

Biotoxinas marinas emergentes en las costas europeas: Estrategias analíticas para su caracterización

Ana Gago-Martínez

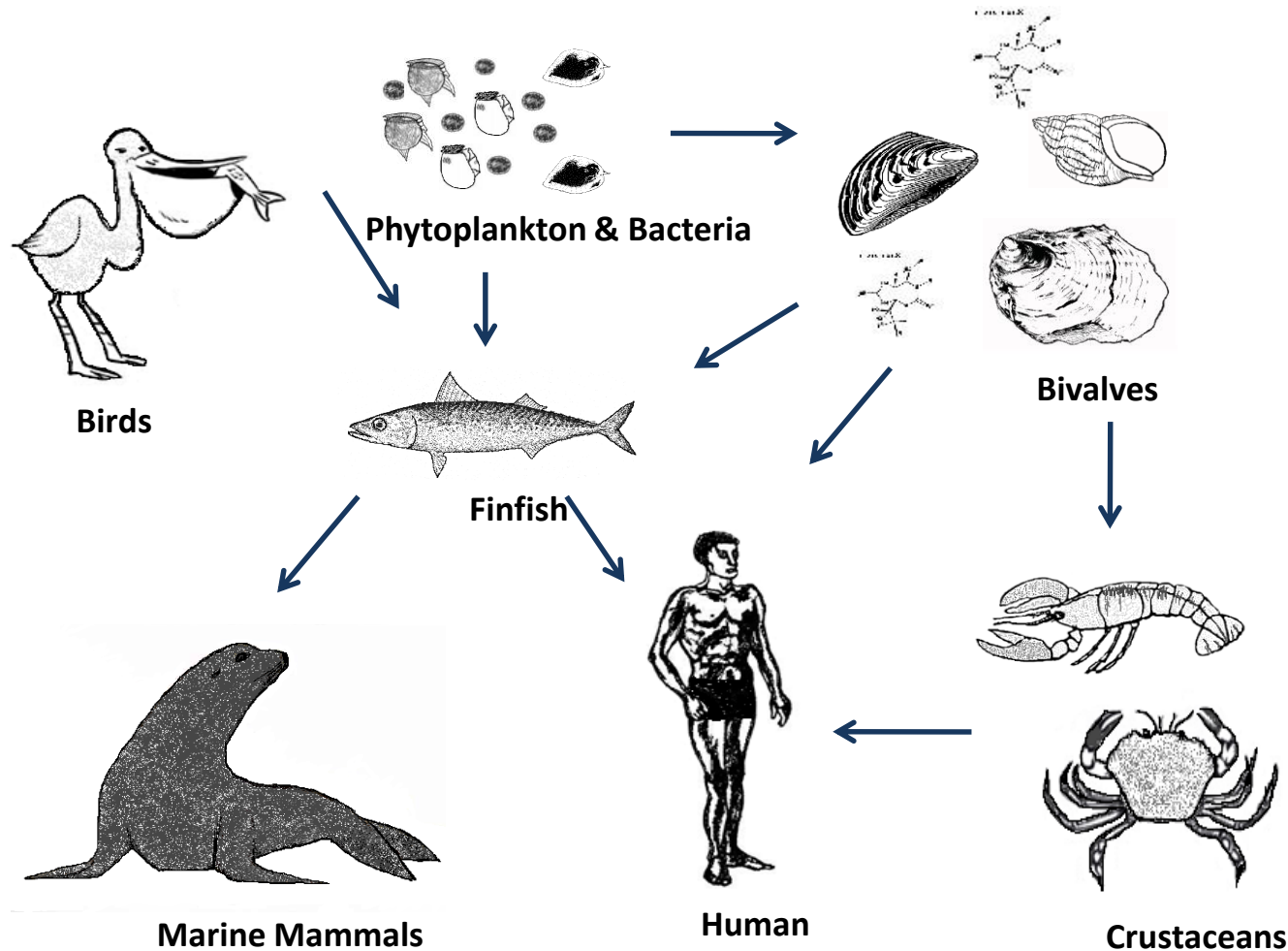
Universidad de Vigo & EURLMB

***I Jornada sobre riesgos emergentes en seguridad alimentaria
Vigo 20 Junio 2018***

RESUMEN

- ***Las Biotoxinas marinas, un riesgo para la salud pública***
- ***Biotoxinas marinas emergentes en la UE***
- ***Investigación en toxinas emergentes***
- ***Retos analíticos***
- ***Perspectivas futuras***

Marine biotoxins/ Food safety/ Human health



Oceans and Climate change:

- *Sea water level increase*
- *Air temperature increase*
- *Temperature of surface water increased*



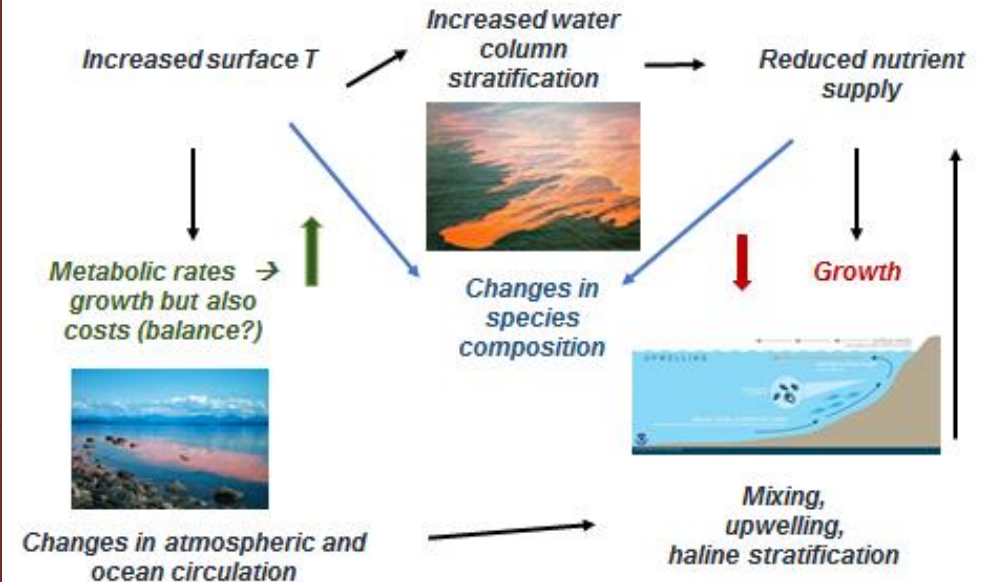
Cambio climático/ biotoxinas marinas

Oceans and Climate change:

- Sea water level increase
- Air temperature increase
- Temperature of surface water increased

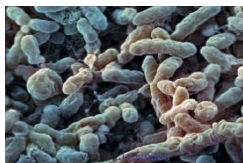
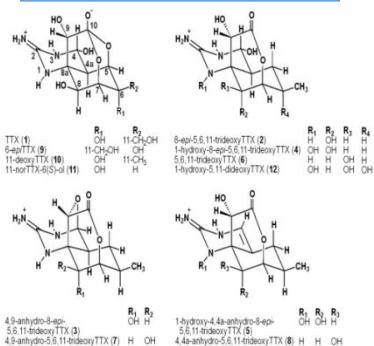


Effects of Climate Change on Marine Phytoplankton

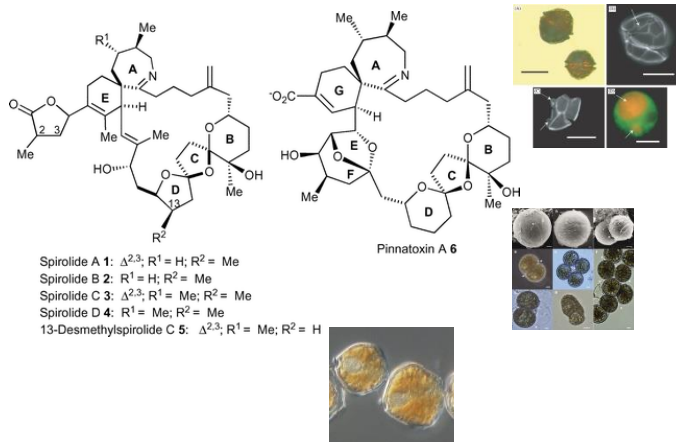


EMERGING MARINE BIOTOXINS

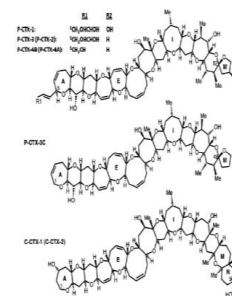
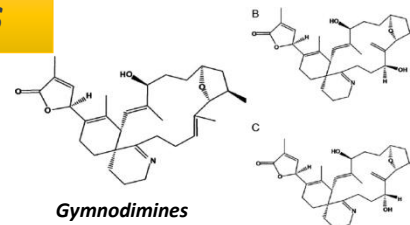
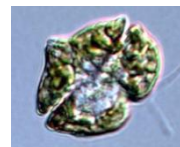
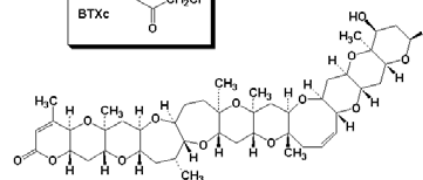
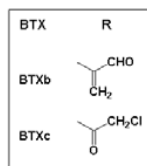
Tetrodotoxins



Cyclic Imines



Brevetoxins



Ciguatoxins



EMERGING MARINE BIOTOXINS

SCIENTIFIC OPINION

Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group¹

EFSA Panel on Contaminants in the Food Chain^{2,3}

European Food Safety Authority (EFSA), Parma, Italy

SCIENTIFIC OPINION

ADOPTED: 15 March 2017

doi: 10.2903/j.efsa.2017.4752

Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods

SCIENTIFIC OPINION

Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Brevetoxin group¹

EFSA Panel on Contaminants in the Food Chain (CONTAM)^{2,3}

European Food Safety Authority (EFSA), Parma, Italy

SCIENTIFIC OPINION

Scientific Opinion on marine biotoxins in shellfish – Cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins)¹

EFSA Panel on Contaminants in the Food Chain (CONTAM)^{2,3}

European Food Safety Authority (EFSA), Parma, Italy

This scientific opinion replaces the earlier version published on 7 June 2010.⁴

Scientific Opinion on marine biotoxins in shellfish – Emerging toxins

Ciguatoxin group (July 2010)

- EFSA Panel on Contaminants in the Food Chain
- EFSA Panel on Contaminants in the Food Chain (CONTAM)

RECOMMENDATIONS

Methods of analysis

Certified reference standards and reference materials for CTX-group toxins need to be provided to allow method development, method validation and the reliable application of analytical methodology in control programmes.

Methods other than the MBA, in particular in vitro (**cytotoxicity and receptor binding**) **assays for screening and LC/MS-MS for confirmation**, should be further developed and optimized, with respect to selectivity and sensitivity for CTX-group toxins in fish tissues. Subsequent (inter-laboratory) validation studies are needed.

Corrigendum to Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organization of official controls on products of animal origin intended for human consumption

(Official Journal of the European Union L 139 of 30 April 2004)

CHAPTER II: OFFICIAL CONTROLS OF FISHERY PRODUCTS

G. POISONOUS FISHERY PRODUCTS

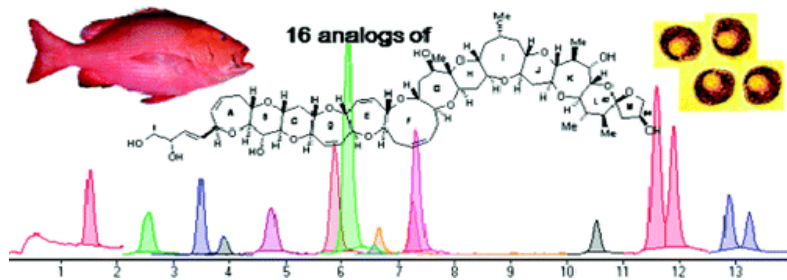
Checks are to take place to ensure that the following fishery products are not placed on the market

2. fishery products containing biotoxins such as *Ciguatera* or other toxins dangerous to human health.

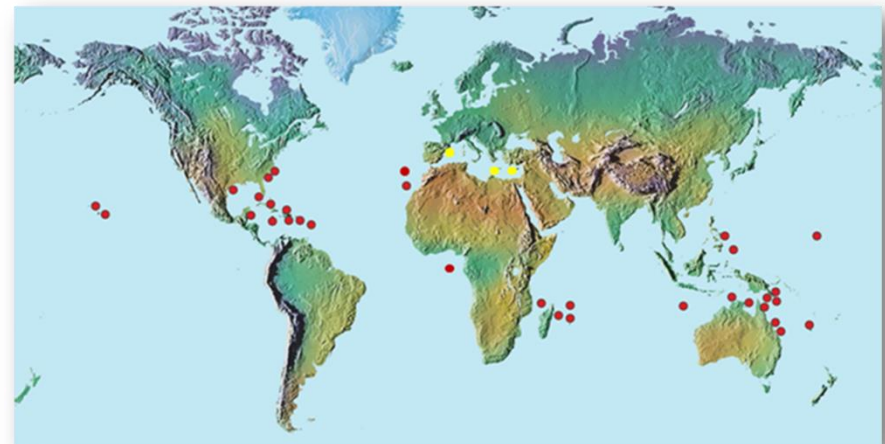
CIGUATERA FISH POISONING (CFP)

- Reports on the presence of Ciguatoxins in the Pacific, Atlantic and Indian Ocean

CFP Worldwide distribution



[Kentaro Yogi††](#), [Naomasa Oshiro†](#), [Yasuo Inafuku†](#), [Masahiro Hiram§](#), [Takeshi Yasumoto*//](#)



INCIDENCE OF CFP IN EU WATERS



Biotoxinas emergentes em águas europeias e novos riscos para a saúde pública

Emergent biotoxins in European waters and new public health risks

Paulo Vale

Instituto Nacional de Recursos Biológicos, I.P. – Instituto de Investigação das Pescas e do Mar, Lisboa, Portugal



<http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20188>

Outbreaks (n=9) and number of cases (n=68) of ciguatera food poisoning, Canary Islands, Spain 2008-2012

Outbreak number	Date	Island	Number of human cases	Fish species	Weight (kg)	Origin
1	15/11/2008	Tenerife	25	Amberjack (<i>Seriola fasciata</i>)	37	Local market
2	29/01/2009	Tenerife	4	Amberjack (<i>Seriola dumerilii</i>)	67	Sport fishing
3	03/09/2009	Gran Canaria	3	Amberjack (<i>Seriola</i> spp.)	Unknown	Unknown
4	19/11/2009	Tenerife	2	Amberjack (<i>Seriola</i> spp.)	Unknown	Sport fishing
5	24/04/2010	Tenerife	6	Amberjack (<i>Seriola</i> spp.)	80	Unknown
6	26/06/2011	Gran Canaria	5	Amberjack (<i>Seriola</i> spp.)	24	Sport fishing
7	28/01/2012	Lanzarote	10	Amberjack (<i>Seriola</i> spp.)	15	Sport fishing
8	04/04/2012	Lanzarote	9	Amberjack (<i>Seriola</i> spp.)	26	Sport fishing
9	05/2012	Tenerife	4	Amberjack (<i>Seriola</i> spp.)	Unknown	Local market

UVIGO/EURLMB RESEARCH ON EMERGING TOXINS

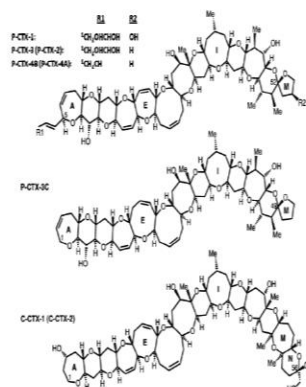
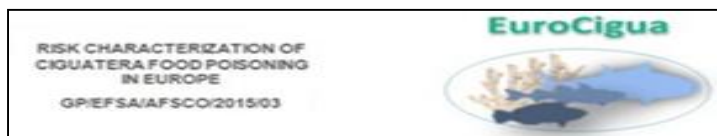
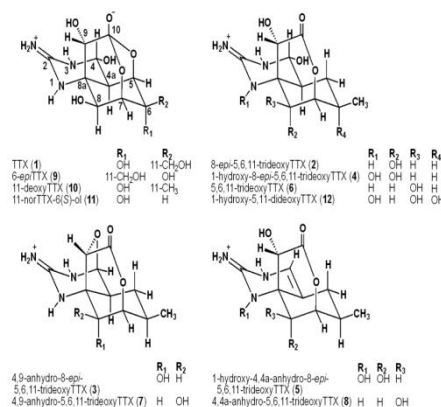
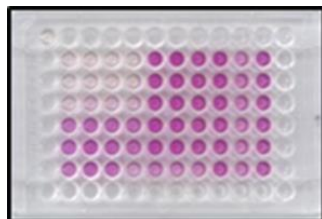


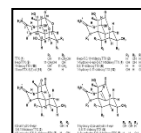
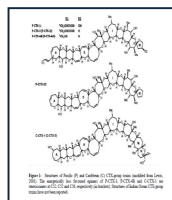
Figure 1: Structures of Pacific (P) and Caribbean (C) CTX-group toxins (modified from Lewis, 2001). The energetically less favoured epimers of P-CTX-3, P-CTX-4B and C-CTX-1 are stereoisomers at C52, C52 and C56, respectively (in bracket). Structures of Indian Ocean CTX-group toxins have not been reported.



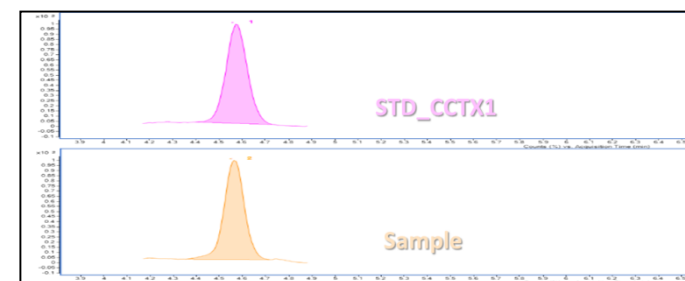
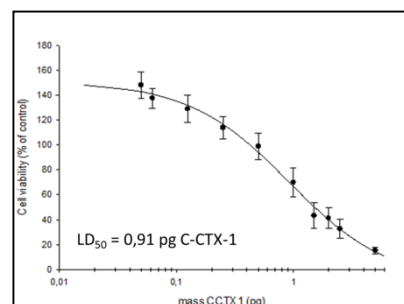
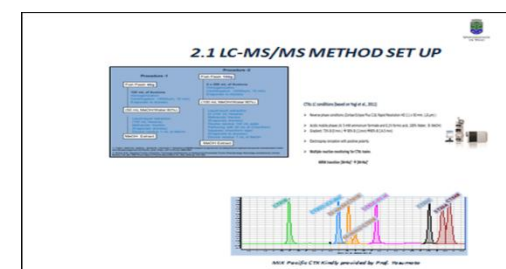
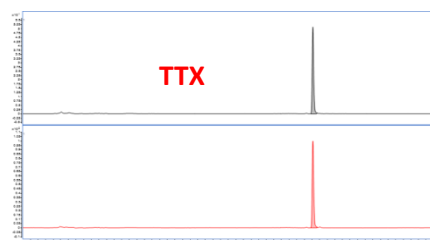
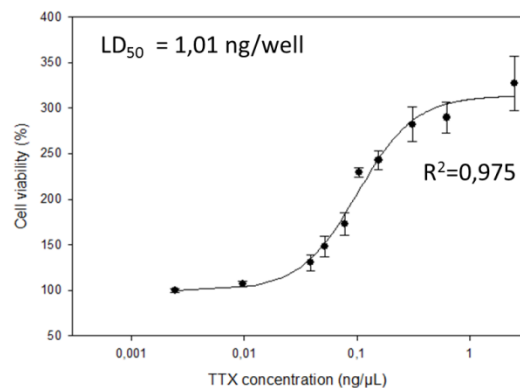
Desarrollo de herramientas analíticas



N2a
(screening)



LC-MS/MS
(Confirmación)



CFP: LC-MS/MS METHOD SET UP

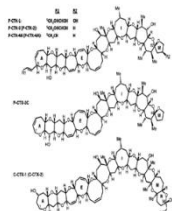
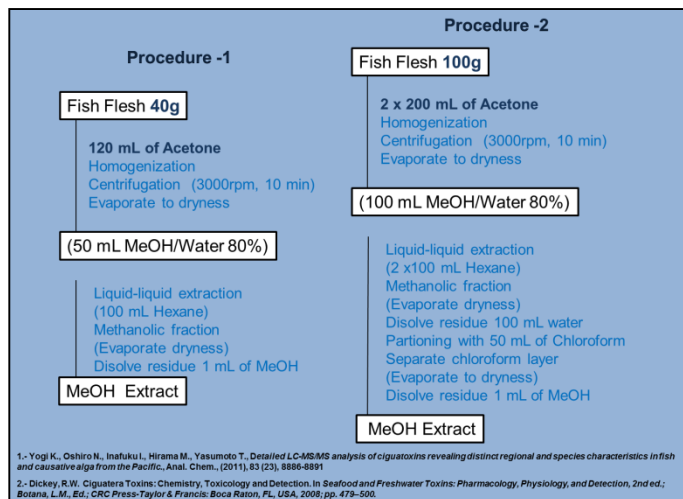


Figure 1. Structures of Pectenotoxin (PTX) and Gibberellin (GIB) and Ciguatera toxin (CTX). The nomenclature for Gibberellin (GIB) and Ciguatera toxin (CTX) are abbreviated as GIB and CTX, respectively (in brackets). Structures of Gibberellin (GIB) and Ciguatera toxin (CTX) are shown.

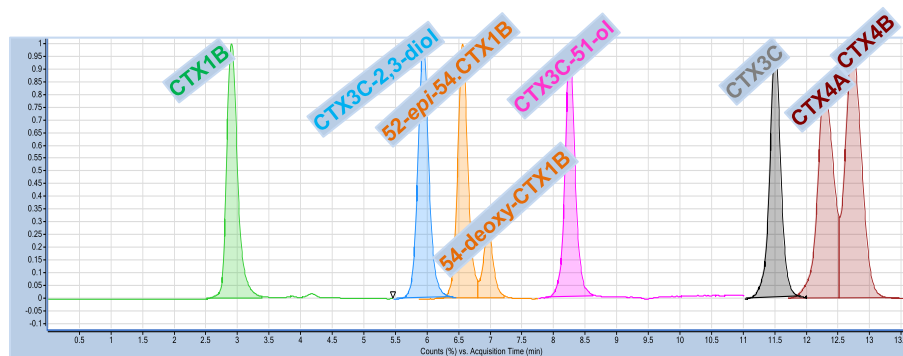


CTXs LC conditions (based on Yogi et al., 2011)

- Reverse phase conditions (Zorbax Eclipse Plus C18, Rapid Resolution HD 2.1 x 50 mm; 1.8 μm)
- Acidic mobile phases (A: 5 mM ammonium formate and 0.1% formic acid, 100% Water; B: MeOH)
- Gradient: 73% B (0 min.) → 90% B (11min) → 90% B (14.5 min)
- Electrospray ionization with positive polarity
- Multiple reaction monitoring for CTXs toxins



MRM transition $[M+Na]^+ \rightarrow [M+Na]^+$



CFP en Europa: EUROCIGUA

RISK CHARACTERIZATION OF CIGUATERA FOOD POISONING IN EUROPE
GPIE/SA/AFSCO/2015/03

EuroCigua



Logos of participating institutions: Ministerio de Sanidad, Consumo y Bienestar Social; aecosan; EFSA; Instituto de Salud Carlos III; IRTA; Universidad de Vigo; ASAE; ipce; ARISTOTELE; BfR; University of Thessaly; Ministry of Health; Ifremer; STATE GENERAL LABORATORY; UNISERV; Universidad de Las Palmas de Gran Canaria; Servicio Canario de Salud; Instituto Nacional de Salud; Region Autonoma de Valencia; Region Autonoma de Murcia; Region Autonoma de Castilla-La Mancha; Region Autonoma de Castilla y León; Region Autonoma de Aragón; Region Autonoma de Cataluña; Region Autonoma de Galicia; Region Autonoma de Asturias; Region Autonoma de Cantabria; Region Autonoma de Extremadura; Region Autonoma de Castilla-La Mancha; Region Autonoma de Castilla y León; Region Autonoma de Aragón; Region Autonoma de Cataluña; Region Autonoma de Galicia; Region Autonoma de Asturias; Region Autonoma de Cantabria; Region Autonoma de Extremadura.

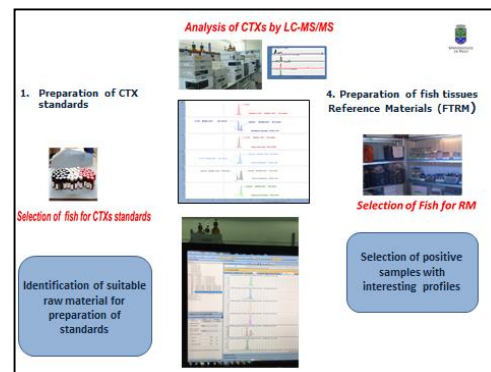
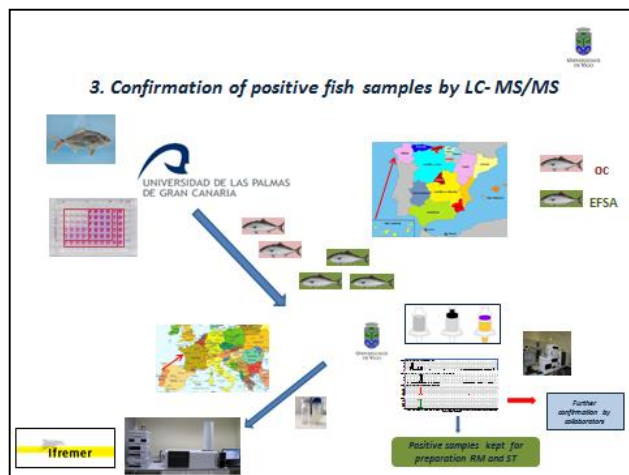
SG4
Coordinated by University of Vigo

To characterize the risk associated with Ciguatera poisoning, by developing an efficient analytical methodology with ability to confirm the identity of the toxins involved in the contamination of phytoplankton and fish samples, as well as developing standards and reference materials to be used for this evaluation and characterization as well as to help participants in the project to set up the evaluated methodologies.

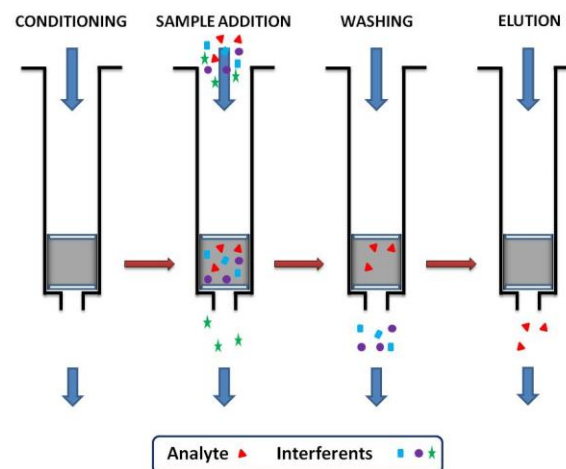


Ifremer

JFRL

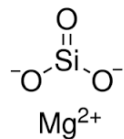


Sample pretreatment: Critical Step



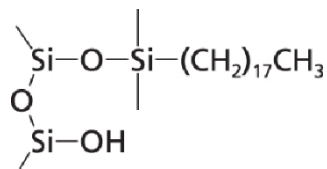
1- Florisil

Critical !!



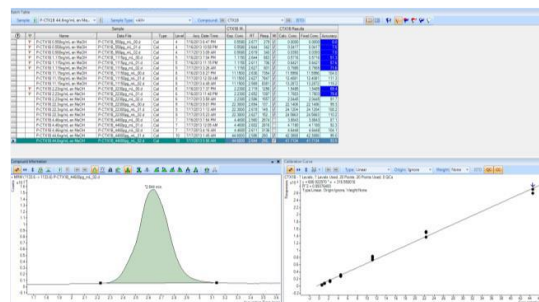
Improved extraction and SPE conditions allowed and increased analyte recovery

2- C18

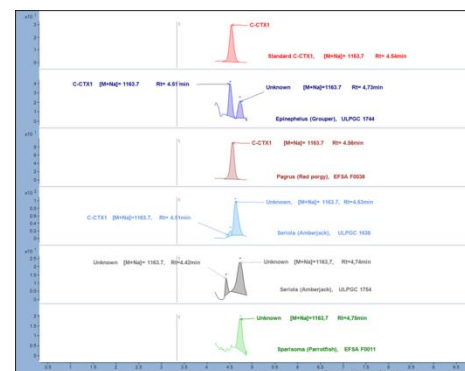
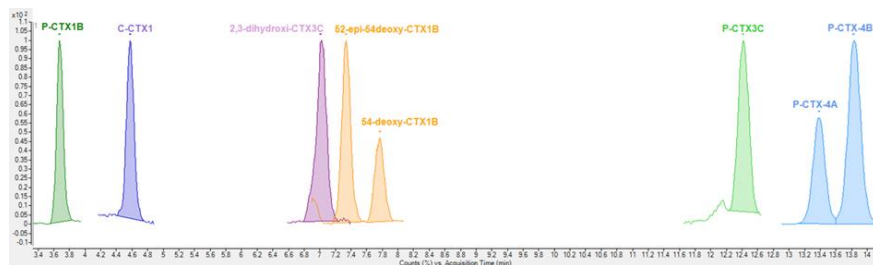
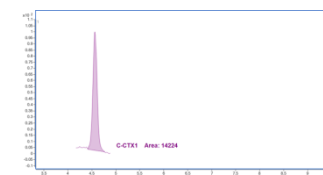


LC-MS/MS evaluation and optimization

Application to naturally contaminated fish samples



ED481A-2018/207

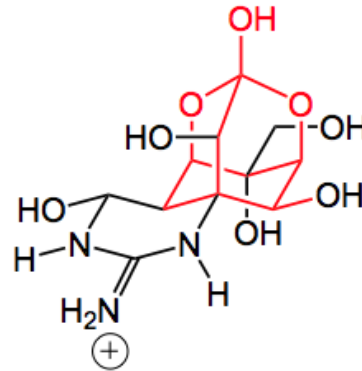


Detection limit*: 0.004 ng/g
Quantitation limit*: 0.015 ng/g
Calibration range*: 0.28 to 27.88 ng/mL
equivalent to 0.010-1.025 ng/g
*Data obtained for P-CTX1B

Implementation of Liquid Chromatography tandem Mass Spectrometry to be applied for the Analysis of ciguatera fish poisoning in contaminated fish samples from Canary Islands

P. Estévez-Bastos, D. Castro, J.M.Leño, Takeshi Yasumoto, R. Dickey, Ana Gago-Martinez* (in press)

TETRODOTOXIN POISONING



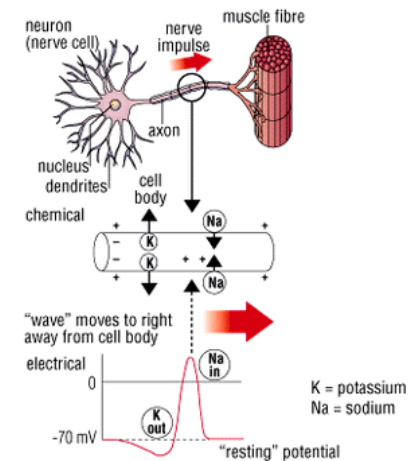
Tetrodotoxin

Tetrodotoxin poisoning

- Tetrodotoxin **causes** this type of poisoning;
- it is found the liver, gonads, intestines, and skin of pufferfish (fugu), as well as in less-commonly eaten animals like parrotfish, frogs, octopus, starfish, angelfish, and crabs
- The disease is potentially deadly.
- **Symptoms** include numbness of the lips and tongue, tingling in the body, and a sensation of lightness. Nausea and vomiting, diarrhea, and belly pain may occur.
- **Treatment** is supportive, and may include giving the person charcoal, pumping the stomach, giving IV fluids, and placing them on a ventilator in severe cases. There is no antidote

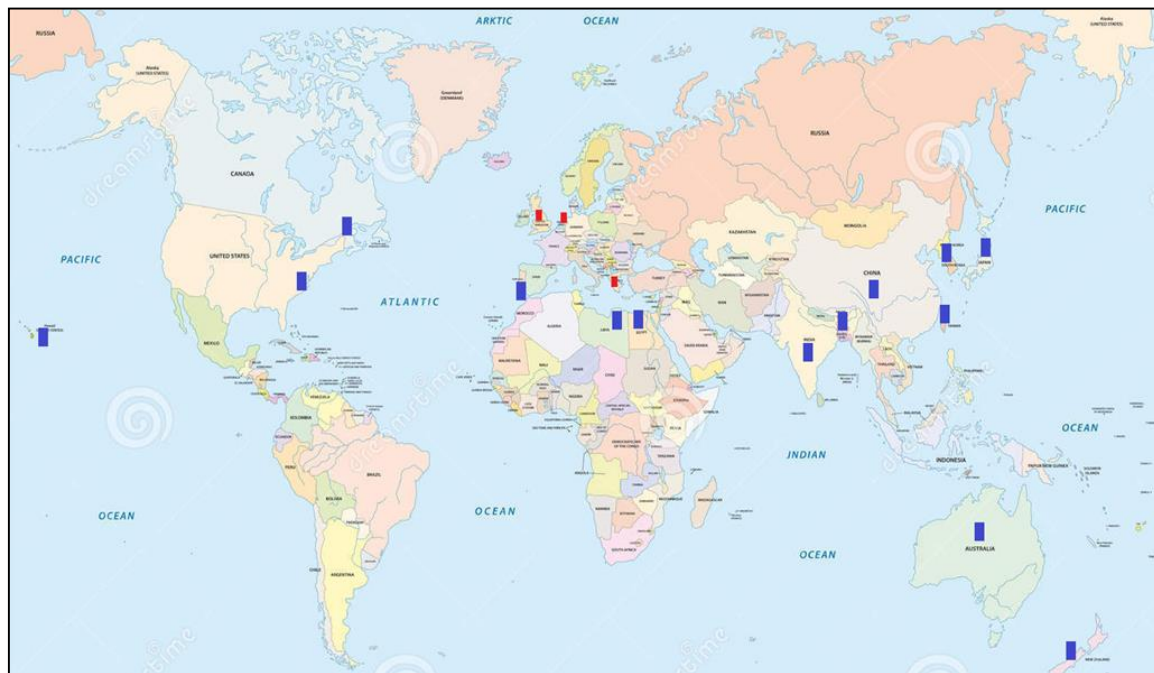
Dr. Shaleesha A. Stanley

7



TTX GEOGRAPHICAL DISTRIBUTION

Mostly associated to Pufferfish or others, few reports on bivalve (JP, NZ, EU)



EFSA OPINION

<https://www.efsa.europa.eu/en/efsajournal/pub/4752>



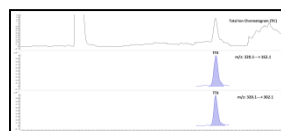
RECOMMENDATIONS

- ❑ More occurrence data on TTX and its analogues in edible parts of marine bivalves and gastropods from different EU waters are needed.
- ❑ Occurrence data should be obtained using EU approved methods.
- ❑ Studies on the sources and critical factors leading to the accumulation of TTX are needed.
- ❑ Further information on the acute oral toxicity of TTX and its analogues is needed. Chronic effects should also be investigated.

22

Development of HILIC – MS/MS for determination of TTX

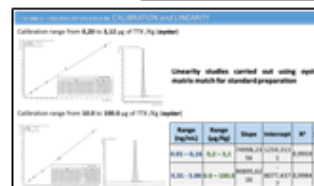
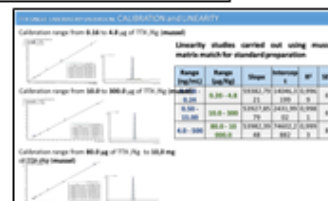
Parameters	HILIC Chromatography
Mobile Phase A	Water with 0.015% (v/v) Formic acid and 0.06% (v/v) of 25% Ammonium hydroxide
Mobile Phase B	Acetonitrile-water (70:30) _{v/v} with 0.01% _{v/v} formic acid
Column	AQUITY UPLC® Glycan BEH Amide Column, 130Å; 1.7µm; 2.1mm x 150mm Lot#0140350431, Waters (Ireland)
Column temperature	60 °C
Injection Volume	2µL
Run time (min)	11 min



SLV VALIDATION EURLMB SOP HILIC-LC-MS/MS for TTX

Analyte: TTX
Matrices: Mussel, Oyster
Standards & Samples
Method: (HILIC) LC-MS/MS

Performance characteristics:
Applicability
Selectivity
Calibration and linearity
Precision
Range
Detection and quantitation limits
Matrix effect
Application to bivalves



(EFSA) More Occurrence data are needed:

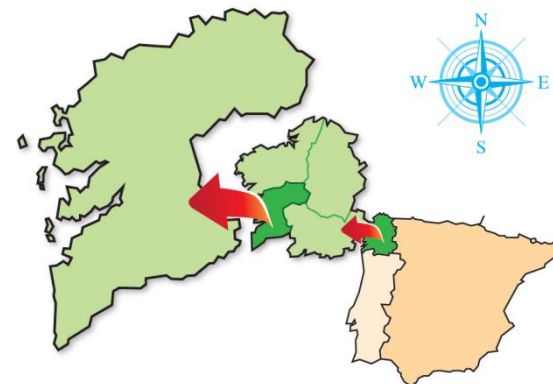
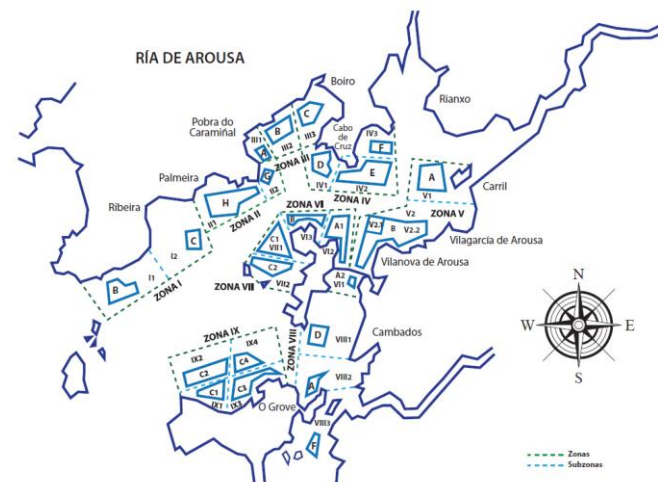
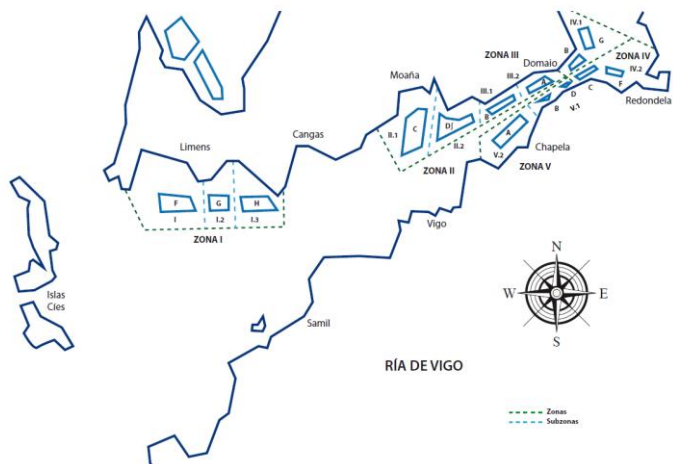
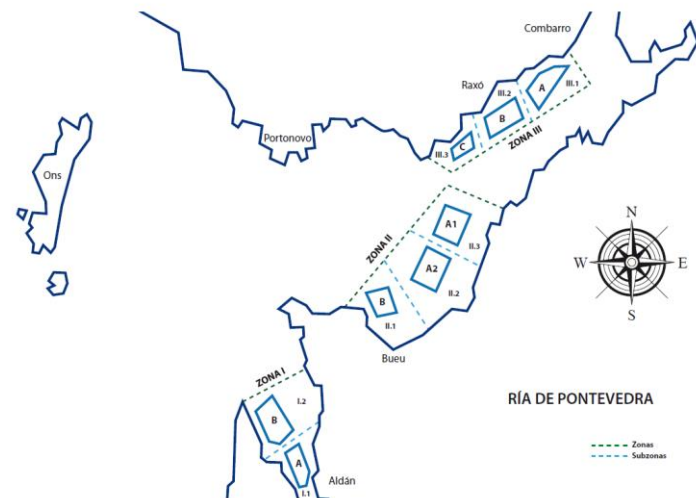
Collaborative research Project Evaluation of TTX in mussels from Galician Rias

- ❖ ***Asociación de productores mejilloneros de Cabo de Cruz***
- ❖ ***Dpt. of Analytical and Food Chemistry & CINBIO, University of Vigo***
- ❖ ***EU Reference Laboratory for Marine Biotoxins***

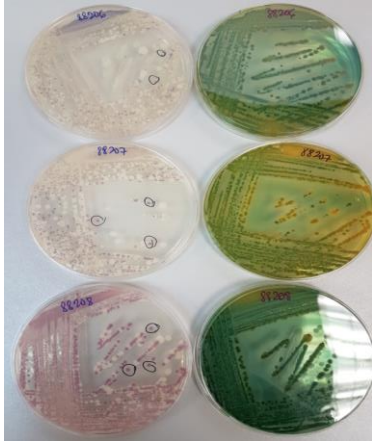


January- June 2017 (ongoing)

227 samples of shellfish from three Galician Rias (Arosa, Pontevedra, Vigo) are being investigated for the presence of TTX .



STRAIN ISOLATION



Isolated bacteria	Number of samples
<i>Vibrio</i> spp	343
<i>Aeromonas</i> spp	4
<i>Citrobacter</i> spp	4
<i>Serratia</i> spp	2
<i>Routella</i> spp	1
<i>Escherichia</i> spp	1
<i>Pseudomonas</i> spp	3
<i>Acinetobacter</i> spp	1
<i>Shewanella</i> spp	1
<i>Salmonella</i> spp	1
<i>Stenotrophomonas</i> spp	1
<i>Comamonas</i> spp	1
<i>Enterobacter</i> spp	1
TOTAL	364

Isolated <i>Vibrio</i> species	Number of samples
<i>Vibrio parahaemolyticus</i>	54
<i>Vibrio alginolyticus</i>	289
<i>Vibrio fluvialis</i>	2
<i>Vibrio cholerae</i>	5
<i>Vibrio vulnificus</i>	1
<i>Photobacterium damsela</i>	1
TOTAL	352



DETERMINATION OF PATHWAY GENES (PKS and NRPS)

STRAIN	PKS	NRPS
<i>Vibrio</i> spp.	1	30
<i>Citrobacter</i> spp.	1	1
<i>Serratia</i> spp.	1	1

NRPS and PKS clusters identified in some strain isolated from shellfish

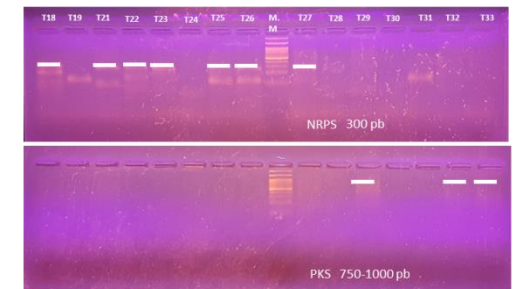
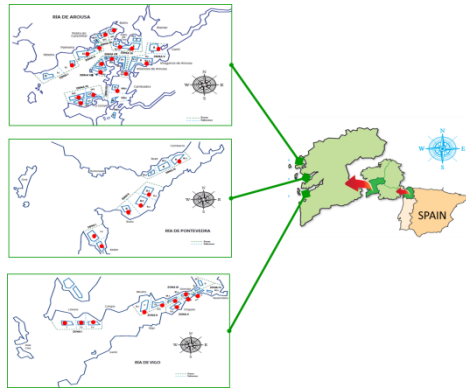
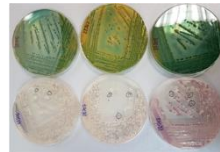


Fig. 1 Gel electrophoresis of the PCR products

First Phase on the Evaluation of TTX in mussels from Galician Rias

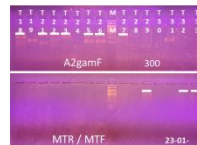


From January- May 2017
(73 samples)

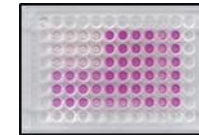


ISOLATION

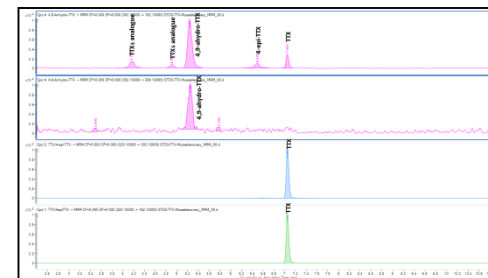
Vibrio spp was the most abundant genus
from the isolated bacteria, in particular
Vibrio alginolyticus



DETERMINATION OF
PATHWAY GENES
(PKS and NRPS)



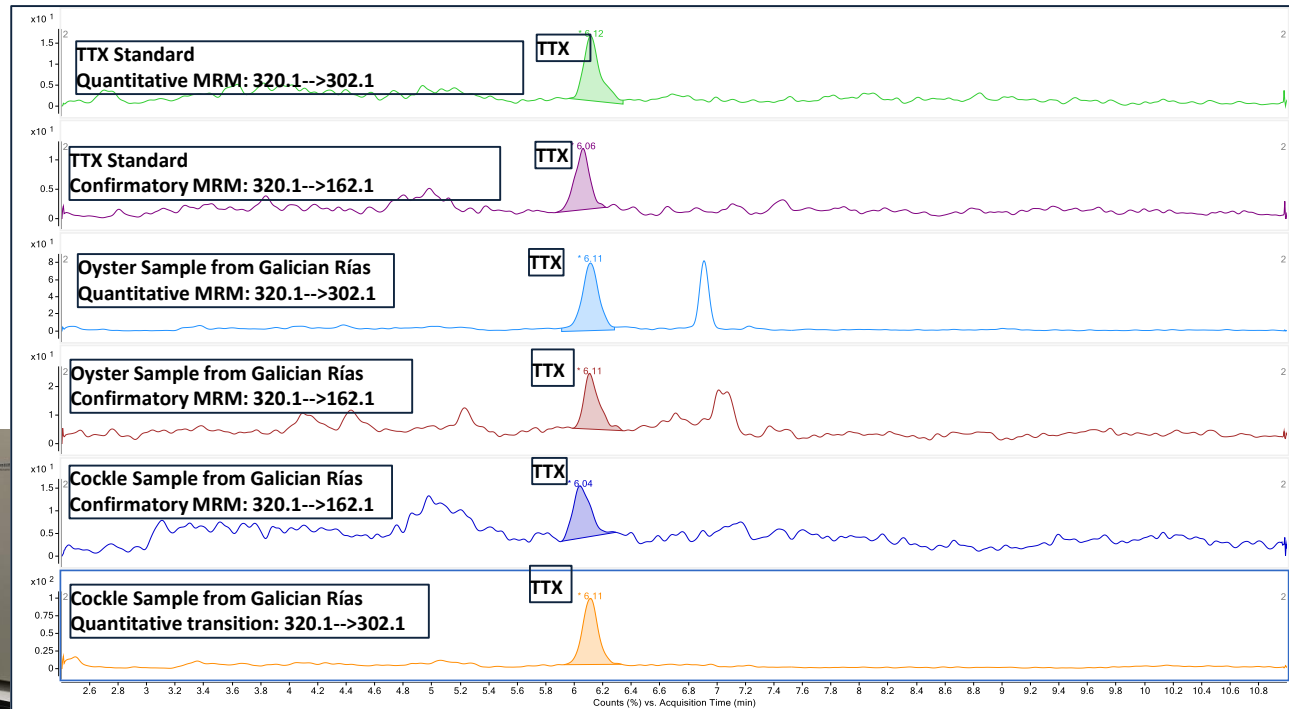
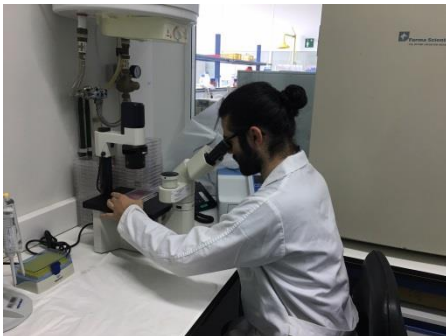
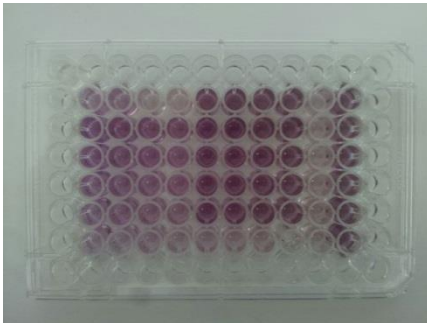
no positive results for TTX by N2a



No TTX was found by LC-MS/MS in
this first phase of the study

Second phase of the study

- N2a Positive samples (clams and oysters)
- Results confirmed by HILIC- LC-MS/MS
(TTX, present in some infaunal samples)

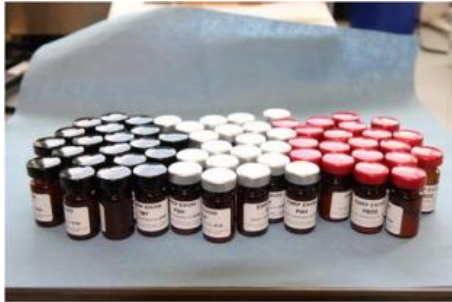


Samples collected from Ría Arosa, Galicia, Spain, June 2017

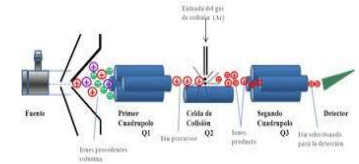
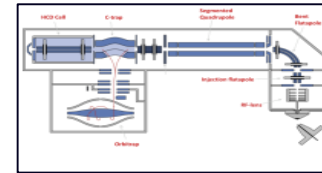
CONCLUSIONES: ESTADO ACTUAL DE LA CARACTERIZACIÓN DEL RIESGO DE CTXS Y TTX EN EUROPA

- Los ensayos de citotoxicidad (N2a) constituyen una herramienta útil para screening aunque presentan limitaciones (tiempo y falta de especificidad)
- Se ha desarrollado un método optimizado de LC-MS/MS suficientemente sensible para la determinación de CTXs
- **Limitación importante:** No disponibilidad de estándares y dificultad para el desarrollo de los mismos, consecuentemente la implementación de métodos y extensión a análogos de CTXs se encuentra también limitada.
- Presencia de C-CTX1 como máximo responsable de toxicidad en Las Islas Canarias, Madeira e Islas Salvajes, se sospecha de la presencia de análogos de la misma (confirmación en curso)
- Necesidad de obtener peces contaminados con mayor contenido en CTXs (localización de “hot spots”)
- Se ha desarrollado y validado un método de HILIC-LC-MS/MS para determinación de TTX
- En la actualidad se dispone de datos (aunque limitados) de ocurrencia de TTX en bivalvos de la UE
- Bajos contenidos de TTX y escasa presencia de la misma en muestras de bivalvos infaunales de las Rías Gallegas
- Necesidad de continuar con la evaluación de la ocurrencia de TTX y su relación con *Vibrio* spp.

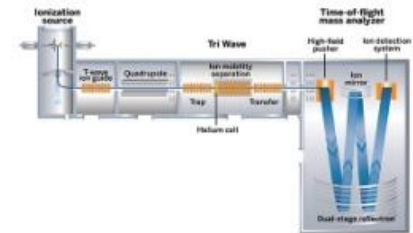
Perspectivas futuras



Standards and Reference materials



analytical tools for quantitation and confirmation



Occurrence data



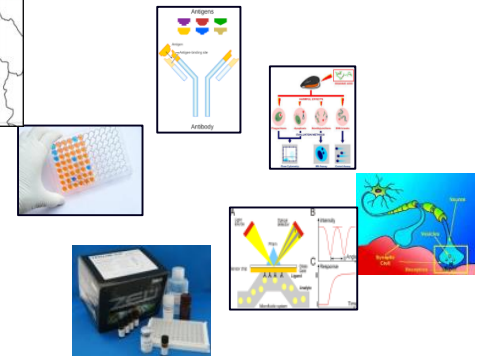
In Vitro



In Vivo



Toxicological evaluation



alternative screening methods is a pending issue.

Research Funding



G. Moreiras



AGL2014-52403-R



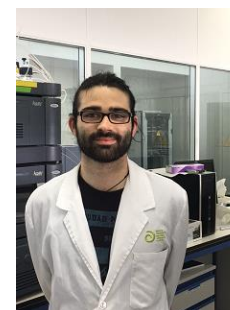
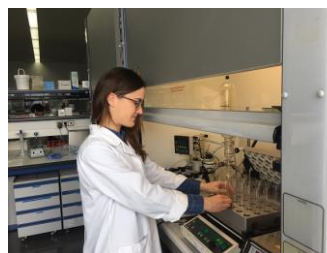
P. Estévez



ED481A-2018/207



D. Castro



J. Giráldez





AGRADECIMIENTOS

