

SCIENTIFIC OPINION

Marine biotoxins in shellfish – Pectenotoxin group¹

Scientific Opinion of the Panel on Contaminants in the Food chain

(Question No EFSA-Q-2006-065C)

Adopted on 27 May 2009

PANEL MEMBERS

Jan Alexander, Diane Benford, Andrew Cockburn, Jean-Pierre Cravedi, Eugenia Dogliotti, Alessandro Di Domenico, María Luisa Fernández-Cruz, Johanna Fink-Gremmels, Peter Fürst, Corrado Galli, Philippe Grandjean, Jadwiga Gzyl, Gerhard Heinemeyer, Niklas Johansson, Antonio Mutti, Josef Schlatter, Rolaf van Leeuwen, Carlos Van Peteghem and Philippe Verger.

SUMMARY

Pectenotoxin (PTX)-group toxins are a group of polyether-lactone toxins. They have been detected in microalgae and bivalve molluscs in Australia, Japan and New Zealand and in a number of European countries. Their presence in shellfish was discovered due to their acute toxicity in the mouse bioassay after intraperitoneal (*i.p.*) injections of lipophilic extracts of shellfish. PTX-group toxins are exclusively produced by *Dinophysis* species. They can be found in filter-feeding bivalve molluscs such as oysters and mussels. To date 15 different analogues have been isolated and characterised. PTX-group toxins are heat stable, but they are easily destroyed under strong basic conditions such as used for hydrolysis of acyl esters of the okadaic acid (OA)-group toxins. PTX-group toxins are also labile under acidic conditions.

PTX-group toxins in shellfish are always accompanied by toxins from the OA-group. This appears to be the basis for grouping PTX-group toxins and OA-group toxins in the European regulation. The Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) concluded that because PTX-group toxins do not share the same mechanism of action as OA-group toxins they should not be included in the regulatory limit for OA-group toxins.

The toxicological database for PTX-group toxins is limited and comprises mostly studies on their acute toxicity in mice. There are no reports on adverse effects in humans associated with PTX-group toxins. The available data on lethality in mice only comprise information following *i.p.* injection and are not sufficient to establish robust toxicity equivalency factors

¹ For citation purposes: Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on marine biotoxins in shellfish – pectenotoxin group. *The EFSA Journal* (2009) 1109, 1-47.

(TEFs). In order to be prudent the CONTAM Panel proposes a provisional TEF value of 1 to be used for PTX1, PTX2, PTX3, PTX4, PTX6 and PTX11, until more robust data become available. PTX7, PTX 8, PTX 9, PTX2 SA and 7-epi-PTX2 SA are much less toxic and were not assigned TEFs.

Following oral administration PTX-group toxins show low systemic absorption and reported toxicity is mainly restricted to the intestinal tract. No data on the chronic effects of PTX-group toxins in animals are available, therefore the CONTAM Panel could not establish a tolerable daily intake (TDI). In view of the acute toxicity of PTX-group toxins, and due to the lack of observations in humans, the CONTAM Panel decided to establish an acute reference dose (ARfD) based on the available animal data on acute toxicity.

Although the oral toxicity is not well defined, the CONTAM Panel considered it appropriate to establish an ARfD on the basis of a lowest-observed-adverse-effect-level (LOAEL) of 250 µg/kg body weight (b.w.) for intestinal toxicity of PTX2 observed in mice. Because the effects were mild and reversible, the CONTAM Panel decided to apply a factor of 3 for the extrapolation from a LOAEL to a no-observed-adverse-effect level (NOAEL). The CONTAM Panel established an ARfD of 0.8 µg PTX2 equivalents/kg b.w., based on a LOAEL of 250 µg/kg b.w. and an overall uncertainty factor of 300.

In order to protect against the acute effects of PTX-group toxins, it is important to use a large portion size rather than a long-term average consumption in the health risk assessment of shellfish consumption. Consumption data for shellfish species across the European Union (EU) were limited, therefore the European Food Safety Authority (EFSA) requested the Member States to provide information on consumption of relevant shellfish species. Based on data, provided by five Member States, the CONTAM Panel identified 400 g of shellfish meat as a large portion size to be used in the acute risk assessment of marine biotoxins.

Consumption of a 400 g portion of shellfish meat containing PTX-group toxins at 160 µg/kg shellfish meat (by analogy with the current EU limit for lipophilic toxins of 160 µg OA equivalents/kg shellfish meat) would result in an intake of 64 µg toxin (equivalent to about 1 µg/kg b.w. in a 60 kg adult). This intake is slightly higher than the ARfD of 0.8 µg PTX2 equivalents/kg b.w. (equivalent to 48 µg PTX2 equivalents per portion for a 60 kg adult) and is not considered to constitute a health risk. Based on current consumption and occurrence data, there is a small chance (approximately 0.2 %) to exceed the ARfD of 0.8 µg PTX2 equivalents/kg b.w. when consuming shellfish currently available on the European market.

The CONTAM Panel concluded that, in order for a 60 kg adult to avoid exceeding the ARfD of 0.8 µg PTX2 equivalents/kg b.w., a 400 g portion of shellfish should not contain more than 48 µg PTX2 equivalents corresponding to 120 µg PTX2 equivalents/kg shellfish meat.

There is no information on the effects of processing (e.g. cooking, steaming, autoclaving) on the levels of PTX-group toxins in shellfish, but it can be assumed that, as for other lipophilic toxins, water loss during processing may lead to an increase in the concentration of PTX-group toxins in shellfish flesh.

The mouse bioassay (MBA) and the rat bioassay (RBA) are the officially prescribed reference methods in the EU for the determination of lipophilic toxins, including PTX-group toxins. The CONTAM Panel noted that the MBA has shortcomings e.g. it has an inherent variability, is not quantitative, has an insufficient detection capability, and is not selective for the PTX-group toxins and thus may give both false negative and false positive results. The RBA detects compounds with diarrhetic effects such as OA-group toxins. PTX-group toxins do not

have diarrhetic properties, therefore the CONTAM Panel concluded that the RBA is not suitable to detect PTX-group toxins.

The current EU legislation permits the replacement of the MBA, provided that the alternative methods have been validated according to an internationally recognised protocol. The evidence available at this moment suggests that the methods based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) have the greatest potential to replace the MBA. These methods also have the possibility for multi-toxin group detection/quantification. At this time however, none of the methods for the determination of toxins from the PTX-group have been validated by interlaboratory studies. The CONTAM Panel noted that, whilst application of single laboratory validation according to recognised international guidelines to demonstrate their fitness-for-purpose can be an impetus for implementation of alternative instrumental analyses of marine biotoxins for regulatory purposes, method performance criteria should be stipulated where possible and validation by interlaboratory trials should be the long-term objective.

Key words: Marine biotoxins, pectenotoxin (PTX)-group toxins, shellfish, bivalve molluscs, mammalian biotests, acute reference dose, portion size, methods of analysis, human health, risk assessment

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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 (BTX), 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 2004². Two additional groups, palytoxins (PITX) 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 dinophysin 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/EC³, are established maximum levels for ASP, PSP and DSP toxins. Annex III of Commission Regulation No 2074/2005/EC⁴ of 5 December 2005 lays down the recognised testing methods for detecting marine biotoxins. Annex II Chapter II (14) to

² ftp://ftp.fao.org/esn/food/biotoxin_report_en.pdf

³ Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. OJ L 139, 30.4.2004, p. 55–205

⁴ Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004 OJ L 338, 22.12.2005, p. 27–59.

Regulation (EC) 854/2004⁵, 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/EEC⁶ 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 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 European Commission (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. 2074/2005⁴ specifies a MBA for the determination of PSP toxins and a MBA or the RBA for lipophilic marine biotoxins. Alternative test methods can

⁵ Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. OJ L 139, 30.4.2004, p. 206–320.

⁶ Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animal used for experimental and other scientific purposes. OJ L 358, 18.12.1986, p. 1–28.

be applied if they are validated following an internationally recognised protocol and provide an equivalent level of public health protection.

Besides PSP toxins, OA, DTXs, PTXs, AZAs and YTXs, 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 DA 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 853/2004³ 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 PSP,
- 20 milligrams of DA per kilogram for ASP,
- 160 micrograms of OA equivalents⁷ per kilogram for OA, DTXs and PTXs in combination,
- 1 milligram of YTX equivalents per kilogram for YTXs,
- 160 micrograms of AZA equivalents per kilogram for AZAs.

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)⁸ for the AZA, OA, STX, DA, and YTX-group toxins, respectively (summarized in the Table 1). The Expert Consultation considered that the database for the cyclic imines, BTXs and PTXs 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.

⁷ 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.

⁸ 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 ⁸	Derived Guidance Level/ Max Level based on consumption of 100g (1), 250g (2) and 380g (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 bodyweight (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. MBAs 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 II⁹ or III¹⁰ 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

⁹ 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.

¹⁰ 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.

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.

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 MBAs should be discussed individually since the level of performance and success differs markedly between the official method for PSP by MBA, the American Public Health Association (APHA) method for BTXs and the multiple MBA “DSP” procedures employed for the other lipophilic toxins such as OA, AZAs 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 the European Food Safety Agency (EFSA) to assess the current European Union (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 acute reference dose (ARfDs) and guidance levels proposed by the Expert Consultation,
- the conclusions of the Codex Committee for Fish and Fishery Products (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 the Community Reference Laboratory (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.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the members of the Working Group on marine biotoxins in shellfish – pectenotoxin group for the preparation of this opinion: Jan Alexander, Diane Benford, Luis Botana, Peter Fürst, Gerhard Heinemeyer, Philipp Hess, Angelika Preiss-Weigert, Gian Paolo Rossini, Hans van Egmond, Rolaf van Leeuwen and Philippe Verger.

ASSESSMENT

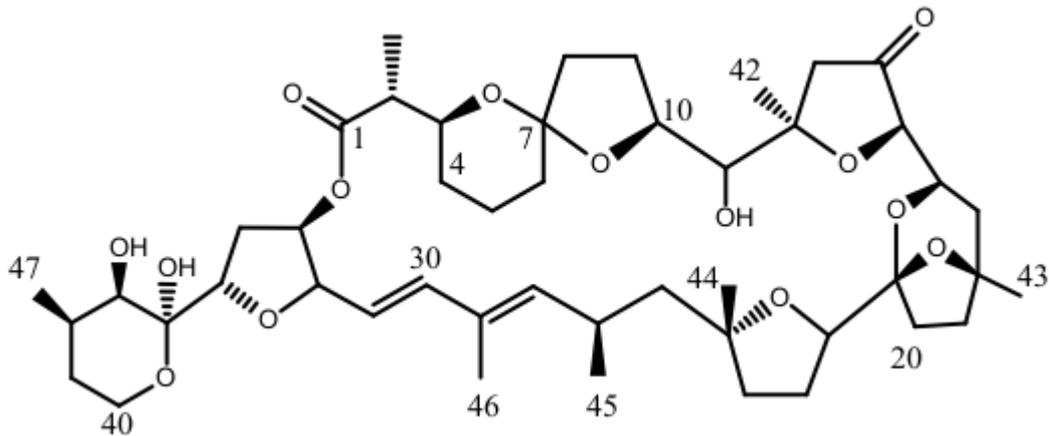
1. Introduction

Pectenotoxin (PTX)-group toxins are a group of lipophilic polyether-lactone toxins. They have been detected in microalgae and bivalve molluscs in Australia, Japan, New Zealand and in a number of European countries. Their presence in shellfish was discovered due to their high acute toxicity in the mouse bioassay after intraperitoneal (*i.p.*) injections of lipophilic extracts of shellfish. PTX-group toxins are exclusively produced by *Dinophysis* species. In shellfish they are always accompanied by okadaic acid (OA)-group toxins (FAO, 2004).

2. Chemical characteristics

PTX-group toxins are heat-stable polyether macrolide compounds, isolated from various species of shellfish and from dinoflagellates of the genus *Dinophysis* (FAO, 2004). A recent review on their biological origin, chemistry and transformations has been provided by Miles (2007). To date, 15 PTX analogues have been isolated and characterised (see Figures 1 and 2 for selected analogues). Their common structural features include a spiroketal group, three oxolanes, a bicyclic ketal and a six-membered cyclic hemiketal (Allingham *et al.*, 2007). PTX2 (produced worldwide by *Dinophysis* spp.) is suspected to be the main precursor, from which many PTX-group toxins are derived through biotransformation during metabolism in the gut of bivalves. Recently, PTX1 has also been identified in plankton directly (Krock *et al.*, 2008). It is suggested that an oxidation of PTX2 occurs, leading to the formation of other PTX-group toxins, including PTX1, PTX3, and PTX6 (Draisici *et al.*, 1996; Yasumoto *et al.*, 2001). PTX1 and -6 are major metabolites in the Japanese scallop (*Patinopecten yessoensis*), while PTX2 is rapidly metabolised into PTX2 seco acid (PTX2 SA) and its epimer 7-epi-PTX2 seco acid (7-epi-PTX2 SA) in mussels and scallops. Sasaki *et al.* (1998) identified PTX4 and PTX7 as spiroketal isomers of PTX1 and PTX6, namely epi-PTX1 and epi-PTX6, respectively (FAO, 2004). Suzuki *et al.* (2006) reported PTX11, as a further analogue (34S-hydroxy-PTX2). Unlike PTX2, PTX11 was not readily hydrolysed to its corresponding seco acid (SA) by enzymes from homogenized green-lipped mussel (*Perna canaliculus*) hepatopancreas.

PTX-group toxins are easily destroyed under strong basic conditions such as those used for the hydrolysis of acyl esters of the OA-group toxins (Yasumoto *et al.*, 2005). PTX-group toxins are also labile under acidic conditions which transform them into the seco acid forms. In shellfish PTX2 SA and 7-epi-PTX2 SA can be metabolised to form corresponding fatty acid esters. Liquid chromatography-mass spectrometry (LC-MS) analysis has indicated that in some extracts these fatty acid esters may be present at 20 fold higher concentrations than the seco acids (Wilkins *et al.*, 2006). But the concentrations of the seco acids are usually very low (see chapter 5). This is also the case for their toxicity (see chapter 10). To quantitate these acyl esters enzymatic hydrolysis may be used to transform them to their corresponding seco acids, in analogy to the method described by Doucet *et al.* (2007) for the enzymatic hydrolysis of DTX3.



Analogue	Substituent	Enantiomeric configuration at C7
PTX1	C43 CH ₂ OH	R
PTX2	C43 CH ₃	R
PTX3	C43 CHO	R
PTX4	C43 CH ₂ OH	S
PTX6	C43 COOH	R
PTX7	C43 COOH	S
PTX11	C34 OH	R

Figure 1. Chemical structures of a selection of PTX-group toxins that have been isolated and characterised.

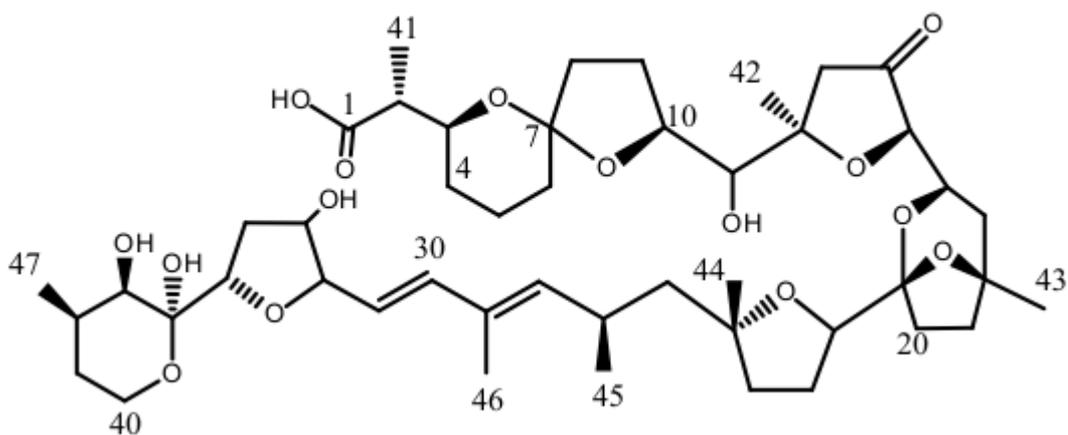


Figure 2. Chemical structure of PTX2 seco acid (PTX2 SA) and 7-epi-PTX2 seco acid (7-epi-PTX2 SA). While PTX2 SA constitutes the R-enantiomer at C7, 7-epi-PTX2 SA exhibits the corresponding S-enantiomer.

3. Regulatory status

For the control of the PTX-group toxins in the European Union (EU), Commission Regulation (EC) No 853/2004³, provides details in section VII: “Live bivalve molluscs”, chapters II and IV. Chapter II: “Hygiene requirements for the production and harvesting of live bivalve molluscs. A. Requirements for production areas” states: “*Food business operators may place live molluscs collected from production areas on the market for direct human consumption only, if they meet the requirements of chapter IV*”. Chapter IV: “Hygiene requirements for purification and dispatch centres. A. Requirements for purification centres” states “*Food business operators purifying live bivalve molluscs must ensure compliance with the following requirements: They must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits: for okadaic acid, dinophysistoxins and pectenotoxins, 160 µg of okadaic acid equivalents per kg*”. The fact that these toxins are grouped together appears to be based on co-occurrence of OA-group toxins and PTX-group toxins rather than on toxicological considerations, because PTX-group toxins do not have the same mechanism of action as OA-group toxins. A group limit for OA-group toxins and PTX-group toxins is inappropriate from an analytical and toxicological point of view. PTX-group toxins should not be expressed as OA equivalents/kg. Instead, separate regulatory limits for OA-group toxins and PTX-group toxins should be used.

Commission Regulation (EC) No 2074/2005⁴ provides details about the “Recognized testing methods for detecting marine biotoxins”. Annex III, Chapter III of this regulation deals with lipophylic toxin detection methods. Biological methods are to be used for the detection of these toxins, and the following details are given: “*A single mouse bioassay involving acetone extraction may be used to detect pectenotoxins. This assay may be supplemented, if necessary, with liquid/liquid partition steps with ethyl acetate/water or dichloromethane/water to remove potential interferences. A mouse bioassay with acetone extraction followed by liquid/liquid partition with diethyl ether may be used to detect pectenotoxins*”. Commission Regulation (EC) No 2074/2005⁴ also states the following concerning alternative detection methods:

“*A series of methods, such as high-performance liquid chromatography with fluorescence detection, liquid chromatography, mass spectrometry, immunoassays and functional assays, such as the phosphatase inhibition assay, shall be used as alternative or supplementary to the biological testing methods, provided that either alone or combined they can detect at least the following analogues, that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection:*

-*okadaic acid and dinophysistoxins: a hydrolysis step may be required to detect the presence of DTX3.*

- *pectenotoxins: PTX1 and PTX2.*

- *yessotoxins: YTX, 45-hydroxyYTX, 1a-homoYTX, and 45-hydroxy-homoYTX.*

- *azaspiracids: AZA1, AZA2 and AZA3.*

If new analogues of public health significance are discovered, they should be included in the analysis. Standards must be available before chemical analysis is possible. Total toxicity shall be calculated using conversion factors based on the toxicity data available for each toxin. The performance characteristics of these methods shall be defined after validation following an internationally agreed protocol”.

Currently there is no detailed guidance on how a non-animal-based method can become an accepted alternative method, i.e. which performance criteria should be fulfilled. In addition, conversion factors have not been established. The regulation also states that “*Biological methods shall be replaced by alternative detection methods as soon as reference materials for detecting the toxins prescribed in Chapter V of Section VI of Annex III to regulation (EC) No 853/2004³ are readily available, the methods have been validated and this Chapter has been amended accordingly*”.

In conclusion the legislation stimulates the replacement of the biological methods, provided that alternatives have been validated according to an internationally agreed protocol. Currently there are no methods for PTX-group toxins, formally validated in interlaboratory studies. The application of single laboratory validation (SLV), according to international guidelines to demonstrate fit-for-purpose of instrumental methods, could offer perspectives and would need to be explored.

4. Method of analysis

Several published methods exist for the determination of PTX-group toxins. The mouse bioassay (MBA) is still applied widely to determine PTX-group toxins, despite growing concern with respect to its use for reasons of animal welfare, its inherent variability and interference from other biotoxins which may co-exist in a sample. Some chemical methods have been developed, but there are no functional assays or biochemical methods for PTX-group toxins yet. The development of analytical methods has been hampered by the scarcity of reference materials, including analytical standards. None of the methods to determine PTX-group toxins have been formally validated in collaborative studies according to the harmonised protocol of ISO/IUPAC/AOAC (Horwitz, 1995).

4.1. Supply of appropriate reference material

PTX2 is available as a certified calibrant solution from National Research Council Canada (NRCC)¹¹. A calibration standard for PTX11 has been purified and characterised at NRCC, release is foreseen in 2009. Even though PTX11 is not regulated, the purified standard can be used to distinguish it chromatographically from its isomer PTX1. NRCC has a calibrant for PTX2 SA in preparation. Currently, there is no PTX1 calibration solution available or in preparation. PTX2 and PTX2 SA are trace constituents of a mussel tissue reference material that may become certified in late 2009 or 2010.

4.2. Mammalian bioassays

Regulation (EC) No. 2074/2005 allows for the use of two types of mammalian bioassays for the detection of the lipophilic toxins, the mouse bioassay (MBA) and the rat bioassay (RBA), neither of which have been formally validated. The RBA detects compounds with diarrhetic effects such as OA-group toxins, but not PTX-group toxins because they do not have diarrhetic properties. Therefore the RBA is not suitable to detect PTX-group toxins.

4.2.1. Mouse bioassay

Historically, MBA has been used extensively in biotoxin monitoring and as such is incorporated into EU legislation (Commission Regulation (EC) No 2074/2005⁴ Annex III,

¹¹ www.nrc-cnrc.gc.ca

Chapter III). The MBA was developed by Yasumoto *et al.* (1978) as an investigative tool for the determination of the causative agents responsible for a food poisoning outbreak associated with the consumption of molluscs in Japan. Essentially, the assay uses acetone extraction of the whole flesh (or the hepatopancreas (HP)) of molluscs followed by evaporation and resuspension of the residue in a 1 % solution of Tween 60 surfactant. Mice are then exposed to the extract via *i.p.* injection and survival is monitored over a 24 hour period (see Figure 3).

In efforts to improve the specificity of the assay, several modifications to the technique (generally involving an additional partitioning step) were developed (Yasumoto *et al.*, 1984; Lee *et al.*, 1987; Botana *et al.*, 2004). Commission Regulation (EC) 2074/2005⁴ allows for the use of different solvents in the liquid/liquid (water) partition step including ethyl acetate, dichloromethane and diethyl ether. A positive result is defined as the death of two out of three mice within 24 hours of injection with an extract operationally equivalent to 25 g whole flesh (including HP). The detectability and selectivity depends on the choice of solvents used for extraction and partitioning.

Clearly it is not ideal for a regulatory method to allow for such procedural variation, so in an effort to harmonise the methodology used within the EU, the Community Reference Laboratory for marine biotoxins (CRL-MB) has developed a standard operating procedure (SOP) based on acetone extraction with either diethyl ether or dichloromethane partitioning against water. The SOP for this method has been available at the CRL web page since 2007 (CRL-MB, 2007)¹².

¹² <http://www.aesan.msc.es/en/CRLMB/web/home.shtml>

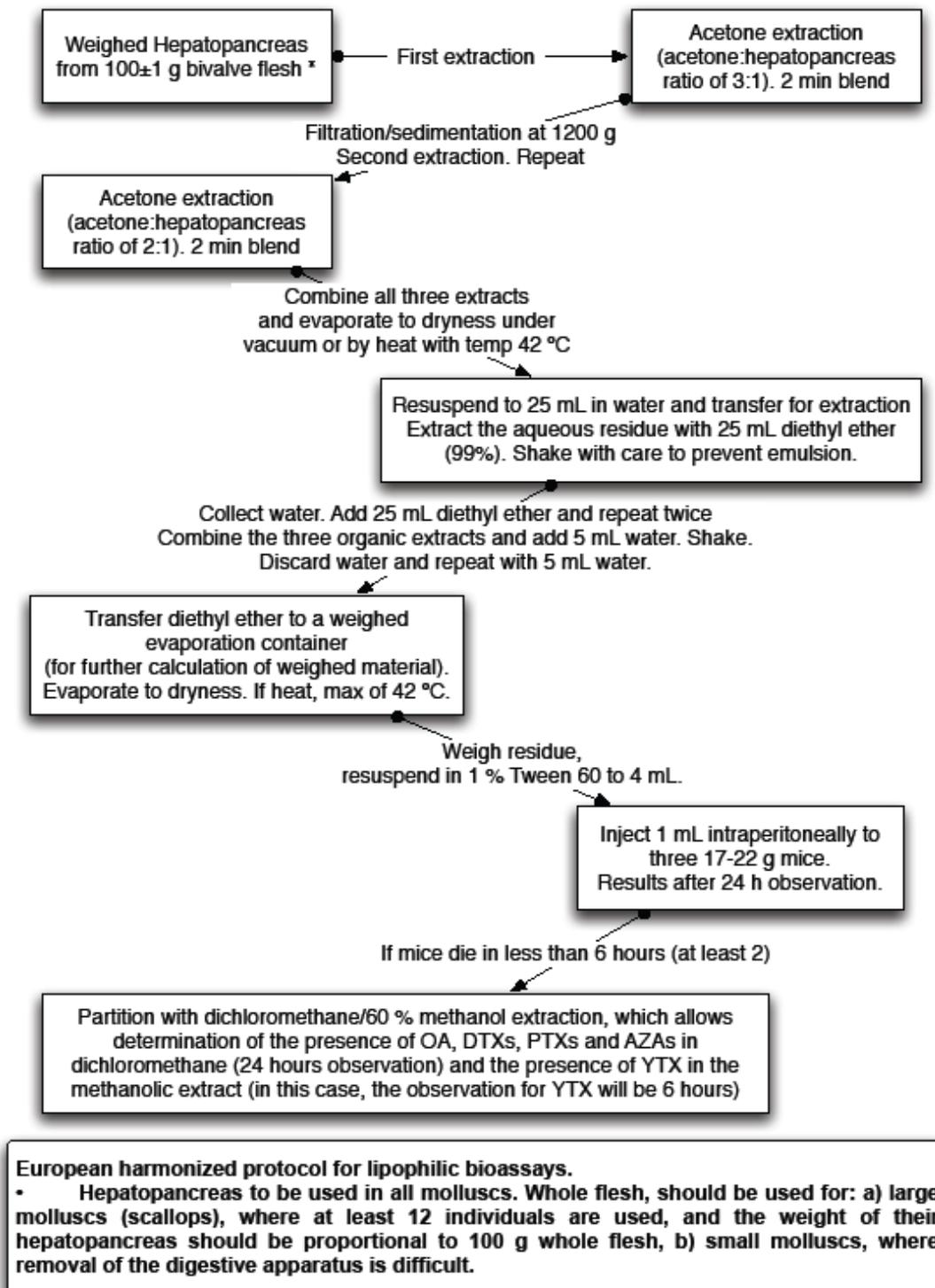


Figure 3. Sample preparation and extraction methods of hepatopancreas for the mouse bioassay (MBA) (CRL-MB, 2007)¹².

The reported *i.p.* lethal dose (LD₅₀) values for PTX-2 in mice are 219 and 411 µg/kg body weight (b.w.). The LD₅₀ is the dose that kills 50 % of the animals. Depending on the PTX analogue, the lethal dose may vary, from 250 µg/kg (PTX1 and PTX11) to 350 µg/kg (PTX3), 500 µg/kg (PTX6), 770 µg/kg (PTX4) and >5000 µg/kg (PTX7, PTX8, PTX9 and PTX2 SA) (Munday, 2008). The residues injected per mouse originate from 25 g shellfish flesh, which would need to contain more than about 5 µg of PTX1, PTX2 or PTX11 in order to kill two out of three 20 g mice (i.e. more than 200 µg/kg shellfish flesh). The sensitivity of the MBA would be lower for other PTX-group toxins.

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.

The main disadvantages of the MBA are:

- the outcome depends on the choice of solvents used;
- it is labour intensive and cannot be readily 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 potential for false positive results due to interferences (e.g. free fatty acids);
- the potential for false negative results;
- it is not selective for solely the PTX-group toxins;
- it is not quantitative;
- the injection volume of one mL exceeds good practice guidelines (less < 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

Several antibodies were obtained to develop antibody-based methods, but at this time no commercial alternative is available. One enzyme-linked immunosorbent assay (ELISA) has been shown to perform appropriately, following in-house validation (Briggs *et al.*, 2005; Briggs, 2008). Interlaboratory validation would be recommended prior to using this methodology in official control.

The main advantages of an antibody-based method are:

- it is very sensitive;
- it is fast, easy to use, and can be applied to screen many samples.

The main disadvantage of an antibody-based method is:

- it does not provide any information on the toxin profile.

4.4. Chemical methods

As ultra violet (UV) absorption is very weak above 200 nm, and no fluorescence methods have been developed, the only physicochemical method of detection is liquid chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS). The mass spectrometric detection of PTX2 in shellfish extracts has been studied in detail and has been shown to be affected by matrix effects, like many other marine toxins. A collaborative study by McNabb *et al.* (2005) focused on the analysis of extracts and found good apparent recovery. Stobo *et al.* (2005), found little matrix effect in the determination of PTX2 by LC-MS, while Fux *et al.* (2008) found between 0 and 92 % signal enhancement, depending on chromatographic conditions. A study by Gerssen *et al.* (2009a) also confirmed that reproducibility is poor for PTX2 compared to other compounds. Some progress was made in a collaborative trial for the LC-MS determination of PTX2 in the 6th Framework project 'BIOTOX', where an acceptable interlaboratory variability (HORRAT of 0.99)¹³ was found for PTX2. However, only 4 laboratories reported data for this parameter and this low number of laboratories is not considered sufficient for the standardization of the method. Further research has been carried out in the BIOTOX project on the clean-up of shellfish extracts prior to LC-MS-MS detection. These results (Gerssen *et al.*, 2009b) are encouraging but they will have to be confirmed in interlaboratory trials. A method including an enrichment of PTX2 based on solid phase extraction (SPE) prior MS detection was also tested in an in-house validation trial (These *et al.*, 2009). The limit of quantification (LOQ) was determined to be 1 µg/kg by applying an enrichment factor of 10.

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

- information on the toxin profile of lipophilic biotoxins can be obtained;
- it is highly specific and sensitive;
- it can be automated.

The main disadvantages of LC-MS/MS based methods are

- it requires costly equipment and highly trained personnel;
- it requires reference material for identification and quantification.

4.5. Summary of methods

At this point none of the methods for the determination of toxins from the PTX-group have been validated by interlaboratory studies. The limit of detection of the mouse bioassay is higher than the current regulatory limit. Additionally, Council Directive 86/609/EEC states that “*Member States may not permit the use of live animals in procedures that may cause pain, suffering distress or lasting harm if another scientific satisfactory method of obtaining the result sought and not entailing the use of live animals is reasonably and practicably available*”.

The evidence available at this moment suggests that LC-MS/MS has the greatest potential to replace the mouse bioassay. Moreover, it is able to detect PTX-group toxins at levels below the current regulatory limit of 160 µg/kg mollusc. The LC-MS/MS methods also have the

¹³ HORRAT: The observed relative standard deviation (RSD) calculated from results generated under reproducibility conditions (RSD_R) divided by the RSD_R value calculated from the Horwitz equation (using the assumption that the repeatability $r = 0.66R$ (reproducibility)). The Horwitz equation is a generalised precision equation which has been found to be independent of analyte and matrix but solely dependent on concentration for most routine methods of analysis.

possibility for multi-toxin group detection/quantification. However, before LC-MS/MS can be used for official purposes, validation results are needed to support their use.

5. Occurrence of PTX-group toxins

5.1. Data Collection

Following a request by EFSA, Germany, Italy, Spain, Norway and United Kingdom (UK) provided data on the occurrence of PTX-group toxins in shellfish. A total of 1220 analytical results were submitted. The number of analyses presented by the countries is considerably different from one country to another. Germany accounts for 58.8 % of the data, Norway for 31.1 %, Italy, Spain and UK for 3.0 %, 2.4 % and 4.7 %, respectively. The data cover the period between 2005 and 2008.

Table 2 shows a summary of the number of data submitted by each country including purpose of testing, analytical method applied, limit of detection (LOD) and limit of quantification (LOQ) of the methods.

Table 2. Data submissions from European Countries for PTX-group toxins in the period from 2005 to 2008.

Country	Year(s) of harvesting	Number of samples	Purpose of testing ^(a)	Method of testing	LOD ^(c) (µg/kg)	LOQ ^(c) (µg/kg)	Number of samples also tested by MBA
Germany	2005-2008	718	pre/post-MC	LC-MS/MS	1-20 ^(b)	-	0
Italy	2007-2008	37	pre-MC	LC-MS/MS	4	12	22
Norway	2006-2008	379	pre-MC	LC-MS/MS	2	20	379
Spain	2006-2007	29	pre-MC	LC-MS/MS	-	5 ^(b)	29
United Kingdom	2006-2007	57	pre-MC	LC-MS/MS	9-39 ^(b)	33-78	57
Total		1220					

Pre/post-MC=pre-market/post-market control, LOD=limit of detection, LOQ=limit of quantification, MBA=mouse bioassay
 (a): PreMC are samples collected at the place of origin, before or during harvesting; PostMC are samples collected at the place of sale or along the distribution chain.

(b): Germany reports only LOD, and this is variable through the years and between different laboratories; Spain reports only LOQ.

(c): The reported LOD/LOQ values are referred to PTX₂, because a calibrant solution is only available for PTX₂.

The LC-MS/MS method is at different levels of development in different countries, as shown by the range in LOD and LOQ values. The ranges in LOD/LOQ show that also within a single country the analytical results differ between laboratories and between different years. It has to be also noted that Germany does not perform a parallel MBA test on its samples, whereas most of the other countries do. Pre-market control samples (pre-MC), which are samples harvested for further processing or direct marketing as prescribed in the respective EU legislation, comprised 628 results and included data from five countries. Post-market control samples (post-MC), which are samples taken from the market (e.g. from stores and supermarkets with unknown but possibly multiple origin), comprised 592 results and included data only from Germany. Post-MC samples represent 82.5 % of the German dataset.

The number of analogues reported by the countries that submitted data varies from 1 to 5. The only analogue that has been reported by all five countries is PTX₂, which is also the only one for which a certified calibrant solution is available. Apart from PTX₂, also PTX₂ SA is sometimes detected, but usually at concentrations in the low µg/kg range. In addition, as discussed later in chapter 10, the seco acids show very low toxicity. Depending on shellfish

species and geographical location other analogues may be relevant (e.g. PTX1, PTX3, PTX6 and PTX11), but due to the lack of data the CONTAM Panel was not able to evaluate their importance. Taking into account these aspects, the CONTAM Panel decided to use only PTX2 for the occurrence and exposure calculations.

5.2. PTX-group toxin concentration in shellfish

Normally the whole shellfish is consumed and therefore the occurrence data for PTX-group toxins need to be expressed as whole shellfish meat. Most of the analyses were performed on whole shellfish meat. In a few samples only hepatopancreas was analysed, in which case a factor of 5 was used to convert the value to whole shellfish meat. This factor, though not representing exactly all individual shellfish species, is considered to represent a good approximation.

A total of 1220 samples were considered for the descriptive statistical calculations. Analytical methods for the detection of marine biotoxins are continuously developing and, therefore, different LODs have been reported in different countries and/or in different periods of time, even though LC-MS/MS was used for all submitted data.

For values reported below LOD or below LOQ the “bounding” approach was applied. The Lower Bound (LB) is obtained by assigning a value of zero (minimum possible value) to all results reported as <LOD or <LOQ. The Upper Bound (UB) is obtained by assigning the value of LOD to results reported as <LOD and LOQ to results reported as <LOQ (maximum possible value). The comparison between UB and LB values (sensitivity test) demonstrates the impact of the approach on the exposure assessment.

The LC-MS/MS data from Spain, Italy and UK are limited and not representative, because in these countries monitoring is routinely performed with MBA. The LC-MS/MS method is only used occasionally, mainly for research purposes. An overview of the basic statistics for the occurrence of PTX2 grouped by country is provided in Table 3.

Table 3. Statistics of relevant data of PTX2 in shellfish in 2005-2008 provided by European countries.

Analytical method/ Country	N	Median	Mean	P95	Maximum	% of samples not quantified	% of values >160 µg PTX2/kg shellfish meat
		LB/UB	LB/UB	LB/UB			
LC-MS/MS							
Germany 2005-2008	718	0/2	0/2	0/10	20 ^(a)	97	0
Italy 2007-2008	37	15	63/66	220	264	49	19
Spain 2006-2007	29	20	27	50	53	0	0
Norway 2006-2008	379	0/2	14/16	79	98	60	0
United Kingdom 2006-2007	57	0/39	20/54	109	418	93	5
ALL	1220	0/2	8/11	57	418	82	1

N=number of samples, P95=95th percentile, UB=upper bound, LB=lower bound

For most of the data no information is available on measurement uncertainty. When two values are given it indicates the respective lower bound (LB) or upper bound (UB) values for samples below the LOD or LOQ. The LB is calculated substituting 0 to all not detected samples. The UB is calculated substituting “<LOD” with LOD value and “<LOQ” with LOQ value. LOD and LOQ are those defined for the specific single analysis.

Due to a low level of contamination and a very high percentage of non-detected results, median, mean, and in some cases even the 95th percentile and the maximum may be influenced by the choice of upper or lower bound approach.

(a): The maximum is due to the presence of a value <LOD with the LOD of 20. This results in the UB approach a figure higher than the maximum reported numerical value of 8.

The basic statistics indicate a generally low amount of PTX2 in the current collection of shellfish samples from European countries ranging from “not detected” to 418 µg/kg. This maximum level was detected in an area where earlier an OA outbreak was registered.

The percentage of analytical results <LOD or <LOQ and therefore without a quantified value varies between countries, ranging from 49 % for Italy in the years 2007-2008 to 97 % for Germany in the years 2005-2008. An exception is Spain, where all samples are above LOQ. The small number of samples and the high proportion of quantified samples show that the data from Spain and Italy are targeted data. The percentage of not quantified results is 82 % for all 1220 analysed samples.

The proportion of samples exceeding 160 µg PTX2/kg shellfish meat varies among countries: 0 % (Germany, Norway and Spain), 5 % (UK) and 19 % (Italy). The value for UK relates to three samples from a single contaminated area; the higher proportion for Italy also depends on the presence of seven targeted samples. Overall, taking into account the objective of the investigation, the time of sample collection (pre- or post-market) and the different number of samples reported, it should be noted that the results shown in Table 3 can be considered as only an approximate “snapshot” of the situation in Europe.

5.3. Difference between species

The limited number of samples and the low level of contamination make a comparison between shellfish species difficult. The distribution of PTX2 analysed in the different shellfish is shown in Table 4. Out of 1220 samples 131 samples were described as shellfish only without any further specification.

Table 4. Statistical descriptors for PTX2 occurrence in different shellfish.

Species	N	Total concentration of PTX2 µg PTX2/kg shellfish meat				% of samples not quantified	% of values >160 µg PTX2/kg shellfish meat
		Median LB/UB	Mean LB/UB	P95 LB/UB	Maximum		
Mussels	897	0/2	8/12	57	418	78	0.3
Others	131	0/1	0/2	0/10	7/10	97	0
Oysters	91	0/2	26/28	182	264	79	7.7
ALL	1220						

N=number of samples, P95=95th percentile, UB=upper bound, LB=lower bound

When two values are given it indicates the respective lower bound (LB) or upper bound (UB) values for samples below the LOD or LOQ. The LB is calculated substituting 0 to all not detected samples. The UB is calculated substituting “<LOD” with LOD value and “<LOQ” with LOQ value. LOD and LOQ are those defined for the specific single analysis.

Due to a low level of contamination and a very high percentage of non detected results, median, mean, and in some cases even the 95th percentile and the maximum may be influenced by the choice of upper or lower bound approach.

Apart from the shellfish species listed in Table 4 a few samples referred to clams (N=40), cockles (N=8) and scallops (N=53). Each of these groups consists of only one quantified sample which does not allow any statistical calculation.

The percentage of samples exceeding the value of 160 µg PTX2/kg varied between 0 % (all species excluding mussels and oysters) to 7.7 % (oysters).

5.4. Influence of type of sampling and origin of the sample

Ideally, the comparison between pre-MC and post-MC samples should refer to the same areas in order to allow evaluation of the effectiveness of the monitoring activities. For PTX-group toxins this is not possible because the available pre-MC and post-MC data refer mostly to different countries. All post-MC data are from Germany, whereas the pre-MC data are from all five countries that submitted data. Moreover, a separate comparison of pre-MC and post-MC data from Germany is not appropriate, since the post-MC samples relate to both locally produced products and imported products. The available data are presented in Table 5.

Table 5. Statistics of LC-MS/MS data of PTX2 in shellfish in pre-market control (pre-MC) and post-market control (post-MC) datasets.

Data groups	N	Total concentration of PTX2 µg/kg shellfish meat				% of samples not quantified	% of values >160 µg PTX2/kg shellfish meat
		Median LB/UB	Mean LB/UB	P95 LB/UB	Maximum		
pre-MC (all countries)	628	0/2	15/20	80	418	67.4	1.6
post-MC (Germany)	592	0/1	0/2	0/7	8/20	97.0	0

Pre/post-MC=pre-market/post-market control, N=number of samples, P95=95th percentile, UB=upper bound, LB=lower bound

When two values are given it indicates the respective lower bound (LB) or upper bound (UB) values for samples below the limit of detection (LOD) or limit of quantification (LOQ). The LB is calculated substituting 0 to all not detected samples. The UB is calculated substituting “<LOD” with LOD value and “<LOQ” with LOQ value. LOD and LOQ are those defined for the specific single analysis.

Due to a low level of contamination and a very high percentage of non detected results, median, mean, and in some cases even the 95th percentile and the maximum may be influenced by the choice of upper or lower bound approach.

The data from the post-market investigations of samples originating from Germany only revealed low levels which might be an indication that the pre-market control prevents effectively the presence of PTX2 in marketed products. This is probably due to the fact that PTX-group toxins are normally found together with other lipophilic toxins, therefore the reduction between pre-MC and post-MC is influenced by the screening also for other toxins.

5.5. Influence of processing

There is no information on the effects of processing (e.g. cooking, steaming, autoclaving) on the levels of PTX-group toxins in shellfish. However, it can be assumed that as for other lipophilic toxins, water loss during processing may lead to an increase in concentration of PTX-group toxins in shellfish flesh.

6. Comparison of LC-MS/MS data with the results of mouse bioassay

A total of 487 samples originating from Italy, Norway, Spain and UK were tested with both MBA and LC-MS/MS. The results are presented in Table 6.

Table 6. Concentration of PTX2 measured by LC-MS/MS in samples comparatively tested by MBA.

Mouse bioassay	N	Total concentration of PTX2 µg/kg shellfish meat				% of samples not quantified	% of values >160 µg PTX2/kg shellfish meat
		Median LB/UB	Mean LB/UB	P95 LB/UB	Maximum		
Positive	164	0/39	32/42	130	418	50.6	4.9
Negative	323	0/2	14/16	79	183	62.2	0.6

N=number of samples, P95=95th percentile, UB=upper bound, LB=lower bound

When two values are given it indicates the respective lower (LB) or upper bound (UB) values for samples below the LOD or LOQ. The LB is calculated substituting 0 to all not detected samples. The UB is calculated substituting “<LOD” with LOD value and “<LOQ” with LOQ value. LOD and LOQ are those defined for the specific single analysis.

Due to the low level of contamination and the large amount of non detected results, median and mean are influenced by the choice of upper or lower bound approach.

Only 0.6 % of the samples tested negative in the MBA had a value higher than the limit of 160 µg PTX2/kg shellfish meat, based on LC-MS/MS results. The proportion of samples tested positive in the MBA but having an LC-MS/MS result <LOD or <LOQ was 50.6 %. The high proportion of samples with positive MBA, but PTX2 level <LOQ indicates a contribution of other lipophilic toxins, such as OA-, AZA-, YTX- or cyclic imine group toxins, or combinations thereof, to the positive response in the MBA.

7. Human consumption of shellfish

Limited consumption data were available for individual shellfish species across the EU. The EFSA concise database does not yet provide sufficient information since there is no differentiation between meal sizes for fish and other seafood. Therefore, EFSA requested the Member States to provide information on shellfish consumption. Data have been submitted by France, Germany, Italy, The Netherlands and the UK. A compilation of the data received is presented in Table 7. The mean portion sizes for consumers only ranged between 10 g (France, bivalve molluscs) and 136 g (The Netherlands). The data from Germany, Italy and the UK are within this range.

The German national food consumption survey performed by a weighing protocol in the late 1980s indicates a minimum meal size of mussels of 2 g (mainly as an ingredient in dishes), a median of 63 g, a mean of 107 g and a 95th percentile of 400 g among mussel consumers. The maximum portion size reported in this study was 1500 g. The French Calipso study differentiated mussels and bivalve molluscs. The maximum portions for mussels (245 g) and all bivalve molluscs (415 g) varied, whereas the mean portions were similar. A survey reported by the UK indicates a mean shellfish meal size of 114 g and a maximum of 239 g. A Dutch study reported a mean portion size of 136 g of shellfish and a maximum of 480 g. These data are for consumers only. The surveys show a large variation in the percentage of the populations consuming shellfish and it is unclear whether the data are related to cooked or uncooked shellfish.

Table 7. Shellfish eating habits in France, Italy, The Netherlands, the UK and Germany, based on national food consumption surveys.

Country	Study	Number of consumers N (%)	Number of eating occasions for consumers/ year	Mean portion weight (g)	95th percentile (g)	Maximum portion weight (g)	Maximum frequency
France (7 days)	INCA 1999	218/1985 (11 %)	N/A	10			N/A
France (FFQ)	CALIPSO 2004 (bivalve molluscs)	962/997 (96 %)	N/A	32	94	415	N/A
France (FFQ)	CALIPSO 2004 (mussels)	862/997 (86 %)	N/A	22	70	245	N/A
Italy (7 days)	INN-CA 1994-96	212/1,981 (11 %)	47	83		1000	4/week
Germany (7 days)	NVS 1985-88	150/23239 (0.6 %)	171	107	400	1500	3/week
UK (7 days)	NDNS 2000-01	212/1631 (13 %)	51	114		239	4/week
The Netherlands (2 days)	DNFCS 1997-98	47/4285 (1.1 %)	39	136	465	480	N/A

FFQ=food frequency questionnaire, 7 days=7 day dietary record, 2 days=2 day dietary record, N/A=not available

INCA=Enquête Individuelle et Nationale sur les Consommations Alimentaires (Volatier, 2000).

CALIPSO=Fish and seafood consumption study and biomarker of exposure to trace elements, pollutants and omega 3 (Leblanc *et al.*, 2006)

INN-CA=Nationwide Nutritional Survey of Food Behaviour (Turrini *et al.*, 2001)

NVS=Nationale Verzehrsstudie (Adolf *et al.*, 1995)

NDNS=National Diet and Nutrition Survey (Henderson *et al.*, 2002)

DNFCS=Dutch National Food Consumption Survey (Kistemaker *et al.*, 1998)

Because PTX-group toxins have acute toxic effects, it is important to identify a large portion size rather than a long term average consumption in order to protect the health of the consumer. In the studies presented in the table above, the maximum reported sizes are in the range of 239 to 1500 g. The CONTAM Panel noted the largest portion sizes of 1000 g and 1500 g, and considered it likely that the shells were included in these weight estimates. Therefore, the CONTAM Panel considered the 95th percentile as a more realistic estimate of the portion size for high consumers. As shown in Table 7 the 95th percentile values range from 70 to 465 g and the CONTAM Panel chose the figure of 400 g to be used as a large

portion size in acute exposure assessments. It should be noted that this figure is at the higher end of the range of the 95th percentile reported by the Member States and is therefore likely to cover a higher percentile for the entire EU. This is in good agreement with the report of the Joint FAO/IOC/WHO *ad hoc* expert consultation on marine biotoxins (FAO/IOC/WHO, 2004a), where 380 g was reported as the largest 97.5th percentile portion size for consumers only.

8. Exposure assessment

8.1. Deterministic estimate of dietary exposure to PTX2

Based on the assumption that products tested negative in the MBA reach the market (Table 6) the dietary exposure can be estimated as in Table 8.

Table 8. Deterministic intake estimate of PTX2 based on samples tested negative in the MBA.

P95 (concentration of samples tested negative in the MBA)	79 µg PTX2/kg whole shellfish meat
Exposure by eating a 400 g portion at 79 µg PTX2/kg	31.6 µg PTX2/person (0.5 µg PTX2/kg b.w.)
Exposure by eating a 400 g portion at 160 µg PTX2/kg whole shellfish meat	64 µg PTX2/person (1.1 µg PTX2/kg b.w.)

P95=95th percentile, MBA=Mouse bioassay, b.w.=body weight

The exposure for a European consumer of a 400 g portion of shellfish meat contaminated with PTX2 at the 95th percentile of occurrence in samples tested negative in the MBA is 0.5 µg PTX2/kg b.w. This represents less than half of the exposure (1.1 µg PTX2/kg b.w.) of a person eating a 400 g portion at the level of 160 µg PTX2/kg whole shellfish meat (current EU limit), and is also less than the acute reference dose (ARfD) of 0.8 µg PTX2 equivalents/kg b.w. proposed in chapter 12.

These results are conservative but not unrealistic estimates of PTX2 dietary exposure in four European countries.

8.2. Probabilistic estimate of dietary exposure to PTX2

A probabilistic estimate of dietary exposure to PTX2 has been performed by a Monte Carlo simulation using the distributions of both the occurrence data (see footnote 14) and the data on the consumption of shellfish. Compared to the deterministic estimate the probabilistic exposure estimate provides information on the chance to exceed a specific exposure level. Because a person eating shellfish does not eat the same portion size containing the same level of toxins each time, the probabilistic calculation includes all the combinations of all different occurrence and consumption data.

For the probabilistic estimate the same concentration data obtained by the LC-MS/MS measurements of the samples tested negative in the MBA (Table 6) were used¹⁴, because it can be assumed that these samples will reach the market.

¹⁴ All samples with quantifiable levels of PTX2 (compare table 6) were characterised by a beta distribution, which has been derived by the best fit analysis of the @RISK tool. This set of data includes 66 samples exceeding the given LOQ of 20

Because insufficient information is available on the distribution of portion sizes, the CONTAM Panel decided to use a triangular distribution as a simple and pragmatic approach. A triangular distribution is characterised by three values, the minimum, the most probable and the maximum. In the case of shellfish consumption a value of 0 was used as a minimum. From the range of 10 to 136 g reported as mean consumption figures in Table 7 the CONTAM Panel chose a value of 100 g to be used as “most probable” value, although there is no evidence that it is the most frequently consumed portion. The better-documented large portion size of 400 g (see chapter 7) was used to represent the maximum.

The resulting probabilistic dietary exposure distribution has a median value of approximately 1.7 µg PTX2/person, a mean of approximately 3.1 µg PTX2/person (corresponding to 0.05 µg PTX2/kg b.w.), and a 95th percentile of approximately 12 µg PTX2/person (corresponding to 0.2 µg PTX2/kg b.w.). A 99th percentile of 27 µg PTX2/person (corresponding to 0.45 µg PTX2/kg b.w.) has been extrapolated from the fitted distribution. The probabilistic exposure estimate is presented in Figure 4 illustrating the chance to exceed a specific level of exposure to PTX2 equivalents when consuming a single portion of shellfish. The chance to exceed an intake of 48 µg corresponding with the ARfD for PTX-group toxins of 0.8 µg PTX2 equivalents/kg b.w. established in chapter 12 is about 0.2 %. The chance to exceed the deterministic dietary exposure estimate of 64 µg PTX2 equivalents per person, corresponding to a consumption of a 400 g portion containing PTX2 equivalents at the level of the current EU limit value, is even lower than 0.2 %.

µg/kg as well as 62 samples where concentrations between 1 and 20 µg/kg have been reported. This fitted distribution function was truncated at the LOQ of 20 µg/kg [=RiskBetaGeneral(0,5867;3,1686;0,053348;230,76;RiskTruncate (20;))]. The samples reported at or below the LOQ (80 % of the total number) were randomly assigned a numerical value by using a discrete distribution [RiskDiscrete({0;1};0.8; 0.2)] reflecting the ratio of non-quantifiable/ quantifiable samples (80 %/ 20 %). This implies that 80 % of the samples reported at or below the LOQ were assigned a “0” (zero). The remaining 20 % of these samples were assigned a value between 0 and 20 µg/kg by using a uniform distribution function [RiskUniform(0;20)]. This matches with the 62 samples having numerical values below the LOQ.

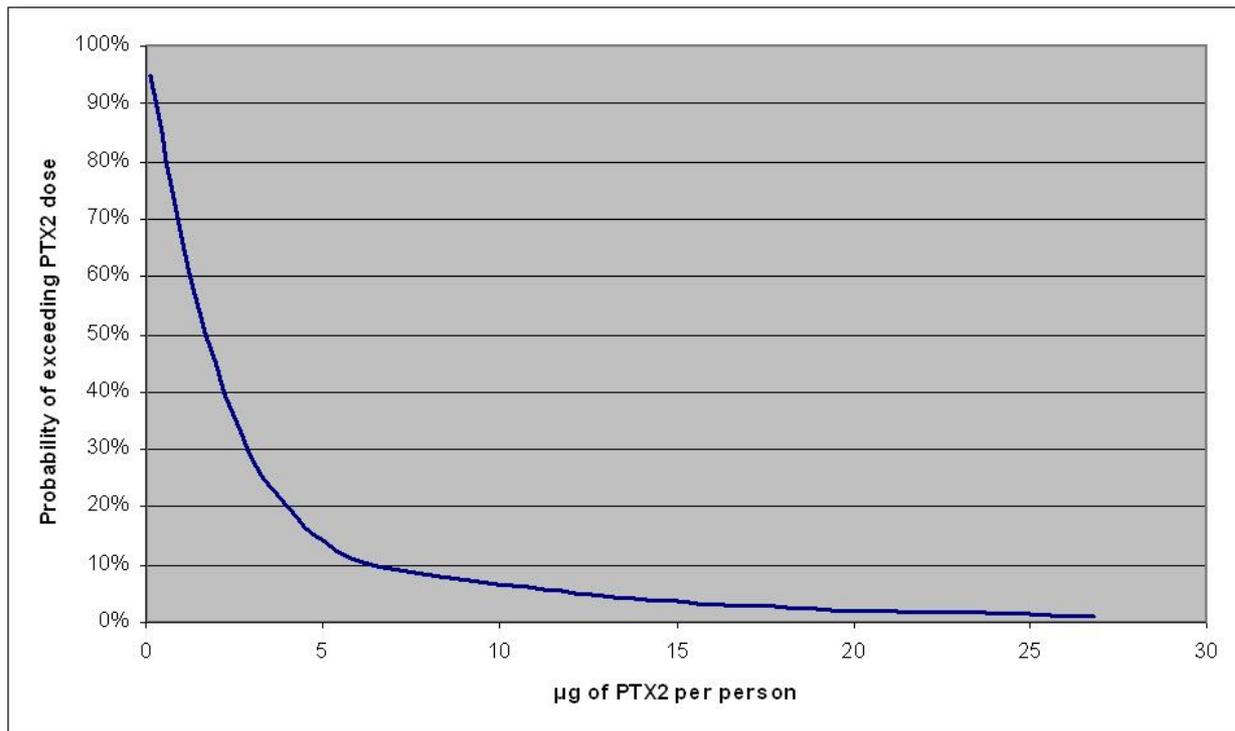


Figure 4. Probability of dietary exposure to PTX2 resulting from consumption of a single portion of shellfish.

9. Toxicokinetics

9.1. Absorption, distribution, elimination and bioaccumulation

Twenty four hours after administration of a single oral dose of 5.7 µg PTX2/animal to mice (n=3) about 1 µg of the parent compound was found in the gastrointestinal content and faeces, with only traces in the gastro-intestinal tissue. No detectable amounts could be found in other internal organs and urine. Total recovery was 19 %, but it should be noted that only the parent compound was determined. In a second experiment a mix of PTX2 and PTX2 SA was administered by gavage and a similar pattern was observed, with the majority of the dose remaining in the gastrointestinal content and excreted in the faeces without being absorbed. Following *i.p.* administration of PTX2 and PTX2 SA these compounds were detected in blood and internal organs and in the gastrointestinal tract and faeces, but also with low recovery (Burgess, 2003).

From an ongoing study in Norway (Espenes *et al.*, 2009), NMRI female mice (ca. 20 g body weight) received a single dose of PTX2 by gavage at 1 or 5 mg/kg b.w., three mice per dose. Twenty four hours after treatment samples were taken from several organs, including stomach, small and large intestines, liver, kidney, heart and whole blood. The amounts of PTX2 found in different tissues were by far the highest in the stomach followed by the intestines. Internal organs and whole blood showed only traces. Although the studies reported by Burgess (2003) and by Espenes *et al.* (2009) are not appropriately designed to determine the bioavailability of PTX-group toxins, they suggest a low gastrointestinal absorption of these toxins in mammals following oral administration. In mice given PTX2 at 5 mg/kg, the following levels were found (rounded figures): stomach (7 µg/g), duodenum (0.27 µg/g),

small intestine (0.13 µg/g), colon (0.05 µg/g), liver, kidney, heart, and whole blood (< 0.007 µg/g). The PTX2 levels in mice receiving the lower dose (1 mg PTX2/kg) showed a similar distribution with accordingly lower concentrations (Espenes *et al.*, 2009).

9.2. Biotransformation

No information on biotransformation of PTX-group toxins has been identified.

10. Toxicity data

10.1. Mechanistic considerations

The molecular mechanism of action of PTX-group toxins has been investigated with reference to original observations that associated this class of compounds with the OA-group toxins of diarrhetic toxins (Yasumoto *et al.*, 1985).

Unlike OA which inhibits some isoforms of serine/threonine phosphoprotein phosphatases at nM concentrations (Bialojan and Takai, 1988), PTX1 does not inhibit these enzymes at doses as high as 10 µM (Fladmark *et al.*, 1998). This finding has provided the molecular basis to distinguish PTX-group toxins from OA-group toxins.

The morphological alterations found in some organs in mice (Terao *et al.*, 1986) and in isolated hepatocytes (Aune *et al.*, 1991) after exposure to PTX1 indicated that PTX-group toxins could affect the cellular cytoskeleton, setting the ground for the hypothesis that their mechanism of action might involve actin, the major protein component of the cytoskeleton.

Several studies (see Table 9) have confirmed that PTX-group toxin alteration of actin-based cytoskeleton is the proximal cause of responses triggered by this group of toxins, and have provided information regarding molecular processes exerted by PTX-group toxins in sensitive systems.

Table 9 summarises the major phenomena recorded in cells exposed to PTX-group toxins.

Table 9. Molecular responses induced by PTX-group toxins *in vitro*.

Effects	Cell type	Analogue	Concentration (M)	Reference
Alteration of actin-based structures	chick embryo hepatocytes	PTX1	$\sim 10^{-7}$	Zhou <i>et al.</i> , 1994
	A10	PTX2	$\sim 10^{-7}$	Hori <i>et al.</i> , 1999
	NRK-52E	PTX2	$\sim 10^{-8}$	Spector <i>et al.</i> , 1999
	BE(2)M-17 neuroblastoma	PTX6	10^{-6}	Leira <i>et al.</i> , 2002
	rabbit enterocytes	PTX6	10^{-6}	Ares <i>et al.</i> , 2005
	BE(2)M-17 neuroblastoma and hepatocytes	PTX2	10^{-7}	Ares <i>et al.</i> , 2007; Espiña <i>et al.</i> , 2008
	n.a. (cell-free)	PTX2	n.a.	Allingham <i>et al.</i> , 2007
Cell death and apoptosis	several human tumour lines	PTX2	10^{-9} - 10^{-6}	Jung <i>et al.</i> , 1995
	rat and salmon hepatocytes	PTX1	10^{-6}	Fladmark <i>et al.</i> , 1998
	HCT116 colorectal cancer	PTX2	10^{-8}	Chae <i>et al.</i> , 2005, 2008
	leukemia cells	PTX2	10^{-8}	Shin <i>et al.</i> , 2008; Kim <i>et al.</i> , 2008a, 2008b; Moon <i>et al.</i> , 2008
	neuroblastoma	PTX2	10^{-9} - 10^{-8}	Cañete and Diogene, 2008

n.a.=not applicable

A study showing that PTX1 disrupts stress fibers provided the first indication that PTX-group toxins alter F-actin (Zhou *et al.*, 1994). This observation was subsequently confirmed with PTX2 (Spector *et al.*, 1999) and led to the conclusion that PTX2 causes actin depolymerization (Hori *et al.*, 1999). Subsequent studies have confirmed that PTX-group toxins induce actin depolymerization in several cellular types (Leira *et al.*, 2002; Ares *et al.*, 2005, 2007).

A detailed description of the interaction between PTX2 and actin (Allingham *et al.*, 2007), and the characterization of structure-activity relationships of PTX-group toxins (Allingham *et al.*, 2007; Ares *et al.*, 2007) have also been obtained. An initial report indicated a 1:4 stoichiometry of PTX1:actin interaction (Hori *et al.*, 1999), but more recent crystallographic data have shown that PTX2 and actin form a 1:1 complex (Allingham *et al.*, 2007). The analyses carried out by X-ray crystallography have also detailed the PTX-actin interaction. F-actin is composed of two helices containing polymers of non-covalently bound actin molecules that take contacts both laterally, providing interchains interactions, and longitudinally, in intrachain associations. PTX2 was shown to associate with actin monomers in a site that is close to the “inner” filament axis, inhibiting the lateral subunit interactions critical for filament assembly, thereby interfering with actin polymerization (Allingham *et al.*, 2007). Furthermore, PTX2 did not show any depolymerizing effect involving severing of G-actin monomers from F-actin (Allingham *et al.*, 2007).

The structure-activity studies have shown that the structure of the PTX molecule ring is a key determinant of actin binding, so that isomerization around the C₇ of the PTX molecule (Allingham *et al.*, 2007), and the rupture of the lactone ring (Allingham *et al.*, 2007; Ares *et al.*, 2007) would interfere with PTX-actin interactions.

PTX-group toxins have been shown to cause cell cycle arrest, cell death and apoptosis. The wide range (10^{-9} and 10^{-6} M) of effective concentrations of PTX-group toxins reported in literature (Jung *et al.*, 1995; Fladmark *et al.*, 1998; Chae *et al.*, 2005 and 2008; Shin *et al.*, 2008; Kim *et al.*, 2008a,b; Moon *et al.*, 2008; Cañete and Diogene, 2008), as well as the apparent resistance of some cell lines to the cytotoxic effect of PTX-group toxins (Leira *et al.*, 2002), indicate the existence of cell-specific factors affecting the sensitivity of biological systems to this group of natural compounds.

The study by Chae *et al.* (2005) provided direct evidence that agents altering the actin-based cytoskeleton activate apoptotic responses, and that PTX2 triggers the intrinsic (mitochondrial) pathway of apoptosis. This indicates that F-actin depolymerization induced by PTX-group toxins is the causative event of subsequent cell death.

Other studies have confirmed that PTX2 induces apoptosis in several cell lines through multiple mechanisms, involving the perturbation of the cell cycle machinery, the NF- κ B system, JNK and ERK protein kinase isoforms, proteins of the Bcl-2 family, as well as caspase proteases (Chae *et al.*, 2005 and 2008; Kim *et al.*, 2008a,b; Moon *et al.*, 2008; Shin *et al.*, 2008). Although multiple molecular events are triggered in cells exposed to PTX2, experimental data would confirm that they are downstream of the alterations induced by PTX-group toxins in the actin cytoskeleton.

On the basis of the available information, obtained with cellular systems and X-ray crystallography, the CONTAM Panel concludes that the PTX-actin interaction and the consequent perturbation of the actin cytoskeleton could be the molecular basis of cellular vacuolization in animal models and cell damage in biological systems exposed to PTX-group toxins.

10.2. Effects in laboratory animals

10.2.1. Acute toxicity

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

The acute lethality following *i.p.* administration of the most toxic PTX analogues. LD₅₀ values for PTX2 vary between 219 and 411 $\mu\text{g}/\text{kg}$ b.w., and limited data indicate that the acute toxicity of PTX-1, PTX-3 and PTX-11 is similar. The animals showed severe general toxicity, became hunched and lethargic, showed laboured respiration, ataxia and cyanosis (Munday, 2008) (Table 10). The toxicity of PTX4 and PTX6 is lower and for the other analogues tested no lethality was observed (lethal doses ≥ 5000 $\mu\text{g}/\text{kg}$ b.w.).

Table 10. Acute lethality of PTX-group toxins in mice following *i.p.* administration (from Munday, 2008).

Compound	Dose µg/kg b.w.	Parameter	Reference
PTX1	250	MLD	Yasumoto, <i>et al.</i> , 1985
PTX2	219	LD ₅₀	Miles <i>et al.</i> , 2004
	411	LD ₅₀	Yoon and Kim, 1997
	192-400	MLD	Yasumoto, <i>et al.</i> , 1985; Yasumoto <i>et al.</i> , 1989; Yoon and Kim, 1997; Miles <i>et al.</i> , 2004
PTX3	350	MLD	Murata <i>et al.</i> , 1986
PTX4	770	MLD	Yasumoto <i>et al.</i> , 1989
PTX6	500	MLD	Yasumoto <i>et al.</i> , 1989
PTX7	>5000	MLD	Sasaki <i>et al.</i> , 1998
PTX8	>5000	MLD	Sasaki <i>et al.</i> , 1998
PTX9	>5000	MLD	Sasaki <i>et al.</i> , 1998
PTX11	244	LD ₅₀	Suzuki <i>et al.</i> , 2006
	250	MLD	Suzuki <i>et al.</i> , 2006
PTX2 SA	>5000	MLD	Miles <i>et al.</i> , 2004; Miles <i>et al.</i> , 2006
7- <i>epi</i> -PTX2 SA	>5000	MLD	Miles <i>et al.</i> , 2006

MLD=minimum lethal dose, LD₅₀=lethal dose – the dose required to kill half the members of a tested animal population, b.w.=body weight

Severe liver pathology was observed in mice 60 minutes after treatment 1 mg/kg b.w. *i.p.* of PTX1 (Terao *et al.*, 1986). Changes included multiple vacuoles of 15 µm in diameter in the periportal region and eosinophilic granules. The severity of the liver pathology was dose dependent. At lower doses, 150 and 200 µg/kg b.w., only minor changes were seen. In mice given PTX2 at 250 µg/kg b.w. major histopathological changes observed were splenic, renal and hepatic congestion (Munday 2008). Similar changes were seen in mice following PTX6 at 500 µg/kg b.w. *i.p.* (Ito *et al.*, 2008). Biochemical changes indicative of liver pathology in mice occurred at doses of 100-200 µg/kg b.w. *i.p.* and at the highest dose a decrease in liver weight was observed in (Yoon and Kim 1997; Munday 2008).

In suckling mice receiving doses of 0.15 to 1 mg/kg b.w. *i.p.* of PTX1 no diarrhoea was observed (Terao *et al.*, 1986). Neither did PTX2 nor PTX2 SA, 7-*epi*-PTX2 SA and PTX11 at 5 mg/kg b.w. cause diarrhoea in mature mice (Miles *et al.*, 2004, 2006; Suzuki *et al.*, 2006).

Repeated *i.p.* administration of 20 or 100 µg/kg b.w. of PTX2 (daily) in mice over a 1- or 2-week period did not cause deaths or changes in clinical chemistry indicative of liver or kidney toxicity, whilst at 200 µg/kg b.w. 50 % of the animals died (Yoon and Kim., 1997). Administration of PTX2 *i.p.* at 100 µg/kg b.w. for 20 consecutive days to nude mice inoculated with tumour cells was without effect on body weight (Chae *et al.*, 2005).

10.2.1.2. Toxicity following oral administration

Oral toxicity of PTX-group toxins studied generally appears to be much lower than toxicity following *i.p.* administration. An exception might be the study by Ogino *et al.* (1997) which found that as little as 25 µg/kg b.w. of PTX2 could be lethal in mice. However, this study using 4 or 5 mice per dose group showed no dose response as the lethality at 25, 100, 200, 300, and 400 µg/kg b.w. was 25, 0, 20, 40 and 25 %, respectively.

In a limited study in mice (n=5), single oral administration of PTX2 or PTX2 SA at doses up to 5000 µg/kg b.w. caused no overt signs of toxicity, including diarrhoea (Miles *et al.*, 2004). In conflict with these results Ishige *et al.* (1988) observed swollen intestine filled with fluid at an oral dose of 250 µg PTX2/kg b.w. in a single mouse tested, and diarrhoea was observed at

doses of 1000 (1/5), 2000 (1/1) and 2500 (1/1) µg/kg b.w. Starting from 250 µg PTX2/kg b.w. vacuole formation was observed in the epithelial cells of the small intestine. Hyaline droplet and vacuolar degeneration were observed in the liver at 1000 µg/kg b.w. and above (Ishige *et al.*, 1988).

Ito (2006) reported that in mice, single oral administration of PTX2 at doses of 400 µg/kg b.w. and above resulted in tissue injury with vacuole formation in epithelial cells and fluid accumulation in the small intestine. No effects were observed at 300 µg/kg b.w. In rats, PTX2 at the lowest doses tested caused intestinal fluid accumulation at 300 or 400 µg/kg b.w. when administered in 2 % lecithin water or in saline, respectively. Notably, no effect was observed when PTX2 or OA were administered separately to mice at a concentration of 300 µg/kg b.w. or 50 µg/kg b.w., respectively, whereas fluid accumulation was observed when the compounds were given together.

In a recent study mice and rats were given single oral doses of PTX6 by gavage (Ito *et al.*, 2008). In neither species diarrhoea was induced. In mice, doses up to 5000 µg/kg b.w. only caused a slight ultrastructural and transient injury at the small intestinal villus tops. Neither were other mouse organs affected. In contrast, in rats small-intestinal injury was observed at a single oral dose of 2000 µg/kg b.w. No other doses were investigated in rats. In neither species did PTX6 cause diarrhoea. In the same study PTX2 caused intestinal fluid secretion in mice and slight fluid secretion in rats at single doses of 500 and 1500 µg/kg b.w., respectively. In mice PTX6 (5000 µg/kg b.w.) did not increase the intestinal fluid secretion when given together with OA (50 µg/kg b.w.) or PTX2 (400 µg/kg b.w.).

PTX1 did not induce diarrhoea in suckling CD-1 mice following oral administration at doses up to 2 µg/mouse (equivalent to about 100 µg/kg b.w.) (Hamano *et al.*, 1985, 1986). No signs of toxicity were observed in mice given an oral dose of PTX11 at 5000 µg/kg b.w. (Suzuki *et al.*, 2006).

There are no data on possible effects of PTX-group toxins following repeated oral administration.

10.3. Relative potency of analogues

Data on relative toxicity of PTX-group toxins only exist following *i.p.* administration in mice and comprise limited information on minimum lethal doses (see Table 10, section 10.2.1.1.). Only for PTX2 and PTX 11 there are LD₅₀ values varying from 219 to 411 µg/kg. Overall, lethal doses for PTX1, PTX2, PTX3 and PTX11 are not distinguishable. Analogues PTX4 and PTX6 appear slightly less toxic, whereas PTX7, PTX8, PTX9, PTX2 SA and 7-epi-PTX2 SA are much less toxic.

In order to be prudent, the CONTAM Panel proposes a provisional toxicity equivalency factor (TEF) value of 1 to be used for PTX1, PTX2, PTX3, PTX4, PTX6 and PTX11, until more robust data become available. PTX7, PTX 8, PTX 9, PTX2 SA and 7-epi-PTX2 SA are much less toxic and were not assigned TEFs.

10.4. Chronic toxicity and carcinogenicity

No data on possible long-term effects of PTX-group toxins have been found. In an experimental nude mouse model, however, where subcutaneous injection of control HCT116 cells (human colon cancer cell line) and their p53-null counterparts led to growth of both kinds of cell implants, it has been shown that a daily *i.p.* injection of PTX2 (0.1 mg/kg b.w.)

for 20 consecutive days reduced the tumour mass composed of the p53-null cells but not of those expressing the functioning p53 protein (Chae *et al.*, 2005).

10.5. Genotoxicity

No data on genotoxicity have been identified.

11. Observations in humans

There are no reports of human illness causally associated with exposure to PTX-group toxins. PTX-group toxins are exclusively produced by *Dinophysis* spp. which also produce OA-group toxins, and therefore they always co-occur with OA toxins (FAO/IOC/WHO, 2004a). This makes it difficult to assess whether PTX-group toxins may contribute to human cases of diarrhetic shellfish poisoning (DSP).

While it had initially been suggested that PTX2 SA and 7-epi-PTX2 SA may have been responsible for outbreaks of human illness involving nausea, vomiting and diarrhea in Australia in 1997 and 2000 (Burgess and Shaw, 2001), the symptoms were later attributed to OA esters (FAO/IOC/WHO 2004b). Mussels implicated in a recent DSP outbreak in the UK contained PTX-group toxins as well as OA-group toxins. The intakes of OA-group toxins in this outbreak were similar to the lowest-observed-adverse-effect-levels (LOAELs) for OA-group toxins identified in other reports, suggesting that the PTX-group toxins made little, if any, contribution to the symptoms.

12. Hazard characterisation

Because of the lack of data relating to repeated oral administration of PTX-group toxins in animals or humans, it was not possible to establish a tolerable daily intake (TDI). In view of the potential for acute toxicity of PTX-group toxins, the CONTAM Panel decided to establish an ARfD. In assessing the acute oral toxicity of PTX-group toxins animal data have to be used as there are no quantitative data in humans. Although the oral data on adverse effects of PTX-group toxins were limited and partly conflicting, to be prudent the CONTAM Panel decided to take into consideration the intestinal toxicity of PTX2 observed in mice and rats at the LOAELs of 250 µg/kg b.w. (Ishige *et al.*, 1988) and 300 µg/kg b.w. (Ito, 2006), respectively. It is noted that PTX2 caused intestinal effects in rats at a single dose of 1500 µg/kg b.w. (Ito *et al.*, 2008). A single oral dose of PTX6 at 2000 µg/kg b.w. caused effects in rats (Ito *et al.*, 2008).

In its derivation of an ARfD, the CONTAM Panel decided to use the LOAEL for PTX2 in mice of 250 µg/kg b.w. Because the effects were mild and reversible, the CONTAM Panel decided to apply a factor of 3 for the extrapolation from a LOAEL to a no-observed-adverse-effect-level (NOAEL). The CONTAM Panel established an ARfD of 0.8 µg PTX2 equivalents/kg b.w. based on a LOAEL of 250 µg/kg b.w. and an overall uncertainty factor of 300.

13. Risk characterisation

Because PTX-group toxins have acute toxic effects, the CONTAM Panel concluded that the identification of a large portion size rather than a long term average consumption is of importance to assess the health risk of the consumers. It considered the 95th percentile as a

realistic estimate of the portion size for high consumers, and identified the figure of 400 g to be used in acute exposure assessments.

Consumption of a 400 g portion of shellfish meat containing PTX-group toxins at 160 µg/kg shellfish meat (by analogy with the current EU limit for lipophilic toxins of 160 µg okadaic acid equivalents/kg shellfish meat) would result in an intake of 64 µg toxin (equivalent to about 1 µg/kg b.w. in a 60 kg adult). This intake is slightly higher than the ARfD of 0.8 µg PTX2 equivalents/kg b.w. (48 µg PTX2 equivalents per portion for a 60 kg adult).

In order for a 60 kg adult to avoid exceeding the ARfD of 0.8 µg PTX2 equivalents/kg b.w., a 400 g portion of shellfish should not contain more than 48 µg PTX2 equivalents corresponding to 120 µg PTX2 equivalents/kg shellfish meat.

As explained in chapter 6, the CONTAM Panel assumed that all shellfish samples showing a negative response in MBAs will reach the market and will thus be consumed. Therefore, the concentration data derived by LC-MS/MS for these samples (Table 6) could be used to estimate the dietary intake of PTX-group toxins.

Consumption of a 400 g portion of shellfish meat containing 79 µg PTX2/kg shellfish meat corresponding to the 95th percentile of the concentration (see Table 6) would result in an intake of 32 µg PTX2 (equivalent to approximately 0.5 µg/kg b.w. in a 60 kg adult). This intake is below the ARfD of 0.8 µg PTX2 equivalents/kg b.w. However, because the data were only for PTX2 and not for other toxicologically relevant PTX-group toxins, it is unclear whether there is a risk to the consumer of shellfish containing the current levels of PTX-group toxins.

From the probabilistic exposure estimate as presented in Figure 4 (Chapter 8) based on the distributions of both the concentration and the consumption data, it can be estimated that there is a small chance (approximately 0.2 %) to exceed the ARfD of 0.8 µg PTX2 equivalents/kg b.w. (48 µg PTX2 equivalents/person for a 60 kg adult), when consuming shellfish containing levels of PTX-group toxins that could be present in shellfish currently available on the European market.

Using the distribution of the concentration data for PTX2 presented in Table 6, the CONTAM Panel estimated that a 60 kg person consuming a portion of 400 g of shellfish meat has a chance of 0.8 % to exceed the ARfD of 0.8 µg PTX2 equivalents/kg b.w.

Because insufficient data for other relevant PTX-group toxins have been reported, the CONTAM Panel could only base its risk characterisation of PTX-group toxins on the exposure to PTX2. At the moment it is unclear however, to what extent this might have led to underestimation of the dietary exposure to PTX-group toxins.

14. Uncertainty

The evaluation of the inherent uncertainties in the assessment of exposure to PTX-group toxins has been performed following the guidance of the Opinion of the Scientific Committee related to Uncertainties in Dietary Exposure Assessment (EFSA, 2006). In addition, the report on “Characterizing and Communicating Uncertainty in Exposure Assessment” has been considered (WHO/IPCS, 2008). According to the guidance provided by the EFSA opinion (2006) the following sources of uncertainties have been considered: Assessment objectives, exposure scenario, exposure model, and model input (parameters).

14.1. Assessment objectives

The objectives of the assessment were clearly specified in the terms of reference and the CONTAM Panel prepared a risk assessment including the derivation of an ARfD, description of the different detection methods, and an exposure assessment for the current situation. The uncertainty of the assessment objectives is considered to be negligible.

14.2. Exposure scenario

The estimate of exposure is based on occurrence data reported from only four European Countries and for PTX2 only. Uncertainty possibly introduced by non-consideration of cooking for quantitative exposure assessment is considered to be negligible as an impact for final conclusions, because these toxins are heat stable. As the majority of the occurrence data are derived from raw shellfish and cooking leads to increased concentrations, the exposure might be somewhat underestimated. On the other hand, since it is unclear whether the consumption data relates to cooked or uncooked shellfish, taking the portion size as uncooked may lead to the overestimation of the exposure.

14.3. Exposure model

The high numbers of samples having levels below LOD may introduce uncertainties to the overall estimate. These uncertainties are considered to be negligible, as they do not have a major influence on the risk characterization.

Uncertainty is also caused by the fact that exposure was based on occurrence data from non representative pre-market and post-market control samples. These samples may not reflect the “real” range of occurrence of PTX-group toxins in shellfish on the market.

14.4. Model input (parameters)

Appropriate calibration standards for PTX-group toxins are currently only available for PTX2. The number of PTX-group toxins measured in the five countries that reported results differ from one to five analogues. As PTX2 was the only analogue that was reported by all five countries, the CONTAM Panel decided to use this analogue for exposure assessment. As some other toxicologically relevant analogues might also occasionally be present, the consideration of only PTX2 may lead to an underestimation of exposure to PTX-group toxins.

The oral toxicity is not well defined and the most sensitive data on toxicity in mice were used. Since other studies showed toxicity only at higher doses and not at doses comparable to the most sensitive data, it is assumed that the ARfD is conservative.

14.5. Summary of uncertainties

In Table 11 a summary of the uncertainty evaluation is presented, highlighting the main sources of uncertainty and indicating an estimate of whether the respective source of uncertainty might have led to an over- or underestimation of the exposure or the resulting risk.

Table 11. Summary of qualitative evaluation of the impact of uncertainties on the risk assessment of the dietary exposure of PTX-group toxins.

Sources of uncertainty	Direction
Uncertainty in analytical results because of different number of analogues measured and lack of certified calibrants	+/- ^(a)
Extrapolation of occurrence data only from five European countries to Europe as a whole	+/-
Incomplete database for shellfish consumption in Europe; data only from limited number of European countries and limited data on shellfish species other than mussels	+
Influence of non-detects on exposure estimate	+
Consideration of shellfish sampled for pre-market control for systematic dietary estimation of exposure	+
Dietary exposure estimates were only based on PTX2 and, due to lack of data, did not include other relevant PTX analogues	-
Uncertainties in the data used for establishing the ARfD	+

(a) + = uncertainty with potential to cause over-estimation of exposure/risk
 - = uncertainty with potential to cause under-estimation of exposure/risk

The CONTAM Panel considered the impact of the uncertainties on the risk assessment of exposure to PTX-group toxins from shellfish consumption and concluded that its overall assessment of the acute risk is likely to be conservative- i.e. more likely to over- than to underestimate the risk.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Hazard identification and characterisation

- Pectenotoxin (PTX)-group toxins are heat-stable polyether macrolide compounds, isolated from various species of shellfish and from dinoflagellates of the genus *Dinophysis*. To date, 15 PTX-group toxins have been isolated and characterised.
- In animals PTX1, PTX2, PTX3, PTX4, PTX6 and PTX11 are acutely toxic following intraperitoneal (*i.p.*) administration, but systemic absorption of PTX-group toxins appears to be low following oral administration and reported toxicity is mainly restricted to the intestinal tract.
- Based on the similar *i.p.* toxicity of PTX1, PTX2, PTX3, PTX4, PTX6 and PTX11, the Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) assigned a toxicity equivalency factor (TEF) of 1 to these analogues. PTX7, PTX 8, PTX 9, PTX2 SA and 7-epi-PTX2 SA are much less toxic and were not assigned TEFs.
- There are no data on adverse effects of PTX-group toxins in humans.
- There are no data on chronic effects of PTX-group toxins in animals and therefore no tolerable daily intake can be established.

- Although the oral toxicity is not well defined the CONTAM Panel considered it appropriate to establish an acute reference dose (ARfD) on the basis of a lowest-observed-adverse-effect-level (LOAEL) of 250 µg/kg body weight (b.w.) for intestinal toxicity of PTX2 observed in mice.
- Because the effects were mild and reversible the CONTAM Panel decided to apply a factor of 3 for the extrapolation from a LOAEL to a no-observed-adverse-effect level (NOAEL). Together with a default factor of 100 for intra- and inter-species differences, the overall uncertainty factor of 300 resulted in an ARfD of 0.8 µg PTX2 equivalents/kg b.w (equivalent to 48 µg PTX2 equivalents for a 60 kg adult).

Occurrence/Exposure

- PTX2 is frequently detected in shellfish and also sometimes PTX2 SA is found when shellfish is contaminated by *Dinophysis* spp.
- Depending on shellfish species and geographical location other analogues (e.g. PTX1, PTX3, PTX6 and PTX11) may also be relevant, but due to the lack of data the CONTAM Panel was not able to evaluate their importance.
- There is a lack of representative data regarding the contamination of shellfish in the different Member States particularly the geographical location and species of shellfish.
- The available occurrence data for PTX-group toxins in shellfish are from non-representative pre-market and post-market control samples submitted only from five European countries. The number of PTX-group toxin analogues reported by these countries differed from one to five. As PTX2 was the only analogue reported by all five countries, the CONTAM Panel decided to use this analogue for exposure assessment.
- Consumption data for shellfish are only available for a few Member States. These data seldomly distinguish between shellfish species nor the type of processing. In addition, different study designs were used in the collection of consumption data.
- From the available data, the CONTAM Panel identified the figure of 400 g as a large portion size to be used in acute exposure assessments.

Risk characterisation

- Consumption of a 400 g portion of shellfish meat containing PTX-group toxins at the current regulatory limit would result in an intake of 64 µg toxin (equivalent to about 1 µg/kg b.w. for a 60 kg adult). This intake is marginally higher than the ARfD of 0.8 µg PTX2 equivalents/kg b.w. (equivalent to 48 µg PTX2 equivalents for a 60 kg adult).
- Based on current consumption and occurrence data for PTX2 there is a small chance (approximately 0.2 %) to exceed the ARfD of 0.8 µg PTX2 equivalents/kg b.w. (equivalent to 48 µg PTX2 equivalents for a 60 kg adult) when consuming shellfish currently available on the European market.
- In order for a 60 kg adult to avoid exceeding the ARfD of 0.8 µg PTX2 equivalents/kg b.w., a 400 g portion of shellfish should not contain more than 48 µg PTX2 equivalents corresponding to 120 µg PTX2 equivalents/kg shellfish meat.

Methods of analysis

- The mouse bioassay (MBA) and the rat bioassay (RBA) are the officially prescribed reference methods in the European Union for the determination of lipophilic toxins, which include PTX-group toxins.
- The MBA has shortcomings and is not considered an appropriate tool for control purposes because of the inherent variability in results and insufficient detection capability. It is not selective for the PTX-group toxins and thus may give false positive results.
- The RBA detects compounds with diarrhetic effects such as okadaic acid (OA)-group toxins, but not PTX-group toxins because they do not have diarrhetic properties. Therefore the RBA is not suitable to detect PTX-group toxins.
- The methods based on liquid chromatography-mass spectrometry, especially in tandem mass spectrometry mode, have the greatest potential to replace the mammalian assays and to detect levels of PTX-group toxins below the current regulatory level.
- Neither the mammalian assays, nor the physico-chemical alternative methods, have been formally validated in interlaboratory studies, following internationally agreed protocols.
- PTX-group toxins are heat stable. There is no information on the effects of processing on the levels of PTX-group toxins in shellfish. As for other lipophilic toxins water loss during processing may lead to an increase in concentration of PTX-group toxins in shellfish flesh.

RECOMMENDATIONS (INCL. KNOWLEDGE/DATA GAPS)

Hazard identification and characterisation

- Further information on the toxicokinetics, oral toxicity and relative potency of individual PTX-group toxins is needed.
- Clarification of the mode of action of PTX-group toxins is needed, including the possible effects on mitotic spindle integrity.
- Information is needed on the oral toxicity of PTX-group toxins when combined with other lipophilic toxins that often co-occur in contaminated shellfish, such as OA-group toxins, azaspiracids and yessotoxins. Bulk amounts (mg to g) of well characterised and purified PTX-group toxins are required for this purpose.
- Because PTX-group toxins do not share the same mechanism of action as OA-group toxins they should not be included in the regulatory limit for OA-group toxins.

Occurrence/Exposure

- Extended information on occurrence of different PTX analogues in shellfish is needed.
- Information on the effects of shellfish processing (e.g. storage, cooking, freezing) on toxin levels is needed.

- The database on shellfish consumption should be extended including data on portion size, frequency and different types of shellfish.

Methods of analysis

- Further reference calibrants at least for those analogues for which TEFs have been proposed (PTX1, PTX3, PTX4, PTX6 and PTX11) and certified tissue reference materials for PTX-group toxins are required to quantitate these analogues in order to evaluate the risk posed by their occurrence and to make enforcement of regulations possible.
- It should be investigated if reference methods can be based on performance criteria, thereby allowing the use of several methods rather than a single specific method. The feasibility of the Single Laboratory Validation concept should be further explored.
- Rapid and cost effective screening methods should be developed and validated to reliably detect PTX-group toxins at the level of interest.

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ABBREVIATIONS

APHA	American Public Health Association
ARfD	Acute reference dose
ASP	Amnesic Shellfish Poisoning
AZA	Azaspiracid
AZP	Azaspiracid Shellfish Poisoning
BTX	Brevetoxins
b.w.	Body weight
CCMAS	Committee on Methods of Analysis and Sampling
CCFFP	Codex Committee for Fish and Fishery Products
CONTAM	Panel on Contaminants in the Food chain
CRL	Central Reference Laboratory
CRL-MB	Reference Laboratory for marine biotoxins
CTX	Ciguatoxins
DA	Domoic acid
DSP	Diarrhetic Shellfish Poisoning
DTX	Dinophysis toxin
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
EU	European Union
HP	Hepatopancreas
HPLC	High-performance liquid chromatography
<i>i.p.</i>	Intraperitoneal
ISO/IUPAC/AOAC	International Organization for Standardization/ International Union of Pure and Applied Chemistry/Association of Analytical Communities
JNK	c-Jun N-terminal kinase
LB	lower bound
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
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
MLD	Minimum lethal dose
NF-κB	Nuclear factor kappa B
NOAEL	No-observed-adverse-effect level
NRCC	National Research Council Canada
NRL	National Reference Laboratory
OA	Okadaic acid
P95	95 th percentile
PITX	Palytoxins
Post-MC	Post-market control
PreMC	Pre-market control

PSP	Paralytic shellfish poisoning
PTX	Pectenotoxin
RBA	Rat bioassay
RSD	Relative standard deviation
SA	Seco acid
SLV	Single laboratory validation
SM	Shellfish meat
SOP	Standard operating procedure
SPE	Solid phase extraction
STX	Saxitoxin
TDI	Tolerable daily intake
TEF	Toxicity equivalency factor
UB	Upper bound
U.K.	United Kingdom
UV	Ultra violet
WG	Working group
WHO	World Health Organization
YTX	Yessotoxin