SCIENTIFIC OPINION

Scientific Opinion on marine biotoxins in shellfish – Palytoxin group[1]

EFSA Panel on Contaminants in the Food Chain (CONTAM)[2, 3]

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ABSTRACT

The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) assessed the risks to human health related to the presence of palytoxin (PlTX)-group toxins in shellfish. PlTX-group toxins have mainly been detected in soft corals of the genus *Palythoa* and in algae of the genus *Ostreopsis*. Blooms of *Ostreopsis* spp. have recently been reported in some European countries. Occurrence of *Ostreopsis* spp. may result in contamination of shellfish intended for human consumption. Currently there are no regulations on PlTX-group toxins in shellfish, either in the European Union (EU), or in other regions of the world. The toxicological database of PlTX-group toxins is limited, comprising only acute toxicity studies for PlTX and ostreocin-D via several routes of administration in various animal species. The oral route was least sensitive. Acute toxicity and deaths have been reported from human outbreaks, but there are no reliable quantitative data on acute toxicity in humans. In view of the acute toxicity and the lack of chronic toxicity data for PlTX-group toxins, the CONTAM Panel was only able to derive an oral acute reference dose (ARfD) of 0.2 µg/kg b.w. for the sum of PlTX and its analogue ostreocin-D. In order for a 60 kg adult to avoid exceeding the ARfD a 400 g portion of shellfish meat should not contain more than 12 µg of the sum of PlTX and ostreocin-D, corresponding to 30 µg/kg shellfish meat. The mouse bioassay (MBA) has been used to detect PlTX-group toxins, but cell based assays have been developed as alternative. However, positive results require confirmation by chemical methods. High performance liquid chromatography-fluorescence detection (HPLC-FLD) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods can be valuable tools for the determination, but method optimisation and validation as well as the development of certified reference materials and standards are necessary.

KEY WORDS

Marine biotoxins, palytoxin (PlTX)-toxin group toxins, shellfish, mussels, sea urchins, mouse bioassay (MBA), acute reference dose, portion size, methods of analysis, human health, risk assessment.

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SUMMARY

Palytoxin (PlTX)-group toxins are marine biotoxins which have mainly been detected in marine zoanthids (soft corals) of the genus *Palythoa* and benthic dinoflagellates of the genus *Ostreopsis*. PlTX-group toxins were first reported in Hawaii and Japan but are currently distributed worldwide. Blooms of *Ostreopsis* spp. have recently been reported in four European countries: France, Greece, Italy, and Spain. Occurrence of *Ostreopsis* spp. may result in contamination of shellfish species intended for human consumption.

PlTX-group toxins are complex polyhydroxylated compounds with both lipophilic and hydrophilic areas. At least 8 different PlTX analogues are known: PlTX, ostreocin-D, ovatoxin-A, homopalytoxin, bishomopalytoxin, neopalytoxin, deopalytoxin and 42-hydroxypalytoxin, but only for PlTX and ostreocin-D the chemical structure has been characterised. The occurrence data reported by European countries were limited and comprised only PlTX and ovatoxin-A in mussels and sea urchins. Currently there are no regulations on PlTX-group toxins in shellfish, either in the European Union (EU), or in other regions of the world.

Signs and symptoms of PlTX-group toxins intoxication are not well-defined, but include myalgia and weakness, possibly accompanied by fever, nausea and vomiting. Fatalities appear to be rare although there are reports of severe cases, in which patients died after about 15 hours.

The toxicological database is limited, comprising only acute toxicity studies via several routes of administration in various animal species. The toxicity of PlTX is strongly dependent on the route of administration; in experimental animals PlTX and ostreocin-D are much less toxic after oral than after parenteral administration. Acute toxicity and deaths have been reported from human outbreaks, but there are no reliable quantitative data on acute toxicity of PlTX-group toxins in humans. PlTX-group toxins are acutely toxic mainly by interference with Na^+/K^+-ATPase ion-pump.

The absence of long term toxicity studies precludes the derivation of a tolerable daily intake (TDI), but in view of the acute toxicity of PlTX-group toxins the Panel on contaminants in the food chain (CONTAM Panel) decided to establish an acute reference dose (ARfD) using the lowest-observed-adverse-effect-level (LOAEL) for oral toxicity (gavage) in mice of 200 µg/kg b.w. as the reference point.

It was noted that sublingual administration of PlTX in the region of 200 µg/kg b.w. caused toxicity of internal organs. Since transmucosal transport of PlTX in the mouth could not be excluded and because mice seem to be less sensitive than other species, the CONTAM Panel decided to apply an extra uncertainty factor of 10 in addition to the default uncertainty factors of 10 for intra- and 10 for interspecies variation and derived an oral ARfD of 0.2 µg/kg b.w. This ARfD applies to the sum of PlTX and ostreocin-D.

In order to ensure protection against the acute effects of PlTX-group toxins, it is important to use a large portion size rather than a long term average consumption in assessing the health risk of the consumers. In line with the previous scientific opinions on shellfish toxins the figure of 400 g shellfish meat was used in the acute exposure assessment as an appropriate estimate of a large portion size. In order for a 60 kg adult to avoid exceeding the ARfD of 0.2 µg/kg b.w., a 400 g portion of shellfish meat should not contain more than 12 µg of the sum of PlTX and ostreocin-D, corresponding to 30 µg/kg shellfish meat.

The CONTAM Panel noted that the available occurrence data of PlTX-group toxins are from mussels and sea urchins sampled in contaminated regions and thus are not representative for PlTX-group toxins in shellfish currently reaching the market. These data however indicate that consumption of a large portion of 400 g of shellfish meat (i.e. mussels) harvested from contaminated regions as a worst case estimate could result in a dietary exposure of about 3 µg/kg b.w. of the sum of PlTX and ovatoxin-A for a 60 kg person. If ovatoxin-A had a similar potency to PlTX and ostreocin-D, this exposure would be well above the ARfD.

The mouse bioassay (MBA) has been used to detect PlTX-group toxins in fish and shellfish, but for reasons of animal welfare and poor specificity there is a growing concern with respect to its use. Alternative assays such as cell based assays have been developed. While cell based assays, taking advantage of certain PlTX functional properties, appear to have the lowest limit of detection (LOD) for PlTX-group toxins, some assays showed interference from other toxins and the positive results should be confirmed by chemical analysis. High performance liquid chromatography-fluorescence detection (HPLC-FLD) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods can be valuable tools for the determination of PlTX-group toxins. The optimisation of these methods for application to shellfish extracts, their (inter-laboratory) validation and the development of standards and reference materials are necessary.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Marine biotoxins, also commonly known as shellfish toxins, are mainly produced by algae or phytoplankton.

Based on their chemical structure, the toxins have been classified into eight groups, namely, the azaspiracid (AZA), brevetoxin, cyclic imine, domoic acid (DA), okadaic acid (OA), pectenotoxin (PTX), saxitoxin (STX) and yessotoxin (YTX) groups, as agreed at the Joint FAO/IOC/WHO *ad hoc* Expert Consultation held in 2004[4]. Two additional groups, palytoxins (PlTX) and ciguatoxins (CTX), may also be considered. STX and its derivatives cause Paralytic Shellfish Poisoning (PSP), and DA causes Amnesic Shellfish Poisoning (ASP). Diarrhetic Shellfish Poisoning (DSP) is caused by OA-group toxins (OA and dinophysis toxins (DTX)), and AZA group toxins cause Azaspiracid Shellfish Poisoning (AZP). These toxins can all accumulate in the digestive gland (hepatopancreas) of filter-feeding molluscan shellfish, such as mussels, oysters, cockles, clams and scallops, and pose a health risk to humans if contaminated shellfish are consumed. Marine biotoxin-related illness can range from headaches, vomiting and diarrhoea to neurological problems, and in extreme cases can lead to death.

To protect public health, monitoring programmes for marine biotoxins have been established in many countries, which often stipulate the use of animal models (for example, the mouse bioassay (MBA) and the rat bioassay (RBA)), for detecting the presence of marine biotoxins in shellfish tissues.

In the European Union (EU), bioassays are currently prescribed as the reference methods. Various stakeholders (regulators, animal welfare organisations, scientific organisations) have expressed their concerns about the current legislation in Europe, not only with regard to the use of large numbers of animals, involving procedures which cause significant pain and suffering even though non-animal based methods are available, but also since the scientific community argues that the animal test may not be suitable for all classes of toxins and that the state-of-the-art scientific methodology for the detection and determination of marine biotoxins is not fully reflected in current practices.

1. Legal framework

In 2004, the purported *EU Hygiene Package* of regulations, bringing together and replacing the existing hygiene regulations for the food sector previously contained in numerous individual vertical Directives was published. In Annex II Section VII Chapter V (2) to Regulation 853/2004/EC[5], are established maximum levels for ASP, PSP and DSP toxins. Annex III of Commission Regulation No 2074/2005/EC[6] of 5 December 2005 lays down the recognised testing methods for detecting marine biotoxins. Annex II Chapter II (14) to Regulation (EC) 854/2004[7], gives the monitoring authorities in the EU Member States the mandate to examine live molluscs for the presence of marine biotoxins. The *EU Hygiene Package* came into effect on 1 January 2006.

2. The Council Directive 86/609/EEC

Council Directive 86/609/EEC[8] makes provision for laws, regulations and administrative provisions for the protection of animals used for experimental and other scientific purposes. This includes the use of live vertebrate animals as part of testing strategies and programmes to detect identify and quantify marine biotoxins. Indeed, the scope of Article 3 of the Directive includes the use of animals for the safety testing of food, and the avoidance of illness and disease.

Directive 86/609/EEC sets out the responsibilities that Member States must discharge. As a result of this use of prescriptive language, Member States have no discretion or flexibility, and most of the

[4] ftp://ftp.fao.org/es/esn/food/biotoxin_report_en.pdf
[5] OJ L 139, 30.4.2004, pp. 55–205
[6] OJ L 338, 22.12.2005, pp. 27–59.
[7] OJ L 139, 30.4.2004, pp. 206–320.
[8] OJ L 358, 18.12.1986, pp. 1–28.

provisions of the Directive must be applied in all cases. It is clear that Member States have to ensure that: the number of animals used for experimental and other scientific purposes is reduced to the justifiable minimum; that such animals are adequately cared for; and that no unnecessary or avoidable pain, suffering, distress or lasting harm are caused in the course of such animal use.

Member States may not (Article 7, 2) permit the use of live animals in procedures that may cause pain, suffering, distress or lasting harm: "if another scientifically satisfactory method of obtaining the result sought and not entailing the use of live animals is reasonably and practicably available". When animal use can be justified, Directive 86/609/EEC specifies a range of safeguards that Member States must put in place to avoid or minimise any animal suffering that may be caused. All justifiable animal use should be designed and performed to avoid unnecessary pain, suffering, distress and lasting harm (Article 8). Member States must ensure (Article 19, 1) that user establishments undertake experiments as effectively as possible, with the objective of obtaining consistent results, whilst minimising the number of animals and any suffering caused.

This latter requirement necessitates the use of minimum severity protocols, including appropriate observation schedules, and the use of the earliest humane endpoints that prevent further suffering, once it is clear that the scientific objective has been achieved, that the scientific objective cannot be achieved, or that the suffering is more than can be justified as part of the test procedure. The EC and Member States are also required (Article 23, 1) to encourage research into, and the development and validation of, alternative methods that do not require animals, use fewer animals, or further reduce the suffering that may be caused, whilst providing the same level of scientific information.

3. Recognised testing methods for marine biotoxins and maximum levels

Commission Regulation (EC) No. 2074/2005[6] specifies a mouse bioassay (MBA) for the determination of paralytic shellfish poisoning toxins (PSP) and a MBA or the rat bioassay (RBA) for lipophilic marine biotoxins. Alternative test methods can be applied if they are validated following an internationally recognised protocol and provide an equivalent level of public health protection.

Besides paralytic shellfish poisoning toxins, okadaic acid, dinophysistoxins, pectenotoxins, azaspiracids and yessotoxins, also cyclic imines, (gymnodimine, spirolides and others which are currently not regulated in the EU), all give a positive response in MBAs.

The reference method for the domoic acid group (the causative agent of ASP) is based on high-performance liquid chromatography (HPLC).

Chapter V (2) (c) and (e) of Section VII of Annex III to Regulation (EC) No 853/2004[5] establishes that food business operators must ensure that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits:

- 800 micrograms per kilogram for paralytic shellfish poison (PSP),
- 20 milligrams of domoic acid per kilogram for amnesic shellfish poison (ASP),
- 160 micrograms of okadaic acid equivalents[9] per kilogram for okadaic acid, dinophysistoxins and pectenotoxins in combination,
- 1 milligram of yessotoxin equivalents per kilogram for yessotoxins,
- 160 micrograms of azaspiracid equivalents per kilogram for azaspiracids.

[9] Equivalents: the amount of toxins expressed as the amount of okadaic acid that gives the same toxic response followed intraperitoneal administration to mice. This applies similarly for the group of yessotoxins and azapiracids, respectively.

4. Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs (Oslo, September 26-30 2004)

Based on the available information, the Joint FAO/IOC/WHO *ad hoc* Expert Consultation suggested provisional acute reference doses (ARfDs)[10] for the AZA, OA, STX, DA, and YTX-group toxins, respectively (summarised in the Table 1). The Expert Consultation considered that the database for the cyclic imines, brevetoxins and pectenotoxins was insufficient to establish provisional ARfDs for these three toxin groups. In addition, guidance levels were derived comparing results based on the consumption of 100 g, 250 g or 380 g shellfish meat by adults. However, the Expert Consultation noted that the standard portion of 100 g, which is occasionally used in risk assessment, is not adequate to assess an acute risk, whereas a portion of 250 g would cover 97.5 % of the consumers of most countries for which data were available.

Available methods of analysis were reviewed for the 8 toxin groups and recommendations made for choice of a reference method, management of analytical results and development of standards and reference materials.

The Joint FAO/IOC/WHO *ad hoc* Expert Consultation, however, did not have sufficient time to fully evaluate epidemiological data and to assess the effects of cooking or processing for deriving the provisional guidance levels/maximum levels for several toxin groups (especially the AZA and STX groups). The Consultation encouraged Member States to generate additional toxicological data in order to perform more accurate risk assessments and to facilitate validation of toxin detection methods in shellfish.

[10] The acute reference dose is the estimate of the amount of substance in food, normally expressed on a body-weight basis (mg/kg or µg/kg of body weight), that can be ingested in a period of 24 hours or less without appreciable health risk to the consumer on the basis of all known facts at the time of evaluation (JMPR, 2002).

Table 1: Summary data used in the derivation of the ARfD and current guidance levels.

Group toxin	LOAEL(1) NOAEL(2) µg/kg body weight	Safety Factor (Human data (H) Animal data (A))	Provisional Acute RfD[10]	Derived Guidance Level/ Max Level based on consumption of 100 g (1), 250 g (2) and 380 g (3)	Limit Value currently implemented in EU legislation
AZA	0.4 (1)	10 (H)	0.04 µg/kg 2.4 µg/adult(a)	0.024 mg/kg SM (1) 0.0096 mg/kg SM (2) 0.0063 mg/kg SM (3)	0.16 mg/kg SM
BTX			N/A		
Cyclic Imines			N/A		
DA	1,000 (1)	10 (H)	100 µg/kg 6 mg/adult(a)	60 mg/kg SM (1) 24 mg/kg SM (2) 16 mg/kg SM (3)	20 mg/kg SM
OA	1 (1)	3 (H)	0.33 µg/kg 20 µg/adult(a)	0.2 mg/kg SM (1) 0.08 mg/kg SM (2) 0.05 mg/kg SM (3)	0.16 mg/kg SM
PTX			N/A		0.16 mg OA equivalents/kg SM
STX	2 (1)	3 (H)	0.7 µg/kg 42 µg/adult(a)	0.42 mg/kg SM (1) 0.17 mg/kg SM (2) 0.11 mg/kg SM (3)	0.8 mg/kg SM
YTX	5,000 (2)	100 (A)	50 µg/kg 3 mg/adult(a)	30 mg/kg SM (1) 12 mg/kg SM (2) 8 mg/kg SM (3)	1 mg/kg SM

SM = shellfish meat, LOAEL = lowest-observed-adverse-effect level, NOAEL = no-observed-adverse-effect level, N/A = not available, EU = European Union

(a): Person with 60 kg body weight (b.w.)

The Joint FAO/IOC/WHO *ad hoc* Expert Consultation also indicated that there were discrepancies between different risk assessments, especially for determining methods of analysis for certain marine biotoxins and in relation to established maximum limits.

Test methods for the eight toxin groups were reviewed and recommendations for Codex purposes made. Mouse bioassays are widely used for shellfish testing but for technical and ethical reasons it is highly desirable to move to new technologies which can meet Codex requirements more adequately. Most currently available methods do not meet fully the strict criteria for Codex type II[11] or III[12] methods and have therefore not been widely used in routine shellfish monitoring. However, the recommendations made by the Expert Consultation represent the best currently available methods. Liquid chromatography-mass spectrometry (LC-MS) has much potential for multi-toxin analysis and has been recommended for consideration and recommendation by Codex. The Joint FAO/IOC/WHO *ad hoc* Expert Consultation is of the opinion that the complexity and chemical diversity of some toxin groups is such that validated quantitative methods to measure all toxins within a group will be extremely difficult. Thus the implementation of a marker compound concept and the use of functional assays should be explored.

[11] A Type II method is the one designated Reference Method where Type I methods do not apply. It should be selected from Type III methods (as defined below). It should be recommended for use in cases of dispute and for calibration purposes.

[12] A Type III Method is one which meets the criteria required by the Codex Committee on Methods of Analysis and Sampling for methods that may be used for control, inspection or regulatory purposes.

5. Working Group Meeting to Assess the Advice from the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs, Ottawa, Canada, April 10-12, 2006

The working group (WG) discussed available reference methods in particular and concluded that they should be highly specific, highly reproducible, and not prone to false positives or false negatives. The methods are expected to be definitive and may well result in significant rejections of products and must therefore withstand the most robust legal and scientific scrutiny.

In considering their weaknesses and merits, the meeting noted that the various mouse bioassays should be discussed individually since the level of performance and success differs markedly between the official method for PSP by mouse bioassay, the American Public Health Association (APHA) method for brevetoxins and the multiple mouse bioassay "DSP" procedures employed for the other lipophilic toxins such as okadaic acid, azaspiracids and others.

Recognizing that the majority of the currently available methods do not meet all Codex criteria for reference methods (Type II), the WG concluded that Codex Committee for Fish and Fishery Products (CCFFP) should consider a variety of biotoxin analytical methods. Wherever possible, reference methods should not be based on animal bioassays. Functional methods, biochemical/immunological and chemical-analytical methods currently in use, and considered to be validated according to Codex standards, should be recommended by CCFFP to the Codex Committee on Methods of Analysis and Sampling (CCMAS) for review and designation as Type II or Type III methods.

Because the Expert Consultation has offered 3 different guidance limits associated with three levels of consumption (100 g, 250 g and 380 g) for most toxin groups, it is important to determine which consumption level is appropriate for the protection of consumers.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Art. 29 (1) (a) of Regulation (EC) No 178/2002, the Commission asks EFSA to assess the current EU limits with regard to human health and methods of analysis for various marine biotoxins as established in the EU legislation, including new emerging toxins, in particular in the light of

- the report of the Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs (Oslo, September 26-30 2004), including the ARfDs and guidance levels proposed by the Expert Consultation,
- the conclusions of the CCFFP working group held in Ottawa in April 2006,
- the publication of the report and recommendations of the joint European Centre for the Validation of Alternative Methods (ECVAM)/DG SANCO Workshop, January 2005,
- the report from CRL Working group on Toxicology in Cesenatico October 2005,
- any other scientific information of relevance for the assessment of the risk of marine biotoxins in shellfish for human health.

ASSESSMENT

1. Introduction

Palytoxin (PlTX)-group toxins have mainly been detected in marine zoanthids (soft corals) of the genus *Palythoa* (e.g. *Palythoa toxica*, *P. tuberculosa*, *P.vestitus*, *P. mammilosa*, *P.carobaeorum*, *P. aff. Margaritae*) and benthic dinoflagellates of the genus *Ostreopsis* (e.g *Ostreopsis siamensis*, *O. mascarenensis*, *O. ovata*). They were first reported in Hawaii and Japan but are currently distributed worldwide (Moore and Bartolini, 1981; Deeds and Schwartz, 2009). Blooms of *Ostreopsis* spp. have also been reported in European countries (Ciminiello et al., 2009), such as in France, Greece, Italy and Spain. Occurrence of *Ostreopsis* spp. may result in contamination of shellfish intended for human consumption, e.g. bivalve molluscs. *O. ovata* mainly produces ovatoxin-A and traces of PlTX.

Signs and symptoms of PlTX-group toxins intoxication are not well-defined, but include myalgia and weakness, possibly accompanied by fever, nausea and vomiting. Fatalities appear to be rare although there are reports of severe cases, in which patients died after about 15 hours (Deeds and Schwartz, 2009).

Currently there are no regulations on PlTX-group toxins in shellfish, either in the European Union (EU), or in other regions of the world.

2. Chemical characteristics

PlTX-group toxins are complex polyhydroxylated compounds with both lipophilic and hydrophilic areas. The basic molecule consists of a long, partially unsaturated aliphatic backbone containing cyclic ethers, 64 chiral centers, 40-42 hydroxyl and 2 amide groups (Figure 1). The primary amino-group at the C-115 end of the molecule accounts for the basicity of PlTX-group toxins (Katikou, 2007). The molecular formula and molecular weight of PlTX analogues differ depending on the *Palythoa* species from which they are obtained (Moore and Bartolini, 1981). The chemical formula of PlTX from *P. toxica* is $C_{129}H_{233}N_3O_{54}$, 115 of the 129 carbons being in continuous chain. Its molecular weight is 2,677 Da and it was found to exist as a dimer in aqueous solution (Uemura, 2006).

Due to their two chromophores, PlTX-group toxins exhibit an ultraviolet (UV) absorption spectrum with a λ_{max} at 233 and 263 nm. PlTX-group toxins are insoluble in nonpolar solvents, sparingly soluble in methanol and ethanol and soluble in pyridine, dimethyl sulfoxide and water. They are heat resistant.

In addition to PlTX, other toxins of the same group are produced by several *Ostreopsis* species. For example *O. siamensis* produces the major toxic constituent ostreocin-D which is a chemical analogue of PlTX, also referred to as 42-hydroxy-3,26-dimethyl-19,44-dideoxypalytoxin. For these two compounds preparative isolation and characterisation by nuclear magnetic resonance (NMR) has allowed elucidation of the planar structure. Five further analogues have been found to co-occur with PlTX in *P. tuberculosa*, e.g homopalytoxin, bishomopalytoxin, neopalytoxin, deopalytoxin and 42-hydroxypalytoxin, but have not been reported to be produced by *Ostreopsis* spp. (Uemura et al., 1985; Ciminiello et al. 2009). *O.ovata* produces ovatoxin-A, for which the molecular mass (2647,5 Da) and structural evidence from mass spectrometry show that it is an analogue of PlTX. Ovatoxin A has two oxygen atoms less than PlTX but it is not known from which part of the molecule the oxygen atoms are missing (Ciminiello et al. 2006, 2008; Guerrini et al., 2009).

3. Regulatory status

Currently there are no regulations on PlTX-group toxins in shellfish, either in the EU, or in other regions of the world. During the first meeting of the working group on Toxicology of the national reference laboratories (NRLs) for Marine Biotoxins (Cesenatico, Italy, 24-25 October 2005), a provisional limit of 250 µg/kg shellfish was proposed (CRLMB, 2005).

4. Methods of analysis

Several published methods exist for the determination of PlTX-group toxins. The mouse bioassay (MBA) is considered a simple way to detect PlTX-group toxins, but for ethical and scientific reasons there are growing concerns with respect to its use. Some functional assays and chemical methods have also been developed. Most of these methods were developed for monitoring PlTX in seawater and phytoplankton (Arzul et al., 1994; Habermann et al., 1981; Igarashi et al., 1999; Neely and Campbell, 2006; Oda et al., 2001; Paul et al., 1995; Rangel et al., 1997) and they have found application for the analysis of shellfish. However, standards are not readily available for PlTX-group toxins, which hampers the method development. None of the methods to determine PlTX-group toxins have been formally validated in interlaboratory validation studies.

4.1. Supply of appropriate reference material

There is a lack of certified standards and certified reference materials for PlTX-group toxins.

4.2. Mammalian bioassay

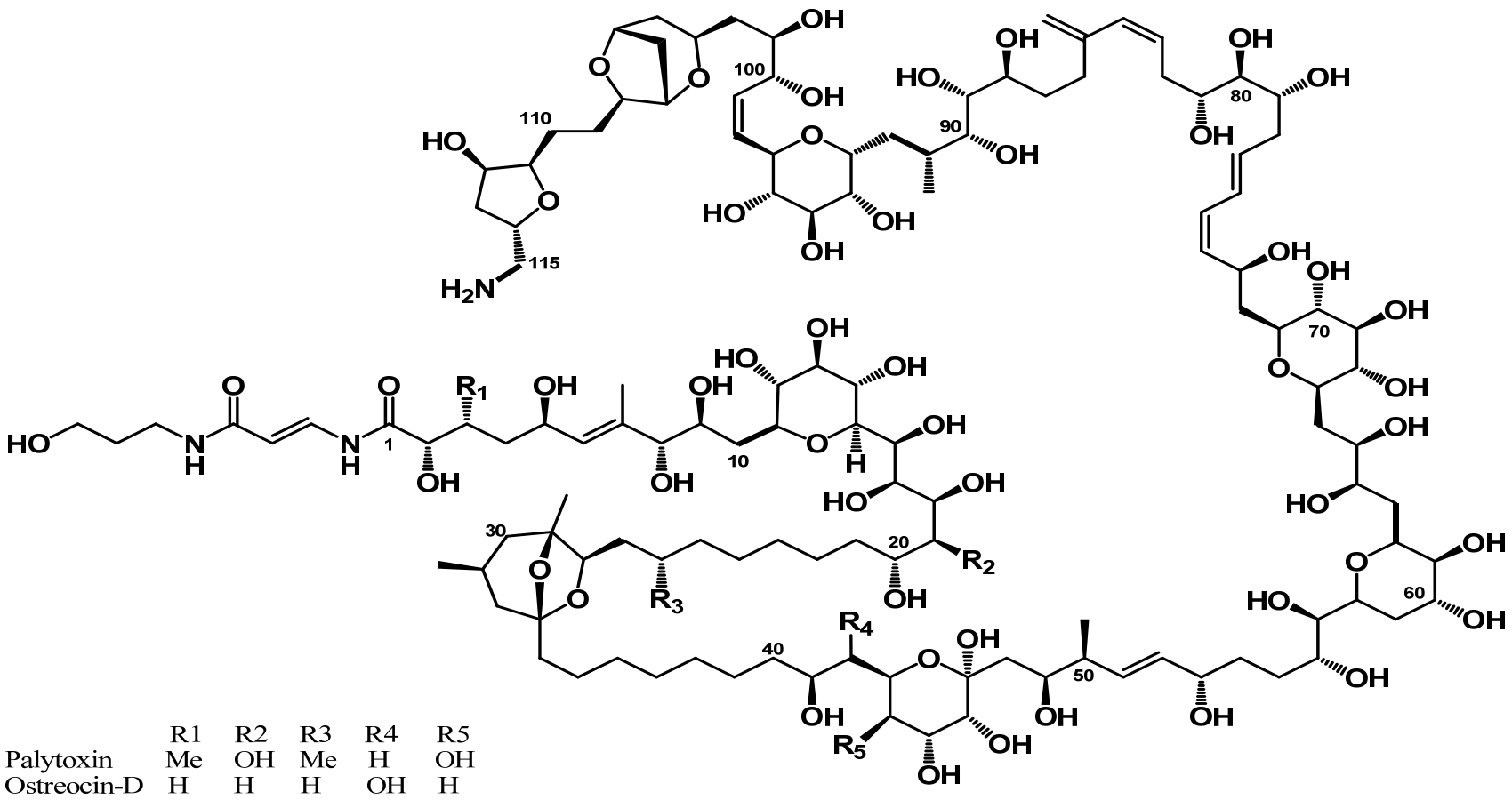
4.2.1. Mouse bioassay

The MBA has been used to detect PlTX-group toxins in fish and shellfish tissues, e.g mussels, oysters, scallops and clams. Two different extraction protocols have been used for the MBA to detect PlTX-group toxins. The first protocol, developed by Yasumoto et al. (1978) is an investigative tool for the determination of the causative agents responsible for a food poisoning outbreak associated with the consumption of molluscs in Japan, and uses acetone extraction of whole flesh or hepatopancreas of shellfish followed by evaporation and resuspension of the residue in a 1 % solution of Tween 60 surfactant. Taniyama et al. (2002) proposed a modified protocol using 75 % aqueous ethanol for extraction of shellfish whole flesh and defattening with diethylether, after removal of the ethanol. This second protocol was found to be more selective for detection of PlTX-group toxins since it allows for discrimination between samples containing okadaic acid (OA) and its analogues, and those containing PlTX-group toxins (Aligizaki et al., 2008). Due to the partitioning behaviour of PlTX-group toxins, as outlined in Aligizaki et al. (2008), the EU harmonised protocol of the MBA (Yasumoto et al., 1985) does not efficiently extract PlTX-group toxins.

In the MBA typical signs for PlTX include jerking and stretching of hind limbs and lower back, weakening of fore limbs, creeping paralysis, ataxia, cyanosis, decreased locomotion, convulsion, gasping for breath and death within 15 minutes. The reported values for the intraperitoneal (i.p.) lethal dose (LD_{50}) of PlTX in mice range from 150 to 720 ng/kg body weight (b.w.) (Onuma et al, 1999; Rhodes et al., 2002; Riobó et al., 2008).

The main advantages of the MBA are:

- the provision of a measure of total toxicity based on the biological response of the animal to the toxin(s);
- it does not require complex analytical equipment.



	R1	R2	R3	R4	R5
Palytoxin	Me	OH	Me	H	OH
Ostreocin-D	H	H	H	OH	H

Figure 1: Chemical structures of the palytoxin (PlTX)-group toxins, PlTX and ostreocin-D.

The main disadvantages of the MBA are:

- a MBA protocol, different from the Yasumoto et al. (1985) protocol is needed to efficiently extract PlTX-group toxins;
- if the Yasumoto et al. (1978) protocol is used, multiple interferences may occur from domoic acid (DA), saxitoxin (STX)-group toxins, yessotoxin (YTX)-group toxins and cyclic imines (CI);
- if the Taniyama et al. (2002) protocol is used, interferences can be reduced, but still include YTX-group toxins and the water-soluble toxins;
- it cannot be automated;
- it requires specialised animal facilities and expertise;
- the inherent variability in results between laboratories due to e.g. specific animal characteristics (strain, sex, age, weight, general state of health, diet, stress);
- the injection volume of one mL exceeds good practice guidelines (<0.5 mL) intended to minimise stress to mice;
- in many countries the use of the MBA is considered undesirable for ethical reasons.

4.3. Biomolecular methods

Biomolecular methods for PlTX-group toxins are based on two different approaches using cell death or antibodies.

4.3.1. Cytotoxicity assays

4.3.1.1. Haemolysis assay (erythrocytes)

Haemolysis assays for the detection of PlTX-group toxins in algal samples and in shellfish are based on the capacity of the toxins to interact with the Na^+/K^+-ATPase converting it into a non specific cation channel. This leads to ion imbalance in the red blood cell resulting in delayed haemolysis. The characteristic delayed lysis and the suppression of haemolytic activity of samples after pretreatment with ouabain (an ATPase blocker), are indicative of haemolytic compounds in the sample.

Several studies (Bignami, 1993; Taniyama et al., 2002, 2003; Riobó et al., 2006, 2008; Lenoir et al., 2004; Aligizaki et al., 2008) used a haemolysis neutralisation assay (HNA) including ouabain to estimate the PlTX-group toxin content of algal, fish or shellfish extracts. The assay developed by Aligizaki et al. (2008) can detect PlTX-group toxins at concentrations equivalent to 1.6 ng PlTX/kg shellfish tissue.

The main advantages of haemolysis assays are:

- they have good detection capabilities;
- they are capable of detecting all biologically active PlTX-group toxins that convert the Na^+/K^+-ATPase into a non specific cation channel;
- the use of microplates enables multiple samples to be analysed in a single run.

The main disadvantages of haemolysis assays are:

- they do not provide any information on the toxin profile;
- they may show interference from other haemolytic compounds possibly occurring in the marine environment.

4.3.1.2. Assays with MCF-7 cells

Bellocci et al. (2008a) developed a cytotoxicity assay using the MCF-7 breast cancer cell line, where the extent of cell lysis is measured by the release of lactate dehydrogenase (LDH) in the culture supernatant.

In this assay cells are exposed to materials to be analysed for the presence of toxins for a short time (1 hour), the culture medium is then removed and cell lysis is induced by the addition of a phosphate buffered saline solution. The removal of culture medium before induction of cell lysis renders the assay more selective for PlTX, allowing discrimination between components acting through the Na+/K+-ATPase, and those that cause cell lysis and/or display cytotoxic activity through a different mechanism of action (Bellocci et al., 2008a). In addition, the removal of culture medium from the cell monolayer at the end of the first incubation is accompanied by the disposal of dead cells and other cellular contents that could have been released into the culture medium, and therefore could be responsible for false positives, due to subsequent detection of LDH activity in culture supernatants.

The assay developed by Bellocci et al. (2008a,b) can detect PlTX-group toxins at concentrations equivalent to about 10 ng PlTX/kg shellfish tissue.

The main advantages of cytotoxicity assays using MCF-7cells are:

- they have good detection capabilities;
- they are capable of detecting all biologically active PlTX-group toxins that convert the Na+/K+-ATPase into a non specific cation channel;
- the use of microplates enables multiple samples to be analysed in a single run;
- they do not show interference from other haemolytic and or cytotoxic compounds possibly occurring in the marine environment.

The main disadvantages of the cytotoxicity assays using MCF-7cells are:

- facilities are needed for maintenance and handling of cell cultures;
- they do not provide any information on the toxin profile.

4.3.1.3. Assays with neuroblastoma cells

Cytotoxicity assays involving the use of neuroblastoma cells and including ouabain pretreatment were developed by several groups (Cañete and Diogène, 2008; Ledreux et al. (2009) and Espiña et al. (2009). Cañete and Diogène (2008) and Ledreux et al. (2009) used Neuro-2a cell-based bioassays in which the effect of PlTX-group toxins was estimated by using the 1-(4,5-dimethylthiazol-2-yl)-3.5-diphenylformazan (MTT) assay for mitochondrial oxido-reductase activity, as an indicator for cell number. A similar assay has been developed by Espiña et al. (2009), using BE(2)-M17 human neuroblastoma cells and adding alamar blue to measure the mitochondrial oxido-reductase activity. The assays developed by Ledreux et al. (2009) and Espiña et al. (2009) can detect PlTX-group toxins at concentrations equivalent to about 50 µg PlTX/kg shellfish tissue.

The main advantages of the cytotoxicity assays using neuroblastoma cells are:

- they have appreciable detection capabilities;
- the use of microplate format enables multiple samples to be analysed in a single run.

The main disadvantages of cytotoxicity assays using neuroblastoma cells are:

- facilities are needed for maintenance and handling of cell cultures;
- they do not provide any information on the toxin profile;
- they are prone to interferences from other toxins (e.g. OA, azaspiracid (AZA)-, YTX-group toxins etc).

4.3.2. Immunoassays

Several antibody-based methods have been used for the detection of PlTX-group toxins, but there have been no recent developments. Levine et al. (1988) developed a radio-immunoassay for the detection of PlTX-group toxins. Bignami et al. (1992) developed five PlTX-specific enzyme-linked immunosorbentassays (ELISA) for the determination of PlTX-group toxins in crude extracts of *P. tuberculosa*. The authors reported that the ELISA methods, although having low detection capabilities, might be subject to matrix interferences. Whether or not matrix interferences could be reduced by dilution is unknown. There is no information about the detection capabilities in shellfish, although the reported limits of detection (LODs) of ELISA methods using PlTX standard were about 1 ng/mL in the assay (Bignami et al., 1992).

The main advantages of the antibody-based methods are:

- they are fast, easy to use, and can be applied to screen many samples for possible further confirmatory analysis.

The main disadvantages of the antibody-based methods are:

- antibodies are not readily available;
- detection capability for PlTX-group toxins in shellfish tissues needs to be clarified;
- they do not provide any information on the toxin profile;
- the accuracy is questionable as the cross-reactivity does not necessarily reflect toxic activity.

4.4. Chemical methods

Several chemical methods are available for the determination of PlTX-group toxins: high performance liquid chromatographic (HPLC) methods with fluorescence detection (FLD) or UV detection, liquid chromatography-mass spectrometric (LC-MS) methods and capillary electrophoresis. Extraction methods for several marine biotoxins in shellfish are available but there is no information on their efficiency for PlTX-group toxins.

4.4.1. HPLC-based methods

4.4.1.1. HPLC-FLD

Riobó et al. (2006) developed a method based on a pre-column derivatisation and solid phase extraction (SPE) clean-up followed by HPLC coupled to FLD and applied it for the determination and quantification of PlTX-group toxins in benthic dinoflagellates of the genus *Ostreopsis*. The results correlated well with those obtained with a haemolysis assay. The instrumental LOD for derivatised PlTX was 0.75 ng standard injected. This instrumental LOD cannot be translated into a LOD for the whole analytical procedure as the method has not been applied to shellfish tissue yet.

The main advantage of the HPLC-FLD method is:

- it can be automated.

The main disadvantages of the HPLC-FLD method are:

- it has not been applied to shellfish tissues yet;
- LOD and limit of quantification (LOQ) information in shellfish tissues is lacking.

4.4.1.2. HPLC-UV detection

PlTX exhibits two UV-absorption peaks (at 233 and 263 nm). HPLC-UV methods have been widely used for the determination of PlTX-group toxins, applying different combinations of columns and mobile phases (Lenoir et al., 2004; Mereish et al., 1991; Oku et al., 2004; Penna et al., 2005; Riobó et al., 2006; Yasumoto et al., 1986). HPLC-UV methods have mainly been used for analysis of

Ostreopsis extracts and, besides the method reported by Yasumoto et al. (1986) concerning determination of crab toxins, no HPLC-UV methods have been published to date for the determination of PlTX-group toxins in shellfish tissues. Reported LODs range from 0.1 to 2 µg injected and are much higher than reported LODs for other chemical methods.

The main advantages of the HPLC-UV methods are:

- they can be automated;
- they are relatively inexpensive and easy to use.

The main disadvantage of HPLC-UV methods is:

- they are about 1000-fold less sensitive than the liquid chromatography-fluorescence detection (LC-FLD) methods, which makes them inappropriate for the detection of PlTX-group toxins in shellfish tissues.

4.4.1.3. LC-MS/MS

Different LC-MS/MS methods have been used for identification and quantification of PlTX-group toxins in seawater and phytoplankton (Lenoir et al., 2004; Penna et al., 2005; Riobó et al., 2006; Ciminello et al., 2006, 2008). Riobó et al. (2006) used FLD and MS/MS detection for analysis of PlTX-group toxins in the benthic dinoflagellate of the genus *Ostreopsis*. The authors showed that the LODs and LOQs of the liquid chromatography-mass spectrometry/mas spectrometry (LC-MS/MS) method were significantly higher than those reported for the LC-FLD method. This may be due to instrumental limitations when using ion trap systems in MS-MS mode over a broad mass range. The detection capability of the LC-MS/MS methods was significantly improved using a triple quadrupole (Ciminiello et al., 2006). This method was applied for the determination of PlTX-group toxins in seawater, plankton and macroalgal extracts (Ciminiello *et al.*, 2006, 2008). Although LC-MS/MS methods are being used by official control laboratories for the quantitative determination of PlTX-group toxins in shellfish, no validation studies on LC-MS/MS methods have been published to date and detailed performance characteristics of these methods are thus unknown. The LODs of LC-MS/MS methods for PlTX in shellfish were reported to be 2 and 36 µg/kg in France and Italy, respectively (Table 2, with differences probably due to differences in instrumentation used and the French protocol being based on analysis of hepatopancreas in mussels and roe in sea urchins). The LOQs were reported as 9 and 120 µg/kg in France and Italy, respectively.

The main advantages of the LC-MS/MS methods are:

- they are rapid;
- they are sensitive, if triple quadrupole instruments are used;
- they can screen and measure PlTX-group toxins individually;
- they give information on the profile of PlTX-group toxins;
- they can be automated.

The main disadvantages of the LC-MS/MS methods are:

- they require costly equipment and highly trained personnel;
- no validation studies have been published and detailed performance characteristics are not known.

4.4.2. High performance capillary electrophoresis

As an alternative to poorly sensitive HPLC-UV methods for the determination of PlTX, high performance capillary electrophoresis (HPCE) methods with UV detection have been developed (Mereish et al., 1991; Lau et al., 1993). The LODs ranged from 0.25 to 0.5 pg/injection of PlTX solution, depending on the wavelength used (Mereish et al., 1991), but the methodology is not suitable for the analysis of shellfish, due to strong matrix effects.

The main advantage of the HPCE methods is:

- they can be automated.

The main disadvantages of the HPCE methods are:

- they have not been developed for the analysis of PlTX in bivalve molluscs (and this may prove difficult due to matrix effects);
- they require highly trained personnel.

4.5. Proficiency tests

PlTX is not a regulated toxin and there is no proficiency testing ongoing for this group of toxins.

4.6. Summary of methods

The MBA has been used to detect PlTX-group toxins in fish and shellfish tissues, e.g mussels, oysters, scallops and clams, but for reasons of animal welfare and poor specificity there is a growing concern with respect to its use. Alternative assays taking advantage of certain PlTX functional properties have been developed. While cell based assays appear to have the lowest LOD for PlTX-group toxins, some of them showed interference from other toxins and positive results obtained with biological methods would require further confirmation by chemical methods. HPLC-FLD and LC-MS/MS methods could be valuable tools for the determination of PlTX-group toxins. The optimisation of these methods for application to shellfish extracts, their (inter-laboratory) validation and the development of standards and reference materials are necessary.

5. Levels of PlTX-group toxins in shellfish from contaminated areas

5.1. Data Collection

EFSA issued in 2008 a call for data on the occurrence of PlTX-group toxins in European countries. Only Greece answered the call, providing analytical results for 11 targeted samples (including 10 mussels and 1 clam) analysed by a HNA and showing estimated values well above the LOD.

Presently, PlTX-group toxins in shellfish do not have regulatory limits, and monitoring data are not available regarding this group of toxins. Nevertheless, *Ostreopsis* spp. have been in recent years increasingly found in the European Mediterranean countries as well as in other temperate areas worldwide (Shears and Ross, 2009).

Monitoring plans and research projects on algae blooming have been initiated and some research projects have also been targeted to evaluate the possible presence of PlTX-group toxins in shellfish. Data obtained from some of these projects were included in the data collection. France[13] provided results for 19 sea urchin and 16 mussel samples, and Italy[14] for 21 sea urchin and 35 mussel samples. Overall, 102 samples from Greece[15], Italy[14] and France[13] were considered. The Greek samples were analysed by HNA while the samples from Italy and France were analysed by LC-MS/MS. All samples from Greece and Italy and sea urchins samples from France represented wild shellfish collected in contaminated regions during *Ostreopsis* spp. bloom. Mussel samples from France originated from experiments where uncontaminated mussels were intentionally placed in areas contaminated by

[13] The data were obtained as part of the French national surveillance network for phytoplankton and phycotoxins (REPHY), as implemented by IFREMER, partly financed by the French Ministry for Food, Agriculture and Fisheries, Directorate General for Food, programme 206.
[14] Italian monitoring project co-ordinated by Agenzia Regionale per la Protezione Ambientale della Campania (ARPAC).
[15] National Reference Laboratory

Ostreopsis spp. in order to study the PlTX-group toxin uptake of the mussels. Table 2 gives an overview of the occurrence data for samples collected between 2004 and 2009.

Table 2: Occurrence data reported on levels of PlTX-group toxins in contaminated areas of the Mediterranean sea (collected between 2004 and 2009).

Country	Year(s) of harvesting	Number of samples	Purpose of testing[(a)]	Method of testing	LOD (µg/kg)	LOQ (µg/kg)
Greece	2004-2006	11	research, pre-MC	HNA	0.0016	-
France	2009	35	research, pre-MC	LC-MS/MS	2	9
Italy	2007-2008	56	research, pre-MC	LC-MS/MS	36	120
Total		**102**				

Pre-MC: pre-market control; HNA: haemolysis neutralisation assay; LC-MS/MS: liquid chromatography-tandem mass spectrometry; LOD: limit of detection; LOQ: limit of quantification;
(a): PreMC are samples collected at the place of origin, before or during harvesting.

In the case of the French samples the digestive gland (hepatopancreas) and remaining tissue were analysed in mussels, and digestive tube and roe in sea urchins. The results were converted to whole shellfish by the reporting laboratory. This procedure generally allows the sensitivity of the method to be improved. In the samples from Italy the whole shellfish was analysed. This fact, together with differences in instrumentation, may explain the different LODs and LOQs shown by the two LC-MS/MS datasets.

5.2. Statistical description of PlTX-group toxins in shellfish

Two different and not directly comparable analytical methods were used: HNA and LC-MS/MS. HNA determines the haemolytic activity of PlTX-group toxins present in the sample, whereas LC-MS/MS can detect separately the different PlTX-group compounds. Italy and France reported PlTX and ovatoxin-A for the samples analysed by LC-MS/MS. The LC-MS/MS method can also detect ostreocin-D but so far only PlTX and ovatoxin-A have been found. PlTX-group toxin concentrations were reported as a sum, assuming identical instrument response factors.

The "bounding" approach was applied for values reported below LOD or below LOQ in order to identify the possible range of the data. The lower bound (LB) is obtained by assigning a value of zero (minimum possible value) to all the samples reported as <LOD or <LOQ. The upper bound (UB) is obtained by assigning the value of LOD to values reported as <LOD and LOQ to values reported as <LOQ (maximum possible value). Table 3 shows the statistical descriptors for the available data grouped by quantified PlTX-group toxins and analytical techniques.

Table 3: Statistical descriptors on levels of PlTX-group toxins in shellfish sampled in contaminated areas of the Mediterranean sea in 2004-2008.

Analytical method/Country	N	Median LB/UB	Mean LB/UB	P95 LB/UB	Maximum	% of samples not quantified[(a)]
			µg/kg shellfish meat			
			PlTX			
LC-MS/MS (Italy, France)	91	0/36	6/30	18/36	373	89.0 %
			Ovatoxin-A			
LC-MS/MS (Italy, France)	91	0/65	82/113	393	625	58.2 %
			sum of PlTX-group toxins			
HNA (Greece)	11	97	102	-[(b)]	300	0.0 %
LC-MS/MS (Italy, France)	91	0/80	84/115	395	625	58.2 %
Total (all techniques)	**102**					

N = number of samples; LB=lower bound; UB=upper bound; P95=95th percentile
For most of the data no information was available on measurement uncertainty. When two values are given it indicates the respective lower (LB) or upper bound (UB) values for samples below the limit of detection (LOD) or the limit of quantification (LOQ). The lower bound is calculated substituting 0 to all not detected samples. The upper bound is calculated substituting "<LOD" with LOD value and "<LOQ" with LOQ value; LOD and LOQ are those defined for the specific single analysis.
(a): Not quantified means no numerical value reported.
(b): Number of samples too small to calculate P95

Most of the samples (91 samples) were analysed by LC-MS/MS, therefore these data were used for further calculations. The LC-MS/MS data included samples from mussels and sea urchins. Statistical descriptors for these two species are reported in Table 4.

Table 4: Statistical descriptors on levels of PlTX-group toxins (sum of PlTX and ovatoxin-A) in sea urchins and mussels analysed by LC-MS/MS.

Species	N	Median LB/UB	Mean LB/UB	P95 LB/UB	Maximum	% of samples not quantified [(a)]
sea urchins	40	0/36	36/61	213	361	77.5 %
mussels	51	48/120	122/158	462	625	43.1 %
All Species	**91**	**0/76**	**84/115**	**391**	**625**	**58.2 %**

N = number of samples; LB=lower bound; UB=upper bound; P95=95th percentile
For most of the data no information was available on measurement uncertainty. When two values are given it indicates the respective lower (LB) or upper bound (UB) values for samples below the limit of detection (LOD) or the limit of quantification (LOQ). The lower bound is calculated substituting 0 to all not detected samples. The upper bound is calculated substituting "<LOD" with LOD value and "<LOQ" with LOQ value; LOD and LOQ are those defined for the specific single analysis.
(a): Not quantified means no numerical value reported.

The number of samples of the two species is limited to 40 (sea urchins) and 51 (mussels). Consequently, the statistical values obtained, particularly the 95th percentile, have a considerable uncertainty and need to be considered with caution.

Because the available occurrence data are from shellfish sampled in contaminated regions and not intended for human consumption, it should be noted that they do not represent levels of PlTX-group toxins in shellfish that currently could reach the market.

5.3. Influence of processing

There are no data on the influence of processing on the levels of PlTX-group toxins in shellfish.

6. Exposure assessment

A reliable exposure assessment for PlTX-group toxins in shellfish in Europe is not possible, due to the lack of occurrence data for shellfish possibly reaching the market. The Panel on Contaminants in the Food Chain (CONTAM Panel) decided therefore to estimate a worst case scenario on the basis of the limited occurrence values available.

Two datasets are available, based on different analytical methodologies HNA and LC-MS/MS. The number of observations in HNA is too limited to calculate a value for 95th percentile, therefore only the LC-MS/MS dataset was considered. The data on sea urchins are not realistic values to be used for exposure assessment, because they were collected during the *Ostreopsis* spp. bloom in summer and not during the commercial harvesting season, which is several months later in the winter time when the *Ostreopsis* spp. bloom does not occur. Therefore the LC-MS/MS results on mussels were chosen to illustrate the consequences of a possible contamination of shellfish with PlTX-group toxins.

A worst case scenario was considered by assuming consumption of 400 g mussels in one meal (the high portion size used in the previous scientific opinions[16]) and contamination levels of 462 µg/kg shellfish meat, corresponding to 95th percentile. Based on these assumptions, the intake per person per meal would be 185 µg of PlTX-group toxins, corresponding to about 3 µg/kg b.w. for a 60 kg person. It should be noted that this relates to the sum of PlTX and ovatoxin-A, with a major contribution of ovatoxin-A.

This scenario represents a worst case deterministic exposure estimate taking into account the 95th percentile PlTX-group toxin concentration recorded in shellfish in contaminated Mediterranean areas. Due to the lack of appropriate occurrence data on PlTX-group toxins in shellfish intended for human consumption, the CONTAM Panel concluded that a probabilistic estimate of dietary exposure to PlTX-group toxins was not feasible.

7. Toxicokinetics

No toxicokinetics studies on PlTX-group toxins were identified in the literature, but limited information on absorption can be inferred from toxicity studies.

Absorption of PlTX and its analogue ostreocin-D through some epithelia is indicated by the systemic toxicity of the compound administered by intratracheal deposition in the rat (see Section 8.3). Oral toxicity data indicated that the absorption of PlTX and ostreocin-D differs in different portions in the

[16] The EFSA Journal (2008), 589, 1-62.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_ej_589_okadaic_acid_en.pdf?ssbinary=true>The EFSA Journal (2008), 723, 1-52.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_ej_723_AZA_en,0.pdf?ssbinary=true>
The EFSA Journal (2008), 907, 1-62.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej_907_yessotoxin_en.pdf?ssbinary=true>The EFSA Journal (2009), 1019, 1-76.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej1019_saxitoxin_marine_biotoxins.pdf?ssbinary=true>
The EFSA Journal (2009), 1109, 1-47.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej1109_pectenotoxins_en.pdf?ssbinary=true>The EFSA Journal (2009), 1181, 1-61.
<http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_op_ej1181_domoic%20acid_marine%20biotoxins_en.pdf?ssbinary=true>

gastro-intestinal (GI) tract, being higher in the oral cavity (sublingual deposition) than after intragastric gavage (see Section 8.3) (Wiles et al., 1974; Ito and Yasumoto, 2009).

8. Toxicity data

8.1. Mechanistic considerations

PlTX causes membrane depolarisation in excitable and non-excitable cells, and contraction of muscle cells (Habermann, 1989). Studies by Habermann and his collaborators have provided the initial indications that the Na^+, K^+-ATPase in the cell membrane represents the target of PlTX, by showing that ouabain, an inhibitor of Na^+, K^+-ATPase inhibits PlTX effects (Habermann and Chhatwal, 1982). Proof for this has been obtained, by the demonstration that PlTX-dependent ionic fluxes are induced upon expression of the Na^+, K^+-ATPase in yeast (Scheiner-Bobis et al., 1994; Redondo et al., 1996). In addition, transmembrane cation fluxes are induced by PlTX in a cell-free system when *in vitro* synthesised Na^+, K^+-ATPase is incorporated in artificial membranes (Hirsh and Wu, 1997).

The normal functioning of the Na^+, K^+-ATPase relies on a regulated access of ions at the two sides of the plasma membrane, so that the ion binding site at only one side of the plasma membrane is accessible at any time. Interaction of PlTX with Na^+, K^+-ATPase, induces a change in the protein conformation which results in the conversion of the Na^+, K^+ pump into a non-specific cation channel, so that the gates on the two sides of the membrane are simultaneously open (Artigas and Gadsby, 2003, 2004, 2006; Reyes and Gadsby, 2006; Takeuchi et al., 2008; Gadsby et al., 2009). This results in Na^+ influx into the cells and K^+ outflow, causing cell depolarisation, as detected in initial studies on the effects of this toxin (Habermann and Chhatwal, 1982; Ikeda et al., 1988; Muramatsu et al., 1988; Habermann, 1989).

In addition to Na^+, K^+-ATPase, other plasma membrane components involved in transport of other ions have been proposed to contribute to molecular responses induced by PlTX (Muramatsu et al., 1984; Ikeda et al., 1988; Sauviat, 1989; Frelin and Van Renterghem, 1995; Vale et al., 2006), but the existence of PlTX targets other than the Na^+, K^+-ATPase is uncertain (Tosteson, 2000).

Secondary effects are triggered by increased sodium entrance into the cell, following PlTX alteration of the Na^+, K^+-ATPase. For instance, the intracellular sodium accumulation induces sodium-dependent transport of calcium ions into the cells by Na^+/Ca^{2+} exchangers (Ikeda et al., 1988; Frelin and Van Renterghem, 1995; Kockskämper et al., 2004; Vale et al., 2006). Similarly, intracellular sodium is exchanged for extracellular protons by Na^+/H^+ exchangers, leading to the lowering of intracellular pH (Frelin et al., 1990; Yoshizumi et al., 1991; Monroe and Tashjan, 1996).

As a consequence of the molecular action of PlTX skeletal, heart and smooth muscle cells are the major targets of PlTX among excitable cells, responding with contraction induced both directly, following the increased intracellular Ca^{2+} concentrations (Ito et al., 1977; Frelin and Van Renterghem, 1995; Kockskämper et al., 2004), and indirectly, through the Ca^{2+}-induced release of neurotransmitters (Nagase and Karaki, 1987; Karaki et al., 1988).

An increased metabolism of arachidonic acid and the production of eicosanoids has long been recognised as a cellular response to PlTX in different cells, such as rat liver cells, mouse clavarie, mouse 3T3 fibroblasts (Levine and Fujiki, 1985; Lazzaro et al., 1987; Miura et al., 2006). This response could be induced by some isoforms of Ca^{2+}-stimulated phospholipase A_2, that would be activated in cells exposed to PlTX and catalyses the hydrolysis of phosphatidylinositols (Habermann and Laux, 1986). The arachidonic acid released from membrane phospholipids would then be metabolised to different prostaglandins (Lazzaro et al., 1987; Nagase and Karaki, 1987; Miura et al., 2006). The increased release of prostaglandin from the endothelium and smooth muscle cells has been shown to determine norepinephrine release and contraction of the rabbit aortas (Nagase and Karaki,

1987). The stimulation of histamine release by mast cells is another effect induced by PlTX (Chhatwal et al., 1982).

PlTX has a possible role in carcinogenesis based on the finding that repeated dermal exposure to PlTX resulted in tumour promotion in the two-stage mouse skin carcinogenesis model (Fujiki et al., 1986). It has been found that PlTX induced the activation of extracellular signal-regulated kinases (ERK), the c-Jun-NH_2-terminal protein kinase (JNK) and p38 kinase (Kuroki et al., 1996; Iordanov and Magun, 1998; Li and Wattenberg, 1998, 1999; Zeliadt et al., 2003). The functional implications of these findings are not fully understood at the moment, but available experimental evidence indicates that an increased activity of these kinases would be part of the cell's response to PlTX (Wattenberg, 2007).

Although it is recognised that the molecular responses induced by PlTX are complex, it can be concluded that the primary action of PlTX is its binding to Na^+, K^+-ATPase. The conversion of Na^+, K^+-ATPase to a non specific cation channel leads to membrane depolarisation and disruption of ion homeostasis in excitable and non-excitable cells, which eventually triggers an array of secondary effects. Among the responses found in animal models, vasoconstriction is the cause of heart failure and the consequent death of animals administered with the toxin (Wiles et al., 1974; Vick and Wiles, 1975; Ito et al., 1982; Habermann, 1989; Tosteson, 2000).

8.2. In vitro toxicity

Cytotoxicity of PlTX-group toxins has been observed in a variety of experimental systems *in vitro*. For instance, the EC_{50} for induction of cytolysis was 5×10^{-10} M in rat erythrocytes and 2×10^{-7} M in cattle erythrocytes, measured after a 4 hours incubation (Habermann et al., 1981).

The cytotoxic activity of other PlTX-group toxins such ostreocin-D and ovatoxin-A has been also recognised, but only limited quantitative data are available. For ostreocin-D, studies directly comparing its potency with PlTX by carring out a full dose-response analysis have shown that ostreocin-D is about 27-fold (Usami et al., 1995) or 67-fold (Bellocci et al., 2008b) less potent than PlTX in mouse erythrocytes and human MCF-7 cells, respectively.

No data are available to compare the relative potencies of ovatoxin-A and PlTX *in vitro*, because no pure ovatoxin-A was available so far. The toxicity of ovatoxin-A *in vitro*, however, is indicated by studies with extracts obtained from field samples of *O. ovata* (Bellocci et al., 2008a; Cagide et al., 2009), that were recognized to contain ovatoxin-A at much higher (about ten-fold) levels than PlTX (Ciminiello et al., 2008; Guerrini et al., 2009).

8.3. Effects in laboratory animals

8.3.1. Acute toxicity

PlTX is one of the most acutely toxic non-protein substances known (Sosa et al., 2009). The dose required to elicit acute toxicity of PlTX is strongly dependent on the route of administration (Ito and Yasumoto, 2009). While there are toxicity data on PlTX and ostreocin-D, a structural analogue of PlTX, there are no such data on the analogue ovatoxin-A, the structure of which yet has not been fully elucidated.

8.3.1.1. Toxicity following intravenous- (*i.v.*), intramuscular- (*i.m.*) and subcutaneous- (*s.c.*) administration

Wiles et al. (1974) examined the toxicity of PlTX by several routes of administration and in various animal species. By i.v. administration the following LD_{50} values were found: 0.025 µg/kg b.w. (rabbit), 0.033 µg/kg b.w. (dog), 0.078 µg/kg b.w. (monkey), 0.089 µg/kg b.w. (rat), 0.11 µg/kg b.w. (guinea pig) and 0.45 µg/kg b.w. (mouse) (Wiles et al., 1974; Vick and Wiles, 1990). The results

indicate large species differences in susceptibility following i.v. administration, with the rabbit, dog and monkey being most susceptible and the mouse the least susceptible species. Dogs and monkeys given PlTX appeared to die from rapid cardic failure due to profound coronary vasoconstriction (Vick and Wiles, 1990).

Following i.m. and s.c. administration of PlTX the LD_{50} values were higher: 0.24 µg/kg b.w. (i.m., rat), 0.40 µg/kg b.w. (s.c., rat), 1.39 µg/kg b.w. (s.c., mouse) and 0.080 µg/kg b.w. (i.m., dog) (Wiles et al, 1974; Vick and Wiles, 1990).

8.3.1.2. Toxicity following intraperitoneal (*i.p.*) administration

PlTX is highly toxic upon i.p. administration in mice with reported LD_{50} values ranging between 0.15 and 0.72 µg/kg b.w., with the latter value determined according to OECD Guideline 425 (Munday, 2006; Rhodes et al., 2002; Riobó et al., 2008). In rats, the 24 hour-LD_{50} was 0.63 µg/kg b.w. (Wiles et al., 1974). The LD_{50} values associated with death of mice within 24 hours were 0.45 µg/kg b.w. (Onuma et al., 1999) and 0.295 ± 0.005 µg/kg b.w. (Riobó et al., 2008). The acute signs of toxicity are characteristic with stretching of hind limbs, lower back and concave curvature of the spinal column, muscle spasms, respiratory distress dyspoea and progressive muscular paralysis (Ito et al., 1996; Riobó et al., 2008). While animals injected with high doses of PlTX (purified by the authors) died within a short time without prominent morphological signs, several internal organs were congested and histopathological changes were observed in the heart, kidney, liver, pancreas, intestines and lymphoid tissues in mice surviving 24 hours. Lymphoid tissues, including spleen and thymus showed necrosis, and observations of peritonitis and congestion, bleeding and severe toxicity to the mucosa in the small intestine were made (Terao et al., 1992; Ito et al., 1996).

In mice receiving 0.25 µg/kg b.w. of PlTX *i.p.* 5 times a week and up to 29 times lymphoid tissues were examined when diarrhoea was seen in 3/5 of the mice. The number of lymphocytes was reduced in tissues and the blood. The mice recovered within a month (Ito et al., 1997).

Usami et al. (1995) reported LD_{50} value for ostreocin-D in mice of 0.75 µg/kg b.w. It should be noted that no details on the purity of ostreocin-D and experimental details on LD_{50} determination were given in the paper. In a very recent study ostreocin-D was given i.p. to male ICR mice (3 to 4 weeks old) at doses of 1-7 µg/kg b.w. (Ito and Yasumoto, 2009). At a dose of 5 µg/kg b.w. the mice died within a few hours, exhibiting similar toxic signs, i.a. paralysed limbs, as seen by Ito et al. (1996) in mice given an *i.p.* dose of 1.5 µg PlTX/kg b.w. At a sublethal dose (4 µg/kg b.w.) wriggling walk and light diarrhoea were observed.

8.3.1.3. Toxicity following intra-tracheal administration

PlTX doses of 1-10 µg/kg b.w. and 1-13 µg/kg b.w. ostreocin-D were given intra-tracheally to male ICR mice (3-4 weeks of age, n=10) (Ito and Yasumoto, 2009). At doses of 2 µg PlTX/kg b.w. and higher the mice died within 2 hours with paralytic signs and lung pathology including extensive bleeding in the alveoli, oedema around blood vessels, gastro-intestinal erosions and atrophy of kidney glomeroli. At a sublethal dose of 1 µg/kg b.w. neurological signs occurred for 1 to 2 hours, which disappeared after 24 hours, but animals still had destruction of alveoli in the lungs, oedema in the gastro-intestinal tract and atrophy of glomeruli in the kidney (Ito and Yasumoto, 2009).

In rats Wiles and co-workers (1974) derived an intra-tracheal LD_{50} of 0.36 µg/kg b.w. In a recent study (Ito and Yasumoto, 2009) the lethal dose of PlTX in male Wistar rats (3 weeks of age, n=3) was 5 to 7.5 µg/kg b.w. At a sublethal dose of 1 µg/kg b.w. (n=1) pathological changes essentially similar to those found in mice (see above) were observed.

The lethal dose of ostreocin-D in ICR mice (3-4 weeks of age, n=3) was 11-13 µg/kg b.w. Bleeding in the lung and stomach was observed, whereas no histopathological changes were observed in the

kidneys. At doses between 4.5 and 9 µg/kg b.w. mice (n=5) showed neurological signs, a pale colour in the eyes, and the lungs had some bleeding areas, but no alveolar destruction or hyperaemic changes in the small intestine were seen (Ito and Yasumoto, 2009).

8.3.1.4. Toxicity following sublingual administration

In the same study as described above, Ito and Yasumoto (2009) also administered single doses of 176 to 235 µg/kg b.w. of PlTX and 172 to 223 µg/kg b.w. of ostreocin-D were administered sublingually to ICR mice (3-4 weeks of age, n=10). PlTX caused inactivity and rapid respiration. Upon autopsy bleeding, interstitial inflammation oedema and alveolar destruction in the lungs, gastrointestinal erosions and kidney glomerular atrophy were observed. Ostreocin-D caused changes essentially similar to those of PlTX, but less severe (Ito and Yasumoto, 2009).

Repeated sublingual doses of PlTX (2-3 daily doses amounting to total dose of 330 and 495 µg/kg b.w.) and ostreocin-D (5 daily doses amounting to a total dose of 954 and 1011 µg/kg b.w.) were given to mice (n=2). With PlTX repeated dosing resulted in scratching and severe pathology, particularly in the heart with general congestions in the stomach and the intestine, but not in the lungs. With repeated dosing of ostreocin-D no scratching, nor aggravation or organ injuries were seen (Ito and Yasumoto, 2009).

8.3.1.5. Toxicity following oral administration

A few studies addressing the acute oral toxicity have been identified (Vick and Wiles, 1990; Munday, 2006; Munday, 2008; Ito and Yasumoto, 2009; Sosa et al, 2009).

Following PlTX administration by gavage in a solution of 1 % Tween 60 in saline with 10 % ethanol an LD_{50} in mice (strain not given) of 510 µg/kg b.w. was reported. When feeding PlTX mixed with a 150 mg portion of cream cheese the LD_{50} was >2000 µg/kg b.w. (Rhodes and Munday, 2004; Munday, 2006). Neither source nor purity of the toxin, nor details on the toxic effects were given.

Sosa et al. (2009) examined the acute oral PlTX toxicity in 4 weeks old female CD-1 mice. Groups of 5 mice received by gavage 300, 424, 600, 848, 1200 or 1697 µg/kg b.w. PlTX. Mice that died during the observation time were autopsied immediately and the surviving ones after 24 hours. PlTX was lethal at a dose of 600 µg/kg b.w. and above and the LD_{50} was calculated to be 767 µg/kg b.w. (95 % confidence limit: 549-1039 µg/kg b.w.). While signs at the two lower dose groups were restricted to scratching, the frequency and severity of signs increases at higher doses and included also spasms, paralysis mainly in hind limbs, respiratory distress and jumping. Clinical blood chemistry showed significant and dose related increased in creatine phosphokinase and lactate dehydrogenase at doses above 600 µg/kg b.w. and in aspartate transaminase at 848 µg/kg b.w. and above. Histopathological alterations were observed in the forestomach, liver and pancreas. Dose related ultrastructural changes were seen in heart and skeletal muscle cells with rounded mitochondria and fibre degeneration. The authors derived a no-observed-adverse-effect-level (NOAEL) of 300 µg/kg b.w.

In the study of Ito and Yasumoto (2009) male ICR mice (3-4 weeks of age) received by gavage single doses of 200 and 500 µg/kg b.w. PlTX in a solution of saline and 200 µg/kg b.w. in solutions with triolein-oil, lecithin, lysolecithin or deoxycholic acid in saline, or in crab or mackerel juice, or in saline to mice with an ulcerated stomach (induced by treatment with 0.3M HCl). At 200 µg/kg b.w. slight changes were observed in the stomach, intestines, lung and kidney, which healed within 24 hours, whereas 500 µg PlTX/kg b.w. caused clear stomach injury. Neither administration of PlTX to mice with ulcers, nor with various vehicles, showed an increase in effects. The lowest-observed-adverse-effect-level (LOAEL) in this study was 200 µg PlTX/kg b.w.

For rats an oral 24 hour-LD_{50} of PlTX >40 µg/kg b.w. was reported (Wiles et al 1975; Vick and Wiles, 1990).

Ito and Yasumoto (2009) also examined the toxic effect of ostreocin-D purified by the group of Ukena et al. (2001) at gavage doses of 200 (n=4), 300 (n=5) and 500 µg/kg b.w. (n=1). The mice receiving doses of 200 and 500 µg/kg b.w. had slight and transient injuries in the stomach and the lung. A LOAEL of 200 µg/kg b.w. of ostreocin-D was suggested.

8.4. Relative potency of analogues

Based on results from experiments in mice described above the potency of ostreocin-D was compared with PlTX. Comparing the i.p. LD_{50} value of ostreocin-D of 0.75 µg/kg b.w. determined by Usami and co-workers (1995) with the most recently determined i.p. LD_{50} values for PlTX of 0.72 µg/kg b.w. (Munday, 2006) and 0.295 µg/kg b.w. (Riobó et al., 2008) would indicate a relative potency of ostreocin-D ranging from 0.4 to 1.0. In a study by Ito and Yasumoto (2009) following oral administration ostreocin-D appeared to be only slightly less toxic than PlTX. Since PlTX and ostreocin-D only show minor structural differences, cause similar signs of toxicity and act on the same target, dose-addition can be anticipated upon co-exposure. Therefore PlTX and ostreocin-D could be considered to be equipotent by the oral route.

There is no information on the relative toxicity of ovatoxin-A.

9. Observations in humans

PlTX has been associated with human illness over the past four decades, but many of the reports are anecdotal and the clinical signs and symptoms and dose response relationship are not well defined. Rhabdomyolysis is one of the most reported signs of PlTX poisoning, characterised by injury to skeletal muscle, muscle breakdown and leakage of large quantities of myocyte contents into plasma. In severe cases renal failure and disseminated intravascular coagulation may be complications of rhabdomyolysis, although the fatality rate is low. Common symptoms include myalgias and generalised weakness, possibly accompanied by fever, nausea and vomiting. Muscle pain and tenderness may also be present, particularly in the calves and lower back. Clinical chemistry findings include elevated serum creatine kinase, hyperkalaemia and myoglobinuria (Deeds and Schwartz, 2009). It may take several months for complete recovery (Taniyama et al., 2002).

PlTX poisoning has also occurred through injured skin (Hoffmann et al., 2008).

There are reports of people suffering symptoms, such as rhinorrhoea, coughing, fever, bronchoconstriction and wheezing, in seaside regions in Italy where *O. ovata* blooms occurred, although there were no associated reports of poisoning due to consumption of PlTX-contaminated fish or shellfish (Gallitelli et al., 2005; Brescianini et al., 2006; Ciminiello et al., 2006).

Cases of poisoning have generally been attributed to the presence of PlTX by means of clinical symptoms, together with MBA and/or other screening techniques without quantification of toxin levels (Kodama et al., 1989; Okano et al., 1998; Taniyama et al., 2002). Co-exposure to ciguatoxins has also been possibly implicated in a case of poisoning due to consumption of *Decapterus Inacrosoma* (mackerel) (Kodama et al., 1989). The algal source of the toxins has generally not been investigated.

Two people suffered severe muscle pain and dyspnoea after eating parrotfish in Aichi, Japan. One victim died four days later. Analysis of the leftover fish suggested that PlTX was the causative agent, but the amount present was not quantified (Noguchi *et al*., 1988, abstract only).

In 1984 in the Philippines, a 49-year-old man experienced symptoms of dizziness, nausea, fatigue and cold sweat within a few minutes of eating a quarter of a hairy crab (*Demania reynaudii*). He later

developed diarrhoea, parasthesias, restlessness, muscle cramps, vomiting, bradycardia respiratory problems and renal failure. He died about 15 hours after eating the crab despite hospital treatment. HPLC analysis of samples of the remaining crab indicated the presence of PlTX, and MBA indicated a content of 77 MU/g of tissue, with the MU defined as the amount of toxin to kill a 17 g mouse in 24 hours by i.p. injection (Alcala et al., 1988). Based on the reported range of LD_{50} values (0.15-0.72 µg/kg b.w.), one MU could be in the range of 2.6-12 ng for a 17 g mouse, in which case the PlTX content of the crab would have been 200-924 µg/kg . Whilst in principle this range could be used, together with assumptions regarding the amount of crab consumed and bodyweight of the victim, to estimate the dose of PlTX, the CONTAM Panel considered that such a calculation would not be robust. This is due to additional uncertainties regarding the analytical results based on the brief detail provided in Alcala et al. (1988): the extraction process was not specific for PlTX, there was an indication of co-eluting substances on the chromatogram, there was no information on conditions or duration of storage of the crab before analysis and whether this may have led to degradation of the toxin. The CONTAM Panel therefore concluded that it would not be appropriate to use these data as a basis for the hazard characterisation.

PlTX has also been linked with a toxic syndrome known as clupeotoxism because of its association with consumption of certain tropical clupeid fish species. In a fatal case of clupeotoxism due to consumption of sardines in Madagascar, a 49-year old woman experienced malaise, then uncontrollable vomiting and diarrhoea within the first two hours, followed by tingling of the extremities, delirium and death within 17 hours. The discarded fish heads were analysed by MBA, a number of *in vitro* approaches and mass spectroscopy. The results indicated that PlTX or an analogue of similar molecular weight was present. *Ostreopsis siatnensis* was inferred as the probable toxin source, but could not be confirmed, which the authors considered to be due to probable decomposition of the microalgae during the storage for nearly two years (Onuma et al., 1999). The available data do not allow estimation of the amount consumed.

10. Hazard characterisation

There are no longterm toxicity studies on PlTX, which precludes derivation of a tolerable daily intake (TDI). PlTX and ostreocin-D are acutely toxic mainly by interference with the Na^+/K^+ ATP-ase ion-pump. The toxins are acutely toxic in experimental animals by several routes of administration and acute toxicity and deaths have also been reported from human outbreaks (see Chapters 8 and 9). Ostreocin-D appears to be close to equipotent with PlTX by gavage administration in mice. Therefore dose-addition could be anticipated by co-exposure.

The toxicity of PlTX is strongly dependent on the route of administration with i.m., i.v. and i.p. routes being the most sensitive followed by the intra-tracheal route. The least sensitive route was by gavage. With sublingual administration a lower dose was required to achieve toxicity than via gavage.

There are no reliable quantitative data on acute toxicity of PlTX in humans.

There is good agreement between studies with regard to oral toxicity of PlTX following administration by gavage as Sosa et al. (2009) found a NOAEL of 300 µg/kg b.w. and Ito and Yasumoto (2009) found only slight and transient changes following 200 µg/kg b.w., which would represent an oral LOAEL. The CONTAM Panel decided to derive an oral acute reference dose (ARfD) for PlTX using 200 µg/kg b.w. as a reference point.

Since the LOAEL was likely to be close to a NOAEL, a small uncertainty factor, such as 3, might have been considered sufficient for extrapolation to a NOAEL. However, it was noted that sublingual administration of PlTX in the region of 200 µg/kg b.w. caused more severe toxicity of internal organs than that at a similar dose by gavage . Since transmucosal transport of PlTX in the mouth could not be excluded and because mice seem to be less sensitive than other species based on the i.v. toxicity data, the CONTAM Panel decided to apply an extra uncertainty factor of 10 in addition to the default uncertainty factors of 10 for intra- and 10 for interspecies variation to derive an oral ARfD of

0.2 µg/kg b.w. Because of a similar mode of action and equipotency of PlTX and ostreocin-D by gavage or sublingual administration the ARfD applies to the sum of PlTX and ostreocin-D.

The CONTAM Panel noted the potent action of PlTX after parenteral administration including intra-tracheal instillation, which implies a high risk to humans of inhaling aerosols of PlTX and aspiration of PlTX containing material as well as contact through injured skin. The ARfD does not apply to these situations.

Because of the lack of *in vivo* toxicity data, the CONTAM Panel could not characterise the hazards of ovatoxin-A and the other PlTX-group toxins.

11. Risk characterisation

Because PlTX has acute toxic effects, the CONTAM Panel concluded that the use of a large portion size is more appropriate than a long term average consumption in assessing the health risk of the consumers. In line with the previous scientific opinions on shellfish toxins[16] the figure of 400 g shellfish meat for the large portion size was used in the acute exposure assessment.

In order for a 60 kg adult to avoid exceeding the ARfD of 0.2 µg/kg b.w., a 400 g portion of shellfish meat should not contain more than 12 µg of the sum of PlTX and ostreocin-D, corresponding to 30 µg/kg shellfish meat.

The limited occurrence data available to support an exposure assessment indicate that consumption of a large portion of shellfish meat (i.e. mussels) harvested from contaminated regions could result in a dietary exposure of about 3 µg/kg b.w. of the sum of PlTX and ovatoxin-A. If ovatoxin-A had a similar potency as PlTX and ostreocin-D, this exposure would be more than 10-fold higher than the ARfD of 0.2 µg/kg b.w. for the sum of PlTX and ostreocin-D. However, it is not possible to determine if such exposure occurs from consuming shellfish that currently reach the market in the EU.

The PlTX-group toxin that was most frequently reported in the available EU occurrence data was ovatoxin-A. Since no information is available regarding the toxicity of ovatoxin-A, the CONTAM Panel could not characterise the respective risk for European consumers.

12. Uncertainty

The limited data on occurrence of PlTX-group toxins in shellfish does not allow a reliable exposure assessment for the European population. In addition, there are limited toxicity data, and the clinical signs and symptoms and dose response relationships following PlTX intoxications in humans are not well defined. Therefore, the CONTAM Panel concluded that the overall uncertainty is large and a detailed consideration of the various potential sources of uncertainty on the impact on the risk assessment is not meaningful.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

General

- Palytoxin (PlTX)-group toxins are complex polyhydroxylated compounds with both lipophilic and hydrophilic areas.

- At least 8 different PlTX analogues are known: PlTX, ostreocin-D, ovatoxin-A, homopalytoxin, bishomopalytoxin, neopalytoxin, deopalytoxin and 42-hydroxypalytoxin, but only for PlTX and ostreocin-D the chemical structure has been characterised.

Methods of analysis

- Extraction methods for several marine biotoxins in shellfish are available but there is no information on their efficiency for PlTX-group toxins.

- The mouse bioassay (MBA) has been used to detect PlTX-group toxins in fish and shellfish tissues, but for reasons of animal welfare and poor specificity there is a growing concern with respect to its use.

- While cell based assays, taking advantage of certain PlTX functional properties, appear to have the lowest limit of detection (LOD) for PlTX-group toxins, some assays showed interference with other toxins and the positive results should be confirmed by chemical analysis.

- High performance liquid chromatography-fluorescence detection (HPLC-FLD) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods can be valuable tools for the determination of PlTX-group toxins. The optimisation of these methods for application to shellfish extracts, their (inter-laboratory) validation and the development of standards and reference materials are necessary.

Occurrence/Exposure

- There is a lack of representative occurrence data on PlTX-group toxins in shellfish in Europe.

- The available occurrence data are from mussels and sea urchins sampled in contaminated regions and not intended for human consumption, therefore they do not represent levels of PlTX-group toxins in shellfish that currently could reach the market.

- Due to the lack of occurrence data a reliable exposure assessment for PlTX-group toxins in shellfish in Europe is not possible. The CONTAM Panel decided therefore to estimate a worst case scenario on the basis of the limited occurrence values available.

- Because PlTX has acute toxic effects, the CONTAM Panel concluded that the use of a large portion size is more appropriate than a long term average consumption in assessing the health risk of the consumers. In line with the previous scientific opinions on shellfish toxins the figure of 400 g shellfish meat was used in the acute exposure assessment as an appropriate estimate of a large portion size.

- The limited data available to support an exposure assessment indicate that consumption of a large portion of 400 g of shellfish meat (i.e. mussels) harvested from contaminated regions

could result in a dietary exposure of about 3 µg/kg b.w. of the sum of PlTX and ovatoxin-A for a 60 kg person.

- There are no data on the influence of processing on the levels of PlTX-group toxins in shellfish.

Hazard identification and characterisation

- PlTX and ostreocin-D are acutely toxic in experimental animals by interference with the Na^+/K^+-ATPase ion-pump.

- Because ostreocin-D appears to be close to equipotent with PlTX following oral administration (gavage) and because of the similar mode of action, dose-addition can be anticipated when co-exposure occurs. In experimental animals PlTX and ostreocin-D are much less toxic after oral than after parenteral administration.

- In humans, rhabdomyolysis is one of the most reported signs of PlTX poisoning, characterised by injury to skeletal muscle, muscle breakdown and in severe cases followed by renal failure and disseminated intravascular coagulation.

- There are no long term toxicity studies on PlTX and because of its acute toxicity the Panel on contaminants in the food chain (CONTAM Panel) decided to derive an acute reference dose (ARfD).

- Because there are no reliable quantitative data on acute PlTX toxicity in humans, the derivation of the ARfD was based on oral toxicity of PlTX in mice. Because the lowest-observed-adverse-effect-level (LOAEL) of 200 µg/kg b.w. was likely to be close to a no-observed-adverse-effect-level (NOAEL), a small uncertainty factor, such as 3, might have been considered sufficient for extrapolation to a NOAEL. It was noted that sublingual administration of PlTX in the region of 200 µg/kg b.w. caused toxicity of internal organs. Since transmucosal transport of PlTX in the mouth could not be excluded and because mice seem to be less sensitive than other species, the CONTAM Panel decided to apply an extra uncertainty factor of 10 in addition to the default uncertainty factors of 10 for intra- and 10 for interspecies variation to derive an oral ARfD of 0.2 µg/kg b.w.

- Because of equipotency and a similar mode of action of PlTX and ostreocin-D by the oral route, the ARfD applies to the sum of PlTX and ostreocin-D.

- The CONTAM Panel noted the potent action of PlTX after parenteral administration including intra-tracheal installation, which implies a high risk to humans of inhaling aerosols of PlTX and aspiration of PlTX containing material as well as contact through skin injuries.

- Because of the lack of data the CONTAM Panel could not characterise the hazards of ovatoxin-A and other PlTX-group toxins.

Risk characterisation

- In order for a 60 kg adult to avoid exceeding the ARfD of 0.2 µg/kg b.w., a 400 g portion of shellfish meat should not contain more than 12 µg of the sum of PlTX and ostreocin-D, corresponding to 30 µg/kg shellfish meat.

- The limited occurrence data available to support an exposure assessment indicate that consumption of a large portion of shellfish meat (i.e. mussels) harvested from contaminated regions could result in a dietary exposure of about 3 µg/kg b.w. of the sum of PlTX and ovatoxin-A. If ovatoxin-A had a similar potency to PlTX and ostreocin-D, this exposure

would be more than 10-fold higher than the ARfD of 0.2 µg/kg b.w. for the sum of PlTX and ostreocin-D. However, it is not possible to determine if such exposure could result from consumption of shellfish that currently reach the market in the European Union (EU).

- The PlTX-group toxin that was most frequently reported in the available EU occurrence data was ovatoxin-A. Since no information is available regarding the toxicity of ovatoxin-A, the CONTAM Panel could not characterise the respective risk for European consumers.

RECOMMENDATIONS (INCLUDING KNOWLEDGE/DATA GAPS)

Methods of analysis

- Certified reference standards and reference materials for PlTX-group toxins are needed.
- Methods other than the MBA should be further developed and optimised with respect to selectivity and sensitivity for PlTX-group toxins in shellfish tissues. Subsequent (interlaboratory) validation studies are needed.

Occurrence/Exposure

- More information is needed on occurrence of PlTX-group toxins in shellfish and other seafood.
- Reporting systems for outbreaks of PlTX-group toxin poisoning in member states should be established to get information on the true incidence and routes of exposure.
- Due to the high acute toxicity of PlTX-group toxins and their emerging occurrence, appropriate strategies to protect human health need to be developed.

Hazard identification and characterisation

- Further information is needed on toxicity of PlTX-group toxins, particularly of ovatoxin-A, to better characterise their oral toxicity and relative potencies.

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ABBREVIATIONS

APHA	American Public Health Association
ARfD	Acute reference dose
ARPAC	Agenzia Regionale per la Protezione Ambientale della Campania
ASP	Amnesic Shellfish Poisoning
AZA	Azaspiracid
AZP	Azaspiracid Shellfish Poisoning
BTX	Brevetoxin
b.w.	Body weight
CALIPSO	Fish and seafood consumption study and biomarker of exposure to trace elements, pollutants and omega 3
CCFFP	Codex Committee for Fish and Fishery Products
CCMAS	Codex Committee on Methods of Analysis and Sampling
CONTAM Panel	Panel on Contaminants in the Food chain
CRLMB	Community Reference Laboratory for Marine Biotoxins
CTX	Ciguatoxins
DA	Domoic acid
DG SANCO	Health and Consumer Protection Directorate General
DSP	Diarrhoeic Shellfish Poisoning
DTX	Dinophysis toxins
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FAO/IOC/WHO	Food and Agriculture Organization of the United Nations/ Intergovernmental Oceanographic Commission of UNESCO/World Health Organization
GI	Gastro-intestinal
HCl	hydrochloric acid
HNA	Haemolysis neutralisation assay
HPCE	High performance capillary electrophoresis
HPLC	High-performance liquid chromatography
HPLC-FLD	High-performance liquid chromatography-fluorescence detection
HPLC-UV	High-performance liquid chromatography-ultraviolet detection
i.m.	intramuscular
i.p.	Intraperitoneal
ISO/IUPAC/AOAC	International Organization for Standardization/ International Union of Pure and Applied Chemistry/Association of Analytical Communities
i.v.	intravenous
JMPR	Joint FAO/WHO Meetings on Pesticide Residues
JNK	c-Jun-NH_2-terminal protein kinase
LB	Lower Bound
LC-FLD	Liquid chromatography-fluorescence detection
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate dehydrogenase
LD_{50}	Lethal dose – the dose required to kill half the members of a tested animal population
LOAEL	Lowest-observed-adverse-effect level

LOD	Limit of detection
LOQ	Limit of quantification
MBA	Mouse bioassay
MTT	1-(4,5-dimethylthiazol-2-yl)-3.5-diphenylformazan
MU	Mouse Unit: the minimum amount needed to cause the death of an 18 to 22 g white mouse in 15 minutes
N/A	Not available
NMR	Nuclear magnetic resonance
NOAEL	No-observed-adverse-effect level
NRL	National Reference Laboratory
OA	Okadaic acid
OJ	Official Journal of the European Union
P95	95th percentile
p-PlTX	PlTX-like compound
PlTX	Palytoxins
Pre-MC	Pre-market control
PSP	Paralytic shellfish poisoning
PTX	Pectenotoxin
RBA	Rat bioassay
REPHY	French national surveillance network for phytoplankton and phycotoxins
s.c.	subcutaneous
SM	Shellfish meat
SPE	Solid Phase Extraction
STX	Saxitoxin
TDI	Tolerable daily intake
UB	Upper Bound
UNESCO	United Nations Educational, Scientific and Cultural Organization
UV	Ultraviolet
WG	Working group
WHO	World Health Organization
YTX	Yessotoxin