Chapter

Instrumental Methods for Detection of Lipophilic Marine Toxins in Endemic Species from Pacific Austral Fjords

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Abstract

Lipophilic marine toxins (LMTs) are a group of marine toxins which in recent years have been consistently identified in the vast majority of shellfish worldwide. One of their main characteristics is having a latitudinal variability and an assimilation/retention specific for each species. LMTs consist of four important groups: okadaic acid group (OA-group), pectenotoxin group (PTX-group), azaspiracid group (AZA-group) and yessotoxin group (YTX-group). These groups have different chemical structures, which has generated an important challenge to establish analytical techniques to identify all toxic analogues from the same toxic matrix. Likewise, in the aquatic environment, shellfish represent the best bio-indicator model that allows for the establishment of levels of toxicities related to LMTs. In this chapter, the evolution for detection of LMTs from mouse bioassay (MBA), enzymatic assays (PP2a), and analytical techniques, such as liquid chromatography tandem-mass spectrometry (LC-MS/MS), are described. These analytical advances have allowed us to determine and identify the characteristic profiles of LMTs produced by marine microalgae, including the prevalence and biotransformation of LMTs in the different endemic species. It is worth mentioning that these techniques have favoured the updating of numerous sanitary standards and the definition of the most appropriate technique for the detection of LMTs in shellfish and endemic species.

Keywords: lipophilic marine toxins, AZA-group, OA-group, PTX-group, YTX-group, shellfish, mouse bioassay, protein phosphatase, liquid chromatographic, risk assessment

1. Introduction

Harmful algal blooms (HABs) consist of a group of cells (dinoflagellates, cyanobacteria and diatoms) that, under the interaction of multiple environmental factors such as luminous intensity, temperature, nutrients and salinity, among others, can increase their cell density when compared to the base population of cells present in the sea, lakes and rivers. At the same time, these HABs are associated with the

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capacity to produce powerful toxins that may affect the marine life of fish, marine larvae, mammals and people [1, 2].

Over the past few years, HABs have been showing a greater frequency and intensity [3–5]. Among the factors that have been proposed to be related to this global increase in HABs (**Figure 1**), the following are highlighted: eutrophication of coastal waters as a result of increased aquaculture and/or from fertilisers derived from agriculture, activities linked to urbanisation, climate change, transport of ballast water from ships and/or the transfer of shellfish populations from already contaminated areas [6, 7].

HABs associated with the number of toxin-producing cells can vary considerably during the year. Periods of exponential growth may occur, which is perceived as a bloom, during changes in weather conditions, water temperature, turbulence (waves), water salinity, and concentration of dissolved nutrients [8–10]. These environmental factors can directly affect the variability and concentration of toxins produced by each of the species involved in a bloom [7, 9, 11].

Due to the above, it is possible to establish that environmental conditions may favour the algal blooms of more than one toxin-producing microalgae, thus generating simultaneous harmful processes in the shellfish and lake ecosystems. Therefore, aquatic organisms can assimilate more than one toxic variety in their tissues, expanding the toxic effects to the living organisms in the system [10, 12, 13].

The main organisms affected by HABs are aquatic filter-feeder organisms, such as bivalves, which are characterised by having a high water filtration capacity (20 L h^{-1}) which allows them to accumulate high levels of nutrients, in addition to the accumulation of toxic phytoplankton in their tissues. All these processes are variable and dependent on endemic species in different areas of the planet [2, 14].

Several studies have provided strong scientific evidence stating that the different endemic species of bivalves identified worldwide are capable of exercising natural control of phytoplankton in coastal waters. This control process is characterised by two stages: (a) Filtration stage: this involves the accumulation of harmful dinoflagellates and their toxins in the digestive glands of shellfish (hepatopancreas); (b) Distribution stage: this involves the distribution of toxins to non-visceral tissues of shellfish, such as the mantle, gills, foot and adductor muscle [15–17]. This

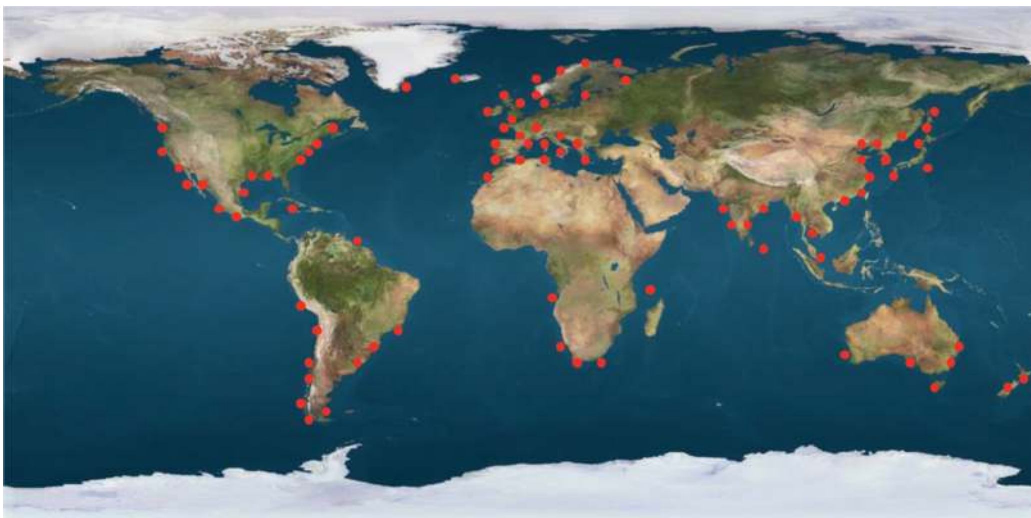


Figure 1.
Geographic distribution of harmful algal blooms associated with the identification of lipophilic marine toxins [1, 3, 5, 14].

distribution to the non-visceral tissues favours the variation of the toxic profiles assimilated in the first stage, through biotransformation pathways that involve enzymatic and non-enzymatic processes [18, 19]. These processes are enhanced by the transfer of toxins through the trophic chain, where an accumulation of toxins with very different profiles is produced in each of the marine organisms involved in the process (zooplankton and whales) [15, 20, 21]. The above mentioned stages are characterised by not causing any apparent damage to the structures and cellular composition of shellfish and bivalves, allowing these toxic compounds to remain in the digestive glands for prolonged periods (months) [22].

The prediction of HABs is very difficult, as there are a number of factors to be considered, such as physical parameters (meteorological and weather parameters, temperature, wind and light conditions, as well as hydrography), chemical parameters (nutrient variability, eutrophication, oxygen availability, anthropogenic pollution and ocean acidity) and biological parameters (evolution of algal communities, grazing and interaction of parasitic microorganisms or viruses) [7].

However, the knowledge of background information related to the temporal and geographical distribution of HAB-producing species is important for the understanding of the problems at a global level [23].

Consumption of seafood products, mainly filter-feeding shellfish contaminated with high amounts of phycotoxins in its visceral (digestive glands) and non-visceral (mantle, gills and foot) tissues, tends to produce severe intoxication when consumed by humans [24]. The number of people intoxicated with these phycotoxins worldwide has reached an average of 60,000 cases per year. This, in turn, produces a large impact on the local economy due to its negative effects on tourism, recreation, and miticulture and aquaculture industries [25, 26]. In Europe, the losses estimated every year in tourism as a result of HABs are approximately € 700 million and about € 116 million in miticulture [1, 4, 27]. Likewise, in order to prevent poisoning caused by the consumption of shellfish or hydrobiological organisms contaminated with phycotoxins, international entities have developed regulations, legislation and follow-up programs for these HABs [28–31].

1.1 Lipophilic marine toxins

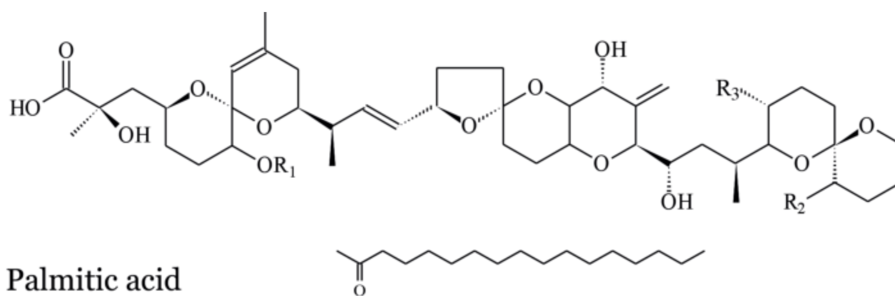
Marine toxins are categorised into different groups, which are characterised by different chemical structures and different mechanisms of toxicity [32]. These groups can be produced by the same or by different species of marine microalgae [31].

Within these groups of toxins, lipophilic marine toxins (LMTs), formed by the following groups, are noted: okadaic acid- (OA), pectenotoxin- (PTX), yessotoxin- (YTX) and azaspiracid- (AZA) group.

1.1.1 Okadaic acid group (OA-group)

The OA-group consists of chemical structures formed by transfused polyethers that at its polar head have a carboxylic acid. The toxins making up this group are called: okadaic acid (OA), dinophysistoxin-1 (DTX-1, 35-R-methyl-OA), dinophysistoxin-2 (DTX-2, 31-demethyl-35-S-methyl-OA) and dinophysistoxin-3 (DTX-3, acyl derivatives of OA, DTX-1 and DTX-2) (**Figure 2**). The OA-group stand out for being potent inhibitors of 1, 2A and 3 serine/threonine protein phosphatases (PP1, PP2a and PP3) [33, 34].

The consumption of shellfish contaminated with this group of toxins produces the medical condition called diarrhoeic shellfish poisoning (DSP), which is characterised by producing symptoms such as diarrhoea, vomiting, nausea and abdominal pain [4, 31].



Name		R^1	R^2	R^3
Okadaic acid	(OA)	H	H	CH_3
Dinophysistoxin-1	(DTX-1)	H	CH_3	CH_3
Dinophysistoxin-2	(DTX-2)	H	CH_3	H
Dinophysistoxin-3	(DTX-3)	Acyl	CH_3	CH_3

Figure 2.
Chemical structures of okadaic acid group and analogues [33, 34].

At the beginning of the 1990s, this group of toxins was also formed by the PTX, YTX and AZA groups, since the process of extraction and evaluation of toxins in bivalves did not differentiate the toxic groups involved in the HABs processes. Thus, the toxic symptoms detected in people were associated with diarrhoeic shellfish poisoning (DSP). Subsequently, the use of analytical processes established that this group was made up of multiple toxins, which had differences from a chemical and toxicological point of view, so that each type of toxin was excluded from the OA group and classified as PTX, YTX and AZA groups [28, 35, 36].

OA-group is present in both planktonic and epibenthic dinoflagellates of the genera Dinophysis and Prorocentrum, where the species *Dinophysis acuminata*, *Dinophysis acuta*, *Dinophysis caudata*, *Dinophysis fortii*, *Dinophysis miles*, *Dinophysis ovum*, *Dinophysis sacculus*, *Dinophysis rotundata*, *Dinophysis tripos* and *Prorocentrum lima*, *Prorocentrum belizeanum*, *Prorocentrum concavum* and *Prorocentrum hoffmannianum* (**Table 1**) are highlighted [4]. The densities of the dinoflagellates associated with OA-group correspond to $<10^4$ L^{-1} cells, with a relative abundance of $<10^3$ L^{-1} cells [37].

The variability of the toxic profile identified in OA-group producing dinoflagellates depends, to a large extent, on the species involved and on the global distribution in which the blooms are identified (**Figure 1**). The variability of the toxin content detected in cells of *Dinophysis acuta* ranges between 0 and 40 OA pg. $cell^{-1}$, 0–0.02 DTX-1 pg. $cell^{-1}$ and 0.3–0.6 DTX-2 pg. $cell^{-1}$, while for *Dinophysis acuminata*, it is 0–160 OA pg. $cell^{-1}$, 0–7.8 DTX-1 pg. $cell^{-1}$ and 0–169 DTX-2 pg. $cell^{-1}$ [38].

At present, data on the chronic effects of OA in animals or humans have been insufficient to determine the Tolerable Daily Intake (TDI). However, a Lowest Observed Adverse Effect Level (LOAEL) corresponding to 50 μg OA equivalent per person has been established, equivalent to ≈0.8 μg OA kg^{-1} of body weight for adults, and a No Observed Adverse Effect Level (NOAEL) corresponding to ≈0.3 μg OA equivalents kg^{-1} b.w. [14].

Considering the lipophilicity of the toxins that make up this group, they are easily accumulated in the tissues of the filter-feeding marine organisms that feed on HABs, spreading quickly to their predators in the food chain [13]. Moreover, depending on the species of molluscs contaminated with the OA-group and their natural clearance, the OA-group toxins tend to be chemically modified in the visceral tissues of the molluscs. In this way, OA, DTX-1 and DTX-2 can be esterified by fatty acids of variable length (C7-C22) in the hydroxyl group (–OH) present in carbon 7 of the structure of toxins (**Figure 2**), palmitic acid (C16:0)

Species	Toxins produced
Dinophysis sacculus	OA
Dinophysis ovum	OA
Dinophysis mitra	DTX-1
Dinophysis tripos	DTX-1
Dinophysis miles	OA, DTX-1
Dinophysis rotundata	OA; PTX-2
Dinophysis infundibulus	PTX-2
Dinophysis caudata	OA, DTX-1, PTX-2
Dinophysis fortii	OA, DTX-1, PTX-3
Dinophysis norvegica	OA, DTX-1, PTX-2, PTX-12
Dinophysis acuminata	OA, DTX-1; PTX-2; PTX-12
Dinophysis acuta	OA, DTX-1, DTX-2, PTX-2, PTX-2sa
	7-epi-PTX-2sa, PTX-12
Prorocentrum concavum	OA
Prorocentrum hoffmannianum	OA
Prorocentrum maculosum	OA
Prorocentrum mexicanum	OA
Prorocentrum belizeanum	OA, DTX-1
Prorocentrum faustiae	OA, DTX-1
Prorocentrum lima	OA, DTX-1
Protoperidinium divergens	OA, DTX-1
Protoperidinium depressum	OA, DTX-1
Protoceratium reticulatum	YTX
Gonyaulax spinifera	YTX
Lingulodynium poliedrum	YTX, homo-YTX, 45-OH-homo-YTX
Protoperidinium crassipes	AZA
Echinochlathria sp.	AZA-2
Azadinium spinosum	AZA-1, -2, -3

Table 1.
List of dinoflagellate species identified as producer species of lipophilic marine toxins [4, 28, 37, 55, 64, 65, 79, 81].

being the most prevalent that is identified in this process [39, 40]. This esterification results in the toxic structures being named acyl derivatives. However, in their first detections, acylation was always associated with DTX-1, acquiring the name of dinophysistoxin-3 (DTX-3) [41]. In Chile, palmitic acid accounts for 90% of the total fatty acids linked to the esterification of toxins in the OA-group, in which 7-O-palmitoyl-dinophysistoxin-1 is the most prevalent toxin detected in endemic bivalves on the coast of Chile [39, 42]. Although the feasibility of other forms of fatty acid derivatives of varying lengths or with different unsaturations may produce acyl derivatives of the OA-group, such as C14:0, C16:1, C16:0, C18:1 and C18:0 [43–45]. All these 7-O-acyl-OA-group esterified analogues have not been detected in any single-cell isolates of *Dinophysis* sp., so detection is only associated with shellfish [46].

The differences observed between the proportions of esterified forms and free forms of OA could arise from the genetic differences in bivalves (endemic species), since the OA esterification is considered to be an enzymatic mechanism associated with the detoxification of bivalves [47]. From a toxic point of view, it has been established that the toxicity of endemic species may depend on the variability in the lipid content present in the digestive glands, which would favour the retention of the toxins, thus explaining its higher toxicity of compartmentalisation if compared to non-visceral tissues, such as the mantle or adductor muscle [19, 48, 49].

The symptoms caused by intoxication associated with the consumption of molluscs contaminated with the OA-group are characterised as starting between 1 and 5 h after the ingestion of contaminated molluscs, symptoms that tend to be reversed 3 days after the toxic symptoms are initiated [50]. The minimum doses of OA and DTX-1 required to produce toxic symptoms in humans have been estimated to be 40 and 36 μg respectively for a 60 kg person [51]. To date, no deaths of people associated with intoxications produced by the OA-group have been recorded. However, it has been clearly established that the OA-group is a potent tumour promoter in animals [52], thus being associated with the risk of gastric cancer among regular consumers of contaminated shellfish with toxins of the OA-group [53, 54]. Therefore, poisonings associated with the OA-group are a latent problem for both public health and the seafood industry [1, 4, 27].

1.1.2 Pectenotoxin group (PTX-group)

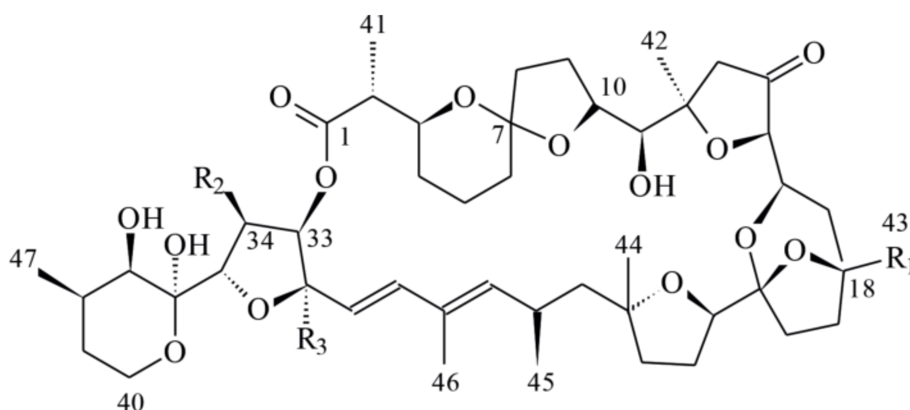
Toxins making up the pectenotoxin group (PTXs) correspond to a family of toxins of polyether macrolides (**Figure 3**) produced by the same species that produce the toxic forms of the OA-group, such as *Dinophysis fortii*, *Dinophysis acuta*, *Dinophysis acuminata*, *Dinophysis caudata* and *Dinophysis norvegica*, which are further detected in heterotrophic dinoflagellates such as *Protoperidinium divergens* and *Protoperidinium depressum* (**Table 1**) [28, 55]. The origin of the name of this group comes from the crustacean *Patinopecten yessoensis* in which it was detected for the first time [33].

For years, the PTX-group was included in the OA-group, since the bioassay tests detected the overall toxicity of both groups. Currently, the PTX-group is classified as a separate group, based on the biochemical effects on which they act, as this group does not inhibit protein phosphatase 2a (PP2a) [14, 56]. To date, no cases of intoxication due to consumption of molluscs contaminated with the PTX-group have been reported [57].

This group of toxins has been identified in countries such as Ireland, Croatia, New Zealand, Portugal, Norway, Japan, Argentina and Chile, showing a direct relationship with the areas which have previously been associated with the presence of the OA-group (**Figure 1**) [28, 40, 58, 59].

The toxic profiles detected in dinoflagellates and shellfish tend to be different, establishing that a metabolic transformation occurs in the bivalves after the filtration/assimilation of toxic algae [46]. Filter-feeding bivalves, when accumulating this type of toxin (PTX-2) in their digestive glands (hepatopancreas), allow for their biotransformation. These biotransformed analogues have been assigned to the seco acid (sa) nomenclature, highlighting the identification in bivalves of pectenotoxin-2 seco acid (PTX-2sa) and 7-epi-pectenotoxins-2-seco-acid (7-epi-PTX-2sa) analogues [28, 43]. 7-epi-PTX-2sa, is the result of the interconversion of PTX-2sa to a thermodynamically more stable analogue. In this way, the conversion of PTX-2 to seco-acid forms could be considered as a protective effect of detoxification by molluscs [41].

There are about 15 analogues identified and associated with the PTX-group, in which the esterified forms of PTX-2sa were the last identified analogues, where



Name	R^1	R^2	R^3	C-7
Pectenotoxin-1 (PTX-1)	CH_3OH	H	H	R
Pectenotoxin-2 (PTX-2)	CH_3	H	H	R
Pectenotoxin-2b (PTX-2b)	CH_3	H	H	S
Pectenotoxin-3 (PTX-3)	CHO	H	H	R
Pectenotoxin-4 (PTX-4)	CH_2OH	H	H	S
Pectenotoxin-6 (PTX-6)	COOH	H	H	R
Pectenotoxin-7 (PTX-7)	COOH	H	H	S
Pectenotoxin-11 (PTX-11)	CH_3	OH	H	R
Pectenotoxin-11b (PTX-11b)	CH_3	OH	H	S
Pectenotoxin-13 (PTX-13)	CH_3	H	OH	R

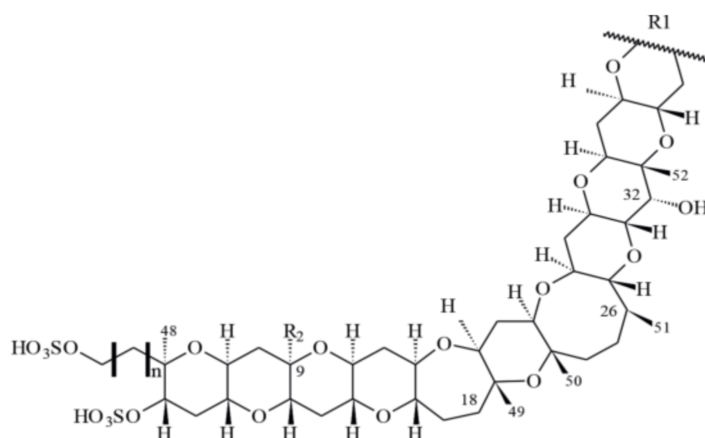
Figure 3.
Chemical structure of pectenotoxin group and its chemical analogues identified in dinoflagellates and bivalve molluscs [28, 40].

37-O-acyl ester-PTX-2-sa, 11-O-acyl ester-PTX-2sa and 33-O-acyl ester-PTX-2sa are noted. All of these analogues were identified in filter-feeding bivalves [60–62].

The available toxicological information on the PTX-group is insufficient at present due to the lack of toxic analogues required to carry out biological studies of interest. However, it has been determined that the lethal intraperitoneal dose of PTX-2 corresponds to 260 μg kg^{-1}. In addition, the results of the toxicity obtained from intraperitoneal (*i.p.*) administration and oral ingestion of PTX in mouse have established that toxicities are comparable, do not produce these symptoms of diarrhoea and are only limited to generate histopathological changes in the liver, stomach and intestine [60]. Furthermore, it has been determined that PTX-2 may potentially be cytotoxic on lung, colon and kidney cell lines. Toxic effects are not extrapolated to analogues such as PTX-2-sa and 7-epi-PTX2-sa, evidencing the importance of the initial structure of the toxin that generates the cytotoxic effects [63].

1.1.3 Yessotoxin group (YTX-group)

Yessotoxin group (YTX-group) corresponds to a group constituted by sulphated polyethers (**Figure 4**), and whose analogues were first identified in the oyster *Patinopecten yessoensis*. Yessotoxin (YTX) is produced by the marine phytoplanktonic microalgae *Protoceratium reticulatum* (Gonyaulax grindley) [64], *Lingulodinium polyedrum* (*Gonyaulax polyedra*) [35, 65] and *Gonyaulax spinifera* (**Table 1**) [35, 66, 67].



Name	R¹	R²	n
Yessotoxin (YTX)		H	1
45-Hydroxy-YTX (45-OH-YTX)		H	1
Homo-YTX (1a-Homo-YTX)		H	2

Figure 4.
Chemical structure of yessotoxin-group and their chemical analogues identified in dinoflagellates and bivalve molluscs [64, 66].

At present, this group of toxins have been identified in different countries worldwide such as New Zealand, Italy, Spain, Norway, Russia, Canada, United Kingdom, Japan, Argentina and Chile (**Figure 1**) [35, 68–70].

Historically, the YTX-group was included in the OA-group, however, this group of toxins do not produce diarrhoea in the mouse bioassay, nor do they produce the inhibition of the protein phosphatase 2a (PP2a) [71, 72]. Although the precise mode of action of the YTX-group is unknown, it has been classified as a potent activator of phosphodiesterase [73].

To date, more than 100 natural YTX analogues have been identified and characterised using nuclear magnetic resonance (NMR) and liquid chromatography coupled with mass spectrometry (LC-MS). Some of the identified analogues are directly related to the producer dinoflagellate, such as norYTX, 41-keto-YTX and 41a-homo-YTX, while other analogues come from processes of biotransformation exerted in the digestive glands of different marine species worldwide that have been evaluated and that involve chemical oxidation pathways such as hydroxylations, carboxylations, desulfations, methylations and amidations [35, 61]. Thus, analogues such as 45-hydroxy-YTX, homoYTX, 45-hydroxyhomo-YTX, carboxy-YTX,

carboxyhomo-YTX, 41a-homoYTX, 45-hydroxycarboxy-YTX, and 1-desulphocarboxyhomo-YTX are the direct result of biotransformation processes that occur in shellfish [28, 46, 74].

The HABs associated with the dinoflagellates producers of the YTX-group are characterised by reaching a cell density of 10^3 L^{-1} cells, where the production of YTXs in dinoflagellates is on average ≈34 pg. $cell^{-1}$ (0–74 pg. $cell^{-1}$), where homo-YTX and YTX are the main toxins forming the profile, whose concentrations are variable and dependent on the areas where blooms have been detected [35].

Symptoms caused by intoxication with the YTX-group in humans are unknown, because no human poisonings associated with this group have been reported [28]. However, the toxic evaluation of YTX in bioassays has determined that an intraperitoneal injection at concentrations of approximately 150 μg kg^{-1} causes difficulty in mobilisation, dyspnoea, jumps, tremors and cramps, with all symptoms starting 4 h after the injection is given [67, 71, 72].

It is noteworthy that YTX oral administration in mouse did not induce any significant difference in haematological and clinical chemistry parameters, including leukocyte percentages and plasma levels of alanine-aminotransferase (ALT), aspartate aminotransferase (AST), creatine phosphokinase (CPK) or lactate dehydrogenase (LDH) [67, 71, 72, 75]. Nevertheless, at an ultrastructural level, changes in the myocardium in *in vivo* studies following both oral and *i.p.* administration have been identified [71, 72, 75–77]. Thus, it has been estimated that YTX intraperitoneal administration induces cardiac damage with a potency >10 times greater than the oral route [76]. This difference could be related to the low adsorption through the gastrointestinal tract and/or biotransformation of toxins associated with the YTX-group [67].

In addition, toxicological data obtained from the evaluation with the YTX and homo-YTX analogues have shown that they have approximately the same toxicity [64], while the other analogues have lower toxicity, especially OH-YTX and carboxy-YTX derivatives because they are ≈5 times less toxic than YTX, while other derivatives associated with the YTX group, such as trihydroxylated amides (41-a-homo-YTX and 1,3-enone isomer of heptanor-41-oxo YTX) have shown no toxicity through intraperitoneal injections in mice at levels >5000 μg kg^{-1} body weight [7, 60, 61].

Naturally, the main vectors of the YTX-group correspond to bivalves (endemic species) characterised by accumulating large amounts of toxins in their digestive glands due to their high filtration capacity. Once they are assimilated and based on the chemical modifications caused by bivalves to toxic analogues of YTX, they can be spread to other non-visceral tissues such as mantle, gills, foot and adductor muscle [62, 74].

The European Food Safety Authority (EFSA) has established that toxic effects may even occur at concentrations below 3.75 mg YTX equivalents kg^{-1} shellfish, which is a limit established as an international sanitary standard for marketing products [14, 36, 78].

1.1.4 Azaspiracid group (AZA-group)

AZA-group corresponds to toxins produced by toxic dinoflagellates, *Protoperidinium crassipes*, *Azadinium spinosum* and the sponge, *Echinochlathria* sp. (**Table 1**) [79–81]. However, in some species it has not been possible to establish a direct relationship between the high toxic levels detected in some species of endemic bivalves and the cell densities of the dinoflagellates producing this group of toxins [82].

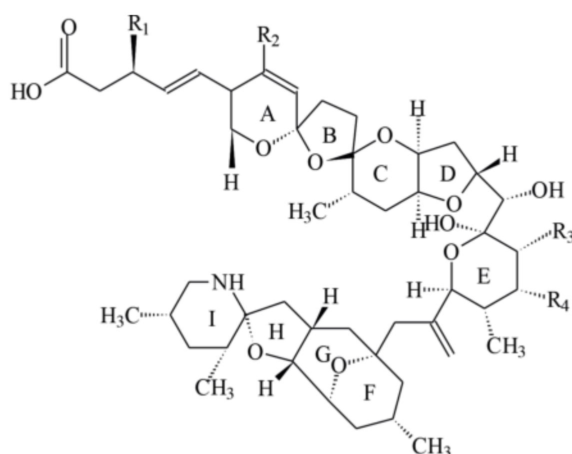
The AZA-group has been identified worldwide in molluscs analysed in Ireland, United Kingdom, Norway, France, Portugal, North Africa (Morocco), Chile and USA (**Figure 1**) [12, 43, 44, 83].

To date, ≈24 toxic analogues belonging to the AZA-group have been identified. The main toxic analogues detected are: azaspiracid-1 (AZA-1), azaspiracid-2 (AZA-2) and azaspiracid-3 (AZA-3) (**Figure 5**) [84, 85].

The mechanisms of action of the AZA-group have not been clearly determined, however studies *in vitro* in mammalian cells show that they produce alterations in the structure of the cytoskeleton of cells and the E-cadherin system, the latter being responsible for the interaction between cells [86, 87]. These experimental results could explain the symptoms associated with human intoxications such as gastrointestinal disorders, abdominal pain and diarrhoea [1]. Although the data obtained from seafood extracts have shown that AZAs do not produce diarrhoea [88].

The intraperitoneal lethal dose determined in mice is directly related to the toxic analogue detected in shellfish extracts. In this way, AZA-1 has a lethal dose of 200 μg kg^{-1}, while AZA-2 and AZA-3 are significantly more toxic, with lethal doses of 110 and 140 μg kg^{-1}, respectively [28]. In addition, it has been established that oral toxicity in mice with AZA-1 at corresponding doses of 900 μg kg^{-1} produces significant damage to the small intestine, while doses of 500 μg kg^{-1} only produce liver damage, which is characterised by an increase in the volume (≈38%) [89, 90].

In addition, toxicological studies have established that the lowest observed adverse effect level (LOAEL) for AZAs corresponds to concentrations between ≈23 and ≈86 μg AZAs kg^{-1} per person, even though it is estimated that at levels of ≈80 μg AZA-equivalents kg^{-1} molluscs would not produce symptoms due to AZA-group intoxication. However, it has been recorded that when ≈400 g of shellfish are consumed, doses corresponding to ≈30 μg AZA-equivalents kg^{-1} have been able to produce AZA-associated intoxication syndrome in humans. These seemingly contradictory data can be explained by the variability of toxic forms subjected to biotransformation, which produces new and different analogues during the accumulation of toxins in the digestive glands of molluscs [91].



Name	R¹	R²	R³	R⁴
Azaspiracid-1 (AZA-1)	H	H	CH_3	H
Azaspiracid-2 (AZA-2)	H	CH_3	CH_3	H
Azaspiracid-3 (AZA-3)	H	H	H	H
Azaspiracid-4 (AZA-4)	OH	H	H	H
Azaspiracid-5 (AZA-5)	H	H	H	OH
Azaspiracid-6 (AZA-6)	H	CH_3	H	H

Figure 5.
Chemical structure of azaspiracid-group and their chemical analogues identified in dinoflagellates and bivalve molluscs [79–82].

Although AZA-1 is the most abundant toxin in this group, the analogues corresponding to AZA-2, AZA-3, 8-methyl-AZA-1 and 22-demethyl-AZA-1 frequently co-occur in different types of bivalves (**Figure 5**). The formation of AZA-3 from AZA-1 in *Mytilus* sp. corresponds to a xenobiotic bioconversion, even though this detoxification pathway results in the formation of a more toxic analogue [81]. The toxicity of molluscs contaminated with AZA-group toxins tends to persist for an average period of ≈5 months post-bloom, allowing toxins to be distributed to other non-visceral tissue. In some cases, these tissues are able to reach toxic levels superior to those described for the digestive glands [12, 92].

In spite of this, the trophic transfer routes through the food web are completely unknown, although to date the AZA-group has been detected in other aquatic species such as crustaceans [43, 93].

2. Results

2.1 Methods of detection

Each group of toxins (OA, PTX, YTX and AZA) present in the different endemic species of bivalves or marine organisms is composed of many toxic analogues, so regulatory levels are represented according to the total toxicity of the analogues studied. Traditionally, regulatory limits have been evaluated using mouse bioassay (MBA), which involves intraperitoneal injection of seafood extracts [14, 91].

Nevertheless, we should consider that one single type of shellfish may contain more than one toxic group, therefore, alternative methods to the bioassays are required for the detection and proper identification of each analogue that may belong to the different described groups [94].

To identify toxic groups, there are also functional tests, which are traditionally defined as methods of detection based on the mechanism of action that each toxin group has in order to establish its quantification, which, in turn, is related to the group toxicity. These assays are usually receptors, proteins or cells [95].

Since shellfish consumption is very important from an economic standpoint, most producer and consumer countries have implemented monitoring systems to prevent these toxins from reaching consumers [94]. These monitoring systems based on detection methods are required to be highly specific, reproducible systems and must not be prone to produce either false positives or false negatives [14].

Thus, some countries differ in the selection of the method required for the identification of toxin groups and three different methods can be used: biological methods (Mouse Bioassay, MBA), biochemical methods (Protein Phosphatase-2a Inhibition Assay, PP2aIA) and chemical methods (Liquid chromatography coupled with tandem mass spectrometry, LC-MS/MS) [96]. Biological and biochemical methods only establish the presence of one group of toxins; they do not identify the toxin involved in a mixture of analogues in a process of contamination or associated with a HAB whereas chemical methods provide a profile of the quantity and variety of analogues in a contaminated sample [94].

At an international level, the identification of LMT has been regulated by establishing maximum permissible concentrations for marine organisms destined for human consumption. The maximum permissible limits are 160 μg of OA equivalents kg^{-1} shellfish meat for OA-group, PTX-group and AZA-group; and 3.75 mg of YTX equivalents kg^{-1} shellfish for YTX-group [29, 30, 78].

It is also worth noting that the OA- and PTX-groups are represented toxicologically together, but this fact is based more on the possible co-occurrence of toxins of the OA-group and PTX-group from a same species of dinoflagellates. However,

it should be considered that these groups do not share the same biological action mechanisms [14, 35, 55].

This regulation allows a series of methods for the detection of these groups, noted among them, immunoassays, functional tests (inhibition of protein phosphatase) and LC-MS/MS [97].

2.1.1 Biological method: mouse bioassay (MBA)

The mouse bioassay (MBA) is a biological assay used for the determination of toxins corresponding only to the OA-group. This bioassay can only provide a response to this group of toxins, however it does not provide any information on the analogues present in contaminated shellfish [7].

To obtain results, this bioassay requires the use of at least three animals per sample, which, in the end, are euthanized regardless of the obtained toxicity result [95]. Thus, the shellfish extract is injected via intraperitoneal (*i.p.*) route to three male mice that must have a weight ranging between 16 and 20 g. A sample is considered positive (contaminated with OA-group) when at least two of the three mice die within 24 h of the injection (**Figure 6**) [95, 98].

The positive result of this bioassay on toxic samples causes diarrhoea in mouse, which is directly proportional to the concentrations of toxins present in the toxic seafood extract. The toxins detected by this MBA may even be present in the faeces of the animals when the concentrations are >700 μg kg^{-1}, with an average lethality exceeding 30% at a dose of ≥1000 μg kg^{-1} [34]. Excretion of OA-group

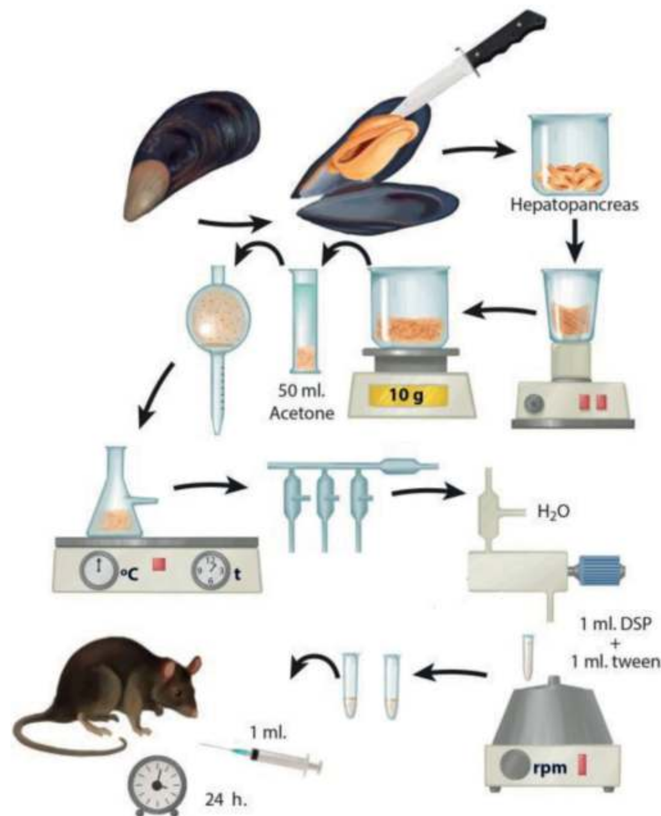


Figure 6.
Diagram of the stages required for the determination of OA-group toxins through the use of the mouse bioassay (MBA) [95, 98].

toxins through faeces indicates that a fraction of the toxin ingested by animals is not absorbed, being eliminated instantaneously, while it is possible to detect low levels in different organs (liver, pancreas, and spleen) as compared to the amount excreted in urine and faeces [34, 99].

In the absence of death (negative assays), mice may similarly develop symptoms specific to OA-group toxins which are significant indicators of a potential contamination and risk [14, 100].

However, a critical factor of the MBA is the body weight of the mouse used, which significantly influences the susceptibility of the toxins associated with the OA-group. The lethality after inoculation of OA at 24 h post-injection reaches 100% in mouse with weights of 14–15 and 16–17 g, while for groups of 19–20 g, it reaches 80% and for groups of mouse weighing between 21 and 22 g, it only reaches 50% [98].

Moreover, all toxins in the OA-group have been shown to be less toxic by oral route compared to an intraperitoneal injection route. The median lethal dose (LD_{50}) via oral route for OA is approximately 400–880 μg kg^{-1}. Specifically, LD_{50} toxicity via *i.p.* for OA averages between 192 and 225 μg kg^{-1} [101], while for DTX-1, LD_{50} toxicity via *i.p.* is ≈160 μg kg^{-1} [102] and for the DTX-3 and DTX-4 analogues, the toxicity ranges from ≈352 to ≈600 μg kg^{-1} [103]. It should be noted that the acylated forms (DTX-3, acyl-derivatives-OA-group) can show a decreased toxicity, stating that these toxic analogues can be ≈20 times less toxic than OA [101]. Additionally, preliminary data on the oral toxicity of DTX-2 samples showed that oral LD_{50} is ≈2150 μg kg^{-1} body weight [104].

These variabilities on acute toxicities of various analogues such as DTX-4 and 7-O-palmitoyl-OA are related to the bioavailability of toxins within the peritoneal cavity, which, at varying ranges, are hydrolysed under acidic or alkaline conditions or by the action of esterases or lipases within the gut allowing for the production of more toxic forms such as OA or DTX-1 [50, 101, 105].

This toxic variability between different analogues means the use of the MBA has a probability of detecting toxins between 40 and 50% associated with the OA-group in the currently established limit of 160 μg OA-equivalents kg^{-1} [7, 10, 14, 102].

Despite the above mentioned, the great advantage of the MBA is that it provides an estimate of the total toxicity of the sample [14], but, at the same time, it has multiple disadvantages:

- It requires animal facilities and expertise (bioterium).
- It cannot be easily automated due to the involvement of animal handling.
- It has a high variability in the results between laboratories, due to characteristics in the mouse used (breed, sex, age, weight, general health status, diet, stress) [98].
- It produces false-positive results due to fatty acid interferences (20,4n-6 and 20,5n-3) [106].
- It can produce false-negative results due to the toxic suppression between groups [96].
- Interfering matrices from heavy metals.
- It is not selective for the OA-group toxins [107].
- It is not a quantitative bioassay and it has a limit of detection ≈200 μg kg^{-1}.

- The *i.p.* injection of the sample is not suitable for the complete detection of the OA-group, since some analogues require hydrolysis to be detected (acyl-derivatives) [105].

- Some countries have banned it for ethical reasons.

Within these disadvantages, it has been established that MBA is able to detect toxins of the OA-, AZA- and YTX- groups. However, the result will never be able to determine the exact group responsible for it, as there is a co-occurrence of these toxins in contaminated endemic species (shellfish) [7, 12, 13, 26].

Toxic evaluations in mice related to the YTX-group have shown that they die (2/3) by injecting them with doses 0.75 mg YTX kg^{-1} body weight, while this increases (3/3) with a dose of 1 mg YTX kg^{-1} body weight. However, mice cannot be killed with oral doses of 10 mg kg^{-1} YTX kg^{-1} body weight (LD_{50} *i.p.* ≈100 µg kg^{-1}) [7]. The differences between intraperitoneal and oral toxicities of YTX would probably be related to the low YTX uptake in the gastrointestinal tract (≈0.02%) [35]; those factors are increased when considering other analogues of the YTX-group that arise from the processes of biotransformation in the tissues of marine organisms (45-hydroxy-YTX, carboxy-YTX) [7].

Regarding the toxic evaluation through MBA with the AZA-group, it has been established that this assay is capable of detecting the toxins associated with AZA-group, producing neurotoxic symptoms in mice such as: slowness, respiratory difficulties, spasms, progressive paralysis and death between 20 and 90 min after the application of the mouse bioassay. Thus, it has been determined that the minimum *i.p.* lethal dose required to cause swelling of the stomach and liver in the mouse, with a reduction in the size and weight of the thymus and spleen is ≈150 µg kg^{-1} [10].

In addition, it has been established that the different analogues of this group have different toxicities, the minimum lethal dose of AZA-2 (8-methyl-azaspiracid) and AZA-3 (22-desmethyl-azaspiracid) corresponds to ≈110 and ≈140 µg kg^{-1}, respectively, suggesting a higher potency in relation to AZA-1. However, AZA-4 and AZA-5 are less toxic, with lethal dose values of ≈470 and < 1000 µg kg^{-1}, respectively. In this way, the toxicity of these analogues through the MBA can be represented as follows: AZA-2 > AZA-3 > AZA-1 > AZA-4 > AZA-5 [10, 16].

In relation to the toxic evaluation of AZA-group by oral route, it has been shown that it does not produce death in mice at concentrations >900 µg kg^{-1} after 24 h. However, post-mortem evaluations have shown various gastrointestinal disturbances, such as accumulation of fluid from the ileum and necrosis of intestinal epithelial cells [10].

For the PTX-group, toxic data related to the MBA establish that they are highly toxic by *i.p.* injection in values averaging between ≈219 and ≈411 µg kg^{-1} [60], which leads to a positive MBA. However, PTXs appear to be of low toxicity by oral route (≈5.0 mg kg^{-1}) and, unlike OA, they do not cause diarrhoea, in addition to the fact that PTX-group is easily destroyed under basic conditions [60, 108].

In this regard, toxicity in mouse with different combined doses of LMTs by oral route has been assessed. Thus, combined oral doses of YTX (1 mg kg^{-1} day^{-1}) and OA (0.185 mg kg^{-1} day^{-1}) show no lethal effects or diarrhoea or any other symptoms of toxicity in mice, as opposed to the results obtained from the toxic evaluation of these groups individually [67].

These data are consistent with the evaluation of combined doses of yessotoxin (1.0 or 5.0 mg kg^{-1}) and AZA-1 (200 µg kg^{-1}) given to mice; those doses do not produce toxic effects on the heart or any other internal organs. It is noted that the absorption of YTX is not potentiated by the co-administration of AZA-1 [67, 109].

Furthermore, the combined evaluation of OA and AZA-1 shows that there is no increase in pathological changes when AZA-1 and OA are administered together, if compared to the administration of OA alone, where a jejunal dilatation is clearly evidenced. It should be noted the reduction of OA absorption in the internal organs when administered together with AZA-1 or vice versa, due to the competence experienced by the weak organic acids through the simple diffusion of the membranes of the gastrointestinal tract should be noted [32].

In conclusion, MBA is not the most appropriate when considering the simultaneity of the toxic bloom processes at a global level and the high capacity of biotransformation of shellfish, which would produce a high variability in the toxicity results through this bioassay.

2.1.2 Biochemical method: protein phosphatase 2a (PP2a) inhibition assay

Biochemical assays are the preferred methods for detecting LMTs in endemic species (shellfish). However, they cannot provide a quantitative measure of all toxic groups (OA-, PTX-, AZA- and YTX-group). This is the main problem of the protein phosphatase 2a (PP2a) inhibition assay, a recognised biochemical method capable of accurately detecting and quantifying only some analogues related to the OA-group [96, 107, 110, 111].

The PP2a inhibition assay (PP2aIA) is a rapid method for the detection of toxins associated with the OA-group, which is based on its functional property of inhibiting type PP2a (**Figure 7**) [112–114]. In general, this method tends to be precise, sensitive, reproducible, simple, and fast [47, 115, 116].

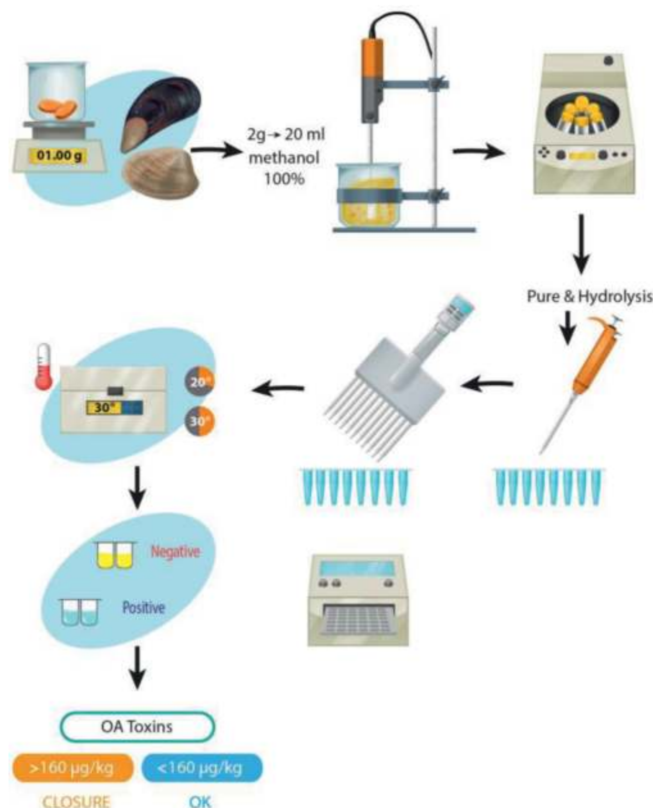


Figure 7.
Stages of the protein phosphatase 2a inhibition assay for the detection of toxins associated with OA-group toxins [107, 122].

Several methods of purification of PP2a have been proposed, including: purification of recombinant PP2a overexpressed in insect cells [114, 117, 118], PP2ac expression in mammalian cells [119], and overexpression of PP2ac in yeast [120]. However, none of these systems is capable of producing a high yield of recombinant proteins [110, 121].

The principle of the assay is based on the fact that PP2a reacts with the p-nitrophenylphosphate (p-NPP) substrate, which is colourless to produce p-nitrophenol (p-NP) by the enzymatic reaction, characterised by having a yellow colour. The toxins associated with the OA-group (OA and DTX-1) inhibit the enzyme, causing a loss of production of the p-NP; this loss is determined through the variability of the absorbance at 405 nm [107]. The amount of enzyme determines the amount of analyte (OA and DTX-1) needed for the inhibition, while the quality of the enzyme ensures the amount of product formed per time unit [122]. In addition, the lack of stability or impurity of the standards used (OA and DTX-1) directly affect the quantification [123, 124].

Regarding the OA-group, it should be noted that DTX-1 is a more potent inhibitor of PP2a than OA [125, 126], whereas DTX-2 is half as potent when compared to OA [102, 112]. The presence of esters in microalgae, such as DTX-4 is ≈500 times less active than OA [103] and acylated forms originated from the biotransformation process in shellfish, such as 7-O-palmitoyl-OA, which is a very weak inhibitor of PP2a, ≈3000 times less active than OA (**Table 2**) [101].

Additionally, the sensitivity of the enzymes (PP2a) for the evaluation of toxins associated with the OA-group may be drastically different. Thus, the choice of the enzyme and the source of origin are crucial for the proper performance of the system. Therefore, one of the main drawbacks is related to the low enzymatic stability of this assay, so, to overcome this problem, some kits have immobilised the enzyme (PP2a), which is a crucial step for the extraction of biosensors [107].

The great advantage of PP2aIA is that it provides an estimate of the total toxicity of the sample; it is a repetitive and fast assay [14]. At the same time, the PP2aIA has multiple disadvantages, among which, the following are found:

- It requires a good quality enzyme (PP2a) [123, 124].
- It requires background knowledge for the interpretation of data.
- It requires interlaboratory validation and standardised protocols.

Toxins	PP2a	
	Substrate	K_i
Okadaic acid (OA)	pNPP	30
Dinophysistoxin-1 (DTX-1)	pNPP	19
Dinophysistoxin-2 (DTX-2) (IC_{50})	pNPP	3.38
7-Deoxy-OA	pNPP	900
7-O-palmitoyl-OA	pNPP	>100 nm
7-O-palmitoyl-DTX-1	pNPP	>100 nm
Methyl-okadaate	pNPP	≫100 nm
2-oxo-Decaborxyl-OA	pNPP	≫100 nm

K_i = dissociation constant.

Table 2.
Inhibition of PP2a by OA-group [112, 122, 127].

- The matrices from marine organisms can underestimate or overestimate the results [124, 129].
- It does not provide any information on the profile of OA-group [101].
- It does not detect acyl-derivatives of OA-group directly [126, 128]
- It does not identify analogues associated with the PTX-, YTX- and AZA-groups [96, 107].

At present, it is widely accepted that the toxic effects of OA-group are caused by the inhibition of protein phosphatases. However, there is no concrete evidence supporting this hypothesis. This is because a pathway from inhibition of PP2a to the toxic effect produced by toxins in the OA-group has not been identified. The OA-group-induced toxic effects are replicated by substances which are not inhibitors of protein phosphatase (*Vibrio parahaemolyticus*), and known inhibitors of protein phosphatase (Microcystin, MC) do not exert the same toxic effects in animals just as the OA-group toxins. Therefore, this method does not have a direct relationship with the systemic effects involved in the processes of intoxication in humans, which are directly limited to the specific ability of the OA-group to inhibit PP2a [101].

However, the results of PP2aIA obtained with OA-group toxins are very well correlated with the results obtained using the MBA. The IC_{50} concentrations for DTX-2 and OA are ≈5.94 and ≈2.81 ng ml^{-1}, respectively, indicating that OA is approximately two times more toxic than DTX-2 [102].

Different origins of PP2a enzymes have allowed for comparisons of the assay, demonstrating that PP2a wild-type is significantly more sensitive to all toxins in the OA-group, relative to that obtained from a recombinant origin, which is ≈1.9-fold lower IC_{50} for OA, ≈1.7-fold lower IC_{50} for DTX-1, and ≈2.2-fold lower IC_{50} for DTX-2 [124]. Thus, the PP2aIA can be considered to detect toxins associated with toxins of the OA-group since its limit of detection (LOD) and limit of quantification (LOQ) are <160 μg OA equivalents kg^{-1} (international maximum limit). Nevertheless, its optimal range of toxic detection is very narrow (between ≈56 and ≈96 μg OA kg^{-1} shellfish) due to the fact that matrices and pigments of bivalves may affect the interpretation of results [14, 123]. It should be considered that samples with DTX-2 only are not able to adequately inhibit PP2a. In this way, and comparing the different types of enzymes according to their origin, it has been generally established that recombinant PP2a tend to underestimate the equivalent contents of OA, whereas the tendency of PP2a wild-type is to overestimate the toxicities [124].

Moreover, the process to establish the total toxicity of a sample of shellfish exposed to HABs associated with OA-group through PP2aIA necessarily involves the hydrolysis of the samples from shellfish, in order to establish the ranges of acyl-derivatives, which are only detected in shellfish and which are also notable for not producing a very low inhibition of the different types of PP2a [126, 128]. Thus, the acylated analogues must be transformed to their base analogue, which could be OA, DTX-1 or DTX-2. This pathway has estimated that the method tends to consider an overestimation through the PP2aIA, due to the non-specific inhibition by components present in the matrices of different endemic species (bivalves), that even at low concentrations (<LOD) produce an effect similar to the inhibition produced by OA. This could be related to the presence of some soluble lipids in methanol, which exert a non-specific inhibitory effect on PP2a or the interaction between the matrix, reagents and/or the temperature step involved in the assay [97, 124, 129].

Furthermore, the high concentrations of matrix from endemic species (shellfish) interfere with the PP2aIA. This is due to the colouring that most of the different shellfish matrices have an effect that, without use of appropriate controls, could erroneously establish the presence of toxins associated with the OA-group [97, 111].

Another limiting factor with this assay is its storage temperature. This is because the substrate is very stable at temperatures <15°C and very sensitive to high temperatures (≥ 37°C), which may lead to underestimation of toxins related to the OA-group. Within this process, it is possible to consider the percentage of recoverability from the different matrices, which has been established between ≈70 and ≈163%, notably affecting the quantification of toxins from shellfish [123].

Finally, one of the biggest disadvantages of PP2aIA is its inability to identify toxins or toxic analogues associated with PTX-, AZA- and YTX-groups [121]. Thus, PP2aIA is only limited to identify the presence of the toxins associated with OA-group, when applicable, even though the co-occurrence of toxins in water or shellfish is evident [12, 13, 26]. Therefore, to identify all toxins in the hypothetical simultaneous presence of all toxic groups in contaminated shellfish, the specific use of enzymatic or cellular assays will be required, considering also that they will never be able to identify the analogues involved in the toxic event accurately [7, 10].

In conclusion, this assay does not tend to be the most adequate to consider the simultaneity of processes of toxic blooms at a global level, in addition to the fact it is not able to provide any information on the profile of the toxins involved in the bloom or contamination of the endemic species (shellfish), which is a crucial stage if we consider that it is the basis of the information in the establishment of risk assessment and management [7].

2.1.3 Chemical method: liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

The emergence of instrumental analytical methods for the detection of LMTs responds to the different questions regarding the MBA and PP2aIA. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is one of the methods that has been highlighted in the last 15 years [130]. This method allows for the quantification of all analogues associated with LMTs (OA-, PTX-, AZA- and YTX-group) when compared to a certified standard for toxins (**Figure 8**) [7, 104].

This chemical method (LC-MS/MS) has been validated and approved by the European Regulation as the new official method for the control of LMTs in shellfish [29, 78, 107, 131]. However, the quantification of toxin analogues is not sufficient for the control and regulatory decision making, since the different analogues have different toxic potencies. For this evaluation, it is necessary to know the relative toxicities of the components making up the mixture of toxins present in a contaminated matrix. For this reason, the term Toxicity Equivalency Factors (TEFs) has been applied, which is defined as the toxicity ratio of a compound from a chemical group that shares the same mode of action of a reference compound in the same group [14, 94, 95, 104].

Thus, the establishment of TEFs in alternative methods to the MBA and PP2aIA for the detection of marine toxins allows, to a great extent, better protection of the consumer in the surveillance programs, since the toxic potential in a mixture of toxins in different endemic species and biological matrices can be better estimated [94, 97, 102, 104].

In this way, the toxin content detected in the different endemic species matrices is expressed as the sum of the equivalents established for each group, considering that for the case of the OA-group, it is necessary to estimate the concentrations of esterified toxins present in the matrices (DTX-3 and acyl-derivatives toxins), a process that requires the evaluation of post-hydrolysis samples of the extract from each matrix [14].

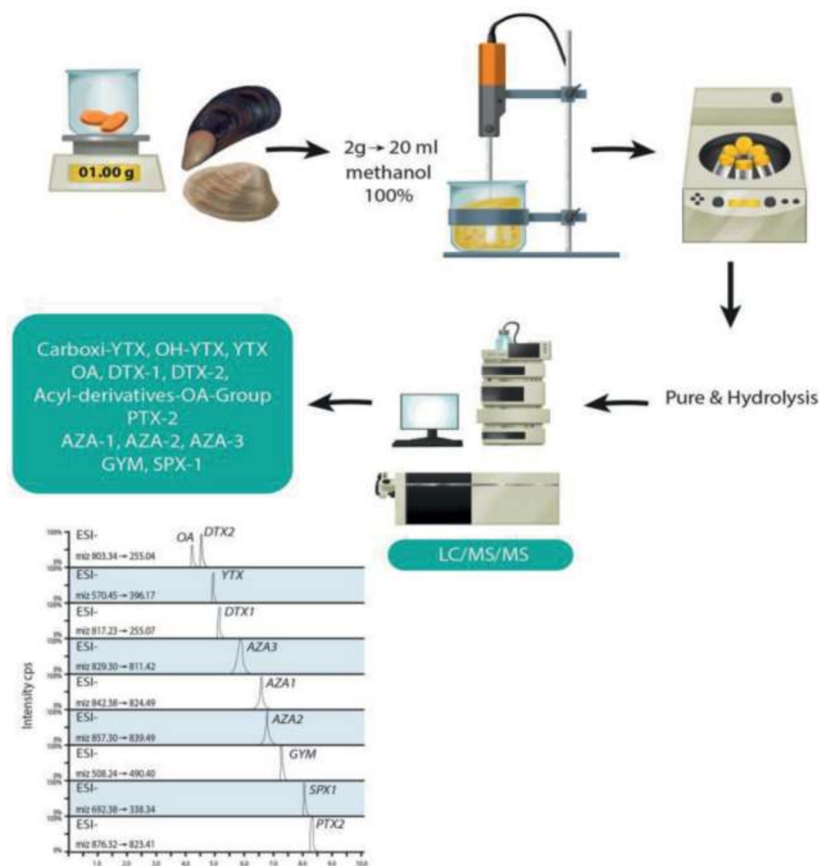


Figure 8.
Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for the detection of lipophilic marine toxins [104, 107, 131].

LC-MS/MS is a method 10 times more sensitive (LOD ≈ 3.0 μg kg^{-1}) than the known 9-anthryldiazomethane (ADAM) fluorescence method (LOD of ≈30 μg kg^{-1}), a chemical method that was initially used to determine OA-group toxins [132, 133] and then proposed to determine toxins of the AZA-group [134]. Nevertheless, for both cases, laborious derivatization steps that increase uncertainty in the analysis process are required. In addition, the ADAM reagent is unstable and should be stored at low temperatures (≤4°C), because its decomposition may induce an incomplete derivatization, interfering with the final analysis [107].

For the use of this analytical chemical method (LC-MS/MS), it is recommended that the analysis of LMTs is started with an initial weight of 150 g of shellfish (without shells), which corresponds to approximately 20–30 bivalves, in order to ensure a representative sample is evaluated [14].

The main advantages of the method include the following [7, 10, 14, 40, 96]:

It is highly specific and sensitive.

- It can individually classify and quantify all toxins of LMTs.
- It provides information on the profiles of all LMTs in the samples.
- It can be automated.
- It provides a reproducible interpretation of the results obtained from the analysis.

- It has no ethical restrictions.

The main disadvantages of the LC-MS/MS method include the following:

- It requires costly equipment and highly trained personnel.
- It requires a wide range of reference standards for identification and quantification.
- The different biological matrices (endemic species) can produce problems in the interpretation of data.

It should be noted that as of July 2011, Regulation (EU) N0. 15/2011 amended the Regulation (EC) No. 2074/2005 in relation to the recognised testing methods for the detection of LMTs in live bivalve molluscs, which establishes the LC-MS/MS method as the official reference method for the detection of LMTs and their use as a routine matter, both for the purposes of official controls at any stage of the food chain, and for the self-controls established by food business operators [29, 30, 78].

All the above mentioned considerations have been established by most countries to improve their quality controls in water and in bivalves from culture centres or endemic species, including water analysis in the location where these cultures are carried out. It should be taken into consideration that the dinoflagellates producers of OA- and/or PTX-, AZA- and YTX-groups at concentrations of ≈2000 cells L^{-1} allow for an adequate accumulation of toxins in bivalves, allowing for the fact that they can be toxic. However, the LOD (sensitivity) of the bioassays for the OA-group in molluscs corresponds to ≈200 μg kg^{-1} shellfish, a value above the internationally established standard of 160 μg OA equivalents kg^{-1} shellfish meat [40].

For the evaluation of all LMTs, the chemical method requires each group to be evaluated in different ionisation modes (**Table 3**) [12, 130, 131, 135].

These ionisation modes allow for the obtainment of suitable ionic fragments and characteristics of each toxic analogue, which allows for the optimal identification of them (**Figure 9**). Nevertheless, it is possible to use both ionisation modes (positive and/or negative mode) for the detection of toxins, but this affects three important

Compound	Ion polarity mode	Precursor ion (m/z)	Product ion 1	Product ion 2	TEF
OA	Negative	803.45	785.4	255.1	1.0
DTX-1	Negative	817.5	785.4	255.1	1.0
DTX-2	Negative	803.5	785.4	255.1	0.6
7-O-palmitoyl-OA	Negative	1041.6	785.4	255.1	1.0*
7-O-palmitoyl-DTX-1	Negative	1055.7	785.4	255.1	1.0*
7-O-palmitoyl-DTX-2	Negative	1041.6	785.4	255.1	1.0*
PTX-1	Positive	892.5	821.5	213.2	1.0
PTX-2	Positive	876.5	823.4	213.2	1.0
PTX-2SA	Positive	894.5	823.4	213.2	1.0
7-epi-PTX2SA	Positive	894.5	823.4	213.3	1.0
PTX-11	Positive	892.5	551.2	213.3	1.0
AZA-1	Positive	842.5	824.5	806.5	1.0
AZA-2	Positive	856.5	838.5	820.5	1.8
AZA-3	Positive	828.5	810.5	792.5	1.4
YTX	Negative	1141.5	1061.7	855.5	1.0
45-OH-YTX	Negative	1157.5	1077.7	871.5	1.0
Homo-YTX	Negative	1155.5	1075.5	869.5	1.0
45-OH-Homo-YTX	Negative	1071.5	1091.5	869.5	0.5

*The TEF of the hydrolysis product of OA, DTX1 or DTX2 would apply [104].

Table 3.
*LC-MS/MS Parameters for determination of lipophilic marine toxins [14, 40, 46, 113, 131, 136, 137]. *The TEF of the hydrolysis product of OA, DTX1 or DTX2 would apply [104].*

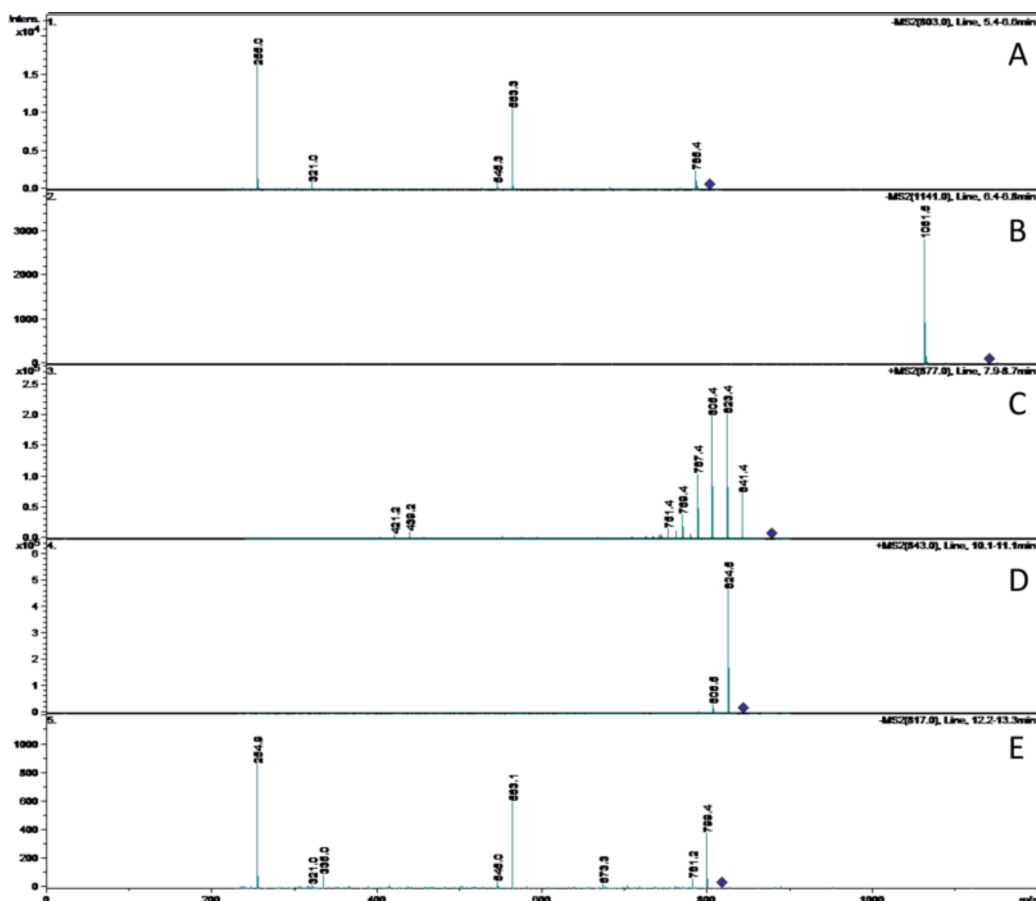


Figure 9.
Mass/mass spectra obtained from a mixture of analytical standards for determination in negative ion mode: AO (A), YTX (B) and DTX-1 (E), and in positive ion mode: PTX-2 (C) and AZA-1 (D) [40, 42, 50].

factors: sensitivity, intensity in the detection of analogues and formation of structural fragments generated by the original toxin, producing a difficult interpretation of the toxic analogues present in the matrices [97, 138]. Thus, the determination of the YTX-group in positive mode produces a very low sensitivity of the ions obtained ($[M-2Na + 3H]^+$) making its practical use unfeasible. While in the case of OA-group, the evaluation of post-hydrolysis matrices in positive mode tends to produce matrix effects that do not allow adequate quantification of toxins related to acyl derivatives [139]. In addition, when the base analogues of the OA-group (OA, DTX-1 and DTX-2) are evaluated, the primary ions, through a positive ionisation, tend to always produce a greater variability, resulting in an overestimation of toxicity under this ionisation mode [7].

Another important factor is the mobile phase to be used. Mobile phases modified with different weak carboxylic acids (formic acid, acetic acid, propionic acid and n-butyric acid) may, in some cases, (formic acid) result in decreased electrospray ionisation (ESI) responses of the negative ions produced from toxins. This is consistent with the idea that acidic conditions decrease the negative-ion ESI response, i.e., the conditions at a low pH do not favour the formation of deprotonated analytes [140].

Given the high variability according to the evaluation from different marine matrices in different endemic species of the world, it is necessary to consider the following validation parameters: accuracy, trueness, precision, linearity, robustness, calibration curve check, limit of detection (LOD), limit of quantification (LOQ), and blank quality control [141–143].

3. Discussion

The use of any of the established methods should always consider the variable clearance rates associated with each potentially toxic marine species; those parameters are species-specific in endemic species, since neither body size nor age play a decisive role in the clearance rate of toxins [96]. Hence, it is extremely important to determine all the groups associated with LMTs.

Moreover, the variability of the results obtained with any of the indicated methods is always conditioned to the following parameters:

Growth conditions: The origin of shellfish from endemic species, on the seabed or from cultures, subtidal or intertidal growth, water depth, and water column mix [7].

Clearance rate: Feeding status, species-specific filtration rates and selectivity, as well as the species of microorganisms that can affect endemic species (bacteria and pathogenic viruses) [12, 26].

Metabolism/detoxification: Species-specific differences in endemic species, metabolic changes in bivalves due to seasonal variation, reproductive status and environmental stress [13].

Thus, in order to ensure seafood safety and minimise potential risks to human health, the development of rapid, sensitive and reliable methods to detect different groups in a preventive manner has been proposed. However, two important factors must be considered:

1. Biological and biochemical methods only allow for the detection of specific groups of toxins, and may exclude some, which may cause a potential toxicity when consuming shellfish seemingly free of toxins [14, 102].

2. EFSA has proposed the reduction of maximum limits of all LMTs associated groups, from 160 μg kg $^{-1}$ to 45 μg OA equivalents kg^{-1} (OA-Group); 30 μg AZA equivalents kg^{-1} (AZA-group) and 120 μg PTX equivalents kg^{-1} (PTX-group); these new limits could only be detected completely by using the LC-MS/MS method [7, 14, 107].

4. Conclusions

The above shows that the MBA and PP2aIA assays for LMTs detection cannot be effectively used to follow the toxic variability in molluscs or endemic species in a quantitative manner, including their detoxification stage, as these assays do not allow producers (shellfish growers) to evaluate the results obtained in order to plan their production activities adequately and in advance, since the use of these assays as screening would have less specificity and a higher cost if compared to the confirmation method (LC-MS/MS) used by marine product importing countries.

The LC-MS/MS method does not show any ambiguous results, since it solves and determines the toxic profiles in different toxic marine endemic species (matrices). In addition, it allows for the exact quantification of each group of toxins by keeping those samples under the established legal limits in the market, which by using other methods could prove to be positive (MBA) or not to be able to establish the toxic group involved in a HABs or toxic processes in shellfish (PP2aIA). Thus, the great advantage of LC-MS/MS is the possibility of differentiating toxins belonging to the OA-, YTX-, AZA and PTX- groups, allowing for the evaluation of the potential health risks through the consumption of shellfish.

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Conflict of interest

The authors declare no conflict of interest.

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