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TESIS DE DOCTORADO

IN VITRO AND *IN VIVO* TOXICITY STUDY OF COMPOUNDS OF NATURAL ORIGIN: MARINE BIOTOXINS

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In vitro and _in vivo_ toxicity study of compounds of natural origin: marine biotoxins

Dª. Paula Abal Camaño

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In vitro and _in vivo_ toxicity study of compounds of natural origin: marine biotoxins

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ABBREVIATIONS AND ACRONYMS

ATC: Acute toxic class method
ARfD: Acute refence dose
ASP: Amnesic shellfish poisoning
AZAs: Azaspiracids
AZP: Azaspiracid poisoning
b.w.: Body weight
BTX: Brevetoxins
CFP: Ciguatera fish poisoning
CI: Cyclic imines
CTX: Ciguatoxin
DA: Domoic acid
DTXs: Dinophysistoxins
DSP: Diarrheic shellfish poisoning
EC_{50}: Half maximal effective concentration
EFSA: European food safety authority
ENS: Enteric nervous system
eq: Equivalents
FDP: Fixed Dose Procedure
GSH: Globally Harmonized System
HAB: harmful algae bloom
HPLC: High performance liquid chromatography
i.g.: Intragastric
i.p.: intraperitoneal
IC_{50}: Inhibitory concentration 50
ICH: International conference on harmonization
IEFs: Inhibitory equivalency factors
LD_{50}: lethal dose 50
LC – MS/MS: Liquid chromatography linked to tandem mass spectrometry
LOAEL: Lowest observed adverse effect level
LOD: Limit of detection
LOQ: Limit of quantification
MBA: mouse bioassay
MTX: Maitotoxins
Na_v: Voltage-gated sodium channels
NOAEL: No observed adverse effect level
NSP: Neurotoxic shellfish poisoning
OA: Okadaic acid
OECD: Organisation for economic cooperation and development
PE: Phycoerythrin
PLTX: Palytoxins
PP: protein phosphatases
PSP: Paralytic shellfish poisoning
PTX: Pectenotoxin
RE: Removal efficiency
RER: Rough endoplasmic reticulum

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RES: Smooth endoplasmic reticulum
s.c.: **S**ubcutaneus
STX: Saxitoxins
TDI: Tolerable daily intake
TEER: Transepithelial electrical resistance
TEFs: Toxicity equivalency factors
TEM: Transmission electron microscopy
TJs: Tight junctions
TTX: Tetrodotoxins
YTX: Yessotoxin
UDP: Up and Down Procedure

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RESUMEN

Los productos naturales suponen una fuente importante de nuevos fármacos con gran potencial terapéutico. La biodiversidad en el medio marino se traduce en un gran espectro de compuestos bioactivos, como los producidos por el fitoplancton. Estas microalgas fotosintéticas se componen de varios grupos taxonómicos, entre los que se encuentran los dinoflagelados. Algunas especies de estos organismos unicelulares tienen la capacidad de producir biotoxinas marinas que provocan serios problemas económicos y sanitarios sobre todo cuando se dividen exponencialmente causando las floraciones algales nocivas (FAN). Éstas se ven favorecidas además por el cambio climático y las actividades antropogénicas, aumentándose su frecuencia de aparición, su severidad e incluso su expansión geográfica.

Las ficotoxinas se clasifican en varios grupos en función de su estructura química y modo de acción. Cada grupo tiene una toxina representativa y varios análogos: las toxinas paralizantes (el compuesto de referencia es la saxitoxina), las toxinas diarreicas (ácido okadaico y dinofisistoxinas), los azaspirácidos, las amnésicas (ácido domoico), las pectenotoxinas, las yesotoxinas, las ciguatoxinas, las maitotoxinas, las brevetoxinas, las palitoxinas (y análogos osteocinas y ovatoxinas) y las iminas cíclicas. En la presente tesis doctoral se presentan estudios de toxicidad de toxinas lipofílicas comúnmente encontradas en la costa de Galicia, el ácido okadaico (OA), sus análogos las dinofisistoxinas (DTXs) y los azaspirácidos (AZAs). Además también se evalúa la toxicidad oral de la toxina hidrosoluble tetrodotoxina (TTX), considerada toxina emergente en nuestras costas.

El OA y las DTXs son compuestos poliéter sintetizados por dinoflagelados de los géneros *Prorocentrum* y *Dinophysis*. Estas microalgas forman parte del alimento de los moluscos, que concentran las toxinas durante el proceso de filtración. La intoxicación alimentaria que se produce tras el consumo del marisco contaminado con este grupo de toxinas causa en el hombre desórdenes gastrointestinales como náuseas, vómitos, diarrea y dolor abdominal. Estos compuestos inhiben específicamente las proteínas serina/treonina fosfatasas (PP), principalmente la proteína fosfatasa 2A (PP2A), pudiendo ser esta inhibición reversible. Sin embargo, esta acción no sería la única responsable de sus efectos diarreicos. Los AZAs producen intoxicaciones con síntomas similares a las del OA y derivados, aunque ambos grupos no comparten el mecanismo de acción, de hecho éste todavía se desconoce para los AZAs. Los organismos productores de esta familia de toxinas también son dinoflagelados, los cuales pertenecen a los géneros *Azadinium* y *Amphidoma*. Sin embargo, el origen de las toxinas del grupo de la TTX es controvertido, se ha relacionado con bacterias principalmente de los géneros *Vibrio* y *Pseudomonas*. La TTX es una potente neurotoxina que bloquea los canales de sodio dependientes de voltaje inhibiendo la transmisión del impulso nervioso. La mayoría de los casos de intoxicación por TTX se deben al consumo de peces con toxina que provoca síntomas como entumecimiento perioral con parestesia de la lengua y labios, parálisis motora temprana, falta de coordinación, dolor de cabeza, vómitos y en los casos más graves la muerte, causada por insuficiencia respiratoria y cardíaca.

Los estudios realizados abarcan las distintas fases a valorar para una completa evaluación del riesgo que entrañan estas toxinas. La eliminación de la fuente tóxica del medio natural mediante la captación de los microorganismos productores de toxinas es un paso crucial que podría solventar las intoxicaciones alimentarias. Por tanto, en primer lugar se investiga el poder de floculación de distintas arcillas naturales frente al dinoflagelado *Prorocentrum lima*,

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organismo productor del OA y las DTXs. El medio marino es simulado mediante columnas de agua marina sobre las que se agrega el cultivo celular de *P. lima* que se encuentra en el momento de máxima producción de toxinas. La adición de arcilla muestra la aglomeración de las partículas minerales en torno al dinoflagelado, haciendo que precipite al fondo con diferente velocidad. Este poder de adsorción se evalúa a lo largo del tiempo realizando recuentos celulares y visualizando los flóculos mediante microscopía óptica, siendo el caolín la arcilla con mayor capacidad de adsorción y cuyas aglomeraciones son visibles incluso a simple vista. Los resultados obtenidos indican que las arcillas naturales bajo estudio son más eficientes en la eliminación de los dinoflagelados cuanto menor es su tamaño de partícula, aunque también es importante su composición mineral.

Las toxinas marinas estudiadas son ingeridas por el hombre mediante el consumo de marisco contaminado en la dieta, por lo tanto investigar el paso de las toxinas a través del tracto gastrointestinal y los efectos tóxicos en sus células es el siguiente nivel de evaluación. Mediante el modelo intestinal humano Caco-2 se evalúa la absorción de la toxina azaspiracido-1 (AZA1), así como sus efectos. Esta toxina no afecta a la viabilidad de la línea celular, como indica el ensayo de citotoxicidad realizado con AlamarBlue a pesar del amplio rango de concentraciones usadas (0.1 – 500 nM) y el largo tiempo de exposición (72 horas). Este reactivo es un indicador de la actividad metabólica celular, las células viables reducen continuamente el indicador convirtiéndolo en fluorescente generando una medida cuantitativa de la viabilidad. Esta línea celular se diferencia a células intestinales y forma una monocapa celular polarizada que simula la barrera intestinal. La integridad de la monocapa se ha determinado midiendo la resistencia eléctrica transepitelial (TEER) que a concentraciones de 10 y 100 nM de AZA1 muestra una disminución comúnmente relacionada con la destrucción de la barrera formada por la monocapa de células Caco-2. Este sistema de cultivo puede crecer sobre una membrana semipermeable contenida en un inserto formando un sistema de dos compartimentos, uno apical que corresponde *in vivo* al lumen intestinal y otro basolateral que simularía el espacio intersticial en contacto con los capilares sanguíneos.

El contenido de ambos compartimentos puede ser recogido para estudiar la cantidad de la toxina que ha pasado a través de la monocapa celular. La detección y cuantificación del AZA1 se realiza mediante el sistema Luminex que se basa en un ensayo inmunológico. Las muestras obtenidas en el ensayo de permeabilidad previamente incubadas con el anticuerpo primario anti-AZA1 son expuestas a las microesferas que llevan unidas la toxina AZA1 dando lugar a un ensayo de competición en el que el anticuerpo anti-AZA1 que no se haya unido a la toxina presente en las muestras se unirá a la toxina de las microesferas. Por tanto, el análisis mediante el sistema Luminex permite conocer la toxina presente en las muestras mediante la cuantificación del anticuerpo primario anti-AZA1 que se une a las microesferas por medio de un anticuerpo secundario marcado con ficoeritrina que identifica al anticuerpo primario. Las microesferas son reconocidas por el sistema de láseres del Luminex que además cuantifica la ficoeritrina unida al complejo. Los resultados obtenidos indican que el AZA1 es absorbido por las células Caco-2 de una forma dosis-dependiente.

El estudio del paso de la toxina a través de la monocapa celular lleva a enfocar la atención en las uniones estrechas, ya que su afectación está relacionada con el aumento de transporte paracelular. La tinción de la proteína ocludina presente en las uniones estrechas celulares indica por microscopía confocal una distribución alterada e incluso su internalización en el citoplasma. Sin embargo, las uniones estrechas que se observan mediante microscopía electrónica de transmisión (TEM) permanecen aparentemente intactas en su estructura. Además, se detectan otros daños a distintos niveles. Las microvellosidades son escasas y de morfología irregular en las monocapas celulares tratadas con AZA1, donde la presencia de autofagosomas es

abundante. También se registran núcleos lobulados con heterocromatina condensada y mitocondrias electrondensas. Todos estos datos indican que tras la ingestión de moluscos con AZA1 la toxina se transporta a través de la barrera intestinal a la sangre causando alteraciones en las células epiteliales.

Los análogos tóxicos que forman un mismo grupo de toxinas se caracterizan por poseer diferente potencia tóxica, de forma que el conocimiento de esta relación es de vital importancia en la evaluación de la toxicidad. Para proteger al consumidor la legislación de la Unión Europea establece como cantidad máxima permitida 160 µg/kg OA eq en moluscos y como método de referencia para la detección de estas toxinas la cromatografía líquida acoplada a la espectrometría de masas en tándem (LC-MS/MS). En este límite se considera la toxina representativa del grupo (OA) por lo que las cantidades de las restantes toxinas legisladas ha de convertirse en equivalentes (eq) de OA en función de su toxicidad. Esto requiere la determinación de los Factores de Toxicidad Equivalente (TEF) definidos como la relación entre la toxicidad de cada análogo y la toxicidad del compuesto de referencia. El análisis comparativo de citotoxicidad del OA y sus derivados muestra que mientras que el OA no causa citotoxicidad en las células Caco-2 en el rango de concentraciones estudiadas, la viabilidad de la línea celular de neuroblastoma SH-SY5Y sí se ve comprometida por la toxina, como también ocurre con la DTX1 aunque para ésta última con mayor potencia. El cocultivo de ambas líneas celulares simula la barrera intestinal junto con la presencia de neuronas productoras de neuropéptidos que regulan su función. El tratamiento con OA produce el descenso dosis-dependiente progresivo del TEER simultáneamente al aumento de la cantidad de OA que atraviesa la monocapa. La distribución de la ocludina también se ve modificada por el tratamiento con la toxina, de acuerdo a la alteración de la barrera intestinal indicada mediante el ensayo de permeabilidad. El estudio de los niveles del neuropéptido Y (NPY) intracelular y el NPY liberado por las células de neuroblastoma revela modificaciones dependientes de la concentración del OA y el tiempo de exposición al tratamiento. Se produce una reducción de los niveles del NPY en el monocultivo tras el tratamiento con OA. Sin embargo se detecta un incremento del NPY al aumentar ligeramente el paso de la toxina a través de la monocapa en el cocultivo. Estos resultados ofrecen una nueva aproximación para establecer la influencia de la acción neuronal del OA sobre sus efectos diarreicos a través de una interconexión entre el sistema nervioso entérico y el intestino mediante la secreción de NPY inducida por OA.

Los estudios *in vivo* apoyan la mayor potencia tóxica mostrada por la DTX1 en los estudios de citotoxicidad *in vitro*. En los ensayos *in vivo* la administración intraperitoneal era la más utilizada para la evaluación tóxica de un compuesto. Sin embargo, las toxinas se ingieren, por lo que en ausencia de datos representativos de intoxicaciones en el hombre los estudios de toxicidad oral en ratón son los más adecuados para establecer los valores de TEF. La Organización para la Cooperación y Desarrollo Económico (OECD) ha desarrollado métodos estandarizados de estudio de toxicidad en animales siguiendo el principio de las tres Rs (reemplazo, reducción y refinamiento). Uno de estos métodos fue el seleccionado para comparar la toxicidad oral aguda en ratón de las toxinas que causan la intoxicación diarreica por marisco (Diarrheic Shellfish Poisoning, DSP), el procedimiento “Up and down” (UDP) de cuatro niveles que proporciona una estimación puntual del valor de la dosis letal 50 (LD_{50}). Según el procedimiento UDP, el número de animales tratados se va incrementando según se aumenta de nivel. Para ello, una única dosis mediante sonda oral se administra a cada ratón. Esta forma de aplicación garantiza la precisa dosificación de los animales. Las dosis administradas se establecen en función de la mortalidad obtenida en los niveles anteriores, siendo las dosis iniciales 1000 µg/kg b.w. (body weight) para DTX1 y OA, y 2000 µg/kg b.w. para DTX2. Las LD_{50} orales obtenidas para OA, DTX1 y DTX2 son 760, 487 y 2262 µg/ kg

b.w., respectivamente. Las LD_{50} muestran el mismo orden de potencia que las IC_{50} relacionadas con el efecto de las toxinas sobre la actividad de la PP2A. Los resultados indican que la DTX1 es el análogo más tóxico, mientras que la DTX2 es el menos tóxico. A partir de las LD_{50} orales se obtuvieron los valores de TEF de OA = 1.0, DTX1 = 1.5 y DTX2 = 0.3. Este es el primer estudio comparativo de la toxicidad oral del OA y sus análogos. Los valores de TEF difieren de los establecidos por la autoridad europea de seguridad alimentaria (EFSA) que se basan en la toxicidad intraperitoneal y que deberían ser reevaluados.

Este es el primer estudio comparativo de la toxicidad oral aguda del OA y sus análogos. La importancia de la reevaluación de los TEF radica no solo en el peligro de envenenamiento, sino también en las consecuencias para la industria pesquera. Los programas de monitorización previenen la entrada al mercado de marisco contaminado por toxinas para proteger a los consumidores en base al máximo nivel permitido por las autoridades sanitarias. Sin embargo, si éstos límites establecidos no se ajustan a la toxicidad real la repercusión económica podría ser bastante importante. En base a nuestros resultados, la mayor toxicidad de DTX1 podría causar un mayor impacto en la industria de la acuicultura en Japón, Canadá y Noruega, donde este análogo es más frecuente. Por el contrario, la disminución del valor de TEF para DTX2 con respecto al actual, supondría un gran beneficio para los productores de marisco en Galicia, así como para Portugal e Irlanda, ya que este análogo se encuentra comúnmente en estas aguas.

La mayoría de los ratones tratados sufrieron diarrea y otros síntomas no específicos de la intoxicación por toxinas DSP, como apatía, piloerección e inmovilidad. Además, el aislamiento de cada ratón en jaulas metabólicas individuales permite monitorizar el consumo de agua y comida. Tanto el OA como sus análogos disminuyen la ingesta de comida y agua, lo que se traduce en una reducción del peso corporal.

El OA, la DTX1 y la DTX2 atraviesan la barrera gastrointestinal, siendo rápidamente detectadas en sangre, orina y heces a lo largo de las 24 horas del experimento. La cuantificación de las toxinas en estas muestras sugiere una dinámica de absorción y excreción diferente entre ellas. Los síntomas y las alteraciones macroscópicas son comunes para las tres toxinas. El examen macroscópico revela estómagos e intestinos generalmente hinchados y con contenido líquido y gaseoso, especialmente en el caso de los estómagos. La distensión observada es progresivamente más severa según se incrementa la dosis de toxina administrada. Estas alteraciones sugieren la inhibición del vaciamiento gástrico, de la motilidad y de la secreción intestinal. Todo esto podría estar ocurriendo mediante cambios en el NPY inducidos por estas toxinas, como sugieren los ensayos con OA *in vitro*.

Se observaron alteraciones microscópicas en múltiples órganos, sin embargo, tras el tratamiento oral con DTX2 éstas se restringen al tracto intestinal, en concreto al hígado e intestinos, siendo además menos severas. A pesar de que los estómagos de todos los ratones tratados con cada una de las toxinas presentan alteraciones macroscópicas, solamente se detecta afectación de las mitocondrias en el tratamiento con DTX1. En el hígado, el tratamiento con cada una de las tres toxinas origina la disminución de los gránulos de glucógeno característicos de este tejido, hinchazón y disrupción de la membrana externa de las mitocondrias e incluso cristolisis en el caso de la DTX2. Además, aparecen autofagosomas desarrollados o en distintas fases de maduración rodeando las mitocondrias de hepatocitos en los tratamientos con DTX2 y OA, respectivamente. La línea epitelial del intestino también se ve afectada, tanto en intestino grueso como delgado con dilatación del retículo endoplásmico rugoso (RER) y mitocondrias. En cuanto al intestino grueso también se aprecian gránulos electrondensos en el interior de las mitocondrias y ensanchamiento del espacio perinuclear tras el tratamiento con OA y DTX2. Las uniones estrechas mantienen su estructura tras los tratamientos con cada una de las toxinas. Sin embargo, las toxinas más potentes, OA y DTX1 afectan al borde en cepillo, observándose

adelgazamientos de la red terminal y degeneración de las microvellosidades, resultando éstas escasas y acortadas.

El bazo y el riñón de los ratones tratados con OA y DTX1 tienen mitocondrias hinchadas mientras que en el cerebro éstas se mantienen aparentemente intactas y son las láminas de mielina de los axones las que aparecen dañadas con extrañas conformaciones y separación de las láminas. En cuanto al corazón, solo se detecta la disrupción de la membrana externa de las mitocondrias tras el tratamiento con OA.

Recientemente han emergido nuevas toxinas en las costas gallegas, como es el caso de la toxina hidrosoluble TTX. La legislación europea vigente impide la comercialización de especies de peces asociados a la presencia de TTX. Sin embargo, estudios recientes han demostrado que la contaminación con distintos análogos de la TTX es posible en varios tipos de marisco. Debido a la escasez de estudios de toxicidad realizados con estas toxinas así como de la evaluación de los efectos tras la administración de la toxina por vía oral, estudiamos la toxicidad oral aguda de la TTX en ratones. Con esta finalidad se utilizó un procedimiento UDP de 4 niveles en el que se evaluó el efecto de la toxina tras las 2 horas de tratamiento. Los valores obtenidos para la LD_{50} y el Nivel sin Efecto Adverso Observado (no observed adverse effect level, NOAEL) son 232 y 75 µg/kg b.w., respectivamente. Estos datos han servido de base para la opinión científica de EFSA en la que se ha propuesto un límite de 44 µg TTX/kg marisco. Tras la administración de la toxina los síntomas que aparecen rápidamente son los espasmos o la parálisis de las extremidades. Los estómagos de la mayoría de los ratones tratados estaban hinchados con acumulación de líquido y gas tras el tratamiento, pero no los intestinos. La absorción de la TTX se confirmó mediante su detección en sangre intracardíaca por LC-MS/MS.

El análisis microscópico por TEM revela que la TTX causa efectos a nivel gastrointestinal. A pesar de que el estómago fue uno de los órganos que se observaron dañados macroscópicamente no se encontraron alteraciones ultraestructurales. Sin embargo, éstas sí se hallaron tanto en hígado como en intestinos y bazo. Mientras que para bazo e intestinos se detectaron hinchazón del RER y mitocondrias, respectivamente, en el hígado se observó un aumento de la presencia de gotas lipídicas. Nuestros resultados indican que aunque la TTX es una neurotoxina, en la evaluación del riesgo de intoxicación en el hombre hay que considerar los efectos gastrointestinales que podrían ocurrir tras la exposición a esta toxina.

Los resultados expuestos ayudarán a completar y actualizar la información disponible para las biotoxinas marinas, además ponen en valor la importancia de realizar una evaluación que cubra todos los aspectos de la intoxicación.

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1 INTRODUCTION

Nature is an unlimited source of bioactive compounds that have always been present in human life. By evolutionary pressure, these molecules have been selected to interact with a wide variety of targets and cause some effect on living organisms across the species (Cimino *et al.*, 2007; Faulkner, 2000; Harvey, 2007). Furthermore, they can modulate several actions of therapeutic value, such as anti-inflammatory, analgesic, antibiotic or anti-cancer (Nagle *et al.*, 2004; Newman *et al.*, 2007; Villa *et al.*, 2010).

In the last three decades discovery of natural products has been reduced in part by the dramatic breakthrough of the chemical synthesis and the design of systems that allow rapid screening of compounds. However it has turned out not to be so productive, because despite the large number of molecules that can be synthesized and the rapid evaluation of their potential, it has been found that few of the compounds obtained are of interest. Natural products research is actually re-emerging as a great source for new drugs, principally due to the development of new technologies for identificate, isolate, purificate and produce them. Some of these compounds have been collected consciously in broad databases (Banerjee *et al.*, 2015).

The importance of natural products lies in that its structures have desirable properties that high differenciate them from synthetic compounds (Feher *et al.*, 2003) such as chemical complexity and diversity or biochemical specificity to bind with macromolecular targets and modulate several cellular processes. Furthermore, they can be used as a model for the desing of new and more active compounds, with what natural products play an essential role in drug discovery (Newman, et al., 2007).

The oceans cover approximately 71% of the total surface area of the Earth, so it is not surprising that they contain a biodiversity far to that of the terrestrial environment. The great competitiveness present in the marine environment has resulted in innumerable bioactive compounds (Blunt *et al.*, 2013; Molinski *et al.*, 2009) created as defense, attack or signalization mechanisms (Paul *et al.*, 2006) among others, specially in those sessile or low mobility organisms. These compounds are released into the water to exert its effect, so they have to be hightly potent, an interesting feature for their use as a therapeutic agent. Marine natural products include a great spectrum of compounds. Stand out as producers organisms sponges, tunicates, algae, cnidarians or microorganisms like fungi, bacteria or phytoplankton receive great attention (Blunt, et al., 2013).

Phytoplankton are photosynthetic microscopic algae, both eukaryotes and prokaryotes. They are the base that supports the food web in aquatic ecosystems as they are the main food of bivalve molluscs, zooplankton and herbivorous fish. Besides, they also provide oxygen to marine life, so any changes in populations will affect a wide variety of life forms. Phytoplankton is composed by several taxonomic groups being cyanotoxins, diatoms and dinoflagellates the most diverse and ecologically significant (Not *et al.*, 2012). They differ in both morphological, physiological and ecological characteristics at the level of phyla, classes and even of species of the same genus. Most of them are single-cell microalgae with different cell shape and size, rate of division and surface features. Some microalgae have a cyst phase, an immobile form that is accumulated on sediment of the seabed that can ascend by currents and leave that phase of dormancy, but cell division tipically occur by binary fission leading to exponential growth. Algal blooms are massive proliferations of phytoplankton linked mainly to changes in environmental factors such as temperature, light, salinity, nutrients, etc. Under favorable

conditions there is an increase in the rate of division of the population of microalgae, thus multiplying exponentially and giving rise to this ecological alteration. Algal blooms are also influenced by certain climatic conditions as water circulation or wind dynamic that can stimulate the upwelling of nutrients from the bottom or provoke cell accumulation or dispersion (Ioc Coi Coi *et al.*). This phenomenon can occur sporadically or present a pattern of repetition at a certain time of the year and area. In some cases, they can be perceived by the naked eye as discolorations of water if the major phytoplankton species is rich in pigments (Figure 1).

Figure 1. Bloom of *Noctiluca scintillans* accumulated in Vigo (Faro de Vigo, https://www.farodevigo.es/gran-vigo/2018/08/31/nueva-marea-roja-playa-bouzas/1953227.html)

This natural phenomenon has appeared in the last decades with greater frequency, intensity and severity due to climate change (Botana, 2016) and the anthropogenic activities. The increase in water temperature that has been globally recorded has favored the geografical expansion of de microalgae, generally from equatorial areas, to reach latitudes that they did not previously inhabit. The increase of nitrogen depositions by urban and industrial effluent sources and agriculture also stimulate the growth of phytoplankton. In addition, the increase of coastal exploitation areas for aquaculture leads to a greater generation of waste that can be used as nutrients by microorganisms (Anderson *et al.*, 2002). These episodes are especially dangerous in areas with a low rate of water renewal, which favors the accumulation of algal biomass. Dispersion is also favored by maritime traffic, being able to colonize new areas or more prolific for its development due to its transport in the ballast water of ships (Hallegraeff *et al.*, 1992). Another example of the antropogenic activites is the lessepsian migration due to the opening of the Suez channel that has supose the expansion of new species (Bentur *et al.*, 2008).

Dinoflagellates, diatoms and cyanobacteria are the taxonomic groups of phytoplankton that trigger massive blooms. The type of bloom triggered differs according to the microalgae producer being able to be harmful (HAB) by the natural production of potent biotoxins or because of the accumulation of algal biomass that cause ecosystem disruptions (Moestrup, 2009 onwards). Planktonic microalgae are distributed mostly in the water column with greatest contribution of light by its condition of photosynthetic microorganism. Therefore, HABs are more abundant in coastal areas and in summer season, but they also occur in the open ocean and in brackish or freshwater systems. Although algal blooms can occur quickly, they can persist several weeks.

Several strategies have been studied for HABs control. They are based in removal of algae population by biological and microbial algaecides or chemical and mechanical processes.

However, only the last two strategies have been applied by the addition of clays (Sengco *et al.*, 2005) or copper (Rounsefell *et al.*, 1958). These processes are favored by the negative surface charges of microalgal cells (Sengco, 2001) what facilitates the surrounding accumulation of organic molecules and therefore the joint agglomeration.

Dinoflagellates represent the vast majority of marine biotoxins producers (Camacho *et al.*, 2007). This group of organisms posses flagella so its displacement is not only restricted to water movements. Marine biotoxins or phycotoxins are natural compounds that can cause harmful effects. They can persist in water or be transmited through the food web to reach upper levels by different organisms that act as vector, triggering different intoxications and even death in humans and animals. Every year close to 2000 cases of human intoxications by marine biotoxins are recorded, with approximately 15% mortality (Hallegraeff, 2014), a great risk to human health.

Several pathways have been described for the transfer of phycotoxins in aquatic systems. They may be release into water or contained within the producing organisms. The transfer occur mostly through the food web and can involve multiple throphic levels (Doucette *et al.*, 2006). Toxins principally accumulate in shellfish, due to their natural filter feeding activity, also some of them have been found in fish. In the case of shellfish, these marine organisms tolerate high levels of algal toxins without apparently suffering any damage. However, mortality of fish and other fauna due to accumulation of toxins has been detected (Pierce *et al.*, 2008). Toxins are distributed in different tissues of the vector organism. While some accumulate mainly in the digestive gland, other toxins accumulate in the reproductive organs, gills or even in the whole body. Producer organisms can be also ingested by herbivorous fish that in turn will be eaten by carnivorous fish, sea birds or big mammals (Doucette, et al., 2006), although there is little information about these last two groups. In regard to humans, poisoning occurs mainly by the consumption of the vector organism, but through other animals included in its trophic web is also possible (Figure 2). Furthermore, certain toxins are also transmitted by inhalation, since several producer species can release aerosolized toxins after cell lysis. In nature, these toxins appear in combination with different analogs from the same family or even from different groups.

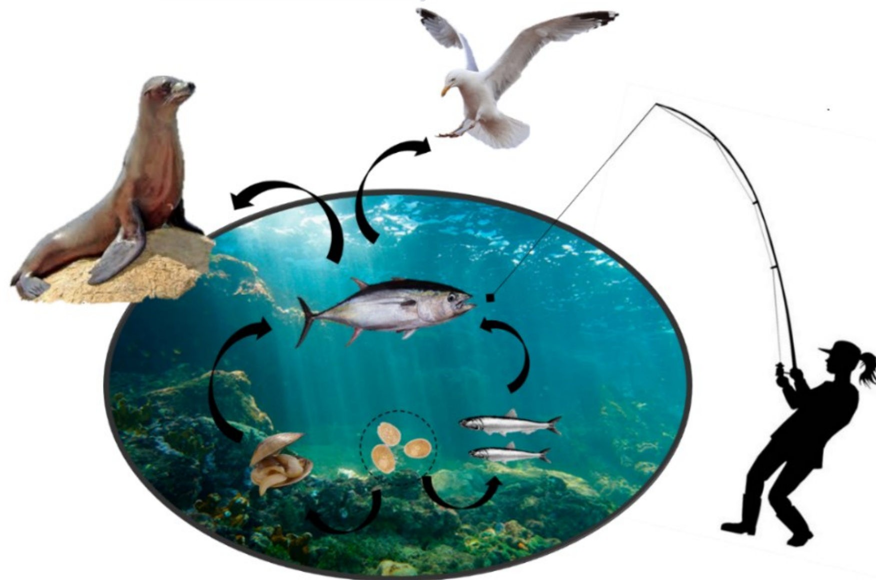


Figure 2. Example of marine food web related with phytoplankton biotoxins.

Marine biotoxins can be classified in different groups according to the syndrome they cause in humans and its chemical structure. However, this classification presented problems with the appearance of new toxins that could not be included in any of the existing groups. In this way, three groups are distinguished according to their solubility: hydrophilic, lipophilic and amphiphilic toxins (Table 1).

Table 1. Classification of marine biotoxins according to relate syndrome, chemical structure or solubility.

Syndrome	**Toxins**	Solubility
PSP	Saxitoxins (STX)	Hydrophilic
ASP	Domoic acid (DA)	
	Tetrodotoxins (TTX)	
DSP	Okadaic acid (OA) Dinophysistoxins (DTXs)	Lipophilic
	Azaspiracids (AZP)	
NSP	Brevetoxins (BTX)	
CFP	Ciguatoxin (CTX)	
	Yessotoxin (YTX)	
	Pectenotoxin (PTX)	
	Cyclic imines (CI)	
	Maitotoxins (MTX)	Amphiphilic
	Palytoxins (PLTX)	

Intoxications produced by these toxins present specific sintomatology, from gastrointestinal (Diarrheic shellfish poisoning, DSP) to neurological afectation. Treatments have been established to fight acute symptoms, although they are largely supportive for some of them such as Paralytic shellfish poisoning (PSP), Amnesic shellfish poisoning (ASP), Neurotoxic shellfish poisoning (NSP) and Ciguatera fish poisoning (CFP). Onset time and duration of the symptoms are also different. Syndromes described in Table 1 are produced by toxins from dinoflagellates, except ASP that is trigger by diatoms and the toxins Palytoxin (PLTX) and Tetrodotoxin (TTX), that have been described to be produced by zoanthid soft corals and bacteria such as *Pseudomonas* and *Vibrio* spp. (Chau *et al.*, 2011), respectively.

As natural compounds, marine biotoxins posses very varied molecular weights and chemical classes. Phycotoxins present a potential use as possible drugs with therapeutic utility with a wide variety of mechanisms of action and great potency. Also, they bind to a wide variety of specific cellular targets on which they can exercise their action, from ion channels essential in the synaptic transmission to proteins of great importance in cellular structure and metabolism. In some cases, such as that of azaspiracids, the toxicity mechanism remains unknown.

Some groups of toxins are globally distributed, while others are limited to specific areas due to more stringent requirements of the producer organisms. Toxin dynamics production varies according to the state of growth in which the cell stands, depending on nutrient conditions and even due to the genetic heterogeneity between strains of the same species (Anderson D. M., 2017). Furthermore, the same producer species can present large differences in toxin content and even in toxin profile depending on the geographical region.

Marine biotoxins show different potency of toxicity between groups of marine biotoxins. The toxicity that a toxin can cause is influenced firstly in the producing microorganism, in relation to the ratio of toxin production and excretion and the rate of cellular division of the microorganism. Although toxin production is high, if excretion and division processes show great activity, toxin concentration will decrease. Phycotoxins can reach the organism vector, either from the water or by assimilation of the producing organism. Modification in toxin levels can occur inside the vector, by its degree of clearance (i.e. 2 L/h in mussels), which is the

difference between the absorption and excretion of toxin, and the possible biotransformation by enzymes of both organisms. Changes in vector biomass produced naturally by fasting or spawning can also influence in the toxin content.

Some of these toxins are resistant to heat, so cooking contaminated food does not reduce the risk, thus it is recommended to only consume authorized seafood according to regulations. The limit of the toxin content in shellfish have been established for the most common toxins by the Codex Committee on Fish and Fishery Products, by the Codex Stan 292: Standard for Live and raw Bivalve Mollusks (2008) in order to protect consumers as a maximun level of the reference toxin equivalent expressed per kg of shellfish meat for every group of toxins (Table 2). The reference toxins are chosen by its greate toxicity or because they were the first of their group to be discovered. The limit value is applicable to live raw and entire bivalve and although toxins show a tendency to cumulate in a specific organ, whole tissue should be analysed. Moreover, the biotoxins can increase its concentration after cooking due to dehydration process or be redistributed within the organs of shellfish, may even be released into the cooking fluids (McCarron *et al.*, 2008).

Table 2. Representative analogue of each group of toxin and its maximum level allowed of the per kg of shellfish meat in the European Union.

Biotoxin group	Maximum level per kg of shellfish meat
Saxitoxin group	0.8 mg of STX equivalent
Okadaic acid group	0.16 mg of OA equivalent
Azaspiracid group	0.16 mg of AZA1 equivalent
Domoic acid group	20 mg of DA equivalent
Brevetoxin group	200 mouse units or BTX1/2 equivalent
Yessotoxin group	3.75 mg of YTX equivalent

Regulatory limits can change in several countries, some toxins may not be reflected in the legislation due to their absence in such areas. This is the case of the so-called 'emerging toxins' which mostly occurred in tropical regions but it is becoming more frequent to detect them in temperate waters. Currently BTX, PLTX, CTX and TTX are non-regulated in the European Union. However, an especial case are CTX and TTX for which it has been said that must be out of the market, but only of the family *Tetraodontidae, Molidae, Diodontidae,* and *Canthigasteridae* overlooking other vectors (2004a; 2004b).

1.1 MARINE TOXINS IN GALICIAN "RÍAS"

The consumption of seafood is an important nutritional contribution in the diet for its rich content of proteins, essential minerals and vitamins and A and D. They also represent a great source of income in coastal areas around the world, being a great example the Galician coast.

Galician "Rías" are coastal identations perpendicular to the coastline dominated by marine processes, resultant from the submersion by the sea of the terminal zone of a fluvial network. They are classified in "Rías Altas" and "Rías Baixas", being the last ones of greater importance in bloom events because they receive more amount of nutrients in summer by direct penetration of the upwelled seawater (Barciela *et al.*, 1999). The abundance of phytoplankton makes this area show great seafood wealth. In order to protect the consumer, legislation and monitoring programs have been developed. They are required to avoid the arrival of contaminated products to consumers. These programs routinely analyze and identify the concentration of toxin producers near the coast as well as toxic content in shellfish to ensure that established limits are

not exceeded. Competent authorities are obliged to close production areas in the case of exceeding the standards for biotoxins and to monitor compliance with these closures until decrease of toxin levels was observed in molluscs, which depurated them naturally by filtration. After following two consecutive results below the regulatory limit that is derived from samples taken more than 48 h apart, the production areas will be re-opening (O'Mahony, 2018).

The appearance of marine biotoxins was linked to downwelling or upwelling relaxation. The upwelling of nutrient-rich water subsurface occur between April and August by a strong atmospheric pressure gradient (Fraga *et al.*, 1993) and quatorward winds along the coast. The responsible species of the bloom probably develops at the open sea and then is entered to the shore and molluscs become affected. After the upwelling season, the “Rías” are prone to red tide outbreaks and the closure of the fishing areas may happen. During the rest of the year, south winds prevail which favour downwelling (Figueiras *et al.*, 1994).

Marine biotoxins from the DSP, PSP and ASP groups are frequently reported in Galician coast (Figure 3) due to the proliferations of dinoflagellates of *Dinophysis* genera especially *D. acuminata* (OA and DTXs) and *Protoperidinium crassipes* (AZAs), dinoflagellates of *Alexandium minutum* and *Gymnodinium catenatum* (PSP), and diatoms of *Pseudo-nitzschia* genera specific *P. australis* (ASP), respectively. Another species that habitually inhabits these waters is the dinoflagellate producer of YTXs *Protoceratium reticulatum*, however these toxins do not reach great concentrations.

Distribution of these groups of toxins can be differentiated according to the type of mollusc cultivated, the geographical zones or the month of the year, although they can also overlap. Thus, in the last year closures in the Galician coast have been recorded both in floating marine farms and infaunal molluscs (Instituto Tecnolóxico para o control do medio mariño de Galicia). The first ones are involved in the culture of mussels and oysters while the second ones include molluscs that live buried in the substrate like clams, cockles or razor shell. Floating farms (Figure 3A) are found in all the estuaries of Pontevedra and only in two of the province of A Coruña. They have closed shellfish areas throughout the year due to the presence of the toxins mentioned. Lipophilic toxins were responsible of closures in cultured zones in all the Galician “Rías” throughout the year, occurring for more days between March and July and being even present in every month in some areas. However, closures derived from the presence of PSP and ASP were much less numerous, while closures by PSP occurred between March and December, being the majority between the months of July and August in the two estuaries located further south (“Ría de Pontevedra” and “Ría de Vigo”). However, the presence of ASP toxins only meant that closing in the months of September and October, first in the “Ría de Arousa” (Figure 3, number 4) and then in the “Ría de Pontevedra” (Figure 3, number 3).

Regarding infaunal mollusc culture (Figure 3B), closures due to lipophilic toxins represented the most extensive closures geographically, since they extend for all the Galician coast, between April and October happening closures during all the months of the year in some zones as it was also seen for the floating nurseries. PSP toxins were the toxins responsible of closures in “Rías” 1 and 2 in July, prolonging some of them until August. ASP toxins were more common in the coast of Lugo and A Coruña, few cases in Pontevedra, between May and December. In this case, it was observed the progressive extension from Lugo in May, June and October but distributing to the south from August.

Lipophilic toxins are the most widespread in the Galician territory, in particular those of the diarrheic symptomatology, as okadaic acid and analogues and azaspiracids, being signaled as the highest risk areas in Spain. Even when these toxins share their solubility, they have different chemical structures and mechanisms of toxic action. No deaths have been reported after consumption of food contaminated with these toxins, although they suppose a significant

risk to human health. Furthermore, it can cause important economic losses to shellfish industry in our coast due to the extensive culture areas.

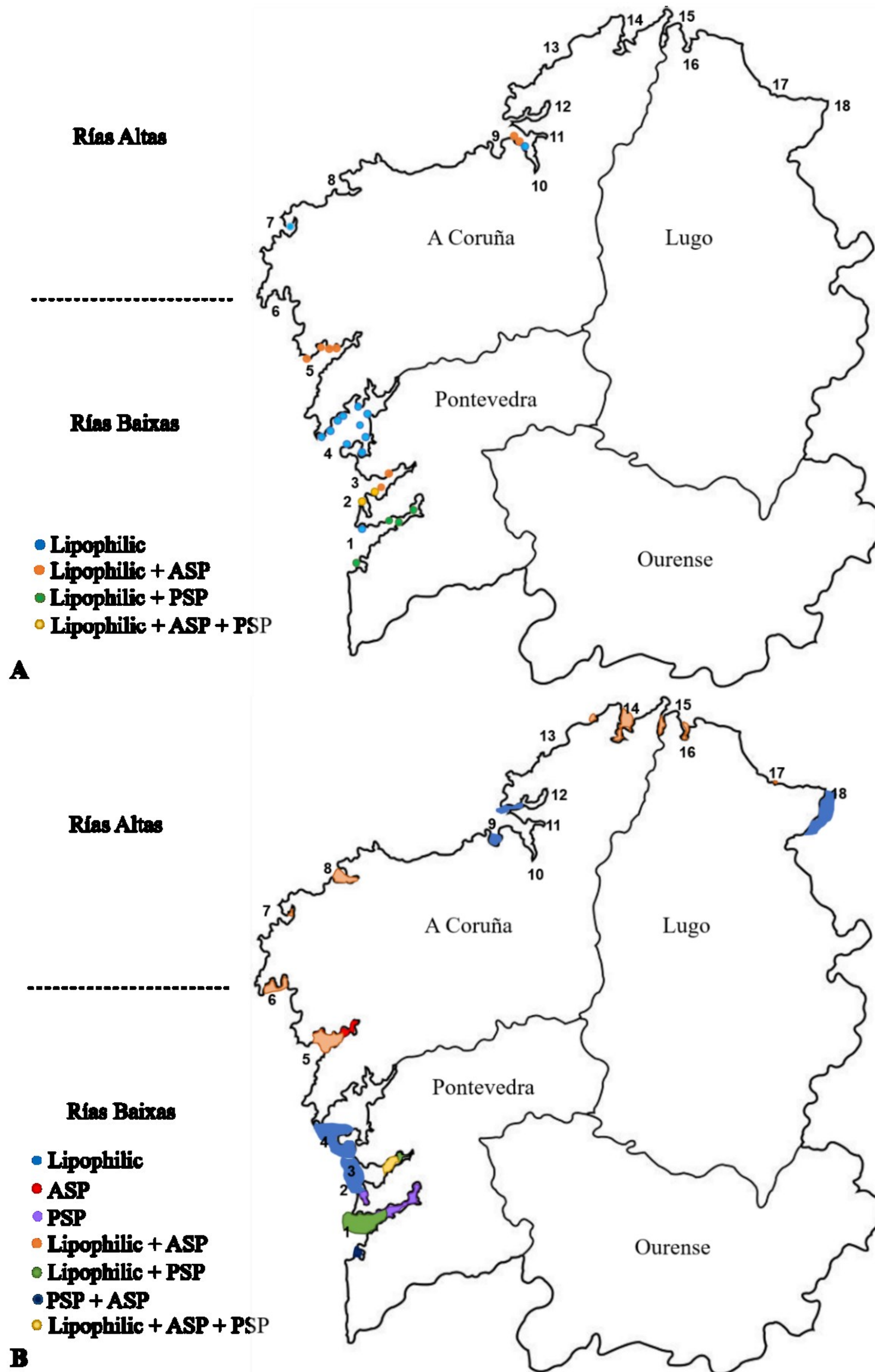


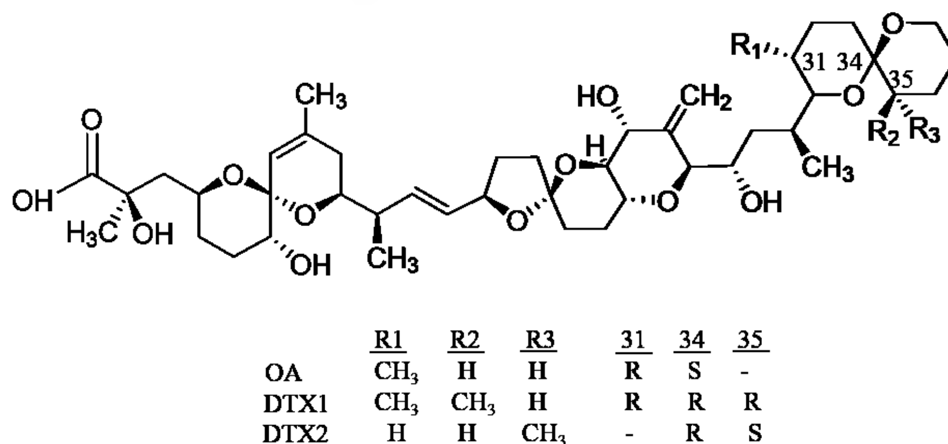
Figure 3. Closures of cultivation areas of rafts (A) and infaunal (B) mollusc in Galicia ocurred sometime during 2018. The presence of toxins of different group is indicated. Data was obtained from Instituto Tecnolóxico para o control do medio mariño de Galicia (INTECMAR) (Instituto Tecnolóxico para o control do medio mariño de Galicia)

As it was mentioned above, the processing of shellfish does not reduce the risk. In the case of these lipophilic toxins can even lead to an approximate 2-fold increase due to water loss. Furthermore, there are several studies on the possible combined effect of these lipophilic toxins. However, there does not appear to be an additive toxic effect with OA and AZA1 mixtures both *in vitro* and *in vivo* models, on the contrary, the absorption of each of them was reduced as well as their effects (Aune *et al.*, 2012). However, *in vitro* studies leave the possibility open that synergy can occur (Ferron *et al.*, 2016).

The emerging toxin TTX also was recently detected in our coast (Leão *et al.*, 2018) after having registered its extension from Asia and detected its presence in several vectors in recent years as indicated by the bibliography (Rodriguez *et al.*, 2008; Silva *et al.*, 2012; Turner *et al.*, 2017a; Turner *et al.*, 2015; Vlamis *et al.*, 2015).

1.1.1 Okadaic acid group

Okadaic acid (OA) and its congeners dinophysistoxins (DTXs) are lipophilic and heat-stable polyether compounds synthesized by dinoflagellates of the *Prorocentrum* and *Dinophysis* genera, although at first the production of OA was attributed to the marine sponge *Halichondria okadai*. Dinophysistoxin-1 (DTX1) (Yasumoto *et al.*, 1980) and Dinophysistoxin-2 (DTX2) (Hu *et al.*, 1992) are the structural analogues of OA, the methylated and the isomeric derivates respectively (Figure 4). Furthermore, a wide range of molecules are included in this group, fatty ester analogues referred as Dinophysistoxin-3 (DTX3) (Yasumoto *et al.*, 1985), although has been proven to be a biotransformation product occurred in shellfish species by acylation of the main toxins (Suzuki *et al.*, 2001) and no toxins produced *de novo* by dinoflagellates. DTX4, DTX5a, DTX5b and DTX5c, the sulfated diesters precursors that hydrolyze to OA and DTXs were also described (Hu *et al.*, 2017). These transformations will vary the toxic potency of the dinoflagellate itself. DSP toxins are accumulated inside the digestive gland of the shellfish or in several organs of crabs (Vale *et al.*, 2002). The digestive process inside the shellfish along with the dinoflagellates enzymes makes these transformations possible. Hydrolysis is usually fast and increase the toxicity of the toxin content. However, acylation supose a process of detoxification, but these formed acyl-derivatives seem to accumulate for a long time in the mussels when the intoxication is prolonged (Moroño *et al.*, 2003).



	R1	R2	R3	31	34	35
OA	CH_3	H	H	R	S	-
DTX1	CH_3	CH_3	H	R	R	R
DTX2	H	H	CH_3	-	R	S

Figure 4. Structure of the main compounds of DSP toxins: OA, DTX1 and DTX2.

This group of toxins cause gastrointestinal disorders such as nausea, vomiting, diarrhea and abdominal pain in a period of about 30 minutes to a few hours after the ingestion of the contaminated vector. The recovery occurs in a few days (Yasumoto *et al.*, 1978). No Tolerable daily intake (TDI) value has been impossible to determine due to the lack of data on the chronic toxicity of these toxins. However, other indices have been established in humans such as the Acute Refence Dose (ARfD) and Lowest Observed Adverse Effect Level (LOAEL), whose values are 0.3 and 0.8 µg OA equivalents/kg body weight (b.w.) for adults respectively (EFSA, 2008).

These toxins were initially described in Japan and Europe, but nowadays are globally distributed. OA and to a lesser extent DTX2, have been routinely found in mussels (*Mytilus galloprovincialis*), clams (*Callista chione*, *Venus gallina*, *Ruditapes decussatus*) and oysters (*Crassotrea gigas*) in the Atlantic coast (Carmody *et al.*, 1996), while DTX1 is commonly found in Japan.

Around ten *Dinophysis* species of more than the 200 described have been identified as the major organisms producers. Furthermore, certain species of the genus *Prorocentrum* also synthesize these toxins, especially *P. lima*, *P. concavum*, *P. belizeanum* (Lopez-Rosales *et al.*, 2014) and *P. maculosum* (Rodriguez *et al.*, 2018). Some strains of dinoflagellates can produce simultaneously OA and DTXs and toxins of the group of PTXs. Both *Dinophysi*s and *Prorocentrum* genera belongs to the *Dinophyceae* class, being planktonic and benthic dinoflagellates, respectively. The benthic species can attach to a substratum via a mucoid holdfast or thread.

The highest indicence of DSP toxins occurs in the Atlantic coast, where the outbreaks starts in spring with species of the *D. acuminata* complex (*D. acuminata*, *D. ovum*, *D. sacculus*), followed by those of *D. acuta* in late summer. Long lasting blooms of *D. acuta*, following previous blooms of *D. acuminata* complex species lead to high accumulation of toxins in late autumn, suposing the worst scenario (Reguera *et al.*, 2014). *D. acuminata* is an early blooming species, the most cosmopolitan specie with a very long growing season.

Cell toxin content in *Dinophysis* spp seems not to be influence by temperature (Kamiyama *et al.*, 2010), light irradiance (Lasse Tor Nielsen, 2012) or food intake (Tong *et al.*, 2011), suggesting that toxicity is subject foremost to strong genetic control. *Dinophysis* species seems to release to the seawater the majority of the biotoxins that produce (MacKenzie *et al.*, 2004).

1.1.1.1 Mechanism of action and toxic potency

DSP toxins specifically and reversibly inhibit the serine/threonine protein phosphatases (PP), mainly protein phosphatase 2A (PP2A), as secondary targets protein phosphatase 1 (PP1) and to a lesser extent on PP2B (Takai *et al.*, 1992) and PP5. The inhibition of the two main phosphatases could affect 90% of the total activity of the PPs in mammalian cells (Cohen, 1997). These phosphatases are involucred in several cellular proceses, since they carry out the dephosphorylation of numerous proteins, being able to affect metabolism, cell cycle progression, gene expression, ion balance, cytoskeletal rearrangements and cell movement (C. *et al.*, 2005; Dawson *et al.*, 1999; Honkanen *et al.*, 1994).

DSP toxins exert inhibition on PP with different potency (Takai, et al., 1992), so that Inhibitory Equivalency Factors (IEFs) were established for the analogues in function of the inhibitory concentration 50 (IC_{50}) of OA on the different PPs. Thus, it was seen that different inhibitory potencies were also found in relation to PP2A, with DTX1 exerting greater toxic power than OA, and DTX2 around half the OA potency (C., et al., 2005). For a long time it was thought that this effect on phosphatases was responsible for triggering diarrhea, the

characteristic symptom of DSP. However, the absence of diarrheic effect of others potent phosphatase inhibitors and the discovery of new effects produced by these toxins, as disruption of the barrier function in intestinal cells and the increase of paracellular permeability (Tripuraneni *et al.*, 1997), could not be explained by this mechanism of action (Valdiglesias *et al.*, 2013). Thus, a reevaluation of the mechanism of toxicity could be necessary (Munday, 2013) and other possible targets can not be discarded.

Since the gut is the main suggested target of the OA group, most studies have focused on these organs. Cytotoxicity assays showed that DTX1 displayed higher toxic effect than OA while DTX2 showed less toxicity than both in intestinal (Ferron *et al.*, 2014) and non-intestinal cells (Solino *et al.*, 2015; Twiner *et al.*, 2016). The disruption of the barrier function in intestinal cells and the increase of paracellular permeability was also described by *in vitro* assay with OA (Tripuraneni, et al., 1997). However, *in vivo* assays with mice showed no clear relationship between reported damage to the intestinal mucosa and diarrhetic symptoms (Aune, et al., 2012; Tubaro *et al.*, 2003).

Furthermore, other effects not related with the digestive tract were disclosed. Several studies reported that OA induced apoptosis (Chen, 2011), inhibit cell growth (Valdiglesias *et al.*, 2011), affect cytoskeleton (Opsahl *et al.*, 2013) and gene expression (Vieira *et al.*, 2013), interleukins production (Pshenichkin *et al.*, 1997), activate caspases (Lago *et al.*, 2005; Rossini *et al.*, 2001) and hyperphosphorilation of tau protein (Arias *et al.*, 1993), assuming that these toxins can be useful tools in Alzheimer disease. OA can also act *in vitro* and *in vivo* as genotoxic and tumoral promoter agent (Cordier *et al.*, 2000; Le Hegarat *et al.*, 2006; Valdiglesias *et al.*, 2010).

In vivo assays demostrate that these toxins are absorbed by the gastrointestinal tract and distributed even through the placenta (Matias *et al.*, 1999) to cause organ afectation, both after intraperitoneal (i.p.) and oral administration of OA and DTX1 (Ito *et al.*, 1994; Ito *et al.*, 2002b; Tubaro, et al., 2003) in acute and chronic toxicology studies. However, there are many discrepancies in the dose inducing effects, perhaps due to the use of non-purified toxins. Results based on i.p. toxicity showed median lethal dose (LD_{50}) values for OA ranging between 185.6 and 225 µg/kg b.w. (Aune *et al.*, 2007; Suzuki *et al.*, 2018; Tachibana *et al.*, 1981; Tubaro, et al., 2003). Body weight (Suzuki, 2014) and mice strain differences in susceptibility to OA were found, whereas this did not happen in terms of sex, even though the males showed more stable results (Suzuki, 2012). For the rest of the analogues the data are scarce and even non-existent, with currently only two values described for DTX1 LD_{50} of 150.4 and 160 µg/kg b.w. (Dominguez *et al.*, 2010; Suzuki, et al., 2018) and whole lethality at the dose of 352 µg/kg b.w. as for OA and DTX3 (Ito, et al., 1994). I.p. LD_{50} was also calculated for DTX2 and DTX4, being 352 (Aune, et al., 2007) and 610 µg/kg b.w. (Hu *et al.*, 1995), respectively.

By the oral route, lethal doses are increased in relation to intraperitoneal, since absorption by intake is lower. Through this via of administration there are large discrepancies in the lethal dose. The LD_{50} for OA described in the bibliography generally range between 400 – 880 µg/kg b.w. (Aune, et al., 2012; Ito, et al., 2002b), despite the fact that in other studies there were no deaths at much higher doses. Thus, no death was found at 610 µg/kg b.w. (Le Hegarat, et al., 2006) while at a higher dose of 1000 µg/kg b.w. only 30% mortality was reached (Vieira, et al., 2013). Oral studies with the rest of analogues are even scarcer than for intraperitoneal toxicity or nonexistent, since the evaluation of toxicity is a fairly recent trend. Contradictory data have been published regarding the lethality of the DTX1, while in some trials an LD_{50} of 300 µg/kg b.w. has been affirmed, in other lethality has not been obtained for values of up to 750 µg/kg b.w. (Terao, 1993).

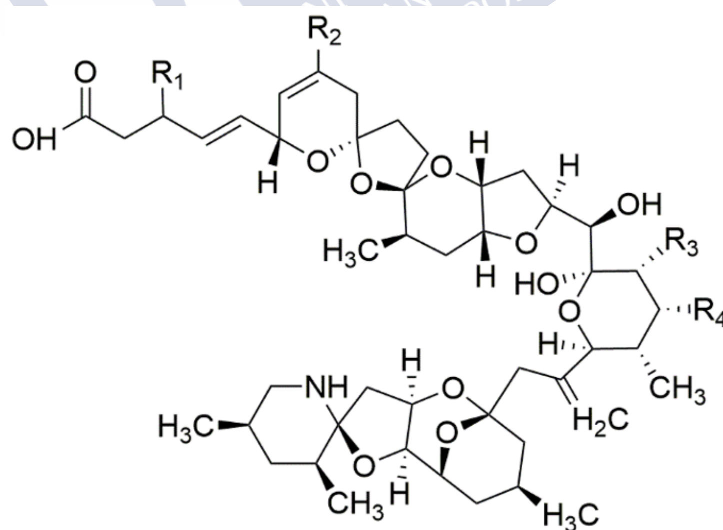
Among the unrelated effects with the diarrheal syndrome triggered by these toxins, it has been seen that they are tumor promoters, and may even induce colorectal cancer in consumers of contaminated shellfish (Manerio *et al.*, 2008), so the study of chronic toxicity is of great importance in this matter.

All those facts justify carrying out further studies on the cellular effects of OA and analogues.

1.1.2 Azaspiracids

Azaspiracids (AZAs) are lipophilic marine biotoxins produced by dinoflagellates of the *Azadinium* (Krock B., 2009; Tillmann *et al.*, 2009) and *Amphidoma* genera (Jauffrais *et al.*, 2012a; Krock *et al.*, 2012). AZAs produce intoxications with a diarrheic symptomatology similar to that of OA, such as diarrhea, nausea, vomiting and stomach cramps, but these group of toxins do not share the same mechanism of toxicity (Aune, et al., 2012). These symptoms start at 3 h after consumption and last approximately 15 h. Human intoxications were observed in the United Kingdom, United States of America, Italy and France.

Azaspiracids conform a family of near 50 polyether toxins, with several stereoisomers and artifacts product of an extraction process (Jauffrais *et al.*, 2012b), all named by numbering in the chronological order of their detection or postulation. Of these compounds, only azaspiracid-1 (AZA1), azaspiracid-2 (AZA2) and azaspiracid-33 (AZA33) to azaspiracid-41 (AZA41) are considered phycotoxins, while azaspiracid-3 (AZA3) to azaspiracid-28 (AZA28) are biotransformation products occurring in shellfish, by hydroxylation and carboxylation reactions. With regard to 37-epi-AZA1 and AZA29 to AZA32, they are considered artefacts (FAO/WHO, 2016). Despite the large number of analogues that compose this family only AZA1, AZA2 and AZA3 (Figure 5) are currently regulated in Europe (2004a).



	R_1	R_2	R_3	R_4
AZA1	H	H	CH_3	H
AZA2	H	CH_3	CH_3	H
AZA3	H	H	H	H

Figure 5. Structures of AZAs regulated in Europe.

AZAs can be accumulated in high concentrations in shellfish, principally in mussels, oysters, scallops, clams and cockles. This presence in shellfish has been recorded in coastal areas of Western Europe, Northern Africa and Southern and Northern America (Alvarez *et al.*, 2010; Furey *et al.*, 2003; Taleb *et al.*, 2006). Furthermore, these toxins have been found in Japanese sponges (Ueoka *et al.*, 2009), Scandinavian crabs (Torgersen *et al.*, 2008) and in plankton (Krock *et al.*, 2008) and seawater (Rundberget *et al.*, 2007). This globally distribution is associated to the wide spread of *Azadinium sp*. (Tillmann, et al., 2009). Several dinoflagellates species were described around the world, but only toxin production was detected by *Az. spinosum*, *Az. poporum*, *Az. dexteroporum* and *Amphidoma languida*.

Differences in the presence of toxin analogues can be due to the different species of the dinoflagellate producer or to different toxic profile within the same species as was seen for *Am. languida* (Tillmann *et al.*, 2017). AZA1 was predominant in mussels (*Mytilus galloprovincialis)* from Galicia (Magdalena *et al.*, 2003), feature proper of *Az. spinosum* and close to that found in Ireland and Norway. However, AZA2 dominance was previously observed in the Atlantic coast of Portugal (Vale *et al.*, 2008), Morocco (Taleb, et al., 2006) and in the south of Spain attributed to *Am. languida* (Twiner *et al.*, 2014) and to *Az. poporum* in other localizations such as China and Argentina (Gu, 2013; Tillmann *et al.*, 2016). Sometimes the synthesis of toxin itself has not been found, but the appearance of fragmentation paterns simillar to those of the known toxins could indicate the production of similar compounds. This is the case of *Am. languida* from Ireland or Irminger Sea (Tillmann *et al.*, 2015), *Az. dexteroporum* from the Gulf of Naples (Percopo *et al.*, 2013; Rossi *et al.*, 2017) and *Az. obesum* from the North Sea (Scotland) (Tillmann, 2010). An exceptional case is AZA11, since it has been seen that occur as a biotransformation product in mussels and by dinoflagellate synthesis (Hess *et al.*, 2014).

AZAs kinetics is characterized by the rapid accumulation and biotranformation in shellfish. The proportion in which these toxins are found in mussel tissue is suggested to be subject to temporal variation, since analogues accumulation differ between tissues and the proportion of metabolites increase proportionally. Toxins were mainly found in digestive gland although its presence was found in other organs such as gills, food, adductor muscle or mantle. In the digestive gland of mussels AZA1 was seen to be the predominat toxin acumulated, while AZA3 does it in the remaining tissues. These differences in toxin content tissue-dependent can be due to migration of the toxins through mussel tissues during the time of acumulation, hindering its clearance (James *et al.*, 2002; O'Driscoll *et al.*, 2014), which has been related to the AZAs interaction with specific proteins (Nzoughet *et al.*, 2008). Furthermore, AZAs clearance can occur prior to any assimilation. In Scandinavian brown crabs AZA1, AZA2 and AZA3 were found mainly in the hepatopancreas and the roe (Torgersen, et al., 2008).

Current EU regulation only includes AZA1-3, the main analogues identified in cooked mussels when the first toxin-specific regulation was formulated. However, this could obstruct the safety controls by underestimated the total amount of AZA present in mussels since monitoring programs do not take into account the possible transformation of the analogues by cooking (Jauffrais, et al., 2012b).

Although the shellfish generally do not show morphological changes that allow to identify the accumulation of toxins inside them, some dinoflagellates can be a possible physiological factor of stress to vectors. Thus, the thickness of digestive gland tubule in mussels containing *A. spinosum* has been recorded. However, recovery was observed after the detoxification period, as was observed for other toxins (Galimany *et al.*, 2008; Pearce *et al.*, 2005).

1.1.2.1 Mechanisms of action and toxic potency

Although the mecanism of action of these toxins remains unknown, several effects were detected by *in vitro* and *in vivo* studies.

In vitro studies have indicated that AZAs produce a wide range of cellular and molecular effects, such as disarrangement and reorganization of cellular F-actin (Twiner *et al.*, 2008), alteration of the cellular pool of the adhesion molecule E-cadherin in epitelial cells (Ronzitti *et al.*, 2007), increase cytosolic calcium levels and cellular concentrations of cAMP (Roman *et al.*, 2002), inhibite cell growth (Vilariño *et al.*, 2006), elevate caspase activity and inhibit neuronal ion flux and bioelectrical activity, these last ones related to neurotoxicity (Cao *et al.*, 2010). Furthermore, AZAs are low/moderate hERG channel blockers (Twiner *et al.*, 2012). These toxins were also shown to prevents endocytosis and causes a pronounced and temporary depletion of ATP (Kellmann *et al.*, 2009), being able to be responsible for numerous cellular alterations. Furthermore, a recent study demostrate a synergic effect between glutaric acid and azaspiracids (Chevallier *et al.*, 2015), since both compounds were found in shellfish both target similar cellular structures (Vilarino *et al.*, 2008) suggesting a combined neurotoxicity (Twiner, et al., 2014). Relative *in vitro* potencies of AZAs have demonstrated to be quite variable, maybe due to different responses in each cell model (FAO/WHO, 2016).

There is a clear difference in symptomatology depending on the route of AZA administration. While intraperitoneal effects are mostly neurological, characterized by slow and progressive paralysis, respiratory difficulties and spasms (Satake *et al.*, 1998), when AZAs are administered orally damage is mainly observed at digestive level.

AZAs are easily absorbed orally and distributed throught several organs (Aasen *et al.*, 2010; Aune, et al., 2012), remaining stable in kidneys with the passing of days while the concentration of toxins dropped in the rest of the organs (Aasen, et al., 2010). Traces of these toxins were detected even in the brain (Pelin *et al.*, 2018).

Scarce information about lethality of AZAs is available and besides that, the term of lethality is not well detailed. The LD_{50} based on i.p. administration were provided by the Marine Institute, the state agency responsible for marine research in Ireland. The results were 74, 117, 164 and 100 µg/kg b.w for AZA1, AZA2, AZA3 and AZA6, whereby AZA1 was found to be the least toxic analogue administered by this route (FAO/WHO, 2016). This difference in toxicity was also previously exposed (Ofuji *et al.*, 1999), although without specifying the degree of lethality and the concrete value, except for AZA1 (Ofuji, et al., 1999; Satake, et al., 1998).

Erosion of epitelial cells and also damage of the lamina propria were observed in mice after oral treatment with AZA1, rarely acompaigned by presence of some watery content although no diarrhea (Ryan, 2008). Using certified AZA1 recovery of these alterations were seen in a short period of time (Botana, 2014), where no lesions in other organs were detected sometimes pale livers were recorded (Aune, et al., 2012). Hepatic lipidosis and necrosis, as well as necrosis of lymphocytes of thymus, spleen and Peyer's patches were also recorded (Aune, et al., 2012; Ito *et al.*, 2002a; Ito *et al.*, 2000). At sublethal doses under prolonged treatments interstitial pneumonia, liver changes, gastrointestinal erosions and lethal effects (Ito, et al., 2002a) were also observed. Furthermore, cardiovascular toxicity (Ferreiro *et al.*, 2016) and heart arrythmogenicity were also detected when administration is intravenous in rats (Ferreiro *et al.*, 2014). Increased prevalence of lung tumor was seen after repeated dosing experiment with AZA1 (Ito, et al., 2002a), although it has been suggested to be related with the high spontaneous incidence of the mice strain used.

Limited data is available on the oral lethality index of AZA1 and analogues and most of the existing studies are characterized by very variable toxic potency with unspecified purity

toxins. AZAs showed a half-life of 24 h in mice (Twiner, et al., 2014). Until recently the studies focused on the AZA1, which lethality was observed ranging from 250 to more than 700 µg/kg in mice (Aasen, et al., 2010; Aune, et al., 2012; Ito, et al., 2002a; Ito, et al., 2000), maybe due to different toxin purities. The LD_{50} was recently calculated in mice, being 443, 626 and 875 µg/kg for AZA1, AZA2 and AZA3, respectively. The absence of pathology in mice organs seen in this study suggest a possible reversible toxic effect although the prevalence of AZA1 and AZA2 was corroborated in almost all the organs and intestinal content after treatment (Pelin, et al., 2018).

1.1.3 Tetrodotoxin

Tetrodotoxin (TTX) is a highly potent neurotoxin. Approximately 30 analogs have been described, but TTXs profiles in shellfish only included TTX, 4-epi-TTX, 4,9-anhydro-TTX, 5,6,11-tri-deoxy-TTX and monodeoxyTTX (Figure 6).

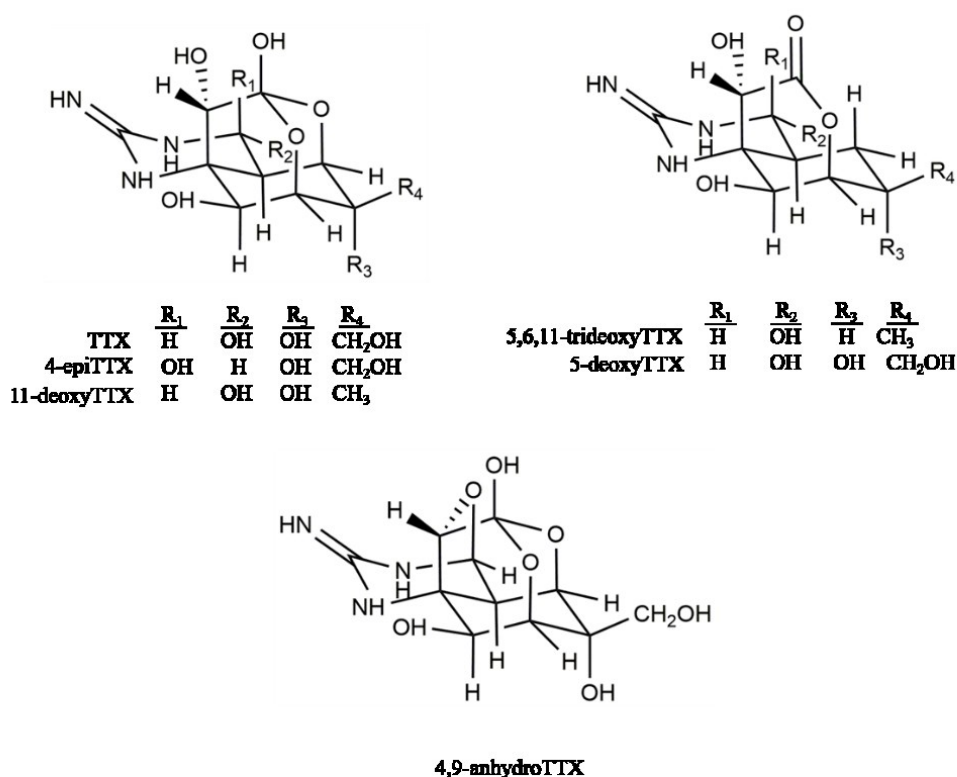


Figure 6. Structure of TTX and analogues found in mollusc.

Intoxication with TTX leads to perioral numbness with paresthesia of the tongue and lips, early motor paralysis, incoordination, headache, vomiting and in severe cases death, caused by respiratory and cardiac failure. Symptoms usually appear within 10–45 min of exposure, although in some cases ocurred between 3 to 6 h after exposure (Noguchi *et al.*, 2001; Yin *et al.*, 2005). The lethal dose of TTX to humans has not yet been established.

As was earlier mentioned, and unlike most marine toxins, the production of TTX toxins is associated to a wide variety of bacteria phyla: *Proteobacteria, Actinobacteria, Bacterioides* and *Firmicutes* (Jal *et al.*, 2015), being some *Vibrio sp* the most common involved species. Furthermore, TTX production was recently suggested to be linked to specific dinoflagellate blooms, such as *Prorocentrum minimum* (Rodriguez *et al.*, 2017; Vlamis, et al., 2015). This

dinoflagellate posses a great physiological flexibility that has allowed it to be widely distributed geographically. This group of toxins used to be confined to Asia so, as was mentioned above, there is no regulation for them in Europe. However, due to its recently emergence a thorough evaluation of the risks involved in their presence is required.

TTX was first isolated from puffer fish ovaries in Japan (Yokoo, 1950), organism in which are mostly found (Suehiro, 1994). TTX and the compounds related are present in marine animals unrelate phylogenetically, including some species of gobies, octopuses (Yotsu-Yamashita *et al.*, 2007), sea stars, crabs, bivalves such as oysters and mussels (Vlamis, et al., 2015) and gastropods (Jal, et al., 2015; McNabb *et al.*, 2010; Rodriguez, et al., 2008; Silva, et al., 2012). Puffer fish lessepsian migrants were first detected on the coast of Turkey in 2003 (Akyol *et al.*, 2005), and since then it seems to have spread progressively, reaching to the Aegean Sea in 2005. However, it was not until 2007 that its arrival in Europe was first detected (Katikou *et al.*, 2009; Rodriguez, et al., 2008). TTX and congeneres were found in gasteropods from Portugal on several occasions, in two of them the same species of the trumpet shell *Charonia lampas lampas* was involved (Nzoughet *et al.*, 2013; Rodriguez, et al., 2008), while in other event were also injured two different species *Gibbula umbilicalis* and *Monodonta lineata* (Silva, et al., 2012). However, only one of these detections resulted in high toxin levels that conduced to single person human poisoning in Spain (Rodriguez, et al., 2008). Affectation of bivalve species was later discovered. TTX was found in clams, mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*) from the English Channel (Turner, et al., 2017a; Turner, et al., 2015), Greece (Vlamis, et al., 2015) and The Netherlands, being maybe the oysters more susceptible (Gerssen *et al.*, 2018). In the last year, TTX was also identified in oysters and cockles of the Galician coast (Leão, et al., 2018) and in mussels from Italy (Dell'Aversano *et al.*, 2019). All these data confirm the spreading in the European Atlantic coastline, so it would not be surprising that at some point TTX become a global problem in the European waters.

Most of the existing studies on the distribution of these toxins in organs and tissues of vectors are related to puffer fish. In this organism, the highest concentrations were found in liver, skin (Tanu *et al.*, 2002), ovaries and muscles (Mahmud *et al.*, 2003). This distribution has been suggested to be analogue specific (Jang *et al.*, 2006). In addition to tissue distribution, it has seen high concentrations in the posterior salivary gland of the blue-ringed octopus and in the ink for some species (Williams *et al.*, 2009). Respect gastropods, the presence in a great variety of tissues was seen, including reproductive tissues, eggs and larvae (Salvitti *et al.*, 2015). However, in bivalves TTX seems to mainly accumulate in the siphon, the digestive system, gills and labial palps (*Paphies australis*, from New Zealand). All these organs are involved in the process of food intake and because the highest concentration was found in the siphons, this seems to suggest an external origin of the toxin (Biessy *et al.*, 2018).

1.1.3.1 Mechanism of action and toxic potency

TTX target voltage-gated sodium channels (Na_v), which are responsible for the voltage-dependent increase in sodium permeability that initiates action potentials. These transmembrane proteins posses different isoforms (Na_v 1.1 – 1.9) according to specific tissue localization, based on the α subunit which is determinant for the affinity of TTX (Catterall *et al.*, 1992). TTX enters the extracellular side of the transmembrane pore and occlude the permeation of sodium ions by binding to receptor site 1 (Cestele *et al.*, 2000) throught complex electrostatics bonds. Channel isoforms can be classified as "sensitive" or "resistant", depending on the binding affinity of TTX. TTX is active on Na_v 1.1, 1.2, 1.3, 1.4, 1.6 and 1.7 isoforms in the nanomolar range (IC_{50} < 10 nM), which are abundant in the nervous system, with the

exception of Na_v 1.4 that is located in the skeletal muscle (Zimmer, 2010). Thus, the absence of conductance of the cations prevent the propagation of action potentials, thereby paralyzing nerve and muscle function (Lee *et al.*, 2008), specially by target the subtype Na_v 1.7 (Alonso *et al.*, 2016; Walker *et al.*, 2012).

There are generally three main perspectives regarding the study of these toxins. In the first place, these were focused on the evaluation of their mechanism of action, but over time the studies were derived in different directions such as characterization of the role and structure of Na_v channels and more recently as therapeutic. The recognition of these channels and the high selectivity showed for some of them raises the possibility that the TTX may have medical application as candidate for anesthetic and analgesic drug design, as local anaesthetics, anticonvulsants and antiarrhythmic drugs (Wang *et al.*, 2015b).

Different toxicity has been described for TTX analogues in function of their structure, being the deoxy and the hydroxyl TTX analogs less or more toxic than TTX, respectively. These differences are due to the enhanced binding to the channels based on the number and position of the hydroxyls groups (Yang *et al.*, 1992a; Yotsu-Yamashita *et al.*, 1999).

The vast majority of studies conducted with this group of toxins are not aimed at assessing their toxicity both *in vitro* and *in vivo*. TTX is generally used to study the potential of others drugs in blockigng the voltage-gated sodium channels. In addition, of the few studies on its toxic action, less are those that evaluate the toxic potency of the rest of analogues mainly because of the lack of sufficient purified toxins.

The potency of some TTX analogues has been determined through the study of the IC_{50} in different systems. *Ex vivo* studies were the first realized, focusing on the inhibition of a single type of channel as they used biological materials such as muscle fibers or axons of animal origin. The IC_{50} values reported for the analogues found in molluscs were 4.1 nM for TTX (Yang *et al.*, 1992b), and much higher with values around 300 – 450 nM for 4,9-anhydro TTX (Kao *et al.*, 1985) and 11-deoxy TTX (Yang, et al., 1992b), respectively.

A recent *in vitro* evaluation of the inhibitory activity of TTX and analogues revealed very large differences according to the compound and the Na_v subtype in HEK293T cells. The same concentration of TTX inhibits to a greater extent Na_v1.6, and slightly lower the Na_v1.1 and Na_v1.3, being the IC_{50} around 3 times higher for these last two channels. However, the activity of Na_v1.2 and Na_v1.7 was inhibited less than 50%, while Na_v1.4 slightly exceeds this potency. Na_v channels have shown to be largely insensitive to several analogues, between them 5-deoxyTTX and 5,6,11- trideoxyTTX which were found in molluscs. Respecting 4,9-anhydro TTX the IC_{50} was greater than 100 nM for Na_v1.4 and Na_v1.5 and close to 300 nM for Na_v1.6 (Tsukamoto *et al.*, 2017). However, controversies have been found in other studies regarding 4,9-anhydro TTX, for which the Na_v1.6 subtype was not insensitive (Rosker *et al.*, 2007).

In terms of the toxic potency in different animal species, equipotency has been suggested for TTX neuronal activity inhibition in some rat and human *in vitro* models (Kasteel *et al.*, 2017). A greater sensitivity of males to TTX has also been suggested in mice (Xu *et al.*, 2003). The values of LD_{50} were described in mice according to different routes of TTX administration, being 10.7, 12.5, 532 µg/kg b.w. for i.p., subcutaneous (s.c.) and intragastric (ig) administration (Xu, et al., 2003), with great controversies especially to what the oral administration refers. However, the lack of clarity both in the procedures and in the origin and purity of the toxin does not allow these results to be definitive. Respecting other congeneres found in mollusc, only i.p. toxicity was evaluated, with results of 10, 70, 64 and 490 µg/kg b.w. for TTX (Noguchi *et al.*, 2011), 11-deoxy-TTX (Bane *et al.*, 2014), 4-epi-TTX (Munday, 2014; Nakamura *et al.*, 1985) and 4,9-Anhydro-TTX (Nakamura, et al., 1985), respectively.

As for the effects of these toxins, it has been detected that TTX induce neurodegeneration, dendrite retraction and loss of dendritic spines in rat cortical neurons after chronic exposure. Neuronal apoptosis was also suggested to be the result of the prolongued suppression of network activity, due to both the rise in the postsynaptic response to glutamate and reduction of calcium clearance (Fishbein *et al.*, 2007). Furthermore, the bladder distension of male and female C57BL/6 mice was seen to be affected by TTX, since much of the afferent responses to this action were mediated by TTX-sensitive Na_v channels (*ex vivo*) and this toxin reduces activation of dorsal horn neurons within the spinal cord *in vivo* (Grundy *et al.*, 2018).

Human instoxications has shed some light on the toxin distribution and permanence in humans body. TTX has been to persist in urine longer than in serum, around 5 days whereas in serum it was only detected in less than 24 hours (O'Leary *et al.*, 2004).

1.2 EVALUATION OF MARINE BIOTOXINS

Several methods are broad applied as routine assays for determination of the presence of phycotoxins and the evaluation of their toxic potencial. They can be generally classified into biological assays and analytical methods.

Biological assays estimate the potency of a drug by the effect caused on biological material. Biological material is understood as cells, tissues or even whole organisms itself, so that they cover both *in vitro* and *in vivo* studies. However, analytical techniques allow to know the composition of a sample in a qualitative and quantitative way, but they do not give information about toxic effect or potency. The combination of all of them would result in a better risk assessment of marine biotoxins toxicity.

Bellow several of the most representative methods are explained, which some of them have been used in this doctoral thesis.

1.2.1 Biological methods

Biological assays involve both *in vitro* and *in vivo* techniques. *In vitro* methods include the establishment of cultured cells or tissues under defined and controlled conditions. They provide important information about concrete effect of toxic substances, with generally fast results and generally allowing to work with high number of samples. More and more complex and specific *in vitro* methods have been developed, seeking the substitution of *in vivo* studies whenever possible for scientific, ethic, legal and economic reasons. However, *in vitro* studies posses severe limitations regarding to some mechanism that can be solved by *in vivo* assays, specially in toxicity evaluation as predictive models for assessing human risk. In order to carry them out, a series of requirements must be fulfilled, which are included in legislation (Directive 2010/63/EU) as well as the application of the Three R’s principle. This concept was introduced 60 years ago by Russel and Burch (Russell WMS, 1959) and seeks the protection of animals and the regulation of their use in the field of experimentation based on the application of replacement, reduction and refinement. Acording to this principle, the replacement of the use of animals can be total, through the development of alternative techniques such as computer models, tissue engineering or chemical methods, but a relative replacement is also admitted, understanding as such the replacement of animals by others with less pain perception, for example. As for the reduction, the use of the minimum possible number of animals is sought with which the greatest possible information and an appropriate statistical analysis for the interpretation of the data can be obtained. The refinement consists of the application of a methodology to improve animal welfare, causing therefore the least anguish and stress from

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birth to death. The European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) is actively involved in search for test methods that respect this principle in a wide variety of research areas, and collect them in a database (Centre).

The greatest difficulty in *in vivo* testing is the handling of animals. Furthermore, as a disadvantage, the great variability in the results depends on the characteristics of animal species used and even on the breed (sex, age, weight, general health status, diet, stress), and unlike *in vitro* studies, the automation of the techniques is not possible.

Bellow different techniques used to study the toxic effects *in vitro* and *in vivo* of marine biotoxins are described. The elected assay should provide clear results, and preferably easy to interpret. Results are commonly expressed in dose-response relationships to estimate potency of the substances whose variability in response can be expressed throught several parameters calculated by mathematical models.

1.2.1.1 *In vitro* assays

Cell-based assays use the response of the cell to a substance, being response very diverse depending on the cell line used and the duration of exposure, from a change in a particular characteristic or even death. Cell lines are one of the main tools in research, since they provide a simpler system to study cell processes with an uniform cell response. Although these techniques may not be specific, they provide information as initial screening to study the mechanism of action of a substante to exert toxicity. This type of assays may involved both one or several cell lines, thus being able to form a cell culture system more accurate that mimic *in vivo* mechanisms and therefore allow a better extrapolation of the results to real situations.

There is a wide variety of cell lines used as animal and human models, being those cancer-derived the most representative. In the present thesis, two standarized intestinal and neuronal cell lines were used in several procedements.

The neuroblastoma cell line SH-SY5Y (ATCC® CRL-2266™) is a thrice subcloned originally derived from a metastatic bone tumor biopsy, widely used as neuronal model. They show piramidal shaped cell body with extend of neurites. The Caco-2 cell line is derived from a human colon adenocarcinoma (ATCC® HTB-37), the most widely used as *in vitro* model of human gastrointestinal tract for the study of absorption and permeability of substaces. These cells in culture are adherent and form a densely populated cell monolayer that in confluence spontaneously differenciate into polarized enterocytes in 15 – 21 days, constituting a monolayer of columnar cells attached by different types of unions.

There are methods of different complexity, while some only inform about whether a compound is toxic or not, others allow to elucidate cellular basis by which a compound exerts its toxicity such as variations in intracellular and extracellular ionic fluxes, changes in spatial distribution of cell proteins or structural alterations of cell organelles among others.

1.2.1.1.1 Cell viability

The reduction of cell proliferation or cell death can be studied by cell viability assays. There has been developed multiple methods to measure cell viability, understanding this quality as the ability of a compound to become toxic. The different techniques principaly differs in the indicator of cytotoxicity used as well as the associated measured method such as fluorescence, absorbance or colorimetric. Indicators can express the type of cell damage or level at which cells are affected, expressing apoptosis, necrosis or cell membrane damage among others. The

most commonly used are associated to cell metabolism activity such as Tetrazolium (MTT) or Alamar Blue assays.

AlamarBlue is an indicator of viable cell metabolism. This reagent is composed by resazurin, which is blue and no fluorescent, hydrosoluble and permeable through the cellular membrane. After entering the cell, this substance is reduced irreversibly to fluorescent pink resorufin (λ = 560 nm excitation, λ = 590 nm emission) (Figure 7) by oxidoreductases inside the mitochondrias of living cells to finally be excreted. This reaction is proportional to the number of metabolically active cells and the time of exposure to the test substance. Thus, cell exposed to a cytotoxic agent will present a decreased metabolic function according to the toxic potency of this one.

The relationship between fluorescence data obtained and the concentrations of the toxic compound used allow to plot a dose-response curve, by which parameters such as the half maximal inhibitory concentration (IC_{50}) can be calculated. This parameter offers a quantitative measure of the potency of a substance in inhibiting by half the methabolism in this case.

The AlamarBlue assay (Espiña *et al.*, 2009) was the chosen method in the present doctoral thesis due to its advantages over another assays frequently used: simplicity, as it is directed added to the treated cells; quickness and sensitivity, as results can be obtained between 1 - 4 hours and differenciate similar conditions, and only shows citotoxicity at long term, alowing therefore continuous monitoring even for several days.

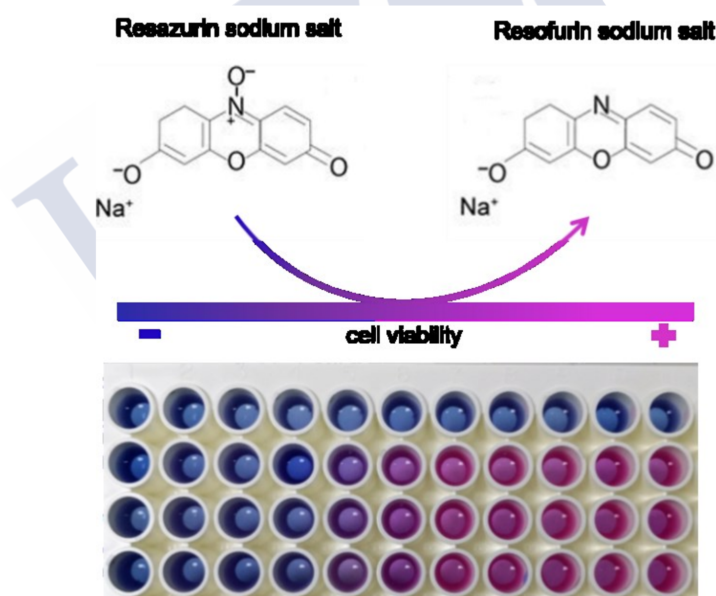

Figure 7. AlamarBlue method for evaluation of cell viability of a citotoxic compound.

1.2.1.1.2 *Transepithelial permeability assay*

Marine biotoxins are mainly incorpored in animals and humans by ingestion, so their toxic potency is determined to a large extent by the bioavailability of these compounds, which depends on the quality of the barrier function of the gastrointestinal tract, a vital function for the physiological activities of the tissue. Absorption and permeability occur through the gastrointestinal tract mainly in the small intestine, where molecules (aminoacids, sugars and fats), water and electrolites are absorbed. The small intestine wall constitute a physical-

chemical barrier being the mucosa the layer directly exposed to the intestinal content, so it is responsible for increasing the inner surface to in turn increase absorption which is achieved through the intestinal villi. These prolongations of the luminal surface are upholstered by a single columnar cell epithelium polarized, composed by different cellular types intermingled such as enterocytes, enteroendocrine cells, goblet cells, Paneth cells and M cells. Of all of them, enterocytes are the most frequent and main cells responsible for absorption. They are lined by microvilli that further increase the absorptive surface of the cytoplasmic membrane. These cells also are involved in the transport across the epithelium (transcellular route) either by active transport or by passive diffusion to the lamina propria, from where the diffusion to the capillaries takes place. However, the most passive transport occurs through the intercellular spaces (paracellular route), regulated by tight junctions (TJs) that are apically located and seal the intercellular spaces of the enterocytes (Figure 8) providing structural integrity to tissues at epitelial and endotelial level.

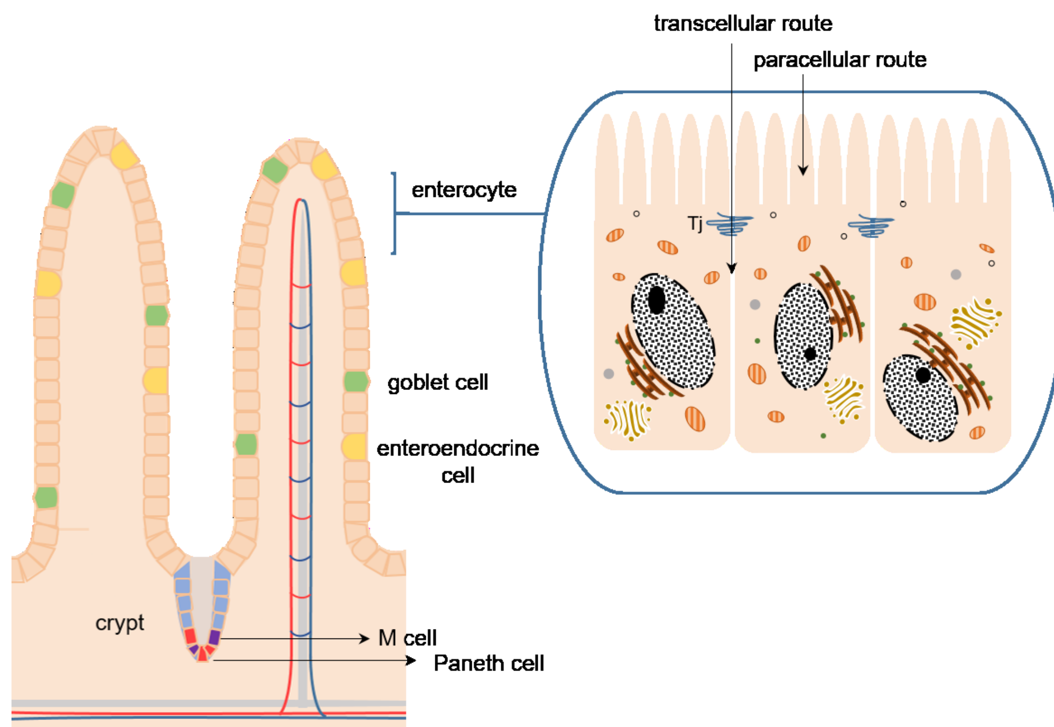

Figure 8. Scheme of small intestine mucosa layer, with detail of enterocytes structure and organelles. Tj: tight junctions.

The maintenance of the highly polarized barriers created by the TJs is essential to homeostasis in vertebrate physiological systems. These structures can be degraded both by regulatory and pathological action. In some cases, as intestinal diseases, these unions are affected and therefore their selective blocking function may be interrupted, thus allowing the mucosal penetration with inespecific passage of substances present in the lumen, such as antigens, microorganisms or toxins (Ma *et al.*, 2000). Therefore, the evaluation of intestinal permeability is crucial to know the state of the membrane and its possible affectation by different toxins, especially those that cause diarrhea. For this, there are several *in vitro* methods, such as measuring of the transepithelial electrical resistance (TEER). This technique provides a quantitative measure of the integrity of the cell monolayer in cell culture models by evaluating the electrical resistance across a cellular monolayer (Srinivasan *et al.*, 2015). Intestinal cell

models are cultured on the surface of a semipermeable membrane contained in an insert where they will grow, leading to a differentiated cells monolayer with characteristics similar to the barrier physiology and functionality, mimic in *vivo*-like conditions (Figure 9). At that moment, the TEER is measured by a voltohmmeter with two electrodes separated by the cellular monolayer, one placed in the upper compartment and the other in the lower one, representing the apical and the basolateral side respectively. This technique also allows the development of a culture system in which several cells lines are present, representing a more reliable intestinal model. Thus, in coculture two different cell lines would be seed separately, in the insert and in the well, so that they could communicate with each other despite the absence of physical contact (Figure 9B).

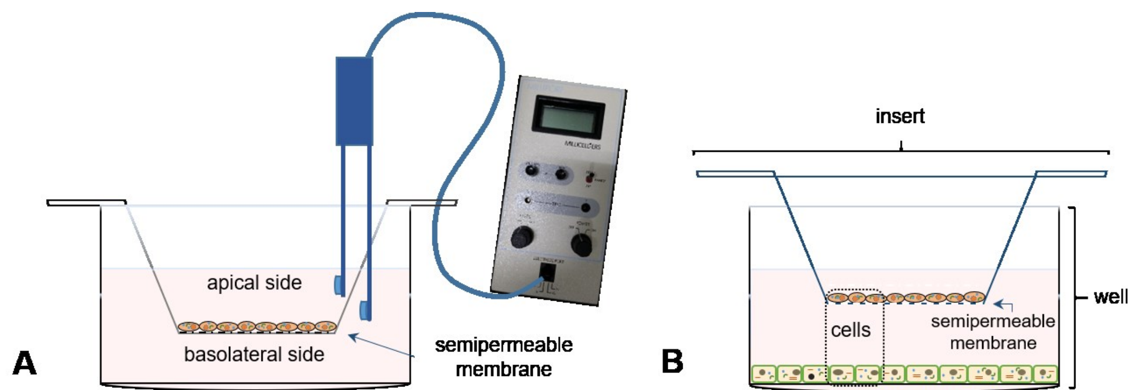

Figure 9. Represenation of TEER measurement by voltohmmeter in cell culture with representation of the apical and the basolateral side (A). Coculture of two cell lines are also represented, indicating insert and well compartiments that form the system (B).

TEER is expresed in $\Omega.cm^2$ units. The resistance of blank insert (inserts without cells) (R_B) must be subtracted to calculate the resistance of the cell monolayer (R_T), and finally relate the result to the area of the semipermeable membrane contained in the insert:

$$\text{TEER} = (R_T - R_B) \times M_{AREA}\ (\Omega.cm^2)$$

The human intestinal model Caco-2 cells provides information of all the routes of transport in enterocytes (Artursson *et al.*, 2001; Espiña *et al.*, 2011). Caco-2 cell monolayers can generate TEER values of 150-400 $\Omega.cm^2$ (Srinivasan, et al., 2015). The half maximal effective concentration (EC_{50}) represents the concentration of a compound which induces the 50% of the maximum response after a specified exposure time. This parameter is commonly used as a measure of potency of a drug. Furthermore, the representation of the relationship between the TEER of treated monolayers and that from controls allow to check the effect of the treatment with a compound on the cell monolayer (Fernandez *et al.*, 2014).

The measure of TEER is a very sensitive and reliable method to confirm the integrity and permeability of the monolayer. Furthermore, it is non-invasive and allow the continuously monitorization of the barrier integrity in live cells during their various stages of growth and differentiation (Srinivasan, et al., 2015). Also, it allows sampling of both compartments individually, in order to study the pass of substances throught the monolayer, the release of compounds in responce to cell stimulation or even cell collection to determine protein alterations.

Several analytic techniques can be used to study the toxin permeability throught the differentiated monolayer by quantification of the toxin content in apical and basolateral compartments (Fernandez, et al., 2014). Furthermore, the collection of the samples allows another type of analysis, such as the quantification of analytes released by the cells as a result of the exposure to toxins. The techniques used for this will be explained in detail in the subsequent corresponding sections.

1.2.1.1.3 Microscopy imaging techniques

Rapid changes in permeability occurs through the cytoskeleton under regulation of proteins from the TJs. This multiprotein complex is located in lipid-rich membrane microdomains (Van De Walle *et al.*, 2010) encircling the cellular borders at the apical side.

TJs are composed by the association of claudin, occludin and ZO proteins, being the latter those that are anchored to the actine cytoskeleton (Figure 10). They are dynamic cell structures, that can interact with a wide variety of molecules and respond to several cell internal and external stimuli. Althought the regulation of the intestinal TJ barrier function remain poorly understood a close relationship has been described between centripetal retraction of some TJ proteins and the loss of the barrier function. Hence, tight junctions critically determine the barrier function and its evaluation is essential to determine the mechanism of drugs absorption.

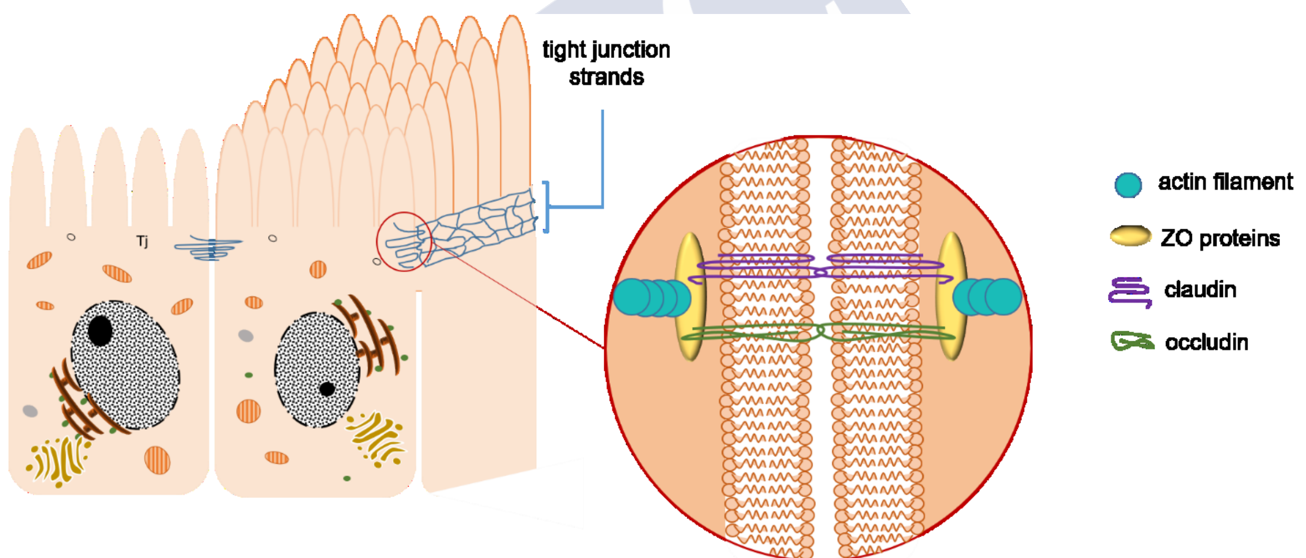

Figure 10. Detail of tight junctions main proteins attaching two enterocytes of the small intestine.

Visualization of cell junctions proteins with specific staining can determine the mechanism by the barrier function is loss. The confocal microscopy generate high-resolution images of material stained with fluorescent probes. This technique allow to obtain three-dimensional images in spatial registration to study structural details of cells.

The barrier characteristics are defined by the specific protein complexes that compose the TJs. Occludin is a transmembrane protein found in all epithelial and endotelial TJs well known to contributes to sustainment and stabilization of their structure and ensuing optimal barrier function in various endothelial cell models (Kuwabara *et al.*, 2001). Occludin is found in several isoforms with different roles and regulatory features according to modification of its domains. The processes that regulate occludin include phosphorylation. Thus, kinases and phosphatases

are of great importance, so the balance between them will determine the phosphorylation state of occludin (Dörfel *et al.*, 2012). Highly phosphorylation events on occludin at serine/threonine (Ser/Thr) residues has been related with intact TJs suggesting a relationship with the assembly of the same (Farshori *et al.*, 1999; Sakakibara *et al.*, 1997). As discussed in previous sections, OA and DTXs inhibit the action of phosphatases, especially PP2A and PP1, which have been found to preferably dephosphorylate occludin in the Thr and Ser residues, respectively (Seth *et al.*, 2007). These residues are abundant in the C-terminal cytoplasmic region, which is essential for occludin interactions with ZO-1. The rupture of this union also affects therefore the filaments of actin, whose depolymerization has been related to induction of occludin internalization by several different endocytic pathways as initial barrier loss (Yu *et al.*, 2008). This migration of occludin and other TJs proteins into the cytoplasm has also been shown *in vitro* and *in vivo* in response to external stimuli like calcium depletion (Ma, et al., 2000) and cytokines (Van Itallie *et al.*, 2010).

Nonetheless alterations located in a thick structure can be taken into account in isolation, since in a cell or tissue an isolated change can trigger a series of alterations in other cellular parts.

Cellular functions are fulfill by a series of highly specialized organelles immersed in the cytoplasm. All these organelles communicate to each other by molecules that release into de cytoplasm loose or encompassed in membranous structures.

The main function of the cellular membrane is to protect and support the cytoplasm, but also contributes to cell compartmentalization and comunication and controls the entry and exit of molecules in cells (Nicolson, 2014). The smooth endoplasmic reticulum (res) is involved in synthesis, storage and transport of lipids as well as degradation of harmful substances or calcium storage, while the rough endoplasmic reticulum (rer) synthesize and transport proteins (English *et al.*, 2009). The Golgi apparatus classify, mature, pack and distribute the molecules synthesized by him or by the endoplasmic reticulum and intervene in glycosilation process (GM, 2000a). Lysosomes posses hydrolitic enzymes that degrade excess or worn organelles, however, enzymes contained in the peroxisomes are specialized in lipid metabolism and protecction against harmful oxidative molecules (GM, 2000b). In mitochondrias occur the most of the reactions of cellular respiration to obtain ATP, they also parcipitate in lipids metabolism and calcium storage (Friedman *et al.*, 2014).

Alterations of the structures of these organelles may evidence changes in their functions due to exposition to a stressor. The transmission electron microscopy (TEM) makes possible to study damage at ultrastructural level (Figure 11). TEM is based on the projection of a high energy beam of electrons through a very thin sample. Interactions between the electrons and the atoms allow to observe features of small scale in the internal structure of cells. As the transmission of electrons through the sample is greater or less, it will be represented in the image as light or dark zones, respectively.

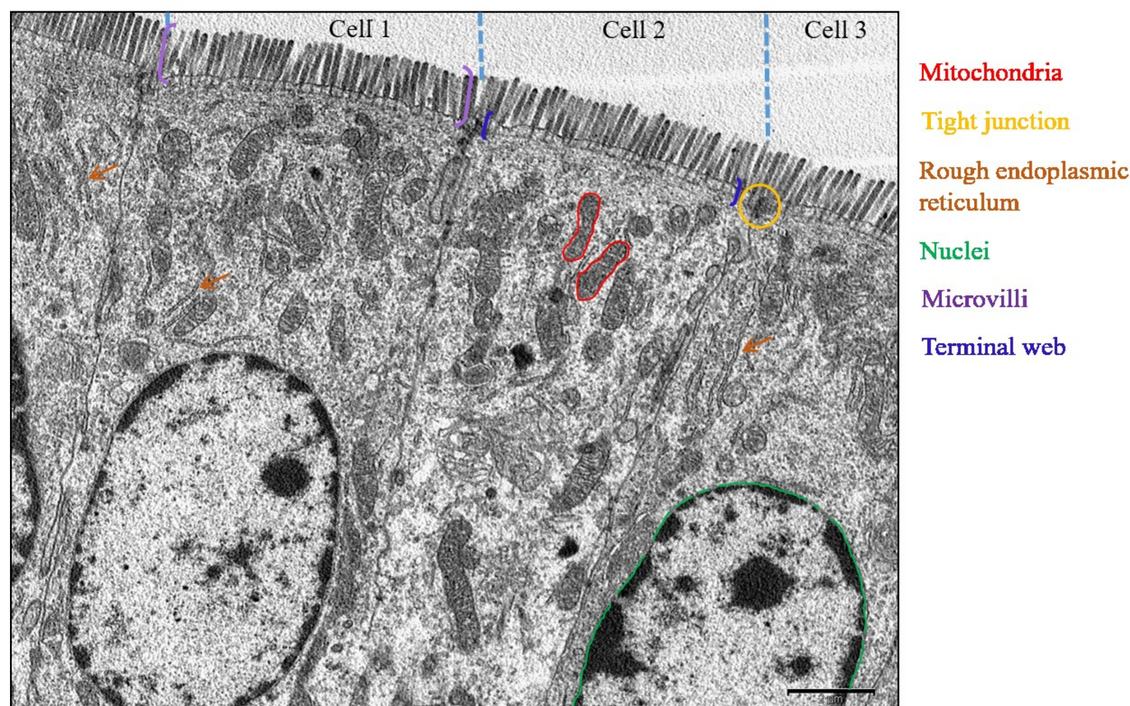

Figure 11. TEM imaging of the epithelial line of small intestine of mouse. The main organelles are signailed.

1.2.1.2 *In vivo* assays

In vivo toxicity studies are conducted to assess the degree to which compounds are toxic for organisms. They were commonly carry out in rodent species, specially in females as they was seen to be generally slightly more sensitive in those cases where differences were observed (OECD, 2002a). The vast majority of *in vivo* studies with marine toxins were perform by mouse bioassay (MBA), that was the reference method for many groups of toxins for many years. This assay involves the i.p. injection of an extract, toxin or a sample of shellfish into mice and survival monitorization over a variable period of time according to the toxin under examination, minutes or 24 h (EU-RL-MB, 2014; Suzuki, et al., 2018). However, it has been seen that this technique does not allow to distinguish between different toxins that produce similar symptoms, such as TTX and STX. Furthermore, this route of administration is not representative for marine biotoxins since most of they are acquired through diet. Besides, MBA requires much lower quantity to see affectation as i.p. absorption is greater than by oral administration, since as seen in previous sections, the gastrointestinal tract is specially designed to control absorption of unwanted or harmful substances. This was an advantage in the past to face the limited availability of toxins and the absence of refining techniques to obtain purified material but currently it implies the need for a new toxicity evaluation. The use of test material well identified and purified must be insured before conducting toxicological tests. This characterization is currently possible through analytical techniques, such as High Performance Liquid Chromatography (HPLC), which will be explained later. Therefore, i.p. administration could be considered an inadequate method for the risk assessment of marine toxins, since it supposes a high estimation of the toxicity of the substances (Tubaro, et al., 2003).

Several methods exist to orally administered a substance: via the diet including drinking water, by gavage, or by encapsulation. However, gavage is the most commonly used as avoids stability problems that may arise when the toxin is contacted with other substances and also

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ensure precise and accurate dosing of animals (Turner *et al.*, 2011). In gavage, toxins are directly administered into the stomach by means of a flexible cannula/ tube or by a stainless-steel catheter with final bulb introduced by the mouth and attached to a siringe (Figure 12). Catheter lenght and administration volumes must be adjusted to animal species and their body weight to avoid cause damage to the mouse or exceed the stomach volumen and that the treatment pass immediately into the small bowel. Thus, the relationship between dose volume and body weight for rodents is 10 mL/kg (Diehl *et al.*, 2001). Furthermore, in toxicology testing animals are usually fasted prior to dosing the test substance as a way of standardizing tests, in order to reduce variability and to ensure a more uniform absorption by preventing mixing test drug with food. The time of fasting depends on the feeding pattern, the cirdadian cycle and the physiology of the species as well as the length of dosing, among others (Jensen *et al.*, 2013; OECD, 2002a). After administration, food and water are provided *ad libitum* (Aune, et al., 2012).

Figure 12. Intragastric administration with a stainless-steel catheter

The choice of the age of the animal is also an important factor, since very young and very old individuals can show deficiencies in rates of detoxification and/or excretion of harmful substances (Vyskočilová *et al.*, 2013).

There are different groups of toxicity studies with regard to duration and number of doses administered. Thus, acute studies determine the effects of a single dose administration in a short period of exposition usually within a 24 hour period, while repeated dose, subchronic, or chronic toxicity studies identify whether toxicity occurs after continuous exposure to a substance (Parasuraman, 2011).

The toxicity studies are used to determine the lethal doses sometimes by dose–response curves obtained, such as LD_{50}. This parameter indicate the dose of a substance that can cause death in 50% of treated animals, expressed in terms of quantity of test substance per unit weight of test animal. Several test guidelines and standardized methods according to the Three Rs have been brought out by The Organisation for Economic Cooperation and Development (OECD) and the International Conference on Harmonization (ICH) for acute toxicity testing (OECD, 2002a). They are the Fixed Dose Procedure (FDP) (OECD, 2002b), the Acute Toxic class method (ATC) (OECD, 2001) and the Up-and-Down-Procedure (UDP). However, only the UDP provides a point-estimate of the LD_{50} value with confidence intervals and allow a test substance to be classified according to all the systems in current use, including the new Globally Harmonized System (GSH) (OECD, 2008). Furthermore, a significant reduction in the number of animals is achieved in this procedure by performing sequential dosing steps. The four-level UDP use increasing number of mice dosed one at a time in each level dose, and the applied dose is established based on the results of mortality obtained in the previous level after the established time of the experiment, decreasing or increasing if more than half of the treated mice die or survive, respectively. The results obtained are fitted to a sigmoidal logistic four-parameter equation, which reflect the establishment of a dose-response relationship that relate the exposures and the spectrum of induced effects, which have to be independent from other exposures. This is represented by a sigmoidal curve. This model has an upward and curvature

at the lower and higher dose levels, respectively, with a middle range close to linearity (Moffett *et al.*, 2007).

Through the UDP procedure, in addition to LD_{50}, others toxicity indexes related to observed effects can be estimated. This is the case of No Observed Adverse Effect Level (NOAEL) or the LOAEL, they have great importance in the conduct of risk assessments. An adverse effect is understood as a biochemical, morphological or physiological change in response to a stimulus that negatively affects an organism to respond to that stimulus (Lewis *et al.*, 2002). NOAEL is the highest dose without significant adverse alteration of animal characteristics distinguishable from those observed in control organisms, while LOAEL represent the lowest dose which causes an adverse effect. Another parameter than can be defined when we work with acute toxicity assessment methods is the ARfD, which refers to an estimate of the amount of a substance in food and/or drinking water that can be ingested in a period of 24 hours or less without appreciable health risk to the consumer. While ARfD is a concept from acute studies, both NOAEL and LOAEL can be calculated according to long term exposition (Park *et al.*, 2011). Repeated-exposure studies allow the definition of another concept for humans, the TDI, defined as an estimate of the amount of a substance in food and/or drinking water that can be ingested daily over a lifetime without appreciable health risk to the consumer. For a full risk assesment, studies on possible effects on reproduction and evaluation of potential carcinogenicity and teratogenicity are required (OECD, 2009, 2015).

All these parameters provide very useful information respect to the toxin toxicity, however there is the possibility of going deeper into more aspects such as the case of toxicokinetic studies. They provide information on absorption, distribution, biotransformation and excretion of a substance and its metabolites to relate the concentration to the observed toxicity. Furthermore, the study of the oral bioavailability determine the dose fraction of a compound that reach systemic circulation without suffer modifications, which mainly depends on hepatic and intestinal metabolism. The analysis of mice samples requires the use of analytical systems that identify and quantify the toxin present in the biological samples. According to the purpose of the study, the individual storage of animals may be required, such as the studies on the dynamics of excretion and absorption. For it, collection of blood samples and also urine and fecal samples following administration of the toxin are needed. The organ extraction provides information about the potential accumulation, biotransformation and persistence of toxins in tissues. The knowledge of distribution could also allow the identification of target tissues and understand of the mechanisms of toxicity.

All these results may be extrapolated to human hazard although we must take into account the species factor as a variable.

1.2.2 Analytic methods

There are several analytical methods used to complement the *in vitro* and *in vivo* toxicity studies. Some of them have even been routinely employed in monitoring programs implemented for each group of toxins to harmonize detection and sometimes quantification. However, only few methods are actually validated in function of the group of toxins to determine and the country of application. Advantages of these methods are the capacity for high throughput analysis and the highly specific results and sensitivity as they allow the identification and quantification of different the toxins unequivocally in a single run.

In addition to detect, these methods allow to quantify the toxins in samples with accuracy results about toxin profile and quantity in a sample. These methods needs standards for each of

the toxin analogues, preferably certified reference materials, since they are only able to detect the presence of specific molecules set as objective. However, only a few are currently available.

1.2.2.1 Target specific assay

Lipophilic toxins of the group of OA target protein phosphatases. Differences in the potency of inhibition on PP have been described according to the toxic analogue and the phosphatase under study, being the enzymes PP1 and PP2A more sensitive inhibited.

The ability of OA and its derivatives to inhibit PPs has been used to develop tests to detect such diarrheic toxins in different samples. An enzyme-based assay has been one of the methods used in Europe for a long time (EFSA, 2008). This test based on the inhibition of the PPs activity determines quantitatively a sum of OA-equivalent material present in a sample of water or mollusc tissue in a very short time.

Several protocols have been developed but generally they consist of the purified catalytic unit of the enzyme phosphatase which can be either of animal, human or recombinant origin (Ikehara *et al.*, 2010), and a specific colorimetric substrate for such enzyme. Mainly there are two variants of this assay, the addition of the phosphatase in solution to the microtiter well or its immobilization in both agarose gel (Sassolas *et al.*, 2011) or encapsulated in the sun-gel and subsequently immobilized in the microtiter well (Hayat *et al.*, 2012). This method of detection allows the coupling of other techniques, such as the conjugation of the PP to magnetic nanoparticles (Garibo *et al.*, 2012) or the use of chromatography to achieve the separation of the PP-toxin complex and the free toxin (Serres *et al.*, 2000).

The transformation of the substrate or the absence of modifications will be the indicator of phosphatase activity, which is usually measured by absorbance (Takai *et al.*, 1991) or fluorescence (Vieytes *et al.*, 1997). The colorimetric assay was the one used in this doctoral thesis based on inhibition of PP2A in order to establish the toxic potency of the different analogues using accurate well-characterized standards. In the absence of toxin, the total hydrolysis of the substrate occurs, whereas if toxin is present in the sample, the hydrolyzed product will be inversely proportional to the toxin concentration may even be null if the activity of the phosphatase is completely inhibited (Figure 13). The quantitative determination of toxin in each sample is obtained by extrapolation of the results with different software throught a dose-response calibration curve.

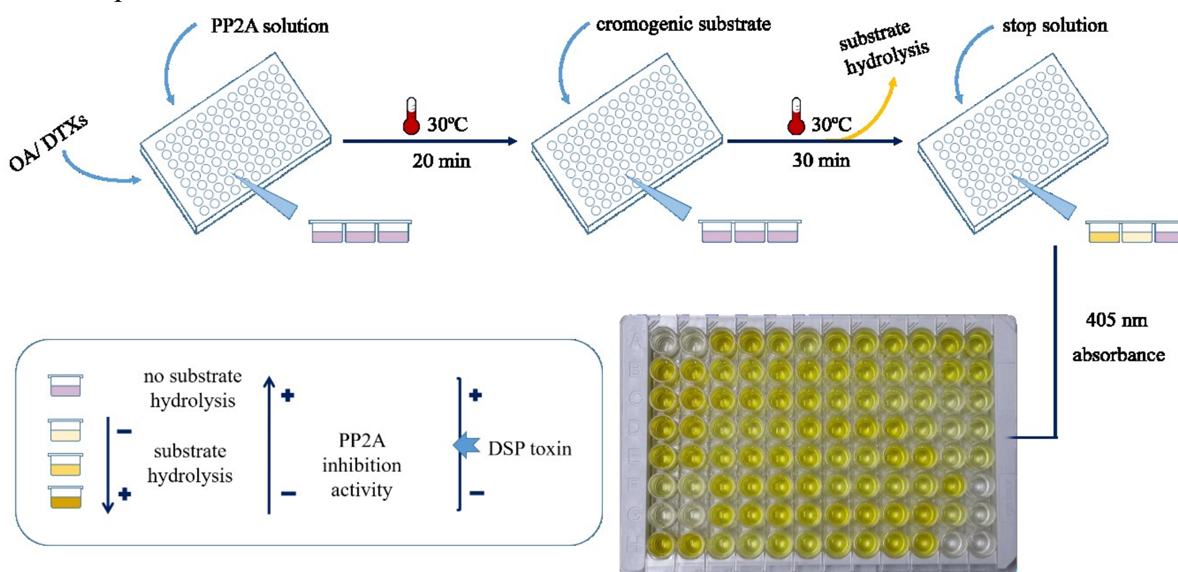

Figure 13. Scheme of enzyme based assay based on the inhibition of PP2A by DSP toxins.

1.2.2.2 Immunological methods

Antibodies capable of recognizing some marine toxins have been developed, specially those lipophilic and paralytic toxins (Vilarino *et al.*, 2013). Several are commercially available and they can be used in immunoassays both in isolation form and in detection kits. Immunological methods are not only used for the labeling of toxins, they can also be applied to measure effects of the same on cells or tissues, both *in vivo* and *in vitro* culture. These tests offer high sensitivity and usually not require highly sophisticated skill sets and training.

Immunoassays measures the presence or concentration of molecules usually through the use of an antibody that have the ability to recognize and bind to them in a specific way, even in presence of complex mixtures. For this, solutions with a known concentration of the analyte are employed to interpret the signal of the samples and detectable labels that indicate this chemical link by means of radiation, fluorescence or light emissions or color changes production so that it can be measure.

Radioimmunoassays (RIA) (Levine *et al.*, 1988) as well as surface plasmon resonance (Le Berre *et al.*, 2015) are immunoassays used for marine toxin detection. However, enzymes are the most common label, and therefore the most developed tests are the enzyme immunoassays (EIAs) (Ling *et al.*, 2015; Liu *et al.*, 2014). In addition to the different formats of existing immunoassays, these tests can be coupled to several analytical techniques such as flow citometry (Fraga *et al.*, 2013). The two immunological tests used in the present thesis are explained in the following sections.

1.2.2.2.1 Competitive fluorescence assay

A recent technique combine the flow citometry to microspheres xMAP internally marked with fluorofores by Luminex technology. In addition, this technique allows the use of a series of antibodies, whereby the final quantification of the analyte is the result of an immunoassay. This method has been successfully used for the detection and multiple simultaneous quantification of several toxins in the same sample, using antibodies specially designed to recognize and bind to these toxins (Fraga, et al., 2013).

The surface of these microspheres admit different configurations according to the functional groups coating them, being the most versatile those with carboxylic groups. The functional groups added to activate the microspheres also provide a greater diversity. Thus, the analytes of interest will be recognized and immobilized specifically by covalent bonds. On the other hand, the samples to be analyzed are incubated together with an antibody that will recognize the analyte in solution, after which this mixture will be put in contact with the microspheres. Then, the competition to bind the antibody will occur between the free analyte in the sample and the immobilized one in the microspheres. A secondary antibody labeled with a fluorophore, commonly phycoerythrin (PE), will recognize the microsphere – Ab1 complex and become part of it. Finally, the samples acquired by the equipment will be guided by the flow and exposed individually at the lasers. The first laser (635 nm) classifies microspheres in regions according to the spectral signal, which corresponds to the ratio of fluorophores contained in the microspheres, while the second laser (532 nm) or quantifier excites the fluorophore attached to the Ab2 bind to Ab1- microsphere (Figure 14). Thus, the measured signals that correspond to complex of the microesphere-Ab1-Ab2-PE can be related inversely to the amount of toxin present in the sample (Fraga, et al., 2013).

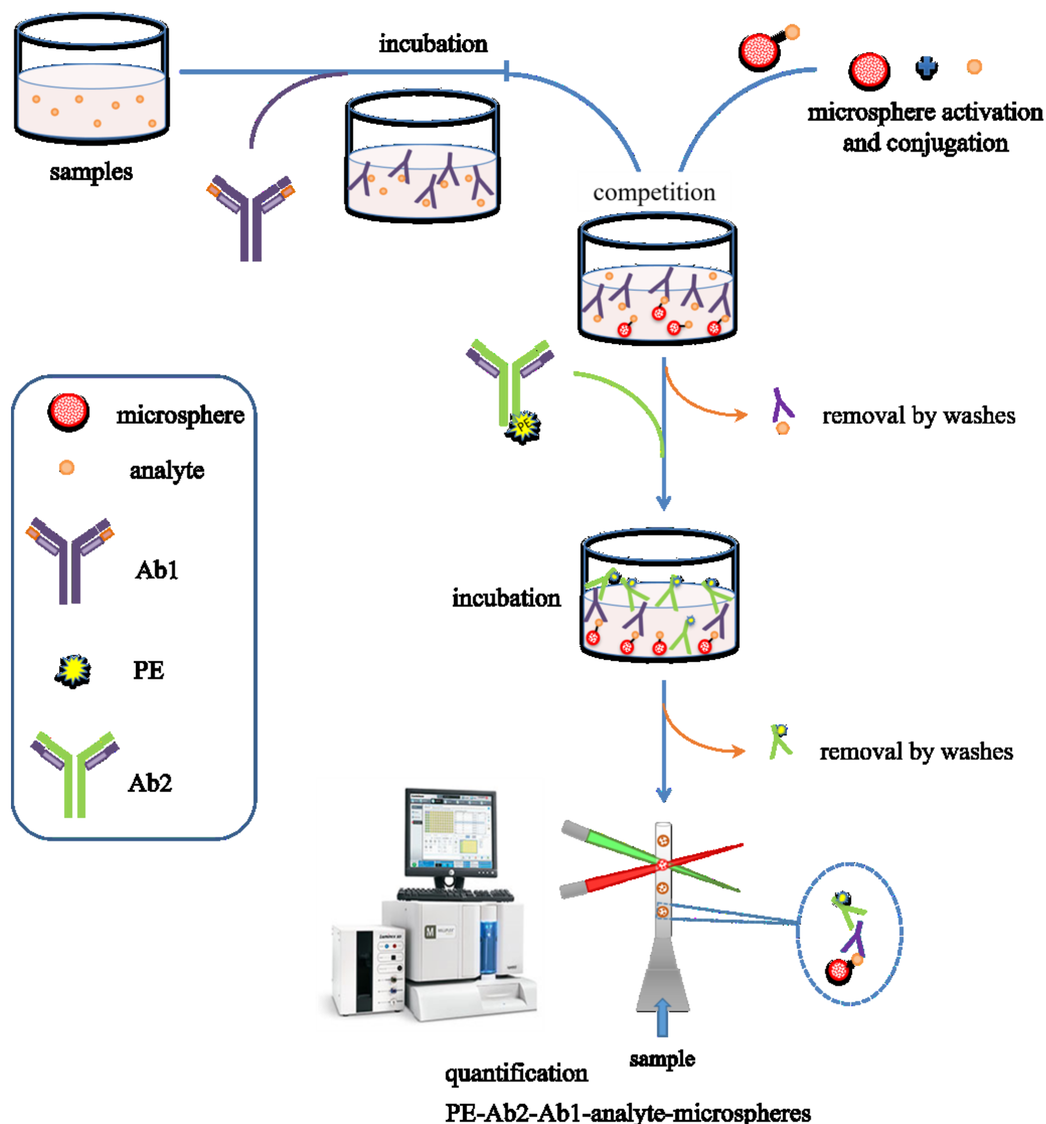

Figure 14. Scheme of general Luminex procedure.

1.2.2.3 Liquid chromatography – mass spectrometry (LC-MS)

The high performance liquid chromatography (HPLC) is a type of column chromatography based in separation of the compounds of a mixture to analyse. For that, the sample is injected and transported in a mobile phase at high pressure throught the stationary phase of the chromatographic column. The chemical interaction ocurred inside the column is determinant for the retention inside of the different components, which is why both the choice of a mobile phase and a column filling according to the nature of the sample is important for a good separation of the components in the elution to identify them. Water, methanol and acetonitrile are the solvents most used to which buffers or salts can be added for a better chromatographic resolution. This parameter is also improved by small size of particles of the columns and high pressures to which the equipe works.

There are different types of techniques coupled to liquid chromatography, several of them used in the analysis of marine toxins. Mass spectrometry (MS) is an analytical tool used for measuring analytes from a sample and determine the elemental composition and molecular

structure by elucidation of their patterns of molecular mass and disintegration, so that it can even be identify those that are unknown.

Actually the official method for lipophilic toxins detection is linked to tandem mass spectrometry (LC-MS/MS) (EU-RL-MB, 2015). The combination of LC-MS allow to achieve separation by ionisation methods using electrical and/or magnetic fields to differentiate ions. With regard to TTX, currently there are no oficial methods for detection and quantification of this group of toxins in Europe, although it is contemplated in Dutch sanitary survey program since 2016, where TTX is monitored by LC-MS/MS, the methodology usually used, till a neuroblastoma assay is validated and accredited (Gerssen, et al., 2018).

A good toxin detection requires the optimization of several parameters. However, the greatest fluctuations may be due to the extraction procedure sometimes required of the samples, since a poorly refined method can introduce a very pronounced matrix effect in the analysis. The factor of matrix effect can mask, diminish or even increase the perception of the toxin (Matuszewski *et al.*, 2003).

Three fundamental modules can be distinguished in mass spectrometers: ionisation source, analyser and detector. The first is the part of the instrument by which the sample is introduced in ionized form after elution in the HPLC, according to the requirements of the sample and mass spectrometry.

Electrospray ionisation source (ESI) is the most widely used for biological molecules (Pitt, 2009). This method suppose the sample dissolution and dispersion as aerosol aided by the introduction of a nebulising gas, usually nitrogen, and a electric field created by high voltage application. However, neutral and low polarity molecules must be ionisated by different methods, like Atmospheric Pressure Chemical Ionisation Source (APCI) or Atmospheric Pressure Photo-ionisation (APPI). While the first uses gas-phase ion–molecule reactions at atmospheric pressure, the last one use photons to ionise molecules after nebulisation (Edmond de Hoffmann, 2007).

Once ionisation have been produced, gas-phase ions are separated according to their masses, which are measured by the mass analyser according to their mass-to-charge ratio (m/z) (Edmond de Hoffmann, 2007). The different types of mass analysers coupled to LC-MS analysis differ in the way in which the separation occurs. The analysers use static or dynamic electric and magnetic fields alone or combined (Edmond de Hoffmann, 2007). Quadrupole analysers are widely used. They separate ions using electric fields of controlled voltages. They work at atmospheric pressure as well as under high vacuum so they can serve to connect high and low-pressure regions of the mass spectrometer and also serve as collision cells (Edmond de Hoffmann, 2007). The ions are accelerated and collide with neutral gas molecules in the collision cell causing analyte fragmentation, being possible the successive ion fragmentation in instruments that combine several quadrupoles. The differents quadrupoles of the mass spectometer perform different functions. Ions reached the first quadrupole (Q1) and then they are directed to the collision cell, the second quadrupole (Q2). The fragmented ions are sent to the third quadrupole (Q3) where they are filtered to enable isolation and examine multiple precursor to product ion transitions (MRMs) to finally reach the detector (Figure 15). The vacuum system and the pumps are essential for the conduction of the ions, as well as the difference of the voltages applied between the quadrupoles and the collision cell (Edmond de Hoffmann, 2007). Ion Trap Mass and Time of Flight (TOF) analysers are also commonly used and combination of different mass analysers is also possible (Pitt, 2009).

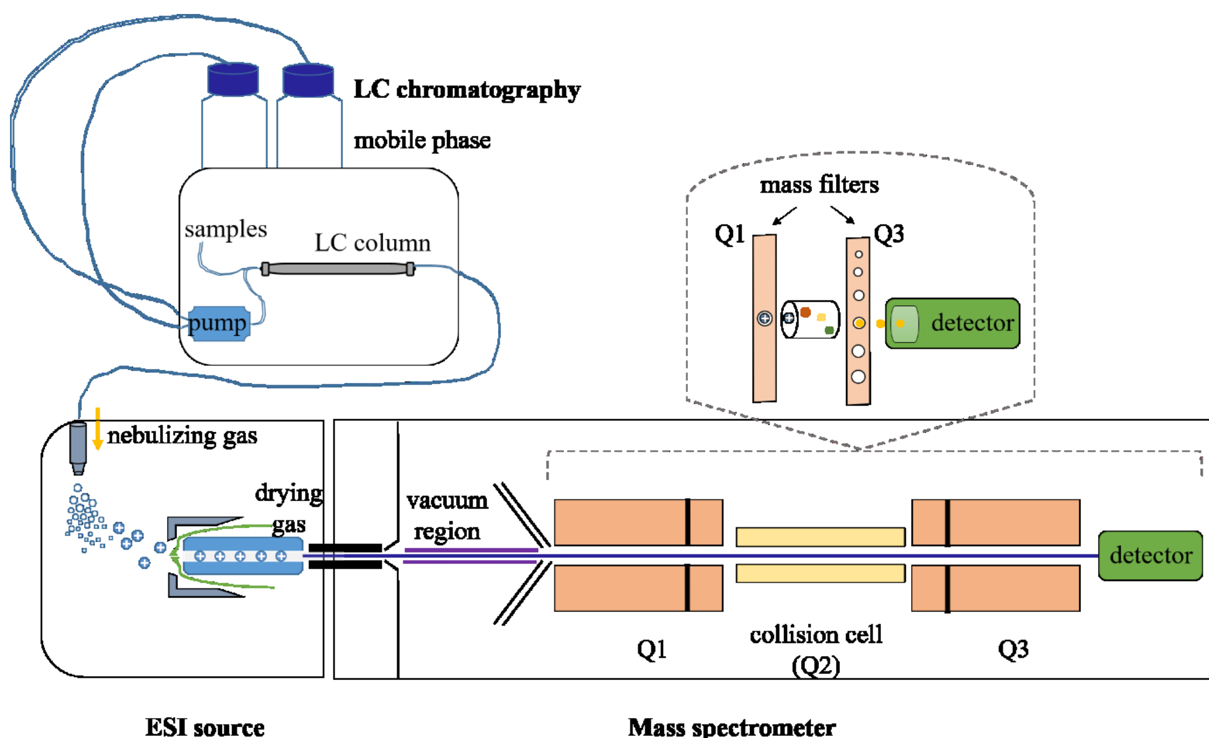

Figure 15. Scheme of LC - MS/MS equipment with three quadrupole in tandem.

The use of the different detectors will depend on the nature of the compounds to be determined, but all detect the ions based on their charge, mass or velocity (Edmond de Hoffmann, 2007). Since the marine biotoxins have different chemical structures, others HPLC methods have also been described coupled to different detectors based on fluorescence (LC – FLD) or ultraviolet (LC-UV) (O'Neill *et al.*, 2015; Turner *et al.*, 2017b). The presence of the toxin should generate a distinguishable signal from that of a blank sample. The signal of the incident ions recognized by detectors is transformed in an electric current proportional to the abundance of the incident ions (Edmond de Hoffmann, 2007), and it is represented by the appearance of a peak at a certain time. The comparation of the results with masses and peaks furnished by the calibrating product allow the identification of the compound of interest.

Combination of two or more MS experiments (MS/MS) allow to measure the fragmented isolated ions and select representative ion transitions to achieve better selectivity and sensitivity. In the most common tandem mass spectrometry experiment a first analyser is used to isolate a precursor ion, which then undergoes spontaneously or by some activation a fragmentation to yield product ions and neutral fragments. A second spectrometer analyses those product ions (Liu *et al.*, 2017).

The limit of detection (LOD) is the smallest concentration of analyte in the test sample that can be reliably distinguished by a certain analytical method from the signal of analytical noise, which is produced in the absence of analyte. To determine this term is basic to know the signal of the analyte, for that a sample of known toxin content at low concentration is previously used. Similarly, there is the concept of limit of quantification (LOQ), that is the lowest concentration of analyte that can be determined.

For the quantification of the toxin present in a sample, a calibration line of known range is used, always including a minimum of points that inferiorly encompass the established LOD. The results of the samples must be within this curve, so that whether they are lower or higher,

the samples must be concentrated or diluted, respectively. However, quantification of toxin analogues is not sufficient for toxicity evaluation since the different analogues have different toxic potencies.

HPLC technology is widely used for the analysis of a large variety of samples. Regarding marine toxins, this type of techniques allows generally the identification of each toxic analogue as well as its quantification present in samples of water and shellfish. The analysis of samples from *in vivo* studies, such as organs, urine, feces and blood allow to determine the dynamics of absorption and excretion, as well as the distribution in the different organs of the mouse. Metabolic transformation can also occur being able to lead to compounds of different toxicity of the original.

1.2.3 Toxicity Equivalency Factors (TEFs)

Marine toxins may appear both together with several analogues from the same group or with toxins from other groups. The different analogs within the same group can show a varied toxic potency. Therefore, for the correct evaluation of the total toxicity in function of one of the toxins of the group. Thus, the Toxicity Equivalency Factors must be calculated in order to obtain a good estimate of the impact of marine biotoxins on human and environmental health. TEFs are the toxicity ratio of a compound from a chemical group that shares the same mode of action of a reference compound in the same group. Therefore the toxicity of the toxin analogue is expressed as a fraction of the toxicity of the reference compound in terms of potency, defining the amount of compound required for a certain effect (FAO/WHO, 2016). The knowledge of the concentration of each compound present in a sample of water or shellfish and its multiplication by the corresponding TEF value allows to determine the total toxicity of a sample.

Because until recently the MBA was the reference method for the study of OA and analogues, TEFs values corresponding to this technique, together with the PP2A inhibition assay are also available (EFSA, 2008). By them, OA and DTX1 have been given the same toxic potency. However, the TEFs values established in agreement with cell based assays reveal differences adjudicating the DTX1 with respect to the OA, a relative power slightly superior to the triple according to cytoxicity tests and also superior in paracellular permeability tests but with greater variability (EFSA, 2008). As for the DTX2, the evaluation tests are very scarce, but for the previous ones, a power of about half of that shown by the OA has been obtained. The comparison of the obtained results can give rise to differences of toxicity according to the technique used, making it possible to underestimate the real toxicity of a sample and thereby compromising human health. Therefore, the marine biotoxins require a correct evaluation of oral toxicity to estimate the TEF values, since an approximation from other techniques seems that they could not provide realistic results.

Oral toxicity studies in animals of each and every one of the analogs are actually considered to be most relevant for updating TEFs, in absence of human data, since it is the route of introxication with most marine toxins. Then, i.p. toxicity, mouse bioassay and *in vitro* data are the following considered in this order of importance (FAO/WHO, 2016). Oral toxicity studies are based on acute toxicology and the LD_{50} is the parameter mostly used to estimate the toxic potency. However, oral toxicity data are not available for many of the marine biotoxins, principally due to the previous trend to the i.p. evaluation and the low availability of toxic material. In cases where oral toxicity has been studied, results obtained without certified standards are often found. In addition, some comercial supplies must be checked to confirm their purity, as they could provide quantification errors (Vieira *et al.*, 2015). The expresion of

toxicity by means of an ambiguously defined parameter is another major problem. This is generalized to all groups of toxins, but the results are especially controversial in the case of DSPs (FAO/WHO, 2016), principally between the OA and DTX1 analogues. For TTX, there are no previous studies of acute oral toxicity of the toxin.

2 OBJETIVES

The present doctoral thesis aims to evaluate the toxicity of the groups of marine toxins found most frequently in the Galician coasts and those which recent arrival has been documented. To this end, different *in vitro* and *in vivo* techniques were used to study their toxicity, allowing us to focus on the following specific objectives:

- Establish the flocculation power of several clays in order to develop a system that eliminates dinoflagellate cells from the marine environment.

- Determine the intestinal permeability of AZA1 using an *in vitro* human intestinal model.

- Study the relationship between the enteric nervous system and the intestine in the diarrheal effect of OA by establishing a coculture with two human models *in vitro*, one intestinal and one neuronal.

- To evaluate the acute oral toxicity of DSP toxins: OA, DTX1 and DTX2 in order to establish the TEFs.

- To study the acute oral toxicity of TTX *in vivo* in order to determine LD_{50} and NOAEL as well as the macroscopic and ultrastructural effects produced by the toxin.

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3 RESULTS

The results obtained in the present doctoral thesis are recorded in this section, along with the metodology used in the experiments as well as the associated discussions. They were published in different international journals. Due to the thematic of the studies, the articles are grouped in different blocks of content.

3.1 SECTION I: APPLICATION OF NATURAL CLAYS IN THE ELIMINATION OF TOXIC PHYTOPLANKTON IN SEAWATER

Article 1. Study of Adsorption and Flocculation Properties of Natural Clays to Remove *Prorocentrum lima*. Maria Carmen Louzao, Paula Abal, Diego A. Fernández, Mercedes R. Vieytes, José Luis Legido, Carmen P. Gómez, Jesus Pais and Luis M. Botana. *Toxins* **2015**, *7*, 3977-3988; doi:10.3390/toxins7103977.

Summary:

Harmful algae blooms (HABs) represent one of the most important sources of contamination in marine environments, as well as a serious threat to public health, fisheries, aquaculture-based industries, and tourism. Therefore, methods that effectively control the phytoplankton populations with minimal impact on marine ecology are required. In the present work the adsorption and flocculation properties of different clays in seawater containing dinoflagellates were evaluated.

The microalgae *Prorocentrum lima* is used in this study. This dinoflagellate can produce OA and DTXs, the marine biotoxins responsible of the human intoxication identified as diarrhetic shellfish poisoning (DSP).

Clays under study were selected for the specific surface and the particle size, distinguishing bentonite and kaolinite. The physical and chemical characteristics of natural clays provide them with absorptive properties that can be applied in the remove of microalgae. The strategy is based on cell removal, a function that is favored by the large specific surface area and high cation exchange capacity shown by natural clays particles.

The experimental procedure consisted in a flocculation assay, in which different water columns were set up with seawater, the corresponding clay and the dinoflagellate culture. The mixtures were allowed to settle by gravity and samples were taken at different times of the experiment to performe the cell count. Bottom samples of each water column were taken at the end of the experiment to visualized them with the microscope.

The experimental results confirmed the cell removal through the flocculation of algal and mineral particles leading to the formation of aggregates, which rapidly settle and further entrain cells during their descent. Cell counts shows the adsorption of *P. lima*. The cell removal efficiency seems to be related with composition and particle size of clays. The adsorption capacity of particles decreases substantially with the increase of size, being sodium bentonite and kaolinite the most effective. Furthermore, in our experiments the removal efficiency of all the clays were close to 90% after the first 30 minutes except for bentonite > 45 µm. The microscopy images of the samples enable to observe the clays forming aggregates of two or

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more cells where the mineral particles were bound to the outer membranes of the dinoflagellates, especially kaolinite. Therefore, this preliminary data offers promising results to use these clays for the mitigation of HABs.

Article 1 link: https://www.mdpi.com/2072-6651/7/10/3977

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3.2 SECTION II: *IN VITRO* STUDY OF THE EFFECTS INDUCED BY MARINE BIOTOXINS.

Article 2. Absorption and Effect of Azaspiracid-1 Over the Human Intestinal Barrier. Paula Abal, M. Carmen Louzao, María Fraga, Natalia Vilariño, Sara Ferreiro, Mercedes R. Vieytes and Luis M. Botana. Cell Physiol Biochem 2017; 43:136-146; doi: 10.1159/000480331.

Summary:

Azaspiracids (AZAs) are marine biotoxins produced by dinoflagellates from *Azadinium* and *Amphidoma* genera, triggering azaspiracid poisoning (AZP). Currently the presence of AZAs is widespread in bivalves from different coastal regions of Europe having a significant economic and health impact. Thus, elucidation of its biological targets and effects in humans to protect shellfish consumers is demanded.

The main aim of the present study was to examine the consequences of human exposure to AZA1 by the study of absorption and effects of the toxin on Caco-2 cells, an *in vitro* model of human gastrointestinal tract. First, it was determined if AZA1 was cytotoxic using the cell viability assay AlamarBlue. The ability of AZA1 to cross the intestinal epithelium was evaluated by transepithelial permeability assay using Caco-2 cells and an accurate well-characterized toxin standard. Changes induced by AZA1 in the integrity of the Caco-2 cells monolayer were evaluated thought measurement of the transepithelial electric resistance (TEER). The passage of the toxin through the cell monolayers was quantified by microsphere-based immunoassay and Luminex 200™. Furthermore, cell alterations and ultrastructural effects produced by AZA1 have been observed with confocal and Transmission Electron Microscopy (TEM).

AZA1 was absorbed by Caco-2 cells evidenced by a time-dependent decrease of toxin in the inserts and increase in the wells. This absorption correlates with a slightly TEER decrease statistically significant in cells after 12 hours of toxin incubation without affecting cell viability at any concentration or incubation time tested. The possible changes in the integrity of the monolayer were corroborated with the changes in the distribution of the occludin detected by confocal microscopy imaging, indicated a possible monolayer integrity disruption. This transmembrane protein showed irregular cell borders with indentations between cells and loss of the netlike morphology at some points. In addition, in some cells occludin was relocalized from TJ to the cytosol. However, TEM imaging revealed tight junctions perfectly shaped and microvilli morphology similar to control samples. Instead, ultrastructural damages at the nucleus and mitochondria with autophagosomes in the cytoplasm were detected.

These results revealed that after the ingestion of molluscs with the AZA1, the toxin crosses the human intestinal barrier and passes to the blood without altering the cell viability but causing ultrastructural changes on epithelial cells.

Article 2 link: https://www.karger.com/Article/FullText/480331

Article 3. Diarrhetic effect of okadaic acid could be related with its neuronal action: Changes in neuropeptide Y. M. Carmen Louzao, Diego A. Fernández, Paula Abal, Maria Fraga, Natalia Vilariño, Mercedes R. Vieytes, Luis M. Botana. Toxicology Letters 237 (2015) 151–160; doi: 10.1016/j.toxlet.2015.06.004.

Sumary:

The enteric nervous system (ENS) plays a crucial role in the regulation of the gastrointestinal tract, being able to affect the epithelial barrier functions by neuropeptides production. Hereby, a relationship could exist between ENS and the effect of OA and DTXs, since diarrheic shellfish poisoning (DSP) they trigger in humans and animals cause gastrointestinal symptoms. In this work, we study the effect of OA in an *in vitro* model that simulates the intestinal barrier. We use a two-compartment co-culture that contains neuronal cells (SH-SY5Y) and a monolayer of intestinal epithelial cells (Caco-2). This cocultured system allow to examine the cytotoxic effect of the toxin, the integrity of cells monolayer, the intestinal permeability of OA and the levels of NPY.

The results indicated that OA is not cytotoxic for Caco-2 cells. However, both OA and DTX1 clearly decreased the viability of SH – SY5Y cells in a time and dose-dependent manner, DTX1 being 4 to 5 times more potent than OA. Almost all the OA treatments decreased TEER significantly and progressively when incubated for 12 h in coculture indicating an alteration in the stability of the Caco-2 cell monolayer. In addition DTX1 is 15-18 times more potent than OA reducing TEER. The occludin immunofluorescent staining in Caco-2 cells incubated with OA presented a redistribution of occludin with diffuse staining and granular appearance for the highest concentrations of the toxin.

The transepithelial permeability assays revealed that OA crosses the Caco-2 monolayer specially in treatments of higher doses and longer incubation time coinciding with the greater reduction of TEER. However, no differences were observed between coculture from those of the Caco-2 cells.

NPY levels of SH-SY5Y in the coculture and SH-SY5Y culture after OA treatment revealed an increase in NPY with low OA concentrations but a reduction of this NPY with highest one. Also when OA induced significant decrease in TEER there was an important pass of the toxin through the cell monolayer which was associated to a decrease in secreted NPY. Then, OA cross the epithelial monolayer and modulates the NPY secretion by neuroblastoma cells, which also affects the permeability of OA. This offers a novel approach to establish the influence of OA neuronal action on their diarrheic effects through a cross talk between ENS and intestine via OA induced NPY secretion. Therefore, the OA mechanisms of toxicity that were long attributed only to the inhibition of protein phosphatases, would require a reevaluation.

Article 3 link:
https://www.sciencedirect.com/science/article/pii/S0378427415002143?via%3Dihub

3.3 Section III: Acute oral toxicity of diarrheic biotoxins OA, DTX1 and DTX2 with high incidence in the Galician 'Rias' and TTX, an emerging toxin in the European coasts.

Article 4. Characterization of the dinophysistoxin-2 acute oral toxicity in mice to define the Toxicity Equivalency Factor. Paula Abal, M. Carmen Louzao, José Manuel Cifuentes, Natalia Vilariño, Ines Rodriguez, Amparo Alfonso, Mercedes R. Vieytes and Luis M. Botana. Food and Chemical Toxicology 102 (2017) 166-175; doi: 10.1016/j.fct.2017.02.023.

Sumary:

Toxicological information related to DSP toxins has been obtained mainly with OA, the representative compound and one of the most frequent marine toxins, so the information about DTX2 toxicity is scarce. The official control method of DSP toxins in seafood is the LC-MS, which provides accurate quantitation of the toxin analogues. However, for risk assessment this analytical method requires applying the relative toxicity of each compound (Toxicity Equivalency Factor, TEF) to estimate the total toxicity of a sample, and one of the parameters often used to determine it is the lethal dose (LD). Therefore, the aim of this study is to characterize the acute oral toxicity of DTX2 in mice, since this type of study was considered the most relevant for updating TEFs both for toxicity and bioavailability. The method selected to study the acute toxicity of DTX2 was a 4-Level Up and Down Procedure (4-Level UDP), following the guidelines of the OECD. Furthermore, DTX2 effects such as feeding activity, symptoms, pharmacokinetics and histopathological changes were characterized. *In vivo* studies were performed with Swiss female mice housed under controlled conditions. Overnight fasted mice were administered with the toxin by gavage (10 mL/kg) and placed individually in metabolic cages for the following 24 h with free access to chow and water.

The 4-level UDP was set up starting at 2000 µg/kg b.w. DTX2 induced a dose-dependent mortality, with an oral LD_{50} estimated of 2262 µg/kg b.w. Considering the oral LD_{50} previously published for OA, DTX2 is less toxic than OA with a TEF value of 0.4. Diarrhea was a quite common symptom, usually occurring between the 30 min and the first hour. Symptoms such as apathy, motionless or reduced level of spontaneous activity were observed. The food and water intake were also reduced, with total suppression in mice treated with concentrations higher than 2250 µg/kg b.w. However, the reduction of body weight observed was non-statistically significant in comparison to control animals.

Necropsy was carried out in all animals immediately after death or euthanasia in mice that survive the whole treatment. Stomach and small and large intestine were the main organs macroscopically damaged. The dilated stomach with partially digested food can be crucial for the hypophagic effect of DTX2. Hypersecretion in the small intestine is related to diarrhea. TEM studies demonstrated unaltered density and height of the microvilli and tight junctions were not disrupted in both intestines, disconnecting the diarrheic effect of DTX2 from paracellular epithelial permeability. Ultrastructural evaluation of intestinal cells indicates that DTX2 induces a remodeling of the endocytotic organelles that can affect the absorption of the toxin. In hepatocytes the lesions manifest as presence of autophagosomes and absence of the glycogen granules.

DTX2 passed the gastrointestinal barrier, was incorporated into the bloodstream and circulates throughout the body being detected in urine and feces by LC-MS/MS analysis, with higher excretion on the last. The toxin detected in urine and feces samples from the first hour to 24 h revealed continuous toxin elimination. This article describes the oral toxicity of DTX2

with the LD_{50} (2262 µg/kg b.w) indicating that this compound is less toxic than OA, therefore a reassessment of the TEF is suggested.

Article 4 link:
https://www.sciencedirect.com/science/article/pii/S0278691517300698?via%3Dihub

Article 5. Toxic Action Reevaluation of Okadaic Acid, Dinophysistoxin-1 and Dinophysistoxin-2: Toxicity Equivalency Factors Based on the Oral Toxicity Study. Paula Abal, M. Carmen Louzao, Toshiyuki Suzuki, Ryuichi Watanabe, Natalia Vilariño, Cristina Carrera, Ana M. Botana, Mercedes R. Vieytes and Luis M. Botana. Cell Physiol Biochem 2018; 49:743-757; doi: 10.1159/000493039.

Sumary:

TEF values established by EFSA for OA and DTXs were based on the relative inhibitory effect on protein PP2A and on acute i.p. toxicity in mice. The purpose of this work is to study the oral toxicity of OA and DTX1 since in the absence of information on intoxications in human these are the most appropriate data to establish TEF.

In vivo studies were performed with Swiss female mice housed under controlled conditions. Overnight fasted mice were administered with toxin by gavage and placed individually in metabolic cages for the following 24 h with free access to chow and water. Acute oral toxicity was studied by a 4-level UDP with the starting dose of 1000 µg/kg b.w. Symptomatology associated to oral intoxication and mice mortality were recorded and macroscopic organ damage and ultrastructural alterations in tissues were evaluated during necropsy and through TEM, respectively. The absorption and excretion dynamics were evaluated by LC-MS/MS analysis of urine, feces and blood samples. Finally, the *in vitro* potency of the OA, DTX1 and DTX2 was measure by PP2A inhibition assay.

Diarrhea is the main symptom that manifests itself in all mice treated with toxins. This symptom appears in most cases in less than an hour and disappears between 3-4 h postdosification. The reduction in food and water consumption was statistically significant and body weight decrease was observed in mice that received OA or DTX1.

Stomachs of mice administered with DTX1 or OA were swollen with liquid and gas content, indicating an inhibition of the gastric emptying. Animals treated with OA presented inflammation and fluid accumulation in the small intestine. Those treated with high doses of OA and DTX1 had dilation of the large intestine. Both alterations indicate that intestinal motility and secretion is altered.

TEM analysis revealed a decrease in glycogen granules and mitochondria alterations in liver cells of mice treated with OA or DTX1. The brush border in small and large intestine appeared with shortened, scarce and disorganized microvilli, with alteration of the terminal web but undamaged tight junction. Therefore the accumulation of fluids in the intestine and diarrhea is not related with paracellular pemeability.

OA and DTX1 have different toxicokinetics and toxic potency although diarrhea is similar. Toxins detected in urine were lower than in feces. OA had a continuous and dose-dependent excretion in feces while DTX1 excretion was irregular and always lower than that of OA at the same administration doses, suggesting a higher intestinal absorption of DTX1 than OA. The absorption of the toxins was checked with the detection of OA and DTX1 in blood at 24h.

Our *in vivo* results allow to estimate oral LD_{50} for OA and DTX1 as 760 and 487 µg/kg b.w respectively, determining an order of potency of DTX1 > OA > DTX2 corroborated with the inhibitory potency on PP2A. Considering the OA as a reference compound, the TEF values derived from oral toxicity are OA = 1, DTX1 = 1.5 and DTX2 = 0.3. This is the first comparative study of acute oral toxicity of DSP toxins. The results indicate that DTX1 is more toxic than OA orally while DTX2 is the least toxic. Thus, the current TEFs should be modified and the accepted toxic mode of action reevaluated.

Article 5 link: https://www.karger.com/Article/FullText/493039

Article 6. Acute Oral Toxicity of Tetrodotoxin in Mice: Determination of Lethal Dose 50 (LD50) and No Observed Adverse Effect Level (NOAEL). Paula Abal, M. Carmen Louzao, Alvaro Antelo, Mercedes Alvarez, Eva Cagide, Natalia Vilariño, Mercedes R. Vieytes and Luis M. Botana. Toxins 2017, 9, 75; doi:10.3390/toxins9030075.

Sumary:

TTX is emerging on European coasts. Its presence in molluscs is quite recent, increasing the risk for seafood consumers since there is no legal limit for TTX content in seafood within the European Union. Thus, information on the oral toxicological properties of TTX is needed.

A 4-level UDP was designed in order to determine for the first time in mice the oral lethal dose 50 (LD_{50}) and the NOAEL using a well characterized standard of TTX. For that, Swiss female mice were fasted overnight prior to dosing by gavage (10 mL/kg). Then, mice were individualy caged for the following 2 h with free access to chow and water. Symptoms were observed during the whole experiment. Organs alterations were recorded during necropsy and blood samples were collected for toxin analysis.

The starting dose of the modified UDP was 1000 µg/kg b.w. The oral LD_{50} and oral LD_{100} of TTX were 232 and 1000 µg/kg b.w, respectively. At this last dose, mice died within minutes usually by cardiorespiratory failure. Our results indicate that symptoms are dose-dependent. These data are supported by the detection of TTX in blood by LC-MS/MS analysis. Apathy was a symptom quickly and more frequently observed. Over time, mice suffered paralysis of extremities and seizures. However, mice treated with doses lower than 125 µg/kg b.w survived the whole experiment without suffer signs of toxicity, allowing to define the NOAEL for TTX as 75 µg/kg b.w.

The LD_{50} and NOAEL values specified in this study can be used in risk assessment processes to determine the levels of TTX allowed in shellfish in the European Union.

Article 6 link: https://www.mdpi.com/2072-6651/9/3/75

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Article 7. Acute Toxicity Assessment: Macroscopic and Ultrastructural Effects in Mice Treated with Oral Tetrodotoxin. Paula Abal, M. Carmen Louzao, Natalia Vilariño, Mercedes R. Vieytes and Luis M. Botana. Toxins 2019, 11, 305; doi:10.3390/toxins11060305.

Summary:

The recent geografical expansion of TTX with new bearing organisms entail an emerging risk for human safety. Limited toxicological data are available at present. The oral LD_{50} was recently proposed, however there is no information available on organ and tissue damages derived from acute oral toxicity. The present study focuses on the macroscopic and ultrastructural alterations in mice derived from TTX oral exposure.

Swiss female mice were treated with different concentrations of TTX according to a 4-level UDP specifically designed for this toxin. Oral administration was done by gavage (10 mL/kg) with a starting dose of 1000 µg/kg b.w. Organs were examined during necropsy and samples of them were separated for their fixation and analysis by TEM.

Necropsy revealed swollen abdomen with liquid content, afecting stomach and small intestine, except in those mice treated with the highest dose, maybe due to the rapid death of these individuals. With regard to TEM, affectation of the gastrointestinal tract was most remarkable. However, despite the macroscopic damage found in stomach, no ultrastructural alterations were detected in this organ. Increase of lipid droplets in hepatocytes was detected while the rest of the organelles remain unchanged. Electron-dense granules inside the matrix were present in mitochondria of small and large intestine together with swelling of rough endoplasmic reticulum. Nevertheless, brush border mantains its structure, even the tight junctions. In spleen, mitochondria were swollen with disintegration of cristae and matrix. Nuclei and cellular membranes were not altered in any of the studied organs.

Affectations detected in the gastrointestinal tract suggested that they must be taken into account when evaluating human TTX poisoning.

Article 7 link: https://www.mdpi.com/2072-6651/11/6/305

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4 DISCUSSION

Algal blooms are massive proliferations of phytoplankton that occur naturally. They have increase their frequency, intensity and severity in the last decades due to climate change and the anthropogenic activities, that had favored the geografical expansion of the microalgae in the different aquatic environments (Bentur, et al., 2008; Botana, 2016; Hallegraeff, et al., 1992). They suppose a greater problem when toxin-producing microalgae are involved due to the dangerous poisonings they can cause. In aquatic systems, phycotoxins may be release into water or remain contained within the producing organisms. In both cases, they can be captured and transferred through the food web (Doucette, et al., 2006). The natural filter feeding activity of shellfish promotes the toxin accumulation inside different tissues of their bodies, and although the intake of producing organisms by herbivorous fish has also been described, shellfish is the most common vector of toxins transmission in humans. Different poisoning syndromes have been described in human according to the ingested toxin, leading to an important risk to public health as well as a significant negative impact on the fishing industries and tourism. There are monitoring programs that detect the presence of toxins and prevent the entry of contaminated seafood into the market, thus guaranteeing the health of the consumer. For that, health authorities have regulated the maximum level allowed for some groups of toxins (2008) and the official detection methods that should be used. However, the new advances and the appearance of toxins colonizing new habitats requires constant research from the control in the environment to the laboratory to get an updated risk characterization.

The occurence of HABs is directly related to the increase of nutrients, and Galician “Rías” are a clear example of this relationship. This region received large amount of nutrients by direct penetration of the upwelled seawater (Barciela, et al., 1999) and the abundance of phytoplankton makes this area show great seafood wealth. The lipophilic toxins are the most frequently detected in this region, in particular those belonging to DSPs and AZAs groups. They were responsible of closures in cultured zones in throughout the year both in floating marine farms and infaunal molluscs (Instituto Tecnolóxico para o control do medio mariño de Galicia) (Instituto Tecnolóxico para o control do medio mariño de Galicia), resulting in significant economic losses to shellfish industry and a significant risk to human health.

Strong atmospheric pressure gradient (Fraga, et al., 1993) and quatorward winds along the coast favor the upwelling of nutrient-rich water subsurface and enable the arrival of the responsible species for bloom from the open sea and finally its proliferation and accumulation in the coastline. Therefore it does not seem unreasonable to think that a prompt action on this accumulation of phytoplankton could minimize the risk in the face of complete exposure. However, the development of effective methods to achieve this is presented as one of the most challenging aspect for HAB management and although several strategies have been studied most of methods remain at the laboratory scale (Magdaleno *et al.*, 2014; Mohamed *et al.*, 2014).

Methods for HABs mitigation encompass biological, genetic, environmental, mechanical and chemical control (Anderson, 2009). The first two are based on the release of organisms (Mohamed, et al., 2014), either predatory or genetically engineered, acting directly against harmful phytoplankton. Environmental manipulation seeks to disfavor the growth of the target species by altering nutrient levels, water circulation or aeration. The chemical control is based on the toxic chemical release (Nagai *et al.*, 2016) and together with the aforementioned methods could undoubtedly had negative consequences leading to changes in the composition of the

phytoplankton community and even effects at higher levels of the trophic web. In comparison, the mechanical method could be the most innocuous. It is based on remove algal cells from the water column by different actions, such us cell harvesting, sediment resuspension or flocculation.

Both chemical and mechanical methods have been the only applied *in situ* against HABs. The first was based on cooper addition (Rounsefell, et al., 1958), while the second type used ultrasonic radiation (Nakano *et al.*, 2001), or clays (Sengco, et al., 2005). However, the difficulty of finding a species-specific chemical compound environmentally safe makes mechanical methods appear as the most promising method, specifically the flocculation processes. This method is a physical process in which aggregates of algae and clay particles are formed by dispersing clay over the water surface. Collisions and consequent adhesions, cause them to sink and settle to the ocean floor. Thus, we perform a flocculation assay with the microalgae *Prorocentrum lima* as representative producer of OA and DTXs, toxins responsibles for the DSP. This dinoflagellate is a marine benthic microalgae, so it tends to sedimentation since these microorganisms live attached to various surfaces, such as rocks, sediments or other organisms. In our experiments, *P. lima* cells cultured under controled conditions during 30 days, which is the time needed to reach maximum toxin production (Pan *et al.*, 2006; Wang *et al.*, 2015a). Then, the dinoflagellate culture was incubated in seawater with different clays, simulating the natural occurence of algal blooms in the marine environment. As flocculating agent we used five natural clays. The cell removal efficiency (RE) of clays seems to be related with its composition, particle size, specific surface area and surface charges (Li *et al.*, 2013; Qi *et al.*, 2014). Furthermore, it is necessary to take into account that in addition to the characteristics of clays, the concentration of its particles and the cellular concentration of the microorganism are decisive.

Our results showed cell RE almost completed at 30 min, in accordance with those results obtained by Wu *et al.*, who reported at the same time slightly lower adsorption of some microcystins (MC-LR and MC-RR) on natural sediments including kaolinite (Wu *et al.*, 2011). Furthermore, the RE after 1 h was higher than 95% with four of the five natural clays used in our experiments. However chitosan-modified sepiolite needs 8 h to reach the same RE value (Pan, et al., 2006). The smaller particle size of clays seems to be responsible of the highest RE observed (Qi, et al., 2014). Thus, kaolinite and bentonites with small particle size could be used as a flocculating agent in the marine environment due to the good adsorption results obtained. Natural or modified clays have already been applied in several coastal waters in Korea and Japan (Na, 1997; Shirota, 1989). For this it is necessary to study the safety of clays, a characteristic that seems to have those used in our study, since microscopy imaging revealed that most of the immobilized cells remained alive. However, this could be a disadvantage in the case of phytoplankton cells with movement capacity, as they could be released and resuspended in water.

The collection of the toxic phytoplankton from the environment or the toxins they produce makes it possible to isolate this toxic material to perform different experiment and thus evaluate the effects derived from exposure to these compounds. Both *in vitro* and *in vivo* studies are necessary to obtain more complete information, since they complement each other and some can supply the limitations of the other. The current trend is to prioritize *in vitro* studies over *in vivo* studies, due to the ethical and economic considerations that the latter entail. The wide variety of available *in vitro* techniques allows studying the effects of these toxins or other compounds on a wide variety of targets and cellular routes.

As previously described, the toxins that are found most frequently in Galicia are lipophilic toxins, especially OA and its derivatives and AZAs. Both groups of toxins are ingested through

the diet, so the evaluation of their intestinal absorption is key to know the effects derived from exposure by eating contaminated food, especially shellfish. If the toxins permeabilize through the intestinal barrier, these can reach a greater extent to the rest of the organs of the body through the bloodstream, being able to exert diverse toxic effects. *In vivo* studies have shown that AZAs are easily absorbed when they are acquired orally (Aasen, et al., 2010; Aune, et al., 2012), causing gastrointestinal symptomatology. Its toxicokinetic evaluation shows that after 24 hours of its oral administration AZAs can be detected in addition to the organs of the gastrointestinal tract in kidneys, lungs and heart (Aasen, et al., 2010; Aune, et al., 2012). There is great uncertainty surrounding the mechanism of action of AZAs, despite the fact that several toxic effects have been described. In our work, besides to assessing the intestinal permeability of AZA1, the way in which this toxin is able to cross the intestinal barrier as well as the effects derived from its exposure are studied. Therefore, the evaluation of intestinal permeability is crucial to know the state of the membrane and its possible affectation by different toxins, especially those that cause diarrhea. In our experiments with AZA1, we selected the transepithelial permeability assay using the Caco-2 human carcinoma cell line, a model frequently used to evaluate the passage of drugs that reach the intestine after oral ingestion (Artursson, et al., 2001; Hubatsch *et al.*, 2007).

In view of the fact that affectation of the cell viability was not seen, the reduction of TEER values and the consequent time and dose-dependent AZA1 permeation suggested an increase of paracellular pemeability. The correlation between TEER decrease and paracellular permeability was previously described in AZA1 treatments at low toxin concentrations (Hess *et al.*, 2007) and for the lipophilic toxin DTX1 (Fernandez, et al., 2014), with no recovery proven in the first case. Paracellular permeability is regulated by TJs, a multiprotein junctional complex. Occludin is an integral transmembrane protein that has been shown important for regulation of paracellular permeability of epithelial monolayers (Al-Sadi *et al.*, 2011; Shiobara *et al.*, 2013). In our hands, this protein was irregularly distributed after AZA1 treatment, suggesting a possible disruption of the TJs structure. However, TEM imaging showed them apparently undamage. Some studies have shown that mice lacking occludin still maintain normal barrier function of intestinal epithelium, with TJs apparently unaffected morphologically (Saitou *et al.*, 2000). Thus, the structure even the function of the TJs does not depend only on the occludin and other components would have to be studied. Furthermore, upregulation dose-dependent of protein claudin-2 was also described after AZA1 treatment (Hess, et al., 2007). This protein is also part of the complex of proteins that structure TJs (Rosenthal *et al.*, 2010) and its increased expression is a frequent regulatory event during intestinal inflammation (Luettig *et al.*, 2015). Both the occludin and the claudins are anchored to the ZO proteins, to which the actin filaments are attached. Disorganization of actin filament bundles in Caco-2 cells after AZA1 treatment was previously described. However, the amount of polymerized actin was no affected, it was only displaced from the edges to the cell centre (Vilariño, et al., 2006), similar to that seen by us for occludin. This disorganization was related to occludin internalization by several different endocytic pathways as initial barrier loss, leading to the rupture of occludin interactions with ZO-1 (Yu, et al., 2008).

The migration of occludin and other TJs proteins into the cytoplasm has also been shown *in vitro* and *in vivo* in response to external stimuli like calcium depletion (Ma, et al., 2000) and cytokines (Van Itallie, et al., 2010), with the consequent intestinal TJ permeability alteration.

AZA1-3 were shown to elevate cytosolic calcium by its release from internal stores what origins Ca^{2+} influx from extracellular medium (Roman, et al., 2002; Roman *et al.*, 2004). This cytosolic calcium is an important secondary messenger involved in a variety of pathways. This ion was further related with the two types of programmed cell dead: apoptosis and autophagy,

sharing some of the signaling pathways (Bootman *et al.*, 2018; Giorgi *et al.*, 2012). Mitochondrial elongation, upregulation of energy metabolism and the presence of autophagosomes detected by TEM are related with an autophagic process (Furey *et al.*, 2010; Gomes *et al.*, 2011; Kellmann, et al., 2009). However, the observed nucleus damages could be related with the first stage of an apoptotic process, whence transition between autophagy and apoptosis could be happening (Thorburn, 2008). Despite these observed ultrastructural alterations in Caco-2 cells, the microvilli and intact TJs suggest that the indications od a possible autofagic process is unleashed may actually be an attempt to maintain cell survival (Gomes, et al., 2011).

After the *in vitro* evaluation of the effects derived from exposure to a marine toxin, *in vivo* experimentation is necessary to obtain a rough extrapolation of the toxicological data at the human level, specifically those derived from the administration by the oral route in mice (Botana, 2012). The incorrect estimation of the toxic dose could cause great problems both by underestimation and overestimation. It might seem that an overestimation could protect the consumer to a greater extent since he would be exposed to a lower risk by indicating a toxic dose lower than the real one. However, as regards the toxins that are commonly found in the Galician Rías, even though they entail risks to human health they do not cause mortality. An overestimation of the risk would undoubtedly be a great detriment to the seafood markets. The closure of production areas by levels of toxin lower than those that really represent a risk leads to significant economic losses for the fishing industry. Another important risk is the appearance of toxins dangerous to health that are not normally on our coasts, as is the case of TTX. (Leão, et al., 2018), the TTX. This toxin together with its analogues involve great risks to human health, in comparaison with DSP toxins, including among them a high mortality rate.

Each group of marine toxins consists of a series of analogues whose presence and abundance may vary according to the geographical area (Anderson D. M., 2017; Instituto Tecnolóxico para o control do medio mariño de Galicia). They can have very different toxic potencies, so a correct individual evaluation is necessary that includes a detailed analysis of the natural sample that contains them as well as *in vitro* and *in vivo* toxicity studies. For a long time the same toxic potency has been granted to the OA and the DTX1, both always more harmful than DTX2. However, greater toxicity has been obtained recently in mice for DTX1 than for OA (Suzuki, et al., 2018) by i. p. administration (MBA) but this route is not representative of the manner in which DSP intoxication occurs and there is no correlation between the results obtained by MBA and the acute toxicity (FAO/WHO, 2016). The knowledge of the amount of toxin congeners and the relative toxicity of each of the congeners is necessary for risk assessment and management, being studies by oral administration considered to be most relevant for updating TEFs (FAO/WHO, 2016).

Test Guidelines Programme of the OECD has developed standardized methods following the Three Rs that are recommended by various regulatory agencies (OECD, 2008). In our experiments, the acute oral toxicity assessment of marine toxins in mice was a 4-level UDP, which complies with the sought reduction in the number of experimental animals. Our results correspond to the first comparative study of oral toxicity of DSPs toxins carried out in 4 weeks Swiss female mice. We obtained an order of oral toxic potency for DSP toxins of DTX1 > OA >> DTX2, in agreement to that obtained by the PP2A inhibitory assay, with the LD_{50} values of 487, 760 and 2262 μg/kg b.w, respectively. The reduced DTX2 toxicity was previously related with its structure and interaction with the binding site of PP2A (Huhn *et al.*, 2009). Our obtained oral LD_{50} values were lower than previously reported, maybe due to differences related to the quality of the marine toxins utilized (Aune, et al., 2012; Ito, et al., 1994; Vieira, et al., 2013). Furthermore, they do not correlate with most of those based on i.p. toxicity and therefore the

oral TEFs currently established (EFSA, 2008) need to be reevaluated. Our findings confer greater toxicity to DTX1 and lower to DTX2 of those described in comparison with OA. Thus, the oral TEFs derived from LD_{50} values obtained are: OA = 1.0, DTX1 = 1.5 and DTX2 = 0.3. This is the first time that TEFs for DSPs are proposed based on oral toxicity *in vivo*. This concept has been developed to support risk assessment of specific complex mixtures and according to our results the toxicity of DTX1 has been underestimated all this time, attributing the same TEF to OA. The importance of the reevaluation of TEF lies not only in the danger of poisoning, but also in the consequences for the market because the underestimation or overestimation of toxicity will vary the limits allowed and therefore this will have a direct impact on the permitted catches. While the highest toxicity of DTX1 could cause a greater impact in the aquaculture industry in Japan, Canada and Norway where this analogue is more prevalent (Lee *et al.*, 1989; Smayda, 1997), the decrease of DTX2 TEF value respect to the currently granted by EFSA would be a great benefit for shellfish producers in Galicia, since this analogue is commonly found in its waters and also in Portugal and Ireland (Hu, et al., 1992; Vale *et al.*, 1999; Villar-Gonzalez *et al.*, 2007).

The severity of toxicity of the different marine toxin groups lies mainly in their mechanism of action and bioavailability. As we previously commented, the DSPs have shown to have very different effects depending on the studied cell type (Chen, 2011; Opsahl, et al., 2013; Pshenichkin, et al., 1997; Valdiglesias, et al., 2011; Vieira, et al., 2013). The wide variety of effects undoubtedly opens the possibility that DSP toxins affect not only to the PPs, since it has also been described that OA can bind to other proteins in marine organisms (Schroder *et al.*, 2006). The differences in toxicity shown by the DSP group are due to the different analogues toxic potency and the absorption by the gastrointestinal tract in a different degree. In fact, these differences have been previously proposed (Fernandez, et al., 2014) using the same *in vitro* intestinal model. According to this study, DTX1 is able to cross the intestinal epithelium at a concentration to which OA and DTX2 do not. Our results indicate that DTX1 is around 15 - 18 times more potent than OA in causing the descent of TEER after 12 and 24 h of incubation, respectively. In our hands a statistically significant decrease in TEER was observed for Caco-2 monolayers treated with 1250 nM OA, with a clear passage of the toxin from the insert to the well compartment at 1750 nM, OA both in monoculture and in coculture system with the neuroblastoma cell line SH-SY5Y.

A different toxicokinetic pathway between OA, DTX1 and DTX2, is also suggested by our HPLC results, even though the trigger of diarrhea was similar. The intestinal absorption of all the DSP toxins was confirmed by their detection in blood and urine. Instead, the presence of toxins in feces may be due both to non-absorption or to the excretion process occurring after absorption. For all of them lower toxin quantity in urine than in feces samples was found. The rapid onset of the diarrheic process has undoubtedly prevented the total absorption of toxins, which were detected in feces after administration in a period that varied between 20 minutes and one and a half hour for OA and DTX2. However, DTX1 presented greater variability in the time of diarrhea onset, even delaying this symptom until 3 h and even between 12-24 h. Instead, in doses close to the LD_{50} calculated for each toxin the occurrence of diarrhea occurs mainly at the same time as indicated for OA and DTX2. In relation to studies by other authors, great controversy has been found regarding the triggering of this symptom by the OA, absent for doses higher than our OA LD_{50} (Ito, et al., 2002b).

The toxins were excreted in both urine and feces during the whole experiment, as was previously seen for OA (Matias, et al., 1999). Nevertheless the amount of DTX1 excreted in feces and urine was always lower than that of OA and DTX2 at the same administration doses. A continuous excretion was detected in urine for all the toxins. This dynamic was repeated in

feces samples for OA and DTX2, however DTX1 excretion appeared to be increased towards 24 h. Differences in OA and DTX1 content in blood also support a distinct absorption and excretion rate between them, in agreement with the LD_{50} data obtained. The toxin absorption and distribution throught the whole body was previously examined for OA (Aune, et al., 2012), been detected in several organs after 24 h postdosification (Le Hegarat, et al., 2006) and even in intestine four weeks later (Ito, et al., 2002b).

The discomfort of the mice after the administration of the toxins was reflected in different symptoms. Symptoms shown by DSP treated mice were as expected (Aune, et al., 2012 27), being common for all analogues, both specific sings and those that are not. The food and water consumption was reduced with a consequent decrease in body weight. This could be due to both the indisposition of the mice or the feeling of satiety that could caused by the swelling of the stomachs. This affectation agrees without a doubt with inhibition of gastric emptying (Jensen, et al., 2013), when this process is associated with stomach volumen and normally is stimulated by distension (Dixit *et al.*, 2006). Intestines were also dilated with liquid and gas content, to a greater or lesser extent according to the toxin dose. This could be due to alteration of intestinal motility, increasing the time needed for the elimination of toxins and enabling the intestinal reabsorption and enterohepatic circulation of them (Matias, et al., 1999).

Neurotoxic effects induced by OA have already reported (Arias, et al., 1993; He *et al.*, 2001), so the effects observed in mice after OA administration could be due to the intervention of the enteric nervous system (ENS). It plays a crucial role in the regulation of the gastrointestinal tract, through release of several neuropeptides. The enteric neurons express NPY (Cox, 2007), which plays an important role at various levels of the gut-brain axis with very varied effects (Holzer *et al.*, 2012; Longhi *et al.*, 2009; Tan *et al.*, 2018; Ushimura *et al.*, 2015). Both, changes in feeding behaviour, gastric emptying and intestinal motility has been related with NPY levels (Clark *et al.*, 1984; Forbes *et al.*, 2012). Recently, the modulation of diarrheic process has been proposed to swell the list of functions modulated by this neuropeptide, by inhibiting the gastrointestinal motility and preventing the secretion of electrolytes and water in the gut (Holzer-Petsche *et al.*, 1991). This reduction of accumulated intestinal fluid inhibits diarrhea (Moriya *et al.*, 2010) by NPY stimulation of the colonic absorption (Cox, 2007). Furthermore, NPY interaction with the immune system promotes the gastrointestinal inflammation and it has been seen to play a role in pain and depression, protecting from the impact of stress on the gut-brain axis (Holzer, et al., 2012). Thus, the physiological effects of NPY could be inhibited by the action of OA. Our *in vitro* study showed that OA can cross the intestinal epithelial barrier and modulate the NPY secretion in the coculture system and this in turn affects the permeability of the toxin. Therefore, such interaction between toxin and NPY could be related with the diarreic effect.

The rest of toxic actions derived from acute exposure to OA have been related with the inhibition of PP, including the tumour promotion, the neuroroxicity and also the diarrheic process (Arias *et al.*, 1998; Cohen *et al.*, 1990; Haystead *et al.*, 1989). However, there is evidence that this inhibition cannot explain all the toxic effects of this toxin (Espina *et al.*, 2010; Kikuchi *et al.*, 1999; Munday, 2013)

Exposure to the DSP toxins resulted in macroscopic alterations mainly in some organs of the gastrointestinal tract, a not surprising fact since when ingested it is the first contact site. However, other alterations were seen in different organs that corroborate the absorption of the toxins and their distribution by the blood stream, but only in the case of the OA and DTX1. Although these alterations did not appear on a regular basis in mice, the microscopic alterations detected supported them. Cardiomyocytes of mice treated with OA showed disruption of the mitochondria outer membrane, even though rigid hearts were also observed for some treatments

with DTX1. Mitochondrial swelling was seen in spleen and kidney in mice treated with OA and DTX1 and lamellar separation and disintegration of myelin of axons in brain. Matrix swelling and outer membrane rupture, along with loss of ionic homeostasis, was related with the opening of permeability transition pores in the inner mitochondrial membrane, which in turn was proposed as a putative target for cardioprotection (Javadov *et al.*, 2007).

The presence of fluid accumulated in intestine was previously related with erosion of the intestinal tract after administration of low doses of OA (Aune, et al., 2012; Yasumoto, et al., 1985) and maybe with induction of paracellular permeability and diarrhea by OA (Hosokawa *et al.*, 1998) by means of the disruption of TJs in epithelial cells (Munday, 2013). Our *in vitro* studies revealed a progressive redistribution of occludin OA dose-dependent. The modification of the TJ complex has been related with the increase of paracellular permeability through phosphorylation and dephosphorylation of their components and the activity of PP2A (Nunbhakdi-Craig *et al.*, 2002; Seth, et al., 2007). However, our *in vivo* studies showed TJ ultrastructure was undamaged in both small and large intestine, but with affectation of microvilli except for DTX2 treatment. Thus, the absence of alteration of the TJ that we have detected disconnects the occurrence of diarrhea from the destruction of these structures or the increase of the paracellular permeability, unlike what had long been believed.

Impairment of microvilli, mitochondria and RER have been previously documented after OA oral administration (Wang *et al.*, 2012). However, gradually and completely recovery of microvilli at 24 h was described, which could explain the absence of affectation in our trial with DTX2. The scarce and irregular microvilli seen by us could be a consequence of reorganization of the cytoskeleton or the proteins associated with it, as is the case of villin (Khurana *et al.*, 2008) whose down-regulation after OA administration in mice was described (Wang, et al., 2012). The decrease of some proteins related with the cytoskeleton have also been correlated with increased apoptosis in several inflammatory diseases of the gastrointestinal tract (Kersting *et al.*, 2004). Induction of apoptosis by OA was related to increasing reactive oxygen species (ROS) and mitochondrial dysfunction in many cell types (Jayaraj *et al.*, 2009; Xing *et al.*, 2009), with significative alteration of several proteins in OA-treated mice (Wang, et al., 2012).

Affectations seen in mitochondria could be related to actin filaments, as it was proposed they could regulate mitochondrial structure and function, but not through the villin since it is only expressed in epithelial cells, such as intestine and kidney (Wang, et al., 2012). This organelle sypply energy to the cell and its renewal is necessary for the maintenance of cellular homeostasis. The mitochondrial dysfunction can contribute to trigger mitophagy, a selective form of autophagy (Hattori *et al.*, 2014) and even cell death by apoptosis or necrosis (Baines, 2010; Javadov, et al., 2007; Leung *et al.*, 2008). Mitophagy activation could also be related with the reduction of glycogen seen in hepatocytes suggesting a reprogrammation of the metabolic profile to favor certain cellular processes (Esteban-Martinez *et al.*, 2017), as could be cell death. The high energy demand of heart makes this tissue present a high rate of mitochondrial renewal by mitophagy, as protective function for the maintenance of cellular homeostasis (Yamaguchi, 2019). Disruption of the membrane of some mitochondria were seen in cardiomyocytes of OA treated mice, but this did not seem to be enough to trigger the process. Swollen mitochondria were also seen in spleen, kidney, intestines and stomach. Furthermore, in large intestine electron-dense granules inside the matrix of this organelle were detected. They has been related to ion accumulations and the consequent water influx (Pelin *et al.*, 2014).

Dilation of RER was found in small and large intestine of DSP mice, being probably the cause of the widening of the perinuclear space since the ER is contiguous with the outer nuclear membrane. RER is involved in synthesis of proteins, whose retention or liberation is closely related to dilation of the organelle, by variations in synthesis, production or transport

mechanism (Wiest *et al.*, 1990). OA was found to induce arrest of the membrane traffic in Hela and CHO cells (Davidson *et al.*, 1992; Lucocq *et al.*, 1991), a process in which the prevention of the access to COPII structures could be involved (Pryde *et al.*, 1998), as they control the exit of proteins from this organelle. This complex has also been suggested to participate in the formation of autophagosomes just like the actin depolymerization (Wei *et al.*, 2018), providing membrane surface to autophagosomes during its formation (Shima *et al.*, 2019). These membranous structuctures are involved in the degradative pathway of cytoplasmic components and they were seen in small intestine of mice treated with OA and DTX1 and in liver after OA or DTX2 treatment in different phases of development, and surrounding mainly mitochondria. The increase of lipid droplets seen in small intestine of DTX2 treated mice may also be related with the afectation of mitochondria, since autophagosome fuse with the lysosome to form the autolysosome for degradation. The emergence of phagophores has been correlated with the contact sites between ER and mitochondria (Wei, et al., 2018), participating in the expansion of the membrane the RER and the outer membrane of mitochondria, among other organelles. Thus, the intervention of these two organelles in the triggering process of autophagy occurs in several ways (Krols *et al.*, 2016).

TTX has been studied for years mainly in the Asian continent for its common presence in the puffer fish, a culinary species much appreciated by the population (Bane, et al., 2014; Jang, et al., 2006; Mahmud, et al., 2003; Nakamura, et al., 1985). A lethal dose for TTX toxin had been proposed previously (Xu, et al., 2003), however, the absence of description in the methodology and the purity of the toxin used makes necessary a new and detailed assessment. Our results correspond to the first definition of oral LD_{50} and NOAEL value for TTX in mice, being these defined as 232 and 75 µg/kg b.w, respectively. These results have served as the basis for the EFSA opinion report (EFSA-CONTAM, 2017) as well as for the update of the situation on TTX in the Netherlands with the inclusion of the TTX safe limit in the Dutch seafood monitoring program (Gerssen, et al., 2018).

In our hands, the mice treated with TTX showed neurological involvement by the appearance of seizures and paralysis of the extremities, which is not surprising since TTX target Na_v preventing the propagation of action potentials (Lee, et al., 2008). The nine isoforms of these channels are categorised as TTX-sensitive (Na_v 1.1 - Na_v 1.4, Na_v 1.6 and Na_v 1.7) or TTX-resistant (Na_v 1.5, Na_v 1.8 and Na_v 1.9) (Catterall, et al., 1992).

Exposure to TTX in our *in vivo* assays also resulted in macroscopic alterations in some organs of the gastrointestinal tract and hearts. The stomach and small intestine swelling seen seems to be inversely to the toxin dose, so that at lower doses the distension is greater. The gastrointestinal tract is an electrically active tissue, being voltage-sensitive ion channels those that generate and regulate electrical and mechanical activity (Cheng *et al.*, 2010). Multiple Na_v isoforms have been suggested to be present in the gastrointestinal tract (Holm *et al.*, 2002; Smirnov *et al.*, 1992) and they have been proposed to be related with functional and motility disorders of the gastrointestinal tract (Beyder *et al.*, 2012) although the TTX-resistant Na_v 1.5 appears to be the major isoform in the gastrointestinal tract (Beyder, et al., 2012). This isoform is also prevalent in cardiac myocytes (Catterall *et al.*, 2005). Death caused by TTX are mainly due to respiratory and cardiac failure (Noguchi, et al., 2001), which agrees with the appearance of rigid hearts in mice treated with higher doses of the toxin. Ultrastructural effects in spleen, hepatocytes, small and large intestines were also found, despite the short time of exposure to the toxin. However, the TEM study did not reveal any further change in cells of stomach and hearts, in spite of the macroscopic alteration found. This absence of affectation agrees with the lack of affnity for Na_v1.5 sodium channels mainly expressed in this organs (Beyder, et al., 2012;

Catterall, et al., 2005). This absence of microscopic alterations in stomach is consistent with results of chronic treatments with low TTX doses but contrasts with the disintegration of heart myofibrils detected (Boente-Juncal *et al.*, 2019). Until now, TTX poisoning has focused on the evaluation of neurological effects, but in view of our results, gastrointestinal effects should be also considered when evaluating human TTX poisoning.

The present studies will help to complete and update the information available for the marine biotoxins studied in order to minimize the risk to which the consumer may be exposed. These studies also demonstrate the importance of conducting an evaluation that covers all aspects of poisoning, from the presence of phytoplankton in the environment to *in vivo* experimentation, through the no less important *in vitro* studies, always using certified material and the techniques that best fit the reality of intoxication.

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5 CONCLUSIONS

1. The use of natural clays, such as bentonite and kaolinite, represent a promising method to remove the dinoflagellate *Prorocentrum lima* from seawater during HABs

2. AZA1 can cross the intestinal epitelial barrier without affect epithelial cells viability but causing mitochondrial damage and increase of autophagosomes in them

3. OA cross the Caco-2 monolayer, modulates the NPY secretion by neuroblastoma cells and affects epithelial permeability, indicating that the toxicity mechanisms of OA are complex and involve several cell targets

4. The DSP toxins administered by oral route cause very similar symptoms, organic changes and ultrastructural changes. Their toxic potency is DTX1> OA> DTX2, and the TEFs obtained from oral LD_{50} OA = 1, DTX1 = 1.5 and DTX2 = 0.3 are more appropriate to protect the health of the consumer than those based on intraperitoneal toxicity

5. The assessment of acute oral toxicity of TTX indicates that oral exposure to this neurotoxin also causes gastrointestinal effects. In this study, oral LD_{50} (232 µg/kg b.w.) and NOAEL (75 µg/kg b.w.) are first determined, whose values can be used in risk assessment processes that lead to establishing a safe TTX level in shellfish

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