	124 people, 9% deaths	[20]

Local	Date	Seafood	Observed Effects/Symptoms	TX	Detection Method	TX Concentration, (mg TX Equivalents/ Kg Shellfish Meat)	Victim Number	Reference
Fenoarivo Atsinanana		Carcharhinus leucas (shark)	Paresthesia of the extremities, dysesthesia, , dizziness, and arthralgia between 2 and 12h after ingestion		CBA	0.09272 P-CTX-1		
Madagascar: Antalaha District	January 1994	*Herklotsichthys quadrimaculatus*(Fish)	Malaise, uncontrollable vomiting, diarrhea, tinglings of extremities, delirium and death	PlTXs	MBA	0.00045 PTXs/ fish [head and esophagus]	Death of one adult	[18]
					Hemolysis assays	0.00002 PTXs/fish (head and esophagus)		
					Cytotoxicity tests	0.00000005 /fish (head and esophagus)		
					Mass spectroscopy	-		

Local	Date	Seafood	Observed Effects/Symptoms	TX	Detection Method	TX Concentration, (mg TX Equivalents/ Kg Shellfish Meat)	Victim Number	Reference
Madagascar : Nosy Be Island	July 1998	-	-	TTXs	MBA	16 MU/g (no data to covert to mg/Kg)	4 people, one death	[41]
Madagascar : Manakara district	November 1993	*Carcharhinus amboinensis*[shark]	Deep coma and death, body rigidity due to loss of cerebral function, myosis, mydriasis, convulsions, Respiratory distress due to acute pulmonary edema, cardiovascular collapse, bradycardia, gengivorrhagia Dehydration, paresthesia on fingertips and toes, dizziness, pruritus, narcosis, faintness, hyperthermia, ataxia asthenia, dehydration, cephalalgia, diarrhea, epigastralgia, laryngeal distress	CTXs	Ciguatera poisoning Symptomology	-	500 people, 20% deaths	[21]

Local	Date	Seafood	Observed Effects/Symptoms	TX	Detection Method	TX Concentration, (mg TX Equivalents/ Kg Shellfish Meat)	Victim Number	Reference
South Africa: Cape Town	May 1978	*Choromytilus meridionlis*[Mussel]	Paraesthesia of en fingers/hands, Circumoral paresthesia, paranesthesia of toes/feet, Vertigo, Floating sensation, Ataxia, Weakness of upper, Weakness of lower limbs and Dysarthria A headache	PSTs	MBA	72.83 STX	17 people, no deaths	[39]
South Africa: Natal coast	December 1957	*Mytilus meridionalis* [Mussel]	peculiar lightness of the body, with a tingling around mouth, finger, and toes; no moving; feeble inarticulate noise;	PSTs	MBA	0.04 STX	5 people and one cat	[40]
South Africa: Table and False Bays	1888	Donax serra [Mussel]	-	-	-	-	-	[37]
South Africa:	April 1948	*Donax serra*[Mussel] and	-	-	-	-	One death	

Local	Date	Seafood	Observed Effects/Symptoms	TX	Detection Method	TX Concentration, (mg TX Equivalents/ Kg Shellfish Meat)	Victim Number	Reference
Cape Town		*Chloromytilus Meridionalis* [Mussel]						
South Africa: Natal coast	December 1957	*Perna perna* [Mussel]	-	-	-	-	5 people, one death	
South Africa: Cape Town	May 1958	*Chloromytilus meridionalis* [Mussel]	-	-		-	One death	
Reunion Island	September 10th, 2013	*Lagocephalus sceleratus*[fish]	peri-oral paresthesia, weakness of both lower limbs, paresthesia all over the body, headache, dyspnea, nausea and vomiting, blurring of vision, and vertigo	TTX	MBA	Liver: 17 TTX Flesh: 5 TTX	10 people	[35]

Final Considerations and Recommendations

African Indian Ocean and the Red Sea coasts have a subtropical and tropical climate, considered optimal for the development and transportation of several HAB-forming species, and consequently, the production of MTs. Paradoxically, studiesrelated to the occurrence and incidence of HABs and MTs are very limited, from South Africa to Egypt. From a few data available in this zone, most describe only the genus and not the full species, making it very difficult to evaluate the occurrence of the toxic species. The most reported HAB phytoplanktons in this region are cyanobacteria, followed by dinoflagellates, and diatoms as potential MT producers. Relative to MTs, the most reported and involved in seafood poisoning episodes include CTXs, PSTs, and TTXs. The scarcity of the data related to MTs suggests the need for further studies and the creation of specific monitoring programs of HABs, particularly for dinoflagellates and diatoms since these constitute the phytoplankton that produces more fatal MTs, though in recent years several genera of bacteria have been described as producers of a potent group of marine toxins, TTXs, which have already been detected on the African coasts of the Indian Ocean and Red Sea. The main MTs that must be monitored in shellfish are presented in Table II.5. Analytical techniques such as LC-MS/MS are advised and recommended as determination and quantification methods due to their higher reproducibility, specificity, sensitivity and capacity to discriminate analogs of given toxins in the sample. The permitted limit of a toxin in shellfish can be adopted from other countries as an example to follow such as the EU region, USA, Japan, Australia, and New Zealand.

Table II.5. Recommended marine toxins to be monitored and suggestion of permitted limit to be used.

Toxin	Syndrome	Permitted Limit, $mgKg^{-1}$	To be adopted from
STX	PSP	0.8 STXeq	EU region
CTX	CFP	0.00001 P-CTX-1eq	USA

Toxin	Syndrome	Permitted Limit, mgKg^{-1}	To be adopted from
YTX	-	3.75 YTXeq	EU region
PTX	-	0.16 OAeq	EU region
TTX	-	2 TTeq	Japan
DA	ASP	20 DAeq	EU region
OA	DSP	0.16 OAeq	EU region
AZA	AZP	0.16 AZAeq	EU region
PlTX	-	0.25 PlTXeq [*]	EU region
PbTx	NSP	0.8 TX-2 eq	USA, New Zealand, and Australia

* This toxin is not monitored and 0.25 PlTXeq was proposed in the first meeting (Cesenatico, Italy, 24–25 October 2005) of the working group on Toxicology of the national reference laboratories [NRLs] for Marine Biotoxins.

For the success of the MT monitoring programs, the integration and intercollaboration of environmental, public health and researches institutions and universities of the all African Countries of the Indian Ocean and the Red Sea is crucial.

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REVIEW ARTICLE - Marine drugs 2019, 17(1), 28: The incidence of tetrodotoxin and its analogs in the Indian Ocean and the Red Sea

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Abstract

Tetrodotoxin (TTX) is a potent marine neurotoxin with bacterial origin. To date, around 28 analogs of TTX are known, but only 12 were detected in marine organisms, namely TTX, 11-oxoTTX, 11-deoxyTTX, 11-norTTX-6(R)-ol, 11-norTTX-6(S)-ol, 4-*epi*TTX, 4,9-anhydroTTX, 5,6,11-trideoxyTTX, 4-CysTTX, 5-deoxyTTX, 5,11-dideoxyTTX, and 6,11-dideoxyTTX. TTX and its derivatives are involved in many cases of seafood poisoning in many parts of the world due to their occurrence in different marine species of human consumption such as fish, gastropods, and bivalves. Currently, this neurotoxin group is not monitored in many parts of the world including in the Indian Ocean area, even with reported outbreaks of seafood poisoning involving puffer fish, which is one of the principal TTX vectors know since Egyptian times. Thus, the main objective of this review was to assess the incidence of TTXs in seafood and associated seafood poisonings in the Indian Ocean and the Red Sea. Most reported data in this geographical area are associated with seafood poisoning caused by different species of puffer fish through the recognition of TTX poisoning symptoms and not by TTX

detection techniques. This scenario shows the need of data regarding TTX prevalence, geographical distribution, and its vectors in this area to better assess human health risk and build effective monitoring programs to protect the health of consumers in Indian Ocean area.

Keywords: Indian Ocean; Red Sea; tetrodotoxin; pufferfish poisoning

Introduction

The tropical and subtropical climates predominant in the Indian Ocean zone, accompanied by industrialization and population increase, are pointed to as the main factors that, together with eutrophication, contribute to the development of toxic phytoplankton blooms—harmful algal blooms (HABs) and bacteria [1]. HABs and some bacteria are marine toxin (MT) producers, turning the Indian Ocean zone vulnerable to this phenomenon [2–5]. One of the main Indian Ocean MTs is tetrodotoxin (a neurotoxin) and its analogs (TTXs), of which the main producers were reported to belong to different bacteria genera [6–15]. Cases of human poisoning are recurrent, especially after consumption of TTX-contaminated fish, with the puffer fish as the most common vector reported since Egyptian times [16–29]. Due to the lack of TTX monitoring programs, the episodes of human seafood poisoning are still common in the Indian Ocean area, since seafood is the most common food for many people living along coastal zones [16–22,24,26,28–38]. Thus, the objective of this paper was to review the incidence of TTX in the Indian Ocean and the Red Sea zones and associated human seafood poisoning incidents. The monitoring of TTXs in this geographic zone is also recommended.

Tetrodotoxin

TTX (Figure II.15) is a potent neurotoxin group [39] that can provoke severe poisoning after consumption of contaminated seafood. Several species of distinct marine organisms of human consumption were identified as TTX vectors: puffer fish [16–29], gastropods [40], crustaceans [41–44], and bivalves [45]. Also, the occurrence of TTXs in terrestrial vertebrates such as *Polypedates* sp., *Atelopus* sp., *Taricha granulosa*, [46] and *Cynops ensicauda popei* [47] was reported [48,49]. TTX is an alkaloid isolated for the first time in 1909 by Tahara and Hirata from the ovaries of globefish [50]. In the

marine environment, bacteria are pointed to as the main producers of this group of toxins, namely *Serratia marcescens* [51], *Vibrio alginolyticus*, *V. parahaemolyticus, Aeromonas* sp. [52], *Microbacterium arabinogalactanolyticum* [13]*, Pseudomonas* sp. [14]*, Shewanella putrefaciens* [6]*, Alteromonas* sp. [8], *Pseudoalteromonas* sp. [10]*,* and *Nocardiopsis dassonvillei* [12]. Physicochemically, TTXs are colorless, crystalline weak heterocyclic basic compounds (Figure II.15 and Table II.6), highly hydro-soluble and also heat-stable [45]; thus, the toxin is not destroyed by cooking procedures.

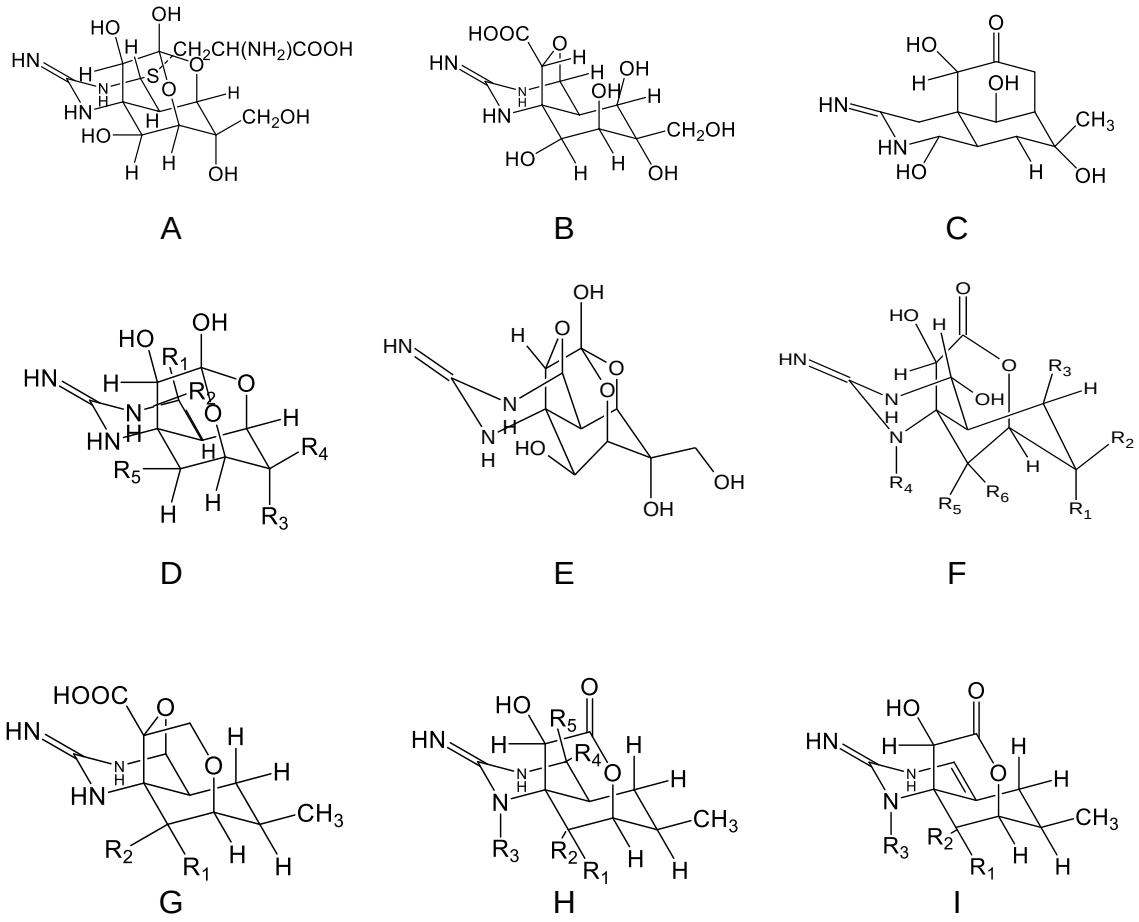

Figure II.15. Tetrodotoxin (TTX) and analogs modified from European Food Safety Authority (EFSA) 2017 [45] and Yotsu-Yamasshita et al. (2007) [15,53,54]. (*) indicates TTX analogs that occur in marine organisms with known relative toxicity. (**A**) 4-cysTTX(*), (**B**) tetrodonic acid, (**C**) 4,9-anhydroTTX(*), (**D**) 1-hydroxy-5,11-

dideoxyTTX, (**E**) TTX and 12 analogs, (**F**) 5-deoxyTTX(*) and three analogs, (**G**) trideoxyTTX and two analogs, (**H**) 4-epi-5,6,11-trideoxyTTX and another analog, and (**I**) 4,4a-anhydro-5,6,11-trideoxyTTX and 1-hydroy-4,4a-anhydro-8-epi-5,5,11-trideooxyTTX (see radicals of the analogs in the Table II.6).

Table II.6. Tetrodotoxin (TTX) and analogs shown in Figure II.15 and modified from European Food Safety Authority (EFSA) 2017 [45] and Yotsu-Yamasshita et al. (2007) [15,53].

E	R1	R2	R3	R4	R5
TTX (*)	H	OH	OH	CH_2OH	OH
4-*epi*TTX (*)	OH	H	OH	CH_2OH	OH
6-epiTTX (*)	H	OH	CH_2OH	OH	OH
11-deoxyTTX (*)	H	OH	OH	CH3	OH
6,11-dideoxyTTX	H	OH	H	CH3	OH
8,11-dideoxyTTX	H	OH	OH	CH3	H
11-oxoTTX (*)	H	OH	OH	$CH(OH)_2$	OH
11-norTTX-6,6-diol	H	OH	OH	OH	OH
11-norTTX-6(R)-ol (*)	H	OH	H	OH	OH
11-norTTX-6(S)-ol (*)	H	OH	OH	H	OH
Chiriquitoxin	H	OH	OH	$CH(OH)CH(NH_3^+)COO^-$	OH
TTX-8-*O*-hemisuccinate	H	OH	OH	CH_2OH	$OOC(CH_2)_2COO^-$
TTX-11-carboxylic acid	H	OH	OH	COO^-	OH
TTX (*)	H	OH	OH	CH_2OH	OH

F	R1	R2	R3	R4	R5	R6
5-deoxyTTX(*)	OH	CH_2OH	H	H	OH	H
5,11-dideoxyTTX (*)	OH	CH3	H	H	OH	H
5,6,11-trideoxyTTX (*)	H	CH_3	H	H	OH	H
8-epi-5,6,11-trideoxyTTX	H	CH_3	H	H	H	OH

G	R1	R2
4,9-anhydro-5,6,11-trideoxyTTX	H	OH
4.9-anhydro-8-epi-5,6,11-trideoxyTTX	OH	H

H	R1	R2	R3	R4	R5

1-hydroxy-8-epi-5,6,11-trideoxyTTX	OH	H	OH	OH	H
4-epi-5,6,11-trideoxyTTX	H	OH	H	H	OH

I	R1	R2	R3
4,4a-anhydro-5,6,11-trideoxyTTX	H	OH	H
1-hydroxy-4,4a-anhydro-8-epi-5,5,11-trideooxyTTX	OH	H	OH

To date, around 28 analogs of TTX were described (Figure II.15 and Table II.6) and some of them were detected in marine organisms [53], with their relative toxicity well known [45] (chemical structures pointed with asterisks in Figure 1): TTX, 11-oxoTTX, 11-deoxyTTX, 11-norTTX-6(R)-ol, 11-norTTX-6(S)-ol, 4-*epi*TTX, 4,9-anhydroTTX, 5,6,11-trideoxyTTX [45], 4-CysTTX, 5-deoxyTTX, 5,11-dideoxyTTX, and 6,11-dideoxyTTX [54–57] (Table 1). Their relative toxicity ranges from 0.01 to 1.0, with 5,6,11-trideoxyTTX and TTX as the least and most toxic, respectively [45], and there are still no available data regarding the toxicity for 4-CysTTX and 5,11-dideoxyTTX. Chemical abstract numbers (CAS) are also listed in Table II.7.

Table II.7. Chemical abstract numbers (CAS) and relative toxicity of TTX analogs [58,59].

TTX Analogs	TEF	CAS Number
TTX	1.0	4368-28-9
11-oxoTTX	0.75	123665-88-3
11-deoxyTTX	0.14	-
11-norTTX-6(R)-ol	0.17	-
11-norTTX-6(S)-ol	0.19	-
4-*epi*TTX	0.16	98242-82-1
4,9-anhydroTTX	0.02	13072-89-4
6,11-dideoxyTTX	0.02	-
5-deoxyTTX	0.01	-
5,6,11-trideoxyTTX	0.01	-
4-CysTTX	-	-
5,11-dideoxyTTX	-	-

* TEF—toxic equivalency factor.

The action mechanism of TTXs occurs through the occlusion of the external pore of site 1 of voltage-gated sodium channels on the surface of nerve membranes, blocking

cellular communication and causing death by cardio-respiratory paralysis [60]. Paralysis occurs by affecting the respiratory system, the diaphragm, skeletal muscles, and tissues in the digestive tract in humans [39]. TTXs normally accumulate in skin, intestines, liver, muscle, gonads, viscera, and ovaries in different species of puffer fish [16,21,22,29,33–37,61–65]. The symptoms that can be used partially as an indication of TTX human poisoning (wt = 50 kg and TTX amount = 2 mg) were grouped into four levels depending on the amount ingested [66] and are described in Table II.8. These symptoms normally appear 40 min after consumption of contaminated food and, in some cases, even six hours after [67].

Table II.8. Characteristic symptoms of TTX human poisoning modified from Noguchi and Ebesu (2001) [66].

Level	Affected System	Specific Symptoms
1	Neuromuscular	Paresthesia of lips, tongue, and pharynx, taste disturbance, dizziness, headache, diaphoresis, pupillary constriction
	Gastrointestinal	Salivation, hypersalivation, nausea, vomiting, hyperemesis, hematemesis, hypermotility, diarrhea, abdominal pain
2	Neuromuscular	Advanced general paresthesia, paralysis of phalanges and extremities, pupillary dilatation, reflex changes
3	Neuromuscular	Dysarthria, dysphagia, aphagia, lethargy, incoordination, ataxia, floating sensation, cranial nerve palsies, muscular fasciculation
	Cardiovascular/pulmonary	Hypotension or hypertension, vasomotor blockade, cardiac arrhythmias, atrioventricular node conduction abnormalities, cyanosis, pallor, dyspnea
	Dermatologic	Exfoliative dermatitis, petechiae, and blistering
4	Respiratory failure, impaired mental faculties, extreme hypotension, seizures, loss of deep tendon and spinal reflexes	

Currently, there is no antidote for TTX; however, some studies indicate that the application of activated charcoal could help in reversing the clinical stage of poisoning victims since it reduces the toxin free amount [68]. Also, alkaline gastric lavage with sodium bicarbonate (2%) is indicated as a treatment within the first hour of the incident, due to TTX instability in alkaline media [69]. Another clinical intervention

recommendation is the use of cholinesterase inhibitors such as neostigmine [28], and mechanical respiratory help may reduce mortality probability by muscle paralysis [38].

TTX Detection Methods

Several methodologies were developed to analyze TTXs and, in recent years, chemical methods became more popular due to their sensitivity with limits of detection (LODs) ranging from 0.9 ng to 0.063 µg. Liquid chromatography with tandem mass spectrometry (LC–MS/MS) techniques, the first choice compared to mouse bioassays (MBAs) and enzymatic methods due to their greater sensitivity and specificity, have the capacity to detect and determine TTXs in complex matrices [70]. Also, due to ethical reasons and lack of specificity, MBA fell into disuse, with the latter reason also attributed to the enzymatic methods. When a poisoning case occurs, it is recommended, when available, to screen the liver, muscle, skin, gonads, and ovaries of the suspected poisoning marine vector samples [28,36,40–42,53,54–56,62,70–88]. Human urine and plasma should also be analyzed for TTX in these cases [80].

Methods for TTX analysis and their respective limits of quantification (LOQs) and detection (LODs) are described in Table II.9 and include the mouse bioassay [12,36,52,89], receptor-based assay [90], immunoassay [31,36,52,73,77,82,89,91–93], thin-layer chromatography [13,72], high-performance liquid chromatography [84,94,95], gas chromatography–mass spectrometry [76,84,95], liquid chromatography coupled to mass spectrometry [33,40,96–98], surface plasmon resonance [30], and liquid chromatography with fluorescence detection (FLD) [15,32,89].

Table II.9. TTX detection methods, their limits of quantification (LOQs), limits of detection (LODs), and toxicity equivalency factors (TEFs) according to the European Food Safety Authority (EFSA). MBA—mouse bioassay; FLD—fluorescence detection; RB—receptor-based; LC—liquid chromatography; MS—mass spectrometry; HPLC—high-performance liquid chromatography; UVD—ultraviolet detection; SPR—surface plasmon resonance; TLC—thin-layer chromatography; GC—gas chromatography.

Analysis Method	LOD	LOQ
MBA [12,36,52,89]	1.1 µg·g^{-1} [89]	-
Enzymatic assays [31,36,52,73,77,82,89,91–93]	2 ng·mL^{-1} [92]	-
TLC–MS [13,72]	0.1 µg [72]	-

HPLC–FLD [84,94,95]	1.27 μg·g^{-1} [94]	
GC–MS [76,84,95]	0.5 μg·g^{-1} [76]	1.0 μg·g^{-1} [76]
LC–MS/MS/UPLC–MS/MS [33,40,96–98]	0.09–16 ng·mL^{-1} [33,40,96–98]	5–63 ng·mL^{-1} [40]
SPR [30]	0.3–20 ng·mL^{-1} [30]	-
HPLC–FLD [15,32,99]	40-100 ng·g^{-1} [15]	-

Geographic Occurrence and Incidence of TTXs in the Indian Ocean and the Red Sea

As described in the introduction section, TTXs were reported in several marine organisms [71], regarding poisoning incidents [71]; the main TTX vectors involved in the Indian Ocean and the Red Sea (Table 10) belong to the Tetraodontidae family: *Arothron hispidus* in India [65], *Takifugu oblongus* in Bangladesh [16,33] and India [35,62]*, Lageocephalus scitalleratus* in Singapure [20]*, Pleuranacanthus sceleratus* in Egypt [21,34,37], Reunion Island [29], and Australia [23,24], *Chelonodon pataca, Sphaeroides oblongus*, *Lagocephalus inermis*, and *Lagocephalus lunaris* in India [35,62]*, Xenopterus naritus* in Malaysia [63]*, Arothron stellatus* in India [64]*, Tetractenos hamiltoni* in Australia [80,100], and *Tetroadon* sp. [17], *Tetraodon nigroviridis,* and *Arothron reticularis* in Thailand [99]. The records of TTX occurrence in other marine species such as mollusks are scarce in the Indian Ocean. Gastropods were reported as TTX vectors in other locations: *Charonia lampas* [85], *Gibbula umbilicalis,* and *Monodonta lineata* on the Portuguese coast [40], *Nassarius* spp. in China [94], *Polinices didyma*, *Natica lineata* [84,101], *Oliva miniacea*, *O. mustelina*, and *O. nirasei* [95] in Taiwan, *Charonia sauliae* [102]*, Babylonia japonica* [86], *Niotha* spp*.* [75,81]*,* and *Tutufa lissostoma* [103] in Japanese crabs, *Demania cultripes, Demania toxica, Demania reynaudi, Lophozozymus incises*, *Lophozozymus pictor*, *Atergatis floridus* [104], and *Atergatopsis germaini* [83]*,* highlightinh these organisms as potential indicator species [11]. Data on these groups are scarce in the Indian Ocean area, suggesting that further studies and monitoring programs for TTXs are needed. Available data regarding this geographic region are displayed in Table II.10.

Table II.10. The incidence of TTXs in the Indian Ocean. NPI—no poisoning incidents, MBA—mouse bioassay; FLD—fluorescence detection; LC—liquid chromatography; MS—mass spectrometry; HPLC—high-performance liquid chromatography; UVD—ultraviolet detection; TLC—thin-layer chromatography; GC—gas chromatography.

Producing Species	Vector	Sample Tissue	Location	Country	Poisoning Date	TTX	Detection	Maximum Concentration	Poisoning Victims	Reference
Australia										
Unknown	Puffer fish *Lagocephalus scleratus*		Close to Fremantle Hospital	Australia	13 May 1996	TTX	Symptomatology	-	3 people	[23]
Unknown	Puffer fish *Lagocephalus scleratus*		Port Hedland	Australia	1998	TTX	Symptomatology	-	1 person	[24]
Unknown	Toad fish *Tetractenos hamiltoni*		New South Wales	Australia	1 January 2001 to 13 April 2002	TTX	Symptomatology	-	11 people	[100]
Unknown	Toad fish *Tetractenos hamiltoni*	Urine Serum		Australia	2004	TTX	HPLC–UVD	5 ng/mL 20 ng/mL	7 people	[80]
Asian countries										
Unknown	Puffer fish		Khulna	Bangladesh	April 18 2002	TTX	Symptomatology	-	45 people	[27]
Unknown	Puffer fish *Takifugu oblongus*	Skin Muscle Liver Gonads	Khulna	Bangladesh	18 May 2002	TTX	MBA	18.9 MU/g 4.4 MU 4.9 MU/g 132.0 MU/g	36 people, 7 deaths	[16]

Producing Species	Vector	Sample Tissue	Location	Country	Poisoning Date	TTX	Detection	Maximum Concentration	Poisoning Victims	Reference
		Viscera categories						37.0 MU/g		
			Natore					-		
			Dhaka							
Unknown	Puffer fish	Liver	Khulna	Bangladesh	24 July 2005	TTX	Symptomatology	-	6 people	[22]
Unknown		Skin	Khulna	Bangladesh	25 March 2006	TTX	LC–MS/MS	25.35 $\mu g \cdot g^{-1}$	NPI	[33]
						Anhydro		7.71 $\mu g \cdot g^{-1}$		
						11-Deoxy		1.12 $\mu g \cdot g^{-1}$		
						Trideoxy		15.31 $\mu g \cdot g^{-1}$		
		Muscle				TTX		1.64 $\mu g \cdot g^{-1}$		
						Anhydro		-		
						11-Deoxy		-		
						Trideoxy		-		
		Liver				TTX		45.71 $\mu g \cdot g^{-1}$		
						Anhydro		29.17 $\mu g \cdot g^{-1}$		
						11-Deoxy		-		
						Trideoxy		9.09 $\mu g \cdot g^{-1}$		
		Ovary				TTX		356.00 $\mu g \cdot g^{-1}$		

Producing Species	Vector	Sample Tissue	Location	Country	Poisoning Date	TTX	Detection	Maximum Concentration	Poisoning Victims	Reference
						Anhydro 11-Deoxy Trideoxy		85.87 µg·g^{-1} 26.00 µg·g^{-1} 2,929.70 µg·g^{-1}		
Unknown	Puffer fish		Dhaka	Bangladesh	2008	TTX	Symptomatology	-	11 people	[25]
Unknown	Puffer Fish		Narshingdi Natore Dhaka	Bangladesh	April and June 2008	TTX	Symptomatology	-	95 people, 14 deaths	[26]
Unknown	Puffer Fish		Dhaka City	Bangladesh	October 2014	TTX	Symptomatology	-	11 people, 4 deaths	[18]
Unknown	Puffer fish	-	Khulna	Bangladesh	-	TTX	Symptomatology	-	37 people, 8 deaths	[28]
Unknown	Puffer fish *Chelonodon patoca* *Sphaeroides oblongus* *Lagocephalus inermis* *Lagocephalus lunaris*	Liver Ovary Liver Ovary Liver Ovary Liver Ovary	Bay of Bengal	India	June 1998 to March 2001	TTX	MBA	25.9 MU/g 183 MU/g 16 MU/g 7.9 MU/g 5.5 MU/g 28.9 MU/g 5.9 MU/g 16.6 MU/g	NPI	[61]
Unknown	Puffer fish *Chelenodon potoca*	Liver Ovary	Bengal coast	India	June 2000 – March 2001	TTX	MBA	27.8 MU/g 156.7 MU/g	NPI	[35]

Producing Species	Vector	Sample Tissue	Location	Country	Poisoning Date	TTX	Detection	Maximum Concentration	Poisoning Victims	Reference
	Takifugu oblongus	Liver						11.75 MU/g		
		Ovary						29.1 MU/g		
	Lagocephalus lunaris	Liver						9 MU/g		
		Ovary						30.1 MU/g		
	Lagocephalus inermis	Liver						5.7 MU/g		
		Ovary						9.64 MU/g		
Kytococcus sedentarius	Puffer fish *Arothron hispidus*	Skin	Annankil fish landings at Parangipettai	India	2010	TTX	MBA	-	NPI	[65]
		Intestine						-		
		Liver						-		
Cellulomonas fimi		Muscle						4.4 MU		
		Liver						4.9 MU/g		
		Gonads						132.0 MU/g		
Bacillus lentimorbus		Viscera categories						37.0 MU/g		
			Natore Dhaka					-		
								-		
Unknown	Puffer fish *Arothron stellatus*	Muscles	Parangipettai	India	2016	TTX	HPLC–FLD, TLC–UVD	Qualitative	NPI	[64]
		Gonads				4-*epi* anhydro				
		Liver								
Unknown	Puffer fish *Takifugu oblongus*	Skin	Kasimedu fishing harbor,	India	2016	TTX	MBA	75.88 MU/g	NPI	[62]
							GC–MS	16.5 MU/g		
							HPLC	18 MU/g		

Producing Species	Vector	Sample Tissue	Location	Country	Poisoning Date	TTX	Detection	Maximum Concentration	Poisoning Victims	Reference
		Liver	Chennai, Tamil Nadu				MBA GC–MS HPLC	143.33 MU/g 32.5 MU/g 48 MU/g		
		Ovary					MBA GC–MS HPLC	163 MU/g 34.5 µg 51 µg		
Unknown	Puffer fish	-	Johor	Malaysia	May 2008	TTX	Symptomatology	-	34 people	[68]
Unknown	*Carcinoscorpius rotundicauda*	Urine	Kota Marudu	Malaysia	June–August 2011	TTX	GC–MS	1.3–602 ng/mL	30 people	[88]
Unknown	Puffer fish *Xenopterus naritus*	Muscle	Manggut Kaong	Malaysia	February and July 2013	TTX	LC–MS/MS	27.19 µg/g 16.09 µg/g	NPI	[63]
Unknown	Puffer fish *Lageocephalus scitalleratus*		Alexandra Hospital	Singapore	2013	TTX	Symptomatology		1 person	[20]
Unknown	*Tetraodon nigroviridis*	Reproductive tissue Liver Digestive tissue	Satun	Thailand	April to July 2010	TTX	LC–MS/MS, MBA	63.57 MU/g 97.08 MU/g 43.33 MU/g	NPI	[36]

Producing Species	Vector	Sample Tissue	Location	Country	Poisoning Date	TTX	Detection	Maximum Concentration	Poisoning Victims	Reference
		Muscle						22.12 MU/g		
	Arothron reticularis	Reproductive tissue						-		
		Liver						2.08 MU/g		
		Digestive tissue						3.16 MU/g		
		Muscle						4.02 MU/g		
African countries										
Unknown	Puffer fish *Lagocephalus lunaris*	Gonads Liver Muscles Digestive tract Skin	National Research Center, Dokki, Cairo,	Egypt	September 1990 through May 1991	TTX	TLC–UVD, MBA	752 MU/g 246 MU/g 127 MU/g 221 MU/g 119 MU/g	NPI	[34]
Unknown	Puffer fish *Lagocephalus sceleratus*	Gonads	Attaka fishing harbor	Egypt	October 2002 and June 2003	TTX	MBA	3950 MU/g	NPI	[37]
Unknown	Puffer fish *Lagocephulus scleratus*	Muscle	Suez Gulf	Egypt	23 December 2004	TTX			7 people	[21]
Unknown	Puffer fish		Nosy Be Island	Madagascar	July 1998	TTX	MBA	16 UM/g	3 people, 1 death	[19]
Unknown	Puffer fish *Lagocephal*	Liver Flesh	Reunion Island	Reunion Island	10 Septemb	TTX	MBA, LC–MS/MS	95 MU/g 5 MU/g	10 people	[29]

Producing Species	Vector	Sample Tissue	Location	Country	Poisoning Date	TTX	Detection	Maximum Concentration	Poisoning Victims	Reference
	us sceleratus				er 10 2013					
Unknown	Puffer fish, *Tetraodontidae family*		Zanzibar	Tanzania		TTX	Symptomatology	-	1 death	[17]

Final Considerations

TTX data in the Indian Ocean and Red Sea are usually related to fatal outbreaks due to seafood poisoning and not to scientific research, indicating the lack of MT monitoring programs. The symptomatology reports and MBA are used to identify seafood poisoning caused by TTX and analogs, indicating the need for analytical methods such as liquid chromatography to obtain better quantitative data. Both symptomatology and MBA in isolation are not enough to conclude that TTXs are the causative agent of seafood poisoning, since there are other toxins (PSTs) with similar action mechanism that overlap in symptomatology with TTX poisoning. Additionally, MBA cannot discriminate between the different TTX analogs. MBA and symptomatology are used in countries of the Indian Ocean and the Red Sea to identify TTX poisoning due to the lack of availability and accessibility to chemical methods and the absence of TTX monitoring programs. Thus, the implementation of monitoring programs using chemical analytical methods such as LC–MS/MS instead of MBA in the Indian Ocean and the Red Sea is urgently needed in different species of shellfish and puffer fish, including *Arothron hispidus*, *Takifugu oblongus*, *Lageocephalus scitalleratus*, *Pleuranacanthus sceleratus*, *Chelonodon patoca*, *Sphaeroides oblongus*, *Lagocephalus inermis*, *Lagocephalus lunaris*, *Xenopterus naritus*, *Arothron stellatus*, *Tetractenos hamiltoni*, *Tetraodon nigroviridis*, *Arothron reticularisand*, *Charonia sauliae*, *Babylonia japonica*, *Niotha* spp., and *Tutufa lissostoma*, since they are most consumed and are already confirmed to be vectors of TTX in the Indian Ocean and the Red Sea. These species can be used as indicators for monitoring programs using the maximum limit permitted of 2 $mg \cdot kg^{-1}$ (from Japan).

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III. SCREENING OF MARINE TOXINS IN SEAFOOD FROM MOZAMBIQUE

Highlights of the chapter

- First report of TTTX, 4-epiTTX, 4,9-anhydroTTX, 11-deoxyTTX, and 11-norTTX-6-(R/S)-ol in pufferfish species (Diodon hystrix and Arothron hispidus) from Mozambican coast.
- Pufferfish presented Tetrodotoxins levels in the muscle above (274.3 µg kg−1) recommended limit stated by EFSA (44 µg TTX equiv kg−1).
- First data of PnTX G, E and F in local shellfish (*Atrina vexillum*, *Pinctada imbricata,* and *Anadara antiquata*) from Mozambique.
- Shellfish presented PnTX G levels (2.4 - 14.3 µgkg−1) bellow the LD50 (36.3- 208 µg kg−1) observed in other previous studies in mice.
- Need of setting-up a program for Tetrodotoxins and PnTXs surveillance in seafood from Mozambique.

COMMUNICATION ï Toxicon 2022, 216, 88-91: Tetrodotoxin and analogs in two local pufferfish species from Inhaca Island ï South of Mozambique: First report in the Mozambican coast.

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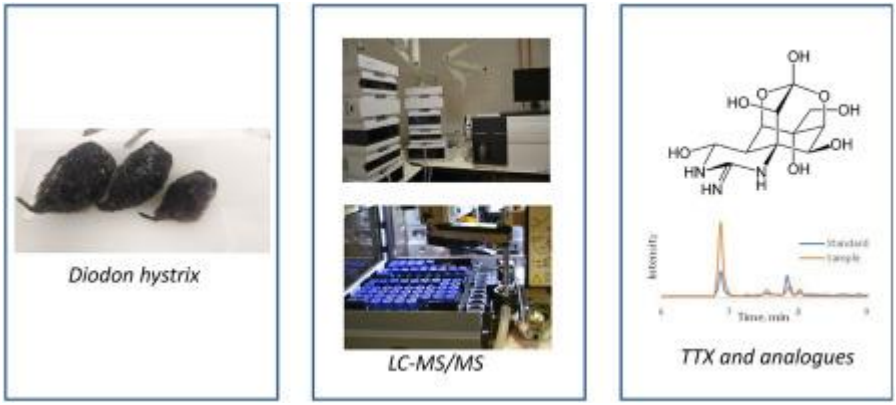
Abstract

Tetrodotoxins (TTXs) were investigated in two local pufferfish species, *Diodon hystrix* and *Arothron hispidus*, from Mozambican coast. TTX and analogues 4-epiTTX, 4,9-anhydroTTX, 11-deoxyTTX, and 11-norTTX-6-(R/S)-ol were found in both species and high level of TTX was found in *A. hispidus* (9522.0 µg TTX kg^{-1}) than in *D. hystrix* (350.9 µg TTX kg^{-1}). The distribution of TTX and their analogues in *A. hispidus* was intestine>liver>skin>>muscle>gonads. This is the first report of TTXs in Mozambican coast.

Graphical abstract



Keywords: Tetrodotoxins, pufferfish, Mozambican coast, marine toxins monitoring, human seafood poisoning, Indian Ocean.

Highlights

- First report of Tetrodotoxins occurrence in the local pufferfish species from Mozambique.
- Pufferfishes presented Tetrodotoxins level above recommended limit in Europe and Japan.
- Need of setting-up a program for Tetrodotoxins surveillance in seafood from Mozambique

Human acute intoxications involving pufferfishes are known from Egyptian times but the toxin responsible (TTX) was isolated for the first time in 1909 from the globefish ovaries (Suehiro, 1994) and its structure was elucidated in 1964 (Mosher et al., 1964). First data confirming that TTXs are the poisoning agent in the pufferfish were most reported in East Asia but currently, TTXs have been detected in other marine organisms (also in terrestrial animals) in other parts of the world, including some African countries of the Indian Ocean (Tamele et al., 2019a, b). Despite the reports of human intoxications involving seafood and awareness in terms of high toxicity and increasing occurrence of TTX in many African countries on the Indian Ocean coast, including Mozambique, the screening of this group of toxins is still not carried out (Dakar), 1998; dos Santos, 2020; Fonseca, 2021; Maputo, 2018).

In this study, TTX and analogues were screened using LC-MS/MS in two local pufferfish species, *Diodon hystrix* (n=4) and *Arothron hispidus* (n=1), collected in the South coast of Mozambique (26°03'28.9"S 32°57'20.7"E) in January and April 2020 by fishery net. The samples were frozen and transported to Portugal for extraction and quantification of TTX in the National Reference Laboratory for Marine Biotoxins at IPMA (Lisbon). Total length and body weight were 13-17 cm and 117.5 -250.5 g for *D. hystrix* and 14 cm and 120.4 g for *A. hispidus*. TTX and analogs were extracted with HOAc according to the method proposed by EURLMB 2017 (EULRMB, 2017). The LC-MS/MS equipment consisted of an Agilent 1290 Infinity coupled to a triple quadrupole mass spectrometer Agilent 6470. All LC conditions were also according with EURLMB 2017(EULRMB, 2017), including the multiple-reaction-monitoring (MRM) transitions from the protonated ions of TTX and TTX derivatives. The system was calibrated with the certified reference material CRM-03-TTXs from Cifga (Lugo, Spain) which

contains TTX and 4,9-anhTTX (certified) and 4-epiTTX and 11-deoxyTTX (non-certified). A five-point calibration curve with a correlation >0.995 was set up for quantification purposes. The limits of detection (LOD) and quantification (LOQ) were evaluated based on the signal-to-noise ratios for TTX with external standard addition. The equivalent toxicity of both pufferfish species was estimated using relative potencies of each analogue of TTX, as reported by the European Food Safety Authority (EFSA) (EFSA, 2017).

TTX was detected in the liver of each pufferfish specimen analyzed. However, much higher concentrations were found in *Arothron hispidus*, 5549.0 µg kg^{-1}, than in *Diodon hystrix*, which varied only from 33.0 to 138.8 µg kg^{-1} (Table III.1).

Table III.1. Concentration (µg kg^{-1}) of TTXs and their analogues (4-epiTXX, 4,9-anhydroTTX, 11-deoxyTTX) detected in the selected tissues of both pufferfish species collected in Maputo Bay - South of Mozambique.

Species	Organ	µg kg^{-1}				Equivalent toxicity, µg TTX eq kg^{-1}
		TTX	4-epiTTX	4,9-anhydroTTX	11-deoxyTTX	
Diodon hystrix	Liver	36.8	6.5	nd	nd	37.8
	Liver	138.8	9.8	202.3	nd	144.4
	Liver	97.5	10.3	nd	nd	99.1
	Liver	33.0	nd	nd	nd	33.0
Arothron hispidus	Liver	5549.0	248.5	3538.8	186.5	5685.6
	Intestine	15164.5	60.8	5646.5	nd	15287.2
	Skin	3575.9	12.4	1165.2	nd	3601.2
	Muscle	274.3	nd	1138.5	nd	297.1
	Gonads	222.2	nd	1978.6	nd	261.8

nd – not detected

After verifying that *Arothron hispidus* liver presented notably higher content of TTX than all *Diodon hystrix* specimens analysed, this species was selected to assess the toxin distribution between organs (gonads, muscle, skin, and intestine). TTX and the following analogues 4,9-anhydroTTX, 4-epiTTX, 11-deoxyTTX, and 11-norTTX-6(R/S)-ol were detected in the liver of *A. hispidus*. These toxins were confirmed by matching sample results with the standard, except for 11-norTTX-6(R/S)-ol, which its identity was deduced from spectral data available in literature (Shoji et al., 2001) (Fig. III.1).

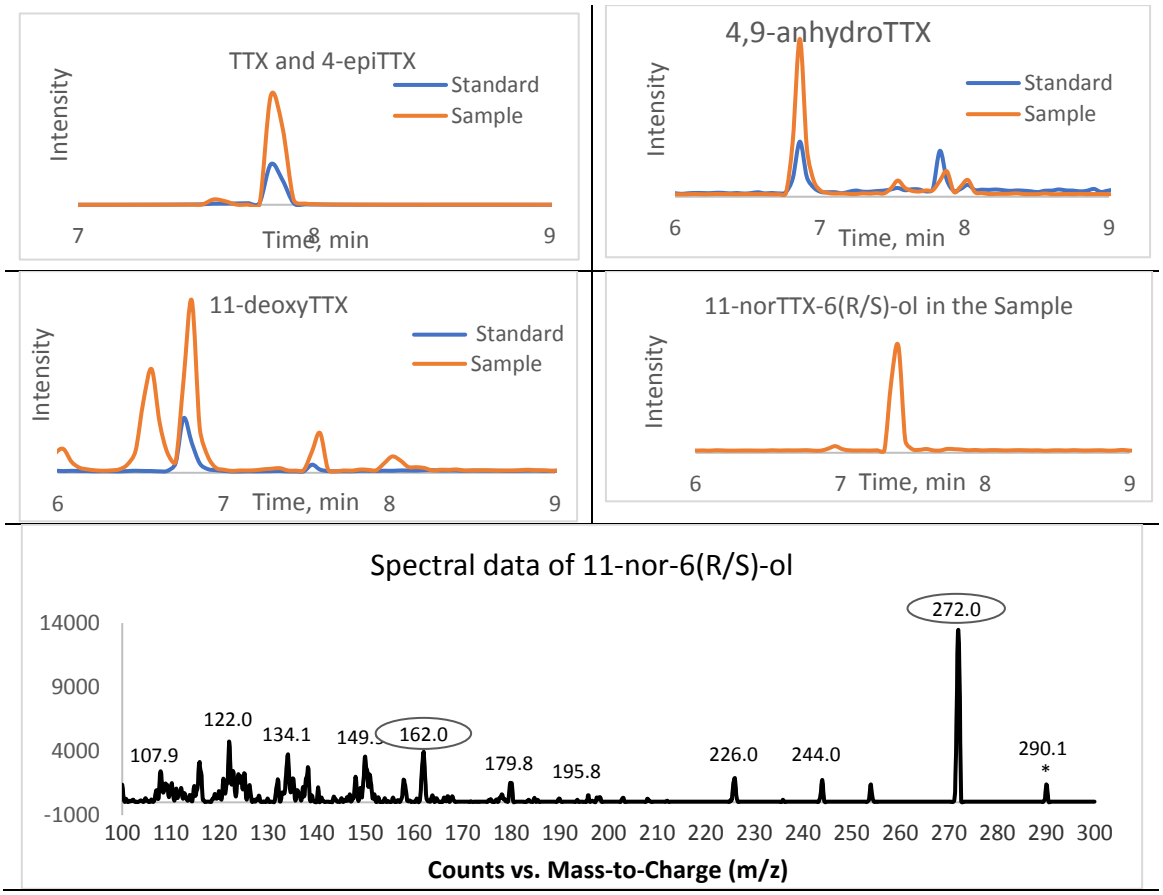


Figure III.1. Chromatograms of the standards used in this study and correspondent toxins found in the samples and spectral data of 11-nor-6(R/S)-ol. The chromatograms correspond to 320>162, 304>162, 302>162 and 290>162 transitions for TTX and 4-epiTTX, 11-deoxyTTX, 4,9-anhydroTTX and 11-nor-6(R/S)-ol respectively.

The present study brings interesting results regarding TTX occurrence in consumed fishes from Mozambique. These results constitute the first data reporting the TTX occurrence in two local species (*Diodon hystrix* and *Arothron hispidus*). Both species *Diodon hystrix* and *Arothron hispidus* are known to bioaccumulate TTXs from other parts of the world. TTX level up to 20800 µg kg^{-1} was found in the liver of *D. hystrix* from Sabah and Sarawak Waters, Malaysia, but no TTX was detected in the muscle (Azman et al., 2014). TTX was also reported in eyes, skin, liver, intestine, and gonads of *D. hystrix* from India for evaluating of genotoxicity in zebra fish(Lokesh et al., 2016). The weak ability of *D. hystrix* for TTX bioaccumulation was also reported in previous studies in which distribution of TTX was evaluated in flesh, skin, liver, gonad, and intestine of

several pufferfish including *A. hispudis*. In that study, no significant TTX level was found in any organs of *D. hystrix*(Khora, 1991). High levels of TTX were found in the liver of *Arothron hispidus* (5549.0 µg kg^{-1}), which may suggest the ability of this species to bioaccumulate more TTX and consequently to be more toxic than *Diodon hystrix* (33.0 – 350.9 µg kg^{-1}).

Among organs of *A. hispudis*, high levels of TTX and 4,9-anhydroTTX were found in the intestine and liver suggesting pufferfish accumulates TTX via dietary route. The skin was the third organ with highest levels of TTX (3575.9 µg kg^{-1}) indicating that pufferfish may allocate TTX and use it as defensive substance to predators, as also suggested from previous studies (Noguchi et al., 2006; Saito et al., 1985). Some studies have highlighted that TTX in some pufferfish species such as *Takifugu rubripes* is being transferred and accumulated from the connective tissue to the basal cells in the skin with young fishes accumulating higher levels than adults fish (Gao et al., 2020).

Lower TTX levels were detected in muscle (274.3 µg kg^{-1}) and gonads (222.2 µg kg^{-1}). The bioaccumulation mechanism in these organs is not well understood. TTX, 4-epiTTX, 4,9-anhydroTTX, 11-deoxyTTX and 11-norTTX-6(R/S)-ol found in the present study, have been also reported in the same species from the Solomon Islands and Okinawa, Japan although with a different distribution among organs. In that study, contrarily to present study, high levels of TTXs were found in the skin (4260 to 51000 µg kg^{-1}) and relatively lower in the intestine and liver(Puilingi et al., 2015). In the present study, 11-deoxyTTX was detected only in the liver at 186 µg kg^{-1} and these results seem similar to those found in the same species from Solomon Islands and Okinawa (Puilingi et al., 2015). In 3 specimens of *A. hispidus* from both Solomon Island and Okinawa, 11-deoxyTTX was found only in the skin at extremely lower levels in some specimens (Puilingi et al., 2015). The lower levels of 11-deoxyTTX found in these studies may suggest that this toxin occurs normally in lower level in *A. hispidus*. Other studies reported also TTX (91µg L^{-1}), 4-epiTTX (12 µg L^{-1}) and 4,9-anhydroTTX (15 µg L^{-1}) in the plasma of *A. hispidus* from Okinawa, Japan (Yotsu-Yamashita et al., 2018).

Regarding to 11-norTTX-6(R/S)-ol, it was not possible to quantify this toxin in the present study because there is no standards available. However, this analogue was already reported in the skin, liver, ovary, testis, stomach, intestine and flesh of *A. hispidus* collected in Solomon Islands and Okinawa (Japan), at levels ranging from < LOQ (30 µg kg^{-1}) to 2230 µg kg^{-1}(Yotsu-Yamashita et al., 2018).

Several previous studies reported occurrence of TTX and analogues in different species of the genus *Arothron* worldwide. TTX and analogues were reported in the different organs of *A. diadematus* (Red Sea(Fouda, 2005)), *A. nigropunctatus* (Japan (Puilingi et al., 2015; Yotsu-Yamashita et al., 2018), Philippines (Sato et al., 2000)); *A. manilensis* (Japan(Yotsu-Yamashita et al., 2018), Philippines (Sato et al., 2000)), *A. immaculatus* (India (Saha et al., 2015)); *A. firmamentum* (Bungo Channel(Nakashima et al., 2004)); *A. mappa* (Philippines(Sato et al., 2000)), *A. stellatus* (Philippines(Sato et al., 2000), India(Joseph et al., 2021)), and *A. reticularis* (Philippines(Sato et al., 2000)). High levels of TTXs in all these *Arothron* species were found in the liver, intestine and skin suggesting that these organs have more affinity to TTXs. TTX levels found in the muscle (274.3 µg kg^{-1}), despite being low compared to other organs, except gonads (222.2 µg kg^{-1}), was higher than the recommended limit stated by EFSA (44 µg TTX equiv kg^{-1})(EFSA, 2017), constituting a potential threat to public health. Regarding to human poisoning involving species of the genera *Diodon* and *Arothron*, it is estimated that 4.1 and 0.6% human cases of TTX poisoning after seafood consumption are caused by fish of the genera *Arothron* and *Diodon* respectively(Guardone et al., 2020). These data of human poisoning involving TTXs from *Diodon* and *Arothron* spp. are very important for TTXs risk assessment in Mozambique since pufferfish species used in the present study are for the human consumption in Mozambique. Despite there are no confirmed cases of human intoxication/poisoning involving TTX, other cases involving fish have already been reported in coastal areas of Mozambique namely in Cabo Delgado, Nampula((Dakar), 1998; Mosse, 2020), and Zambezi (Fonseca, 2021; Maputo, 2018). On another side, TTXs seafood poisoning episodes have already been confirmed in countries of the Channel of Mozambique, in the south of the Indian Ocean, such as Tanzania(Chopra, 1967), Reunion Island(Puech et al., 2014), and Madagascar(Ravaonindrina et al., 2001). These data of human

intoxication/poisoning, some with fatalities, from fishes may suggest involvement of MT including TTXs since this group of toxins was reported in other countries near to Mozambique. The present research reports new data, which although very preliminary due to several aspects such as the reduced number of individuals and species analyzed, species were collected in one point and one period, points out the need to improve knowledge on TTX occurrence in other marine organism of human consumption in Mozambique. More data are needed in order to provide more relevant information for implementation of monitoring program in Mozambique. As reported in this study, consumption of pufferfishes represents a great risk to public health and danger awareness campaigns regarding to consumption of pufferfishes are strongly recommended in Mozambique.

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ARTICLE ï Journal 2022, 10(9), 1215: First report of Pinnatoxins in bivalve molluscs from Inhaca Island (South of Mozambique) ï South of the Indian Ocean

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Abstract:

The objective of this work was to screen the EU-regulated lipophilic and cyclic iminetoxins in four bivalve species (*Atrina vexillum*, *Pinctada imbricata*, *Anadara antiquata*, and *Saccostrea cucculata*) from the Mozambican coast in the Indian Ocean. Toxins were extracted and analyzed according to the EU reference method for the determination of lipophilic toxins in shellfish via LC–MS/MS, but no regulated toxins were found in the analyzed species. However, pinnatoxins

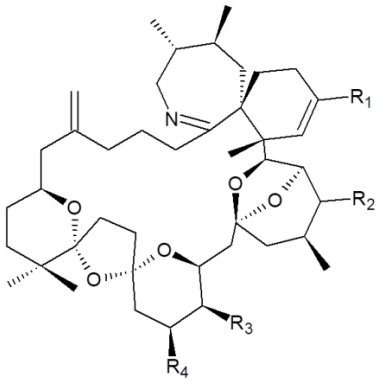
(PnTX G, E, and F) were detected in *A. vexillum*, *P. imbricata*, and *A. antiquata*. Higher levels of the PnTX G were determined for *A. vexillum* (7.7 and 14.3 $\mu g \cdot kg^{-1}$) than for *P. imbricata* (1.6 and 2.4 $\mu g \cdot kg^{-1}$), and for *A. antiquata* (4.5 and 5.9 $\mu g \cdot kg^{-1}$) with both hydrolyzed and non-hydrolyzed extracts, respectively. The higher levels of PnTX G determined in the hydrolyzed extracts indicate the high potential of this species to esterify pinnatoxins, in particular PnTX G.

Keywords: pinnatoxins; bivalves; Mozambican coast; marine toxins monitoring; human seafood poisoning; Indian Ocean

Introduction

Lipophilic marine toxins (LMTs) are produced by several harmful algae species that proliferate in marine environments worldwide [1,2]. They constitute one of the great threats to public health since they can be accumulated in marine organisms for human consumption such as bivalves, crustaceans, and pufferfishes [2]. The most reported LMTs include okadaic acid (OA), dinophysistoxins (DTXs), pectenotoxins (PTXs), yessotoxins (YTXs), and azaspiracids (AZAs). Currently, at least 1000 metabolites from marine microorganisms are LMTs, including the class of cyclic imines (CIs), such as pinnatoxins (PnTXs), pteriatoxins (PtTXs), gymnodimines (GYMs), spirolides (SPXs), prorocentrolides, spiro-prorocentrimine, and portimine [3]. CIs are an interesting group of LMTs (emerging toxins group), with its toxicological profile being poorly understood [4]. They are macrocyclic compounds with imine and spiro-linked ether moieties and are produced by several species of dinoflagellates (*Alexandrium* spp., *Gymnodium* spp., *Vulcanodinium rugosum*), except PtTXs, which are products of biotransformation from PnTXs via shellfish metabolic and hydrolytic transformation [2,4,5]. Among CIs, PnTXs (Figure III.2), which were discovered in 1990 in extracts of the bivalve mollusk *Pinna attenuate*, have received special attention due to their increased occurrence worldwide overtime [6]. PnTXs are emerging toxins, and their toxicological data are very limited; however, they act as potent neurotoxins inhibiting both the nicotinic and muscarinic acetylcholine receptors in the central and peripheral nervous system and at the neuromuscular junction [4,7], which are kept even after cooking procedures [2]. There are no reports of PnTXs in humans yet, but the symptoms

observed in animals (mice) include respiratory arrest, mobility decreasing, hind limb paralysis, breathing difficulties, tremors, and jumps [8].



Pinnatoxins				
Name	R_1	R_2	R_3	R_4
PnTX A	—COOH	OH	H	H
PnTX B/C	COOH, NH_2	OH	H	H
PnTX D	COOH, O	H	OH	CH_3
PnTX E	COOH, OH	H	OH	CH_3
PnTX F	O, O	H	OH	CH_3
PnTX G		OH	H	H

Figure III.2. Chemical structure of pinnatoxins.

The prevalence and occurrence of LMTs were already reported in several species of marine organisms for human consumption as well as human intoxication worldwide. Fortunately, some LMTs are already monitored, and a maximum limit in seafood was fixed in many parts of the world depending on the prevalence and incidence of a given toxin group [2]. Although harvesting restrictions are imposed when shellfish present levels of toxins above the safety limit, cases of human intoxication are still reported nowadays, possibly due to the lack of monitoring programs in some regions (mainly African countries) or due to disrespecting of the health authorities' regulations [1,2]. In African countries of the Indian Ocean, including Mozambique, where this study was focused, data regarding LMTare very limited. Few studies reported the occurrence of OA in *Haliotis asinine*, *Crassostrea gigas,* and *Choromytilus meridionalis* from Europa Island, Mayotte, and Reunion Island, South Africa, Mauritius [2,9,10]. Cases of human intoxication caused by ciguatoxins (CTXs), another class of algal toxins, were already recorded in Madagascar involving 124 (2 deaths) and 500 (100 deaths) people in 2013 and 1993, respectively [11,12]. On the other hand, cases of human intoxication may be attributed to non-legislated LMTs (emerging toxins) in countries where traditional toxins are already monitored [13,14]. In Mozambique, due to the lack of marine toxin monitoring programs coupled with the increasing demand for shellfish for human consumption, further investigations to guarantee the consumption of safe bivalve mollusks are required. This study aims to

investigate the presence of both EU-legislated (okadaic acid, azaspiracid, and yessotoxin group toxins[15]) and non-legislated (toxins whose maximum limit has not yet been set in the EU) lipophilic toxins in four bivalve species—*Atrina vexillum, Pinctada imbricata, Anadara antiquata,* and *Saccostrea cucullate*— collected in the Inhaca Island, south Mozambique.

Material and Methods

Sampling

Four local bivalve species—*Atrina vexillum*, *Pinctada imbricata*, *Anadara antiquata*, and *Saccostrea cucculata* (Table III.2)—were collected in Inhaca Island, south of Mozambique (26°03'28.9"S 32°57'20.7"E) (Figure III.3) which is the growing area of these species.

Table III.2. Number of individuals and weights of the sample used in this study.

Species	Individuals	Weigh (g)
Atrina vexillum	5	17.2–43.1
Pinctada imbricata	28	30.9–51.4
Anadara antiquata	3	23.5–27.4
Saccostrea cucculata	40	34.4–63.8

The sampling was carried out in January and April 2020, which corresponds to the summer season in this region. According to the local population, these species are among the most consumed bivalves locally. The species were dissected and stored at −20°C in the laboratory of the chemistry department of Eduardo Mondlane University (Maputo, MZ) and later were transported to Portugal for toxins analysis in the National Reference Laboratory for Marine Biotoxins Monitoring at IPMA.

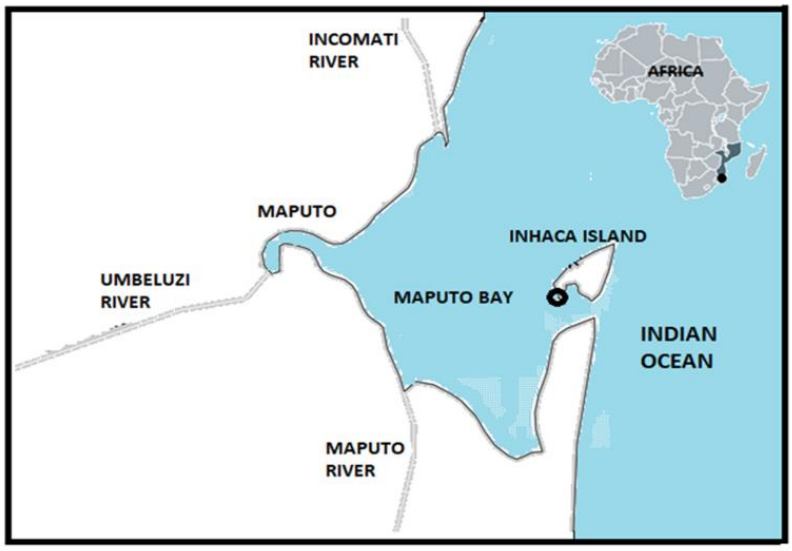


Figure III.3. Map of Maputo Bay, Mozambique. Black circle indicates the location site in the Inhaca Island.

Chemicals

Ammonium formate (LC–MS grade, Fluka Analytical, Steinheim, Germany), acetonitrile (LC–MS grade, Merck, Darmstadt, Germany), water (LC–MS grade, J.T. Baker, Center Valley, PA, USA), formic acid (LC–MS grade), methanol (LC–MS grade). OA, AZA1-3, YTX, PTX and related reference standard solutions were purchased from CIFGA (Lugo, Spain). PnTXG, GYM, and SPX1 reference standard solutions were purchased from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council (NRC, Canada).

Extraction of the Toxins

The extraction of EU-regulated and cyclic imines toxins was carried out according to the method proposed by the European Union Reference Laboratory for Marine Biotoxins (EURLMB) [15]. Two g of homogenized tissues of pooled samples (Table 1) were mixed with 9 mL of absolute methanol using vortex (Vortex Genie 2) for 3 min at the maximum speed level. The resultant mixture was centrifuged for 10 min at 2000 g, 20°C, and the supernatant was transferred to a 20 mL volumetric flask. This procedure was repeated by adding another 9.0 mL of

methanol to the remaining tissue pellet, and it was subsequently vortexed for 1 min and then centrifuged under the same conditions while combining both supernatants, and the final volume was made up to 20 mL with methanol. An aliquot was filtered through a 0.2 µm syringe filter, and 5 µL was injected into the LC–MS/MS system.

An alkaline hydrolysis step was carried out to convert acylated compounds, which may result from shellfish metabolism, into their respective parental toxin. The hydrolysis was started by adding 313 µL of 2.5 M NaOH to a 2.5 mL aliquot of the sample extract in a test tube, which was homogenized for 30 s in the vortex and heated at 76°C for 40 min in a heating block. The sample was allowed to cool down until reaching room temperature and neutralized with 313 µL of 2.5 M HCl. The sample was vortexed for 30 s, and an aliquot was filtered through a 0.2 µm syringe filter, and 5 µL was injected into the LC–MS/MS system.

LCï MSMS Analysis

Determination of lipophilic toxins in both hydrolyzed and non-hydrolyzed extracts was carried out via liquid chromatography with tandem mass spectrometry (LC–MS/MS) detection following the standardized operating procedure (SOP) for the determination of marine lipophilic biotoxins in bivalve mollusks of the EURLMB [15]. The LC–MS/MS equipment consisted of an Agilent 1290 Infinity chromatograph coupled to a triple quadrupole mass spectrometer Agilent 6470 (Agilent Technologies, Germany). The chromatographic separation was conducted with a Zorbax SB-C8 RRHT column (2.1 × 50 mm, 1.8 µm) protected with a guard column (2.1 × 5 mm,1.8 µm). Mobile phase A was water with 2 mM ammonium formate and 50 mM formic acid, and mobile phase B was 95% acetonitrile with 2 mM ammonium formate and 50 mM formic acid. An elution gradient at a flow rate of 0.4 mL min^{-1} was used as follows: 0–3 minutes, gradient from 88 to 50% eluent A; 3–6.5 minutes, gradient 50 to 10, 183% eluent A; 6.5–8.9 minutes, 10% eluent A; 8.9–10 minutes, gradient 10 to 88% eluent A. The detection was carried out in Multiple Reaction Monitoring (MRM) acquisition mode. Two MRM transitions were monitored, one being used for quantification and the other for confirmation (supplementary material).

For PnTX G quantification, a six-point calibration curve (Signal = 2330.8927C − 24.6694; R^2 = 0.9993) with a concentration of PnTX G ranging from 0.5 to 24.0 ng·mL^{-1} was set up for quantification purposes. The lowest calibration point was considered as the quantification limit. The level of esterification was calculated using the formula % esterified = 100 × (1−NH/H), where NH and H mean concentration of the PnTX G in non-hydrolyzed and hydrolyzed extracts, respectively.

Results

The screening of EU-legislated lipophilic toxins did not reveal the presence of these toxins in any of the analyzed species. These results may not be conclusive for risk assessments of lipophilic toxins since the samples were collected in a single location and in one time frame period. Regarding non-EU legislated lipophilic toxins, PnTX G, E, and F were found in *Atrina vexillum, Pinctada imbricata*, and *Anadara antiquata*.

PnTXG was confirmed using commercial standards available in the lab. PnTX E and F were deduced by comparing spectral data of the ion product of *m/z* 784.6 and *m/z* 766.3, respectively, with available data in the literature [16]. Figure III.4 shows chromatograms of the PnTX E and F in the samples and PnTX G both standard and in the samples. The spectral data of the PnTX E and F are illustrated in Figure III.5, with the fragments $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-3H_2O]^+$, and diagnostic fragments at *m/z* 164.1 and 446.0.

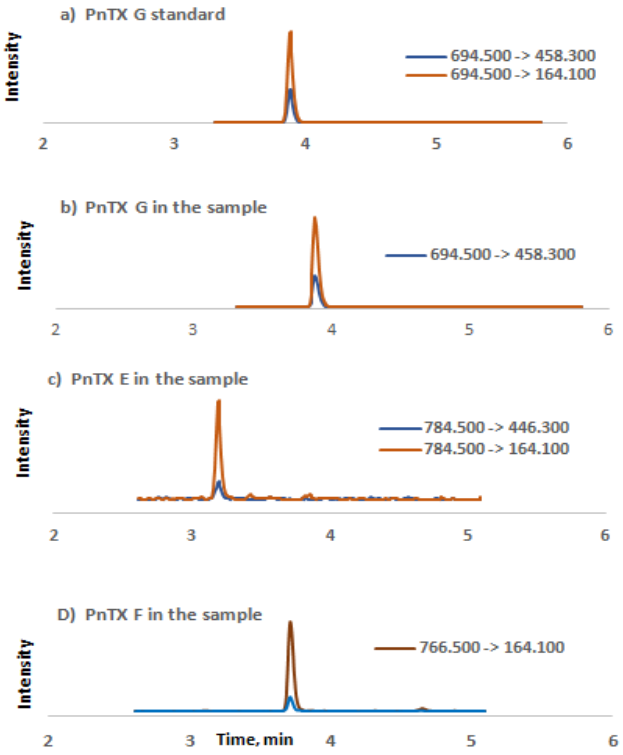


Figure III.3. Multiple reaction monitoring (MRM) chromatograms of the PnTX G (a,b), PnTX E (c), and PnTX F (d) found in this study. All chromatograms of the samples were obtained from the nonhydrolyzed extract of bivalve Atrina vexillum from Inhaca Island (South of Mozambique).

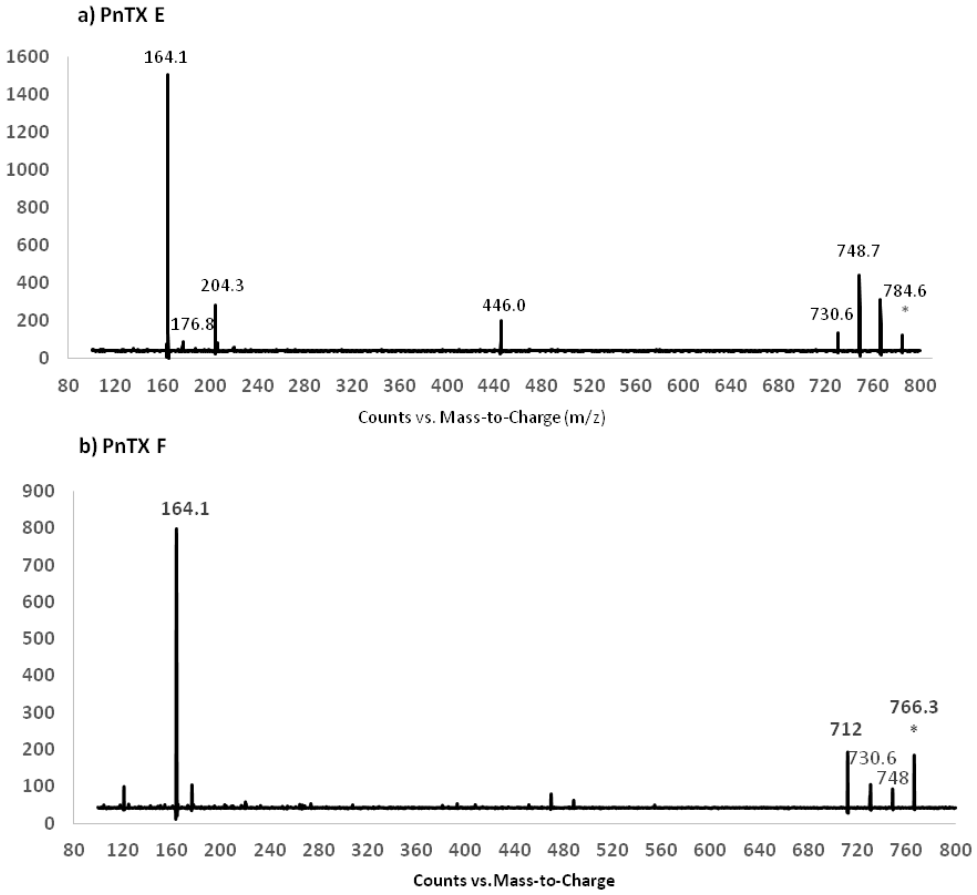


Figure III.4. Product ion spectra of (**a**) m/z 784.6 of PnTX E from the hydrolyzed extract, and (**b**) m/z 766.3 of PnTX F from the non-hydrolyzed extract. * indicates the molecular mass of the toxin.

The highest levels of PnTX G were observed in the hydrolyzed extracts, and this suggests that these species easily esterify PnTX G. Among species, *Atrina vexillum* presented higher levels of PnTX G in both non-hydrolyzed and hydrolyzed extracts (7.7 and 14.3 $\mu g \cdot kg^{-1}$) followed by *Anadara cucculata* (4.5 and 5.9 $\mu g \cdot kg^{-1}$) and *Pinctada imbrica* (1.6 and 2.4 $\mu g \cdot kg^{-1}$). Regarding esterification levels, *Atrina vexillum* showed 46% of the compounds in the esterified form, and contrarily to the levels of PnTX G in the extracts, *Pinctada imbrica* (33%) presented higher levels of esterification than *Anadara cucculata* (24%). PnTX G was detected in both hydrolyzed and non-hydrolyzed extracts, while PnTX A and E were found in non-hydrolyzed and hydrolyzed extracts, respectively.

Discussion

Reports of PnTXs date since 1990 and were discovered in the extracts of the bivalve mollusk *Pinna attenuata* by Chinese investigators [6]. Nowadays, there have been reports of PnTXs in other species of bivalves for human consumption [16-25], putting at risk public health. In this study, three PnTXs were detected, PnTX G, E, and F, in three species *Anadara antiquata, Pinctada imbricata,* and *Atrina vexillum*. As proposed in the previous studies, all PnTXs are formed from PnTX G and F as precursors since they are primary toxins produced by *Vulcanodinium rugosum* [16]. PnTX E is formed readily from PnTX F, in which the lactone ring of PnTX F is opened by hydrolysis forming PnTX E via metabolic and hydrolytic transformations in shellfish and water, and they are also available to be taken by bivalve species [26-29]. This means that the PnTX E detected in this study could be formed from PnTX F produced by an algae species present in seawater or by shellfish metabolism, or both. The rate of conversion of PnTX F to E may vary from species to species. In this study, it was not possible to quantify PnTX F and E due to the lack of reference standards. However, their detection was deduced from product ion spectral data analysis by the screening of *m/z* 784.7, which corresponds to PnTX E, and *m/z* 766.3, which was attributed to PnTX F, and their spectral data were similar to data available in the literature [16,29].

PnTXs below quantification limits found in *Saccostrea cucullata* may suggest a very low ability or even inability to bioaccumulate PnTXs. For PnTX G, the high level found in *Atrina vexillum* when compared with other species, suggests that this species could be considered very suitable to be used as bio-indicator of PnTXs, among the three analyzed species, on the Mozambican coast, but further study is required.

A higher content of PnTX G in hydrolyzed extracts appears to be in agreement with findings reported from extracts of mussel (*Mytilus edulis*) samples from Eastern Canada, in which higher levels of PnTX G were found in hydrolyzed (0.7 to 108 $\mu g \cdot kg^{-1}$) than in non-hydrolyzed samples (0.3 to 3 $\mu g \cdot kg^{-1}$) [19]. The notable difference in PnTX G levels between the hydrolyzed and non-hydrolyzed samples suggests that these species may contain considerable amounts of esters of PnTX G.

PnTXs are emerging toxins that are not regulated yet worldwide, [5] and this complicates the associated risk assessment for public health based on the PnTX G levels found in this study. Previous studies focused on PnTXs in species used in this study are very limited. However, the occurrence of PnTXs in *Atrina vexillum* was expected since *Atrina* sp. are closely related to *Pinna* sp. [30], for which PnTXs were reported for the first time (*P. attenuate, P. murica,* and *P. biclor*) in China, Japan, and Australia [31-36]. Comparing this study with others, the levels of PnTX G found in this study are not different from those found in previous studies in other species in some parts of the world. Similar levels were reported in 35% of European commercial seafood (flat oyster: *Ostrea edulis*, clams: *Ruditapes decussatus*, mussels: *Mytilus galloprovincialis*, blue mussels: *Mytilus edulis*) collected in Spain, Slovenia, Italy, Ireland, and Norway, which were contaminated by PnTX G at levels up to 12 $\mu g \cdot kg^{-1}$) [23]. In Chile, one of the major mussel producers worldwide, PnTX G at concentrations ranging from 2.9 to 5.2 $\mu g \cdot kg^{-1}$ was found in the cooked mussel *Mytilus chilensis* [18]. Samples of *Mytilus edulis* from six locations in Eastern Canada were also contaminated by both PnTX G and A, with levels varying from 0.6 to 108 and 0.3 to 2.5 $\mu g \cdot kg^{-1}$, respectively, with PnTX G being the major toxin in all locations studied [19]. Contrary to this study, high levels of PnTX G were recorded in mussels (*Mytilus galloprovincialis*) and clams (*Venerupis decussata*) from In Ingril, a French Mediterranean lagoon, during a period between 2009 and 2012 [21]. In that study, the concentration of PnTX G varied from 40 to 1200 $\mu g \cdot kg^{-1}$ and 17 to 95 $\mu g \cdot kg^{-1}$ for *Mytilus galloprovincialis* and *Venerupis decussata*, respectively, and in a recurring way during the study period. The higher levels of PnTX G found in *Mytilus galloprovincialis* (than *Venerupis decussata,* with the ratio of mussels/clams varying from 3 to 16 during all 4 years of the study) may suggest this species as a good candidate to act as a sentinel species for PnTX G. Based on these findings, the French Agency for Food Safety (ANSES) recommend the implementation of a monitoring program for PnTXs [37]. Blue mussels (*Mytilus galloprovincialis*) and Pacific oysters (*Crassostrea gigas*) from the shellfish harvesting areas of Catalonia, Spain (NW Mediterranean Sea) were tested for PnTX G at concentration ranging from 2 to 60 $\mu g \cdot kg^{-1}$ [17]. In Mozambique, to date, there are no reports of PnTX occurrence in bivalves, neither are there confirmed cases of human intoxication involving PnTXs. This is the first study of

PnTXs in bivalve species from Mozambique, although it is very preliminary due to the reduced number of specimens analyzed, and sampling was performed at a single point.

Conclusions

PnTX G, E, and F were found in the local *Atrina vexillum, Pinctada imbricata,* and *Anadara antiquata* collected in the Mozambican coast in the Indian Ocean. No EU-regulated lipophilic marine toxins were found in all analyzed species, and no PnTXs were found in *Saccostrea cucculata*. On the other hand, PnTX G was determined to be at considerably high levels in *Atrina vexillum*, followed by *Pinctada imbricata* and *Anadara antiquata* in both hydrolyzed and non-hydrolyzed extracts, respectively. In addition, PnTX E and PnTX F were also detected. The high level of PnTX G found in *Atrina vexillum*, when compared with other species, suggests that this species could be used as a bio-indicator of PnTXs, among the three analyzed species, on the Mozambican coast, but further study is required. This is the first study showing PnTXs in bivalve species from the Mozambican coast.

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IV. PROPOSAL FOR A MARINE TOXINS MONITORING PLAN IN MOZAMBIQUE

Highlights of the chapter

- The *Ministério do Mar, Águas Interiores e Pescas* may be responsible institution for MT monitoring in Mozambique through one of its fishery institutes namely *Instituto Nacional de Investigação Pesqueira* and *Instituto Nacional de Inspecção do Pescado*.
- The sampling process may be carried out seasonally in selected sites along Mozambican coast, one in summer (October to March) and another in winter (April to September) in order to assess a possible seasonality of the MT.
- Permitted limit of MT in seafood can be adopted from countries that Mozambique keeps seafood trading such as EU region, China, South Africa, among other.

OPINION ï Mozambican Journal of Appleid Science 2023: Management of marine toxins risk in Mozambique ï A monitoring program is needed

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Abstract

Accumulation of marine toxins (MT) in seafood constitutes a great threat to public health and the local economies of coastal countries, such as Mozambique. Considering the concerns raised by the global climate change where MT are expected to become increasingly frequent and abundant, implementing effective operational measures to control the risk posed by MT is timely needed. A synthesis of up-to-date information on the risk associated with toxic algal blooms, MT occurrence data in seafood and human poisoning cases involving marine fish in Mozambique is presented as an opinion paper with the final goal of recommending the implementation of a Marine Toxins Monitoring Program (MTMP) to protect public health and improve the safety of marine products.

Keywords: Mozambique; marine toxins monitoring; public health; fishery industry; economy, seafood poisoning.

Significance: This work will help the Mozambican authorities for implementation of marine toxins monitoring plans to protect public health in Mozambique and secure exports of marine products.

Introduction

The Fishery Industry is one of the main sectors for development in Mozambique. It constitutes one of the main food sources for Mozambican population and relevant for the national economy. Mozambique (Figure IV.1) has a coastline of 2700 km, and 30 million habitants in which seafood consumption per capita was approximately 16.4 kg/year in 2020[1]

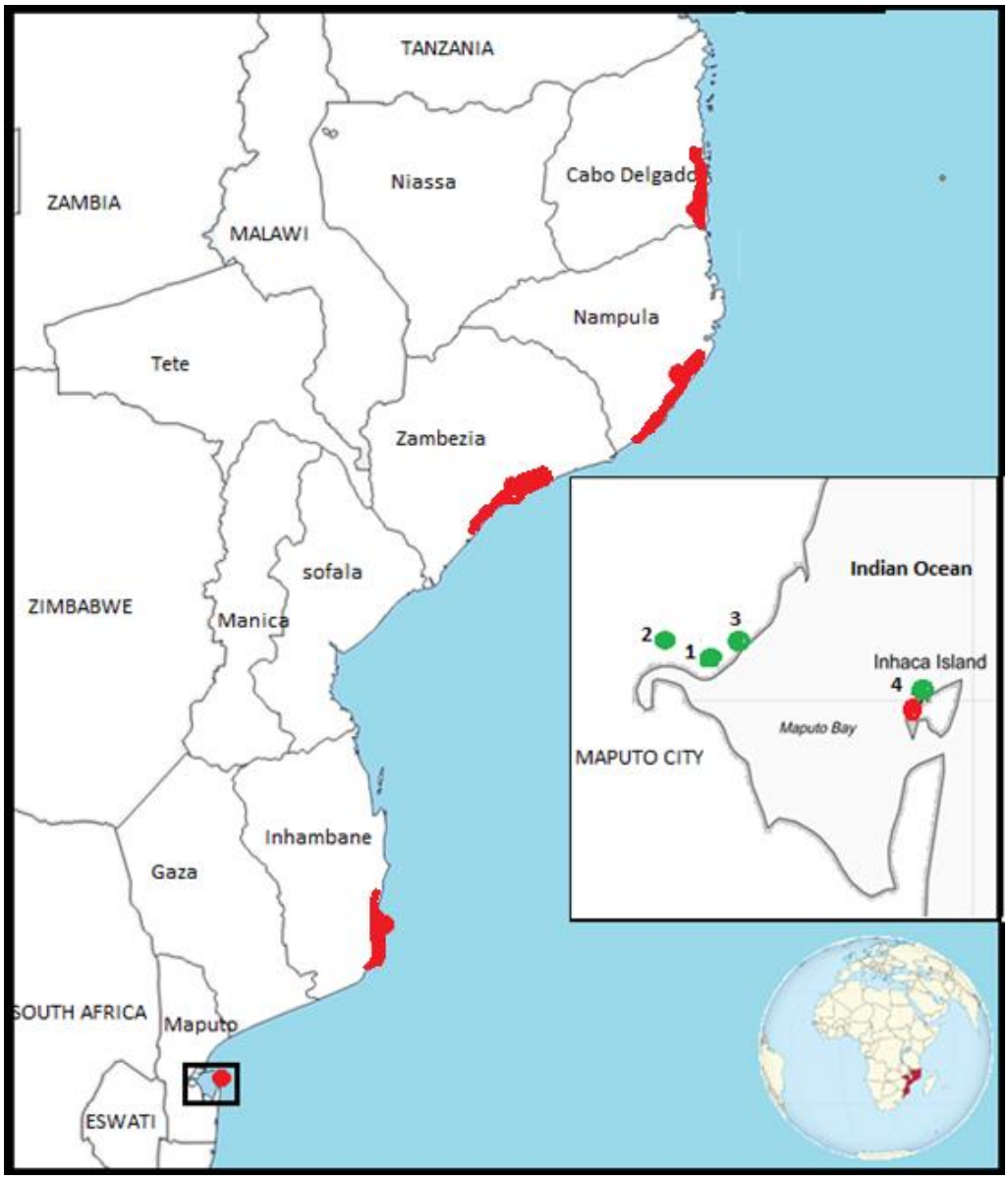

Figure IV.1. Green circles indicate the institutions where can be allocated the labs for marine toxins. 1 - *Instituto Nacional de Investigação Pesqueira*, 2 - *Laboratório Nacional de Higiene de Águas e Alimentos* 3 – *Instituto Nacional de Inspecção de Pescado*, 4 - *Estação de biologia marinha da Universidade Eduardo Mondlane*, 4 –. Red color indicates the sites where human poisoning cases involving marine fish or MT were reported.

In 2020, the annual production of seafood in Mozambique was 434 569 tons (431 257 from fishery and 3 312 aquaculture), being 9 229 tons exported, to Europa (2 790 tons), Asia (3 997 tons), USA (86 tons), Southern Africa (2 344 tons), and other countries (9 229 ton) [1]. The contribution to public finances was approximately 29 376 494 000 Mozambican metical (USD 500 million) from

fishing licenses and fish inspection [1]. However, one of the great threats to the fishery industry is the presence of marine toxins (MT) in seafood that may lead to acute intoxications or/and poisoning in humans, which in the most severe cases may cause fatalities [2, 3]

MT are secondary metabolites produced by different microalgae species that under certain favorable environmental conditions may suddenly increase their cell concentration and affect other marine organisms. This phenomena is widely designated as harmful algal blooms (HAB) [4] and the most reported HAB species include dinoflagellates (*Prorocentrum* spp. [5], *Dinophysis* spp. [3], *Phalacroma rotundatum* [6], *Gambierdiscus toxicus*, *Ostreopsis siamensis* and *Prorocentrum lima* [7], *Alexandrium* spp. [8, 9], *Gymnodium* spp.[10], *Vulcanodinium rugosum* [11], *Karenia* spp. [12] *Protoceratium reticuatum* [12], *Lingulodinium polyedrum* [12] and *Gonyaulax polyhedral* [12], *Dinophysis acuta* [13], *Azadinium spinosum* [14] and *Protoperidinum crassipes* [15], *Alexandrium* spp. [16], *Gymnodinium catenatum* [16], *Pyrodinium bahamense* [16], and cyanobacteria *Trichodesmium erythraeum* [17]), diatoms (*Pseudo-nitzschia* spp. [18]), cyanobacteria and some species of bacteria (*Serratia marcescens, Vibrio* spp., *V. Aeromonas*, *Microbacterium arabinogalactanolyticum* [19], *Pseudomonas* sp. [20]. *Shewanella putrefaciens, Alteromonas* sp. [21], *Pseudoalteromonas* sp. [22] and *Nocardiopsis dassonvillei*) the most reported causative species [3].

Some MT are biotransformation products from other MT via metabolic and hydrolytic transformation in shellfish [3, 23]. Chemically MT can be grouped as lipophilic or hydrophilic according to their solubility. The lipophilic toxins that are regulated in several countries around the world, such as the EU, Chile, Australia & New Zealand, include the okadaic acid (OA), dinophysistoxins (DTX), pectenotoxins (PTXs), yessotoxins (YTXs) and azaspiracids AZAs. Other lipophilic toxins, such as ciguatoxins (CTX), cyclic imines (CIs) [spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs)] and brevetoxins (PbTxs) although not consistently regulated by countries directives, may noticeably accumulate in seafood and affect their consumers. Hydrophilic toxins comprise domoic acid (DA) and analogs, paralytic shellfish toxins (PSTs), tetrodotoxins (TTXs) and palytoxins (PlTXs) [3]. Each group of toxins has a specific mechanism of action, symptomology and intoxication signals in humans

(diarrheic shellfish poisoning – OAs, ciguatera shellfish poisoning – CTXs, neurologic shellfish poisoning – PbTXs, Amnesic Shellfish Poisoning - DAs, paralytic shellfish poisoning – PSTs, azaspiracid shellfish poisoning – AZAs, tetrodotoxin shellfish poisoning -TTXs [3]. These toxins can be detected by several methods (Table IV.1) such as chemical (liquid and gas chromatography), enzymatic, and cytotoxic being the chemical methods most recommended for monitoring in seafood

Table IV.1: Marine toxins and their permitted limit in some countries where they are monitored. PSP—paralytic poisoning, DSP—diarrheic shellfish poisoning, ASP—amnesic shellfish poisoning, AZP—azaspiracid shellfish poisoning, CFP—ciguatera fish poisoning, NSP—neurologic shellfish poisoning, TSP – tetrodotoxin shellfish poisoning, OA – okadaic acid, CTX – ciguatoxins, SPXs – spiralizes, PbTX – brevetoxins, PTX – pectenotoxins, YTX– yessotoxins, AZA – azaspiracids, DA – domoic acid, TTX – tetrodotoxins, PlTX – palytoxins LC – Liquid Chromatography, FL – Fluorescence detection. UV – Ultraviolet detection, EU – European Union region, USA – United States of America, NZ – New Zealand, SA – South Africa

Syndrome	Toxin	Detection	Permitted limit
DSP	OA	LC-FL[24]	160 µg OA eq. kg^{-1} in EUNZ, SA
-	YTX	(*)LC-MS/MS[25]	3,75 mg YTX eq. kg^{-1} shellfish in EU[25, 26]
AZP	AZA	(*)LC-MS/MS[27]	160 µg AZA eq. kg^{-1} shellfish in EU[27]
PSP	STX	LC-FL[28]	800 µg STX eq. kg^{-1} fish in EU, USA, SA, ZN [28]
ASP	DA	HPLC-UV[29-31]	20 mg DA kg^{-1} shellfish in EU, Canada, USA[32, 33], SA, NZ, Australia[34, 35]
CFP	P/C-CTX-1	LC-MS/MS[36, 37]	0.01 µg (P-CTX-1) kg^{-1} fish and 0.1 µg (C-CTX-1) kg^{-1} in USA [37]
-	SPX	LC-MS/MS[38]	400 µg SPX kg^{-1} shellfish in EU[38]
NSP	BTX-2	LC -MS/MS[34, 35]	800 µg BTX-2 kg^{-1} shellfish in USA[39], NZ and Australia [34, 35]
-	PTX	LC-MS/MS[40]	160 µg OA eq. kg^{-1} shellfish in EU[40], NZ, Australia [34, 35]
TSP	TTX	LC-MS/MS[41]	44 µg TTX eq. kg^{-1} shellfish in EU[17], 2 mg TTX kg^{-1} in Japan[41]
-	PlTX	LC-MS/MS, LC-FL, LC - UV[42]	250 µg (PlTX) kg^{-1} shellfish in EU[42]

Many cases of seafood poisoning, some with fatalities, have been reported worldwide, including African countries of the Indian Ocean and the Red Sea. Several of these cases have been associated with seafood contamination with CTXs, PlTXs, TTXs, and PSTs [2, 3]. Intoxication cases occurred after the consumption of marine animals such as turtles, sharks, fishes, and mussels, both in restaurants and at home. Currently, some MT are monitored in countries such as South Africa, Japan, New Zealand, the USA, and the European Union, among other parts of the world (Table IV.1) [3]. In Mozambique, however, MT are not monitored, and this scenario puts all the 30 million Mozambicans and tourists vulnerable to seafood intoxication or poisoning cases. Additionally, MT in significant concentrations (above the permitted limit in many parts of the world) can negatively affect the national economy of Mozambique since the exports of seafood can be severely impacted and interdicted if validated hazards controls are not in place. The food security and safety of seafood will also be affected. The main goal of this study is to elucidate and recommend the Mozambique authorities with responsibilities at food safety level for the implementation of an effective Marine Toxins Monitoring Program (MTMP) to protect public health and improve the fishery industry. Aspects such as bioindicators species, permitted concentration of MT in seafood, and detection methods are suggested. The work is based on collecting data regarding both MT occurrence and human poisoning cases involving marine fish in Mozambique.

Risk assessment of marine toxins in the Mozambican Public Health

The presence of MT in seafood is barely considered a threat to public health in many countries of the Indian Ocean such as Mozambique. A recent literature review concluded that only South Africa has a specific monitoring plan [2, 3]. MT poisoning cases on the Indian African coast are reported from Egyptian times involving TTX after consumption of Lagocephalus sceleratus, which are one of the main TTX vectors in the Indian Ocean [43]. Over time, several human poisoning cases have been reported in African countries such as South Africa, Tanzania, Madagascar, and Comoros, among others.

In Mozambique, there are no confirmed cases of human poisoning involving MT. However, according to the WHO, more than 500 000 cases of diarrhea were

reported, of which 100 cases correspond to dysentery and 7 to cholera [44] and others are unknown. These data indicate that many people of Mozambique consume unsafety food including seafood. Unclarified fish poisoning cases have ever been reported in Mozambique by health authorities (Figure IV.1 and Table IV.2).

Table IV.2: Cases of human poisoning involving marine seafood in Mozambique. Data obtained from national and international media and local health authorities.

Local	Date	Victims	Marine seafood	Symptoms	Reference
Cabo Delgado and Nampula	November 1998	700 people (100 deaths)	Fish	Diarrhea (with and without vomiting)	[45]
Cabo Delgado and Nampula	November 1998	91 deaths	Fish	Diarrhea	[46]
Zambeze	October 2018	5 people (1 death)	Fish	No data	[47, 48]
Nampula	July 2020	12 deaths	Turtle	No data	[49]
Nampula	September 2021	4 deaths	Fish	No data	[50]

100 deaths and about 600 cases of illness in Cabo Delgado and Nampula provinces have been reported by health authorities in November 1998 after the consumption of marine fish [45]. It was suspected that the fish was contaminated by pesticides, but no scientific study was conducted to confirm it, suggesting that other chemical agents such as MT may be also responsible.

On 15 october 2018, health authorities of Zambeze province confirmed the death of one child and hospitalization of 4 adults, and on 23 september 2021 the death of 4 people after consumption of marine fish [48, 50]. According to health staff, these cases are associated with fish poisoning. Unfortunately, the source of the fish was not identified, and no food remains were available for subsequent biological and chemical analyses [47]. There are no data on symptoms presented

by victims. Other cases were reported in the northern provinces of Cabo Delgado and Nampula, on 24 november 1998, where at least 91 people died from diarrhea and related ailments attributed to the consumption of poisoned fish [46]. Once again, there are no details of the species of fish, local of acquisition, and autopsy results in all reported episodes. 12 people of the same family died after consumption of unknown marine turtle species was reported in the Nampula province on 3 july 2020 [49]. Some marine turtles of the Indian Ocean, such as *Eretmochelys imbricata* [51], among others, are well known as MT vectors [52]. The main food in these provinces is seafood since they are bathed by the Indian Ocean. Their relationship with sea suggests that the consumed fish may be cached locally by fishermen.

The possible involvement of MT in any of these cases may be emphasized by confirmed MT human poisoning in Mozambican adjacent countries. In South Africa, many fatalities were reported in 1837 [53], 1888, April 1948, December 1957 [54], May 1958, and May 1978 [55] after consumption of different marine species of fish and mussels (*Donax serra, Chloromytilus meridionalis, Perna perna* [53], *Choromytilus meridionlis* and *Mytilus meridionalis*). In the cases cited above, PSTs were at least suspected of being the causative agent, and concentrations up to 72.8 mg kg^{-1} were determined [54, 55]. In Tanzania, fatalities were confirmed after the consumption of pufferfish, a well-known TTX vector [56], and other cases were reported (June and August 2015) involving a toxic blue-green alga on the seaweed farms that caused dermatological problems [57]. In Madagascar, several human poisoning including deaths were reported, and the marine organism species involved include sharks *Carcharhinus amboinensis* and *Carcharhinus leucas harboring* CTXs, and the fish *Herklotsichthys quadrimaculatus* that acted as vector of PlTXs and TTXs [2, 3]. Human deaths involving TTX (up to 95 Mug^{-1}) after consumption of turtle *Eretmochelys imbricate* and fish *Lagocephalus sceleratus* were also recorded in Comoros (December 2012) [51] and Reunion Islands (September 2013) [58].

MT can also affect the marine ecosystem, killing marine animals such as seabirds, fishes, marine mammals putting in risk their survival [59-61]. For example, in the South Africa coast, several marine animal poisoning cases involving MT have been reported. Dead seabird's black oystercatcher

(*Haematopus moquini*), southern blackbacked gull (*Larus dominiccus*) and hartlaub's gull (*Larus hartlaubii*) were found in the Lambert's Bay and Bloubergstrand. It was suspected the seabirds consumed black mussels (*Choromytilus meridionalis*) and wedge clams (*Donax serra*) contaminated by PSTs and YTXs since considerable density of their producers respectively Alexandrium catenella and Protoceratium reticulatum were found in the local [60, 61]. In 2017, several million animals deaths in the abalone farms were reported in Cape Town and the causative agent was YTX produced by *Lingulodinium polyedra* [62]. Many other cases have been reported in Madagascar [63] and Reunion Island [59]. All these cases in these African countries of the Indian Ocean suggest, undoubtedly that Mozambique should be also highly vulnerable to MT.

Recently, studies carried out by Tamele et al. (2022) confirmed the presence of TTX, 4-epiTTX, 11-deoxyTTX, 4,9anydroTTX, and 11-norTTX(R/S)-ol in two pufferfish species (*Diodon hystrix* and *Arothron hispidus*) from the Mozambican coast. Moreover, pinnatoxins, namely PnTX G, F and E, were recently determined for the first time in Mozambique in local shellfish species: *Atrina vexillum, Pintacta imbricata* and *Anadara antiquata* [64]. Trace amounts of PST including dcSTX, GTX2+3, and STX were also detected in *Atrina vexillum*. The species were collected in January and April 2020 on Inhaca Island, frozen and transported to Portugal (IPMA - National Laboratory of Marine Biotoxins Monitoring) for toxins analysis [64, 65]. TTXs were found in the liver of *Diodon hystrix* (33 to 138.8 µg TTX kg^{-1}; 6.5 to 10.3 µg 4-epiTTX kg^{-1}; 202.3 µg 4,9-anhydroTTX kg^{-1}) and *Arthron hyspidus* (5559.9; 248.5; 186.5; and 3538,8 for TTX, 4-epiTTX, 11-deoxyTTX and anhydroTTX kg^{-1}, respectively) [64, 65].

Other organs of *Arthron hyspidus* also presenting high amounts of TTXs were the skin (3575.9 µg TTX kg^{-1}; 12.5 µg 4-epi TTX kg^{-1}; 1165.2 µg 4,9-anhydroTTX kg^{-1}), the intestine (15164.5 µgTTX kg-1; 60.8 µg 4-epiTTX kg-1; 5646.5 µg 4,9-anhydroTTX kg^{-1}), the gonads (222.2 µgTTX kg^{-1}; 1978.6 µg 4,9-anhydroTTX kg^{-1}) and the muscle (274.3 µgTTX kg^{-1}; 1138.5 µg 4,9-anhydroTTX kg^{-1}) [64]. Toxin content in these species is higher compared to the permitted limit used for monitoring in countries where these toxins are regulated such as Japan (2 mg kg^{-1}) [41] or the EU region (44 µg TTX kg^{-1}) [17, 64, 65]. The total levels of free

PnTXs were 14.3, 5.9, and 2.4, µg of PnTX G kg^{-1} for *Atrina vexillum, Anadara antiquata*, and *Pintacta imbricata*, respectively [64]. PnTX E and F were detected but not quantified due to the lack of certified reference material [64]. PnTX G is considered an emerging MT since its occurrence was just discovered in 2008 in the digestive glands of Pacific oysters *Magellana gigas* from South Australia and their structures were elucidated by NMR spectroscopy and mass spectrometry [23].

Other studies confirmed the presence of Domoic acid and *Pseudo-nitzchia* spp. blooms in the waters of Praia do Tofo – Inhambane province, southern Mozambique, from January 2017 to August 2018. The maximum DA concentration determined was 50 pg L^{-1} of filtered seawater in June [66]. From this study, it was concluded that between May 22 and June 10, 2017, DA concentration and coastal Chl-a significantly increased with the decrease of the sea surface temperature, suggesting potential coastal upwelling within the region [66].

These results (TTXs, PnTX G, and DA) and others reported in South Africa, Madagascar, Tanzania, Comoros, and Reunion highlights that MT poisoning cases may have occurred/occur in Mozambique. The lack of trained health and environment staff to recognize MT symptoms and the absence of MT monitoring become Mozambicans vulnerable to MT poisoning. MT may affect both security and safe seafood in Mozambique since many coastal communities, and tourists in coastal provinces such as Maputo, Gaza, Inhambane, Sofala, Zambeze, Nampula, and Cabo Delgado, consume fish and shellfish as the main food due to their high nutritional value.

Risk assessment of marine toxins in the Mozambican Economy

The presence of MT in seafood may negatively affect the Mozambican economy. The fishery industry significantly contributes to the GDP and is the income source of most of all the people living along the coast. However, data regarding this issue are very limited, which can be caused by several aspects including the lack of consistent data on market sectors, sporadic frequency of HABs, difficulty to know the number and dimension of the area affected, lack of MT data in poor coastal countries, among other reasons [67]. On the another hand, the impact of MT on

the economy is very complex because takes several transversal areas such as public health (medical and hospitalization expenses including cost of transport to hospital and loss of productivity due to the dead or sick people), fishery industry (fish and shellfish mortality, price increasing and demand reduction), tourism (tourism income reduction) and impact monitoring and management (water and marine animal sampling and staff training for recognizing MT poisoning[68, 69]

Even with the limitations described above, MT negative impacts were observed in some African countries of the Indian ocean that have coastal interaction with Mozambique. In South Africa, MT caused the reduction in 80% of month sales in 1994 [70], the loss of 2000 tons (corresponding to US 50 million dollars) of rock lobster in 1997, not estimated loss of oysters *Crassostrea meridionalis* in 2008 [71], close of 12 farms [72] and the loss of 250 ton of abalone species in 2017 [62]. An economic loss of 415 tons of rock lobster *Jasus lalandii* corresponding to 6 US dollars million was also registered in 2015 due the presence of the dinoflagellate *Prorocentrum triestinum* [73]. Economic loses were also reported in Tanzania in the third most important employing people with. 20,000 farmers and annual production of 15,087 tons. In this farmer, considerable amounts of seaweed *Eucheuma denticulatum* died and the farmers became ill in 2012 – 2013 [57, 74]. This case caused considerable economic losses due to the seaweed mortalities and farmer's medical expenses. The suspected causative agents were *Gymnodinium* spp. toxins [57, 74]

In Mozambique, there are no data regarding economic losses related to HABs and/or MT. However, according to economic losses experiences from South Africa, Tanzania, and other countries, Mozambique may or will also suffer considerable future economic losses due to the algal bloom and MT. The occurrence of HABs and MT is increased by climate change worldwide mainly in tropical and subtropical environments such as the Indian Ocean including the Mozambican coast. The Indian coast is considered an endemic area for MT such as CTX which is responsible for many poisoning cases. This scenario threatens the Mozambican fishery and tourism industry and consequently the Mozambican economy. Currently, the fishery industry contributes 10.3% to GDP in Mozambique and most of the seafood catches include crab, fish, shrimp, tuna fish (Sofala and Maputo), among other [75]. The fishery industry revenues come

from national fleet (64%), foreign tuna fleet (26.5%), fish inspection fees (4.6%) and own recipes (4.8%) [76]. Other fishery industry socio-economical contribution including tourism (in coastal areas) and creation of jobs in areas of accommodation, restaurants, travel agencies and other tourist activities. In 2018, the tourism sector in Mozambique raised US 41.8 million dollars of which a portion is an indirect revenue from the fish industry [77]. These fishery industry economic contributions may reduce and negatively affect the Mozambican economy if an effective MTMP is not implemented.

Final considerations and recommendations

The adequate intervention to avoid, minimize and manage intoxication and poisoning cases caused by MT in Mozambique is to implement an MTMP following the example of many coastal countries. Countries with specific MTMP are described in table 1, including the regulatory limit of each group of toxins. Implementation of the MTMP is not difficult, but it may be complex because it needs the collaboration of many parts of the regions to be monitored (Mozambican and Indian coast in general). The detection and sampling methods, the regulatory limit of MT in fish and shellfish, sampling seasonality, and specific MT legislation must be detailed because they are crucial aspects of the MTMP success. Since 2.12% of fish and fishery products are exported (according with 2020 data) to Europa (30.20%), Asia (43.33%), Southern Africa (25.40%), and America (0.93 %) [1], the regulator limit of MT in seafood can be adopted from these countries as it descried in the in table IV.1

Since in Mozambique, there are two institutions responsible for fishery research (Instituto Nacional de Investigação Pesqueira and Instituto Nacional de Inspenção de Pescado) (Figure 1) with provincial delegations in all provinces, the MTMP can be delegated to one of them, or sharing competences. In the first phase, the laboratory of MT analysis may be in Maputo city, due to the availability of the chemical analysis of MT (LC-MS/MS) compared to other provincial delegations and the easy logistic and experience changes with university research centres such as Estação de Biologia Marinha da Universidade Eduardo Mondlane (Eduardo Mondlane University), Laboratório Nacional de Higiene de Águas e Alimentos (Ministry of Health). The sampling process must carry out

seasonally in selected sites, one in summer (October to March) and another in winter (April to September) to assess the possible seasonality of the MT. The full and detailed sampling process may be discussed specifically by the selected institution for MTMP. The recommended chemical method for MT analysis is LC-MS/MS(EFSA), and there are alternative methods such as cytotoxicity, enzyme techniques, and thin layer chromatography, among others, that can be used by seafood producers to carry out their auto-control of MT variability in their area/production [2, 3].

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V. GENERAL DISCUSSION, CONCLUSIONS AND FINAL CONSIDERATIONS

Highlights of the chapter

- Emerging marine toxins (TTXs and PnTXs) were detected in seafood from the Mozambican coast.
- The *Ministério do Mar, Águas Interiores e Pescas* may be delegated for MT monitoring in Mozambique.
- Permitted limit of MT in seafood can be adopted from countries that Mozambique keeps seafood trading.

African Indian Ocean and the Red Sea coasts have a subtropical and tropical climate, considered optimal for the development and transportation of several MT producers, and consequently, the production of MTs [1,2]. The few data available for this geographic region, most of which describing only the genus and not identifying the potential harmful algae at the species level, makes it very difficult to evaluate the occurrence of the toxic species. The most reported HAB species in this region are cyanobacteria, followed by dinoflagellates, and diatoms as potential MT producers. Relative to MTs, the most commonly reported and associated with seafood poisoning episodes are the CTXs, PSTs, and TTXs [1]. In this thesis, TTXs and PnTXs were found in pufferfish (*Arothron hispidus and Diodon hystrix*) and shellfish (*Atrina vexillum, Pintacta imbricata,* and *Anadara antiquata*) from Inhaca island – South of Mozambican coast indicating that Mozambican are vulnerable to MT from seafood. No EU legislated lipophilic MT were found in these species of bivalves. The results found in this thesis are the first data regarding MT in seafood from Mozambique and they point out a threat to public health. Further studies are needed to provide more relevant information in order to improve knowledge on TTX and PnTXs as well as other MT such as PST, CTX, DA, OA, PlTXs which were already reported in the other African countries of the Indian Ocean [1,2].

In Mozambique, the most relevant MTs that must be monitored in shellfish were discussed in the table V.1. including the permitted limits of toxins in shellfish. The

detection methods are already discussed in this thesis and include LC -MS/MS which are recommended [3-7]. Other methods such as cytotoxicity, enzyme techniques, thin layer chromatography, among other [1,8]) may be used alternatively.

Table V.1. Proposal of permitted limit of MT in seafood from Mozambique.

Toxin	Permitted limit
OA	160 µg OA eq. kg^{-1}
YTX	3,75 mg YTX eq. kg^{-1} shellfish
AZA	160 µg AZA eq. kg^{-1} shellfish
STX	800 µg STX eq. kg^{-1} fish
DA	20 mg DA kg^{-1} shellfish
CTX	0.01 µg (CTX-1) kg^{-1}
SPX	400 µg SPX kg^{-1} shellfish
BTX	800 µg BTX-2 kg^{-1} shellfish
PTX	160 µg OA eq. kg^{-1} shellfish
TTX	44 µg TTX eq. kg^{-1} shellfish
PlTX	250 µg PlTX kg^{-1} shellfish

The Mozambican authorities that may be delegated for MT monitoring were also described including sampling strategies

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