SCIENTIFIC OPINION

Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group[1]

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

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ABSTRACT

The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) assessed the risks to human health related to the consumption of ciguatoxin (CTX)-group toxins in fish. CTX-group toxins occur in fish as a result of biotransformation of precursor gambiertoxins produced by the benthic dinoflagellate *Gambierdiscus toxicus*. CTX-group toxins cause ciguatera fish poisoning. They are mainly found in Pacific, Caribbean and Indian Ocean regions and are classified as Pacific (P), Caribbean (C) and Indian Ocean (I) CTX-group toxins. Recently CTX-group toxins were identified for the first time in fish in Europe. Currently there are no regulatory limits for CTX-group toxins in fish in Europe, but the regulation requires that no fish products containing CTX-group toxins are placed on the market. The toxicological database for CTX-group toxins is limited, comprising mostly acute toxicity studies. In view of the acute toxicity of CTX-group toxins the CONTAM Panel considered establishing an acute reference dose (ARfD). However, due to the very limited quantitative data both in experimental animals as well as related to human intoxications, the CONTAM Panel concluded that the establishment of an oral ARfD was not possible. Based on case reports on human intoxications it appears that a concentration of 0.01 µg P-CTX-1 equivalents/kg fish is expected not to exert effects in sensitive individuals when consuming a single fish meal. The mouse bioassay (MBA) has been widely used to detect CTX-group toxins. However, due to insufficient detection capability and ethical concerns the MBA is not considered an appropriate method. *In vitro* (cytotoxicity and receptor binding) assays have been developed as alternative, but they need further development. Liquid chromatography-tandem mass spectrometry methods can be of value for the quantification of CTX-group toxins, but certified reference standards and reference materials need to be provided to allow method development and (inter-laboratory) validation.

KEY WORDS

Marine biotoxins, emerging toxins, ciguatoxin (CTX)-group toxins, fish, methods of analysis, human health, risk assessment.

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SUMMARY

Ciguatoxin (CTX)-group toxins are marine biotoxins which occur in fish as a result of biotransformation of precursor gambiertoxins produced by the benthic dinoflagellate *Gambierdiscus toxicus*. They are mainly found in Pacific, Caribbean and Indian Ocean region and they are classified as Pacific (P), Caribbean (C) and Indian Ocean (I) CTX-group toxins. Recently CTX-group toxins were identified for the first time in fish caught in Europe.

CTX-group toxins cause ciguatera fish poisoning (CFP). This is a complex syndrome characterised by a wide variety of symptoms and signs such as gastrointestinal (e.g. vomiting, diarrhoea, nausea), neurological (e.g. tingling, itching) and cardiovascular (e.g. hypotension, bradycardia) effects. In severe cases the symptoms may begin as soon as 30 minutes after ingestion of contaminated fish, while in milder cases they may be delayed for 24 to 48 hours. Fatalities may occur due to cardio-respiratory failure. At present, CFP is the most common type of marine biotoxin food poisoning worldwide with an estimated number of 10 000 to 50 000 people suffering from the disease annually. CFP is primarily associated with the consumption of large predator fish that have accumulated CTX-group toxins by feeding on smaller contaminated coral reef fish.

Although also other toxins such as gambiertoxin and maitotoxin have been isolated from *G. toxicus* and have been associated with CFP, this opinion only deals with CTX-group toxins because the other toxins have a different mode of action.

CTX-group toxins are lipid-soluble polyether compounds. Chemical structures of more than 20 analogues of P-CTX-group toxins have been identified. For two C-CTX-group toxins (C-CTX-1 and C-CTX-2) chemical structures have been characterised and several analogues have been identified. Only four closely related I-CTX-group toxins have been identified.

Currently there are no regulatory limits for CTX-group toxins in the European Union (EU), but the EU regulation states that checks are to take place to ensure that fishery products containing biotoxins such as ciguatoxin are not placed on the market.

The toxicological database for CTX-group toxins is limited and comprises mostly studies on their acute toxity following intraperitoneal (*i.p.*) administration. Based on the available information it can be concluded that binding of CTX-group toxins to voltage-gated sodium channels and the consequent disturbance of ion conductance through these channels is the major molecular mechanism of action of CTX-group toxins on nerves and muscle fibres.

Until better information is available the Panel on contaminants in the food chain (CONTAM Panel) adopted the following toxicity equivalency factors (TEFs) for CTX-group toxins based on their acute *i.p.* LD_{50} in mice: P-CTX-1 = 1, P-CTX-2 = 0.3, P-CTX-3 = 0.3, P-CTX-3C = 0.2, 2,3-dihydroxy P-CTX-3C = 0.1, 51-hydroxy P-CTX-3C = 1, P-CTX-4A = 0.1, P-CTX-4B = 0.05, C-CTX-1 = 0.1 and C-CTX-2 = 0.3. These TEFs should be applied to express individual analogues identified with quantitative detection methods as P-CTX-1 equivalents.

There are no long term studies in experimental animals that would allow establishing a tolerable daily intake (TDI). In view of the acute toxicity of CTX-group toxins the CONTAM Panel considered establishing an acute reference dose (ARfD). However, due to the very limited quantitative data both in experimental animals as well as related to human intoxications, the CONTAM Panel concluded that the establishment of an oral ARfD was not possible. In addition, it concluded that an ARfD may also not be adequately protective to humans exposed several times to CTX-group toxins even when incidents occurred months apart.

The CONTAM Panel noted that a number of publications state that cases of CFP in the Pacific mostly occur following the consumption of fish containing the equivalent of 0.1-5 µg P-CTX-1/kg of fish flesh. In line with the approach of FAO (2004), the CONTAM Panel applied an uncertainty factor of

10 to the lowest concentration 0.1 µg equivalents of P-CTX-1/kg in fish associated with mild symptoms to indicate a concentration of 0.01 µg equivalents of P-CTX-1/kg of fish, which is expected not to exert effects in sensitive individuals. This concentration should be taken as 0.01 µg P-CTX-1 equivalents/kg fish, to cover all CTX-group toxins that could be present in fish.

Because of the very limited occurrence data, the CONTAM Panel could not comment on the risk associated with the exposure to CTX-group toxins in fish that could reach the European market.

The mouse bioassay (MBA) has been widely used to detect CTX-group toxins in fish, but for reasons of animal welfare there is a growing concern with respect to its use. Due to its poor specificity and insufficient detection capability the CONTAM Panel considered it as not an appropriate detection method for CTX-group toxins. Alternative assays such as *in vitro* (cytotoxicity and receptor binding) assays provide sufficient detection capability and they can detect all active analogues. Although they also do not provide information on toxin profiles, they could be further developed to be applied as screening methods for CTX-group toxins. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods allow specific detection of individual analogues of P-, C- and I-CTX-group toxins and they would be of value for their quantification in fish extracts. None of the current methods of analysis to determine CTX-group toxins in fish has been formally validated. The CONTAM Panel noted that certified reference standards and reference materials for CTX-group toxins need to be provided to allow method development and (inter-laboratory) validation.

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

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

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

1. Legal framework

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

2. The Council Directive 86/609/EEC

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

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

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 EC and Member States are also required (Article 23, 1) to encourage research into, and the development and validation of, alternative methods that do not require animals, use fewer animals, or further reduce the suffering that may be caused, whilst providing the same level of scientific information.

3. Recognised testing methods for marine biotoxins and maximum levels

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

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

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

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

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

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

- 160 micrograms of azaspiracid equivalents per kilogram for azaspiracids.

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

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

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

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

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

Table 1: Summary data used in the derivation of the acaute reference dose (ARfD) and current guidance levels.

Group toxin	LOAEL(1) NOAEL(2) µg/kg body weight	Safety Factor (Human data (H) Animal data (A))	Provisional ARfD[10]	Derived Guidance Level/ Maximum 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 body weight (b.w.)

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

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

[11] A Type II method is the one designated Reference Method where Type I methods do not apply. It should be selected from Type III methods (as defined below). It should be recommended for use in cases of dispute and for calibration purposes.
[12] A Type III Method is one which meets the criteria required by the Codex Committee on Methods of Analysis and Sampling for methods that may be used for control, inspection or regulatory purposes.

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

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

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

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

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

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

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

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

ASSESSMENT

1. Introduction

Ciguatoxin (CTX)-group toxins are marine biotoxins which occur in fish as a result of biotransformation of precursor gambiertoxins produced by the benthic dinoflagellate *Gambierdiscus toxicus* (Murata et al., 1989; Murata et al., 1990; Lehane and Lewis, 2000; Lehane, 2000). They are mainly found in Pacific, Caribbean and Indian Ocean regions and they are classified as Pacific (P), Caribbean (C) and Indian Ocean (I) CTX-group toxins. Recently CTX-group toxins were identified for the first time in fish caught in Europe.

CTX-group toxins cause ciguatera fish poisoning (CFP) which is a complex syndrome characterised by a wide variety of symptoms and signs such as gastrointestinal, neurological and cardiovascular effects. Fatalities may occur due to cardio-respiratory failure. At present, CFP is the most common type of marine biotoxin food poisoning worldwide with an estimated number of 10 000 to 50 000 people suffering from the disease annually (De Fouw et al., 2001; Lehane, 2000). CFP is primarily associated with the consumption of large predator fish that have accumulated CTX-group toxins by feeding on smaller contaminated coral reef fish. Although in addition to gambiertoxin also other toxins such as maitotoxin have been isolated from *G. toxicus* and have been associated with CFP, this opinion only deals with CTX-group toxins because the other toxins have a different mode of action.

2. Chemical characteristics

CTX-group toxins are lipid-soluble polyether compounds consisting of 13-14 rings fused by ether linkages into a rigid ladder-like structure (Figure 1). They are odourless and tasteless. CTX-group toxins are relatively heat-stable molecules that remain toxic after cooking and freezing, and exposure to mild acidic and basic conditions (Lange 1994; FAO, 2004). The main CTX-group toxins in Pacific areas, P-CTX-1, P-CTX-2 and P-CTX-3, are present in fish in different relative amounts (Lewis et al., 1991; Lewis, 2001; Lehane and Lewis, 2000). The chemical structures of more than 20 P-CTX analogues, such as P-CTX-3C (Figure 1) (Murata et al. 1990; Lewis et al., 1991; Satake et al., 1993a,b, 1997,1998; Lehane and Lewis, 2000) have been identified. Structural modifications are mainly seen in both termini of the toxin molecules and mostly by oxidation (Naoki et al., 2001; Yasumoto et al., 2000). 52-epi P-CTX-3 (= P-CTX-2) and 52-epi P-CTX-4B (= P-CTX-4A) are energetically less favoured epimers of P-CTX-3 and P-CTX-4B, respectively. 2,3-Dihydroxy P-CTX-3C and 51-hydroxy P-CTX-3C have also been isolated from Pacific fish. Molecular masses of the P-CTX-group toxins are reported in Table 2.

The chemical structures of two Caribbean CTX-group toxins (C-CTX-1 and C-CTX-2) were revealed in 1998 (Lewis et al., 1998). Pottier et al. (2002a) later identified 10 C-CTX analogues or isomers. 56-epi C-CTX-1 (= C-CTX-2) is an energetically less favoured epimer of C-CTX-1 (Lewis, 2001) (Figure 1). C-CTX-1 is less polar than P-CTX-1 (Vernoux and Lewis, 1997). C-CTX-1 is the major CTX-group toxin found in horse-eye jack (*Caranx latus*) (Vernoux and Lewis, 1997). Molecular masses of the C-CTX-1 and C-CTX-2 are reported in Table 2.

The isolation and initial characterisation of Indian Ocean CTX (I-CTX) from reef fish was first reported by Hamilton et al. (2002a). Later the authors clarified that instead of one I-CTX, actually four closely related Indian CTX-group toxins (I-CTX-1, I-CTX-2, I-CTX-3 and I-CTX-4) were identified, I-CTX-1 and I-CTX-2 being the major ones and I-CTX-3 and I-CTX-4 being the minor I-CTX-group toxins (Hamilton et al., 2002b). It was found that the I-CTX-1 and I-CTX-2 have the same molecular mass as C-CTX-1 suggesting that these compounds have closely related chemical structures (Hamilton et al., 2002b). I-CTX-group toxins differ from P-CTX-group toxins (FAO, 2004). However, chemical

structures have not been reported for I-CTX-group toxins. Molecular masses of the I-CTX-group toxins are reported in Table 2.

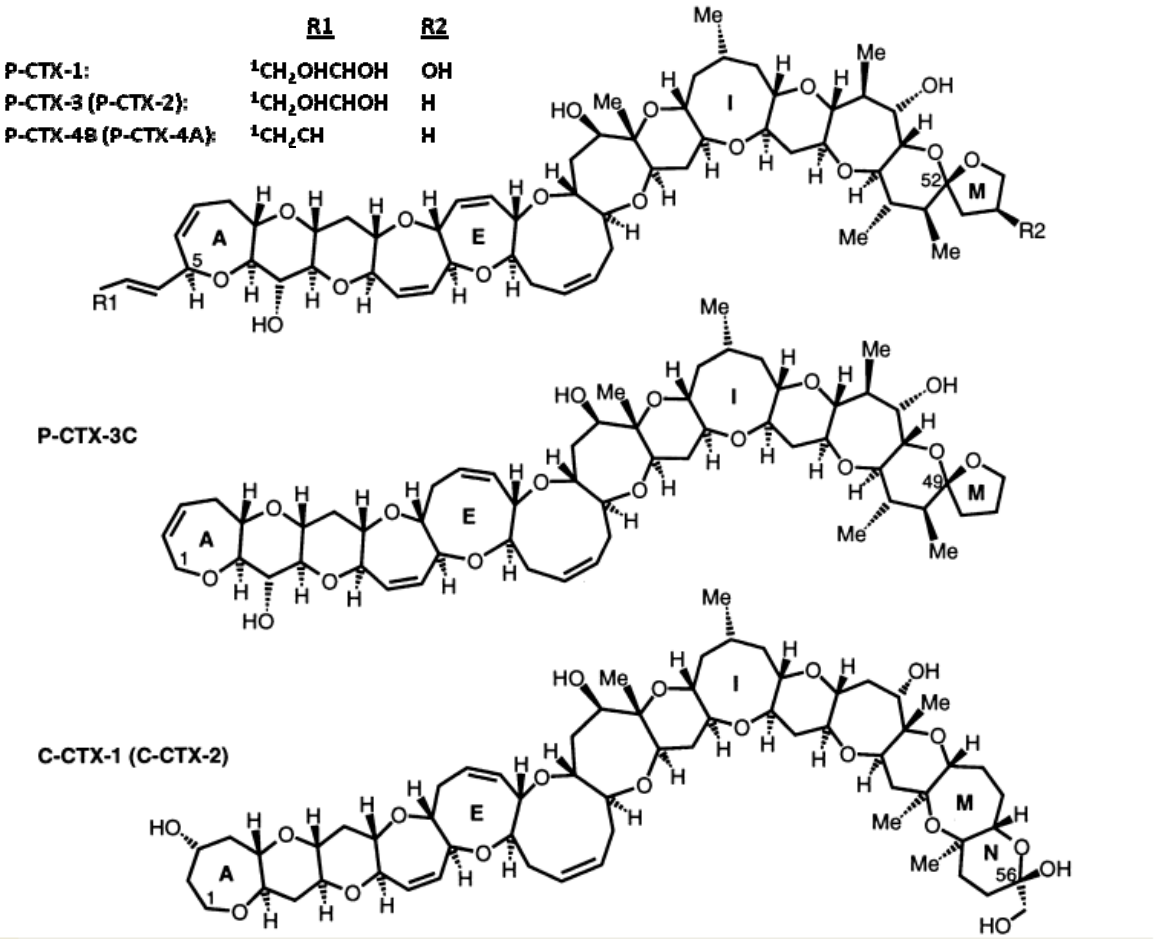

Figure 1: Structures of Pacific (P) and Caribbean (C) CTX-group toxins (modified from Lewis, 2001). The energetically less favoured epimers of P-CTX-3, P-CTX-4B and C-CTX-1 are stereoisomers at C52, C52 and C56, respectively (in brackets). Structures of Indian Ocean CTX-group toxins have not been reported.

Table 2: Molecular masses of CTX-group toxins (revised from Lewis (2006)).

CTX-group toxin	Molecular weight/Da	Reference
P-CTX-1	1110	Murata et al. (1990); Lewis et al. (1991)
P-CTX-2[(a)]	1094	Lewis et al. (1991)
P-CTX-3	1094	Lewis et al. (1991)
P-CTX-3C	1022	Satake et al. (1993b)
P-CTX-4A[(b),(c)]	1060	Satake et al. (1997)
P-CTX-4B[(c)]	1060	Murata et al. (1990)
2,3-Dihydroxy P-CTX-3C	1056	Satake et al. (1998)
51-Hydroxy P-CTX-3C	1038	Satake et al. (1998)
C-CTX-1	1140	Vernoux and Lewis (1997)
C-CTX-2[(d)]	1140	Vernoux and Lewis (1997)
I-CTX-1[(e)]	1140	Hamilton et al. (2002a)
I-CTX-2[(e)]	1140	Hamilton et al. (2002b)
I-CTX-3[(f)]	1156	Hamilton et al. (2002b)
I-CTX-4[(f)]	1156	Hamilton et al. (2002b)

(a): stereoisomer of P-CTX-3; (b): stereoisomer of P-CTX-4B; (c): formerly known as gambiertoxins (GTX-4A and GTX-4B) (Nicholson and Lewis, 2006); (d): stereoisomer of C-CTX-1; (e): possibly epimers (Hamilton et al., 2002b), (f): possibly epimers (Hamilton et al., 2002b).

3. Regulatory status

For the control of CTX-group toxins in the European Union (EU), Commission Regulation (EC) No. 854/2004[13] provides details in Annex III: "Fishery products", chapter II: "Official controls of fishery products". This chapter states: "Checks are to take place to ensure that the following fishery products are not placed on the market: fishery products containing biotoxins such as Ciguatera or other toxins dangerous to human health". Limits are not stated and no specific details or requirements about the analytical methodology to be used are given.

In other parts of the world some countries have regulations or management guidelines for CTX-group toxins in fish. For example the United States Food and Drug Administration (US FDA) has proposed guidance levels of ≤0.1 µg/kg C-CTX-1 equivalents and ≤0.01 µg/kg P-CTX-1 equivalents for the 4th edition of the U.S. Food and Drug Administration (US FDA) Fish and Fishery Products Hazards and Controls Guidance (CDC, 2009). At the time of writing the guidance is under the official FDA review. In Australia for domestic management of CTX-group toxins, the Food Standards Australia New Zealand (FSANZ) publication "Safe seafood Australia" provides guidelines on the susceptible fish species and local areas where fish may be toxic (FSANZ, 2006). For export, Australian Export Control (Fish and Fish Products) Orders (Australia export control orders, 2005) require that "All practical measures for harvesting fish of a species that can be affected by ciguatoxin (being measures that are necessary to minimise the risk of the harvest and preparation of fish and fish products for export of food being affected by ciguatoxin) must be taken. Practical measures could for example include controls on fish size and location of harvest." However, there are no specific regulations for CTX-group toxins in fish in either Australia or New Zealand. Japan has a ban on domestic sales of barracuda fish (MHWL, 1953) and several other fish species that are associated with CFP are either banned fully from importation to Japan or are conditionally permitted for importation to Japan (MHWL, 2001).

[13] OJ L 155, 30.4.2004, pp 206-320.

4. Methods of analysis

Several methods are available for the determination of CTX-group toxins: the most important ones being the mouse bioassay (MBA), biomolecular methods and chemical methods.

4.1. Supply of appropriate reference material

The analysis of fish for CTX-group toxins is complicated by the fact that suitable certified reference calibrants and materials are not readily available.

The CTX-group toxins isolated in the Pacific, the Caribbean and the Indian Ocean all differ slightly and therefore caution should be taken in using reference materials or tests developed from another region (WHO/SEARO, 2006).

4.2. Mouse bioassay

The MBA based on the method described by Banner et al. (1960) is presently the most widely used assay for the detection of CTX-group toxins in fish. The assay is described by Yasumoto et al. (1971, 1984) and it has been extensively used in the Pacific area (Juranovic and Park, 1991). This assay has been described for the detection of CTX-group toxins in the flesh of fish. A diethyl ether extract containing CTX-group toxins is suspended in 0.5 mL 1-5 % Tween 60/0.9 % saline solution and injected intraperitoneally (*i.p.*) into mice (20 ± 2 g) of either sex. Mice are observed continuously for the first two hours, after which checks are performed regularly until 24 hours after dosing. The limit of quantification (LOQ) is 0.5 nmol/kg (approximately 0.56 µg/kg) for P-CTX-1 (Lewis and Sellin, 1993). The relationship between dose and time to death is used for quantification and total lethality is expressed in mouse units (MU).[14] For the mixture of CTX-group toxins found in carnivorous fish this relationship is approximated by log MU = 2.3 log ($1 + T^{-1}$), where MU is the number of MUs of CTX-group toxins injected and T is time to death in hours (Lewis et al., 1991; Lewis and Sellin, 1992). For P-CTX-1, P-CTX-2, P-CTX-3, P-CTX-3C and P-CTX-4B the MU equivalencies have been reported as 5, 9, 18, 26 and 80 MU (ng), respectively (Guzmán-Pérez and Park, 2000). Median lethal *i.p.* doses have been reported as 0.25 µg/kg b.w. for P-CTX-1 (Lewis et al., 1991) and 3.7 µg/kg b.w. for C-CTX-1 (Dickey, 2008) indicating limits of detection (LODs) of 0.2 and 3.0 µg/kg shellfish, respectively. Modified extraction procedures have been developed to reduce interference from maitotoxins (Yokayama et al., 1988; Holmes et al., 1991; Legrand et al., 1992; Holmes and Lewis, 1994).

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:

- no specific information is provided on individual toxins;
- it is not sensitive enough to detect relevant levels of CTX-group toxins;
- it cannot be automated;
- it requires specialised animal facilities and expertise;

[14] One mouse unit (MU) is the LD_{50} for a 20 g mouse, which is equivalent to 5 ng P-CTX-1 (Lewis et al., 1991).

- the inherent variability in results between laboratories due to e.g. specific animal characteristics (strain, sex, age, weight, general state of health, diet, stress);
- it has not been validated;
- in many countries the use of the MBA is considered undesirable for ethical reasons.

4.3. Biomolecular methods

There are three different types of biomolecular methods for CTX-group toxins: cytotoxicity assays, receptor-binding assays and immuno assays.

4.3.1. Cytotoxicity assays

Cytotoxicity assays for the detection of CTX-group toxins in fish tissues are based on the capacity of the toxins to bind to sodium channels, causing them to open at normal cell resting membrane potentials. This results in an influx of sodium ions, cell depolarisation and the appearance of spontaneous action potentials in excitable cells. This sodium influx can be enhanced by the addition of sodium channel activator toxins through an allosteric mechanism. The reported cell based assay for the CTX-group toxins (Manger et al., 1993, 1994, 1995) takes advantage of this phenomenon to produce an assay that is highly sensitive to CTX-group toxins and other sodium channel activator toxins. The LOQ of this assay for CTX-group toxins is at pg/kg shellfish level.

A fluorimetric method developed in 2001 for saxitoxin (STX)-group toxins (Louzao et al., 2001) was later adapted to detect CTX-group toxins (Louzao et al., 2004). This method is based on the capability of the fluorescent dye bis-oxonol to detect changes in membrane potential in excitable cells. Cell viability was measured by using 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT assay) or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium in the presence of phenazine methosulfate (PMS) (MTS assay), where the absorbance of the reduction product formazan is measured (Manger et al., 1995; Dechraoui et al., 1999; Bottein Dechraoui et al., 2005a, 2007). Manger et al. (1995) reported that results obtained from the assay using neuroblastoma cells to detect CTX contaminated finfish extracts correlated with results obtained from MBA. Bottein Dechraoui et al. (2005a) reported a LOQ of 0.039 µg/kg C-CTX-1 in barracuda fish tissue. Such a protocol was adopted in the US FDA and National Oceanic Atmospheric Administration laboratories (NOAA) for in vitro assay of CTX-group toxins in fish tissues (Dickey, 2008). The MBA (used 1990-1994) was replaced by the in vitro cell assay (used from 1994). It now coexists with liquid chromatography-mass spectrometry (LC-MS), which is used as a confirmatory method for positive sodium channel assays (Dickey, 2008).

The main advantages of the cytotoxicity assays are:

- they can be automated;
- they are simple;
- they are adequately sensitive to detect levels relevant to lowest observed effect levels.

The main disadvantages of the cytotoxicity assays are:

- in their current format they are unlikely to be cost-effective for routine screening of individual fish;
- they do not provide any information on the toxin profile;
- they have not been validated.

4.3.2. Receptor-binding assays

Lombet et al. (1987), Legrand and Lotte (1994), Dechraoui et al. (1999) and Bottein Dechraoui et al. (2005a) have used a receptor-binding assay to detect and quantify CTX-group toxins in fish. The assay measures the inhibition of the binding of [^{3}H]-brevetoxin-3 to sodium-channels in rat brain synaptosomes in the presence of CTX-group toxins, using a rapid filtration method with glass fiber filters. Bottein Dechraoui et al. (2005a) reported that for C-CTX-1, the receptor binding assay is 12 times less sensitive than the cytotoxicity assay. For radioreceptor assay, the LOQ was reported to be 0.16 µg P-CTX-3C equivalents/kg fish sample by Darius et al. (2007).

The main advantage of the receptor-binding assays is:

- they are adequately sensitive to detect levels relevant to lowest observed effect levels;
- they are more specific than MBA

The main disadvantages of the receptor-binding assays are:

- they require the use of radioactive [^{3}H]-brevetoxin compounds;
- they are highly dependent on the receptor source;
- they do not provide any information on the toxin profile;
- they can not be easily automated;
- they have not been validated.

4.3.3. Immunoassays

Immunochemical methods such as a radioimmunoassay (RIA) (Hokama et al., 1977), a competitive enzyme immunoassay (EIA) (Hokama et al., 1983, 1984, 1986), a rapid enzyme immunoassay stick test (Hokama et al., 1987), and an enzyme-linked immunosorbent assay (ELISA) (Campora et al., 2008a,b) have been developed. Problems with these immunochemical methods are the different cross-reactivities for the various CTX-group toxins and the cross-reactivities with other polyether compounds. Antibody detection methods, which are being developed based on antibodies raised against P-CTX-1 or P-CTX-1 fragments, may not be suitable for detecting all of the Pacific, Caribbean or Indian CTX-group toxins (Vernoux and Lewis, 1997).

4.3.3.1. Radioimmunoassay

In 1977, a radioimmunoassay (RIA) was developed for the detection of CTX directly in contaminated fish (Hokama et al., 1977). In practice, the method was time-consuming, expensive and required special radioisotope facilities and hence turned out to be impractical for routine field analysis of fish samples (Hokama et al., 1998a).

4.3.3.2. Enzyme-linked immunosorbent assay (ELISA)

Hokama et al. (1983) developed an EIA for the detection of CTX-group toxins. The assay was shown to be similar in efficacy to the RIA developed earlier, but less expensive and more practical. However, it was still tedious and therefore abandoned as a detection method. Speed, practicability and specificity were all combined when the technology of monoclonal antibodies was incorporated into the stick test procedure (Hokama et al., 1989). This method has been used extensively for surveys and for clinical confirmation.

A membrane immunobead assay (MIA) was developed by Hokama et al. (1998a), which uses a monoclonal antibody, prepared against purified moray eel, coated onto coloured polystyrene beads. Overall, the MIA showed a LOD of approximately 32 ng P-CTX-1/kg fish flesh (Manger et al., 1995; Hokama et al., 1998b; Campora et al., 2008a). However, the specificity of the MIA for individual CTX-group toxins was not described. The Hokama et al. (1998a) method was subjected to a semi-quantitative collaborative study of Association of Official Analytical Chemists (AOAC) International in 1999. The study collaborators received dried fish samples, non-spiked or spiked with standard extract containing CTX. Due to difficulties in interpreting test results and lack of information concerning the antibody characteristics, the method was not approved by AOAC International (FAO, 2004).

To address the shortcomings of the existing assays, a sandwich ELISA was recently developed for detection of CTX in fish tissue (Campora et al., 2008a,b). The assay utilises two antibodies, chicken immunoglobulin Y and a mouse monoclonal immunoglobulin G-horseradish peroxidase conjugate, which are specific to the CTX-molecule. The method was developed for CTX-positive fish extract. The individual CTX-group toxins were not specified in the extract.

The main advantages of the immunoassays are:

- fast and easy to use except of the radioimmunoassay;
- they are more specific than MBA;
- can be applied to screen many samples for possible further confirmatory analysis.

The main disadvantages of immunoassays are:

- antibodies based on region-specific CTX-group toxins may not be suitable to detect CTX-group toxins from other regions;
- antibodies are not readily available;
- they do not provide any information on the toxin profile;
- due to cross-reactivities positive results need to be confirmed;
- the uncertainty does not allow reliable quantification;
- information about detection capabilities is scarce;
- successful international validation studies do not exist.

4.4. Chemical methods

High performance liquid chromatographic (HPLC) methods with fluorescence detection (FLD) and liquid chromatography-tandem mass spectrometric (LC-MS/MS) methods are available for the determination of CTX-group toxins.

4.4.1. HPLC-fluorescence detection methods

CTX-group toxins do not possess a suitable chromophore for selective spectroscopic detection but some analogues contain a relatively reactive primary hydroxyl group through which (after appropriate clean-up) a fluorescent label could be attached prior to detection. In a preliminary study Yasumoto et al. (1993) used fluorescent 1-anthroylnitrile for derivatising CTX prior to HPLC separation and fluorescence detection. The authors concluded that further work is required to develop efficient

cleanup procedures. Subsequently, HPLC coupled to fluorescence detection has been used to detect CTX-group toxins in fish after derivatisation with fluorescent reagents (Yasumoto et al., 1995). Dickey et al. (1992) derivatised CTX-1 using a coumarin-based fluorescent reagent but the yield of derivatised CTX-1 did not provide a LOD at µg/kg levels. There are no reports of more recent development of HPLC-fluorescence methods.

The main advantage of the HPLC-fluorescence methods is:

- cheaper than mass spectrometry based methods;
- partially specific for CTX-group toxins.

The main disadvantages of the HPLC-fluorescence methods:

- CTX-group toxins lacking primary hydroxyl group can not be detected;
- they require derivatisation of the CTX-group toxins;
- they do not provide low enough LODs;
- no validation studies have been published and detailed performance characteristics are not known.

4.4.2. LC-MS/MS methods

LC-MS/MS allows specific detection of individual analogues of P-, C- and I-CTX-group toxins (Lewis et al., 1999; Hamilton et al., 2002a,b; Pottier et al., 2002a,b). Determination of toxins from crude fish extract could be established, but for quantification of clinically relevant levels the LOD has to be lowered towards a concentration of 0.1 µg/kg or less (Lewis, 2001). The capability to reach this LOD could be demonstrated for P-CTX-1 by using an enrichment step like solid phase extraction (SPE) prior to MS detection (Lewis et al., 2009). This method was slightly modified by Stewart et al. (2009) and has been established as a referee analysis method by the public health laboratory for the state of Queensland, Australia. The LOD was determined to be 0.03 µg/kg fish flesh with an average recovery of 53 % (range 27-75 %) for various kinds of spiked fish species (n=10).

Due to the lack of certified standards and reference materials and the limited amounts of contaminated material available for method development, the validation status of LC-MS/MS methods is very restricted and up to now no collaborative study has been undertaken.

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

- very specific and thus superior to be used as a confirmatory method;
- they are adequately sensitive to detect levels, relevant to lowest observed effect levels.

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

- expensive;
- highly trained personnel is needed.

4.5. Proficiency tests

There is no ongoing proficiency test for CTX-group toxins.

4.6. Summary of methods

The MBA has been widely used to detect CTX-group toxins in shellfish but for reasons of animal welfare there is growing concern with respect to its use and it has shown insufficient detection capability. *In vitro* assays provide sufficient detection capability, and can detect all active analogues of CTX-group toxins. However, they cannot give information about toxin profiles. Assays based on immunochemical technology have been developed, but they have not resulted in applicable tests. Recent studies have also focussed on the development of chemical methods, such as LC coupled with MS for the detection and quantification of CTX-group toxins. LC-MS/MS methods would be of value for the quantification of CTX-group toxins. Optimisation of these methods for application to fish extracts, their (inter-laboratory) validation and the development of standards and reference materials are necessary.

5. Occurrence of CTX-group toxins

In recent years the presence of *Gambierdiscus* sp. has been recorded in the eastern Mediterranean Sea. The first report on occurrence of *Gambierdiscus* sp. in Crete Island is from 2003 (Aligizaki and Nikolaidis, 2008). The presence of this genus was also confirmed in the Canary Islands and in Madeira, suggesting a spread of it into new areas as shown in Figure 2 (Aligizaki et al., 2008).

The presence of CTX-producing organisms in the Mediterranean has also been confirmed by the contamination of coastal fish species found in Israel (Bentur and Spanier, 2007). These findings suggest a possible future concern about CTX-group toxins in fish and seafood originating from Europe. Very limited occurrence data were obtained following two recent ciguatera outbreaks in Madeira and the Canary Islands.

In January 2004 a fisherman's family showed symptoms of CFP after eating part of a 26 kg Amberjack (*Seriola Rivoliana*) captured during scuba diving along the coasts of Canary Islands. A 150 g sample of the fish that was kept frozen at fisherman's home was analysed by a sodium channel-specific *in vitro* assay and LC-MS/MS. The assay results were positive and the CTX content of the fish sample was estimated to be 1.0 µg/kg. The presence of C-CTX-1 in the fish was confirmed by LC-MS/MS (Pérez-Arellano et al., 2005).

Figure 2: The circles indicate the areas where *Gambierdiscus* sp. was recently found in Europe.

CTX-group toxin intoxications were reported in Madeira in 2008 (Gouveia et al., 2009). *Seriola dumerili*, a large fish of 70 kg and a small *Seriola fasciata* were identified as the intoxication sources. Five parts of *Seriola dumerili* and only a part of caudal muscle of *Seriola fasciata* were analysed for CTX-group toxins by using the LC-MS/MS method (Otero et al., 2010). The quantification of P-CTX-

1 was performed by using a non-certified standard of P-CTX-3C. For the other CTX-group toxins that were found, the same response factor was assumed. The results are reported in Table 3.

Table 3: CTX-group toxin levels in fish analysed by using LC-MS/MS method.

Fish species /sample	C-CTX-1 and C-CTX-2[(a)] µg/kg	P-CTX-1 µg/kg	P-CTX-4A and P-CTX-4B[(a)] µg/kg	P-CTX-3C µg/kg	51-OH-P-CTX-3C µg/kg
Seriola dumerili					
Caudal muscle	7.8	<LOD[(b)]	1.8	0.8	39.4
Seriola fasciata					
Caudal muscle	4.4	1.2	1.1	1.1	25.3

LOD: limit of detection; (a): these are isomers and are not distinguishable in LC-MS/MS analysis;
(b): LOD = 0.04 µg P-CTX-3C/kg;

These few data do not allow any estimation of the occurrence of CTX-group toxins, but they give some indication of the levels of CTX-group toxins that are high enough to cause human outbreaks and that can be found in fish in Europe.

5.1. Influence of processing

There are insufficient data to draw conclusions on the influence of processing on the levels of CTX-group toxins in fish.

6. Human consumption of fish

CTX-group toxins cause acute intoxication and therefore consumption figures for single meals, particularly the high consumption percentiles, are relevant for the exposure assessment. The EFSA Concise European Food Consumption Database provides information on consumption of fish and fish products in 16 European countries[15]. However, the methodology applied in the consumption surveys is not uniform and the reporting periods for the amounts consumed by a single individual per food category vary from 1 day to 28 days. As a consequence, when the survey spans over several days, the reported value is the average consumption of the period and does not necessarily represent a single consumption occasion. This may underestimate the single meal portion in case of foods with low probability of daily consumption.

Table 4 shows a summary of the "Fish and Fish Products" (Category 11B of the EFSA Concise Food Consumption Database[15]) consumption in the subgroup "consumers only" in 16 European countries. The subjects in the subgroup "consumers only" are consumers that in the survey period reported at least one meal with "Fish and Fish products". The mean, 95th percentile and maximum consumption values are reported for each country. The countries are grouped based on survey methodology and the number of survey days is given. Mean and median across countries are calculated for the statistical descriptors in each survey group.

In countries applying a 1-day 24 hours recall survey method, the 95th percentile of consumption among fish consumers ranges between 250 g/day and 422 g/day, with a median across countries of 300 g/day. The 97.5th percentile in the same countries ranges between 300 g/day and 500 g/day, with a median across countries of 322 g/day. Assuming consumption of no more than one fish-based meal per day, the data from this group of countries would most probably represent a distribution of single meals.

[15] http://www.efsa.europa.eu/en/datex/datexfooddb.htm

In countries applying a 2-days survey method (24 hours recall or dietary record), the 95th percentile of consumption among fish consumers ranges between 140 g/day and 178 g/day, with a median across countries of 167 g/day. The 97.5th percentile in the same countries ranges between 176 g/day and 225 g/day, with a median across countries of 202 g/day. Assuming consumption of no more than one fish-based meal over the two days, the reported value would represent a single meal averaged over two days. Using this assumption the second group of countries (from Belgium to the Netherlands (see Table 4)) would show single fish-meal consumption in line with those of the first group of countries (from Austria to Slovakia (see Table 4)). For longer survey periods no assumptions can be reasonably made and the values reported in Table 4 are difficult to interpret with respect to a single portion size. In addition to the data from the seven-day survey, the United Kingdom (UK) has made separately available a table on statistics at the level of single consumption occasions per fish species available separately. The statistics were calculated based on the same data that are included in the EFSA Concise food consumption database (UK National Diet and Nutrition Survey, 2000-2001) (Henderson et al., 2002). The statistics for the fish species with more than 20 consumers are reported in Table 5.

Table 4: Consumption statistics based on individual consumption figures from the group 11B "Fish and Fish Products" of the EFSA Concise Food Consumption Database, calculated among consumers who declared at least one meal of "Fish and Fish Products" (consumers only) in 16 European countries. The individual values are average values over the survey period and do not necessarily represent single consumption occasions. The countries are grouped by survey methodology and group-level statistics (mean and median).

Country	Survey method	Days	Subjects	Number of consumers	% of consumers	Mean g/day	P95 g/day	P97.5 g/day	Max g/day
Austria	24 hours recall	1	2123	168	7.9	174	300	322	520
Bulgaria	24 hours recall	1	853	85	10	198	422	500	848
Iceland	24 hours recall	1	1075	312	29	111	250	302	495
Poland	24 hours recall	1	2692	331	12.3	154	389	440	864
Slovakia	24 hours recall	1	2208	101	4.6	142	250	300	1011
Mean						**156**	**322**	**373**	**748**
Median						**154**	**300**	**322**	**848**
Belgium	24 hours recall	2	1723	552	32	55	140	176	290
Czech Republic	24 hours recall	2	1751	368	21	79	178	225	325
The Netherlands	Dietary record	2	4285	633	14.8	70	167	202	500
Mean						**68**	**162**	**201**	**372**
Median						**70**	**167**	**202**	**325**
Hungary	Dietary Record	3	927	118	12.7	71	152	200	267
Denmark	Precoded food diary with open fields	7	3150	2665	84.6	18	50	62	159
France	Dietary record	7	1195	935	78.2	37	93	113	287
Ireland	Dietary record	7	1373	895	65.2	35	86	102	206
Italy	Dietary record	7	1544	1256	81.3	41	101	122	287
Sweden	Dietary record	7	1088	831	76.4	32	77	103	169
United Kingdom	Dietary record	7	1724	1094	63.5	33	79	91	256
Germany	Dietary-history	28	3550	2939	82.8	22	54	69	254

Consumers: individuals declaring at least one fish-based meal during the survey; subjects: number of individuals participating to the survey in one country; % of consumers: percentage of the consumers of each particular species among all the interviewed subjects; P95: 95th percentile of consumption; P97.5: 97.5th percentile of consumption; max: maximum reported consumption.

In the UK table the 97.5th percentile of consumption among fish consumers ranges across fish species between 185 g/day and 369 g/day, with a median across fish species of 258 g/day (Table 5). These values are comparable to those calculated for the 1-day surveys in Table 4. Based on the available data for the 97.5th percentile from the 1-day surveys and considering the data from the 2-days survey as

well as the additional UK data, a large portion size of 350 g fish per meal can be assumed to reflect high consumers.

Fish consumption of 500 g per meal was taken as reference in the FAO Food and Nutrition Paper on Marine Biotoxins (FAO, 2004). According to the available data, this figure could be interpreted as maximum consumption level for European countries.

Table 5: Consumption statistics based on individual consumption figures from the group "Fish and Shellfish" of the UK National Diet and Nutrition Survey (Henderson et al., 2002). The statistics are calculated on a per day basis and include only fish consumers.

Item name	Number of consumers	% of consumers	Mean g/day	Median g/day	P97.5 g/day	Maximum g/day
Cod	574	33	82	79	207	338
Tuna - canned	513	30	75	63	185	265
Tuna - fresh	38	2	97	100	258	305
Haddock (Melanogrammus)	352	21	82	70	225	340
Salmon	319	18	107	96	340	610
Sardine/Pilchard (Sardina)	82	5	85	80	237	360
Trout	50	3	152	154	369	460
Mackerel (Scomber)	83	5	101	97	239	486
Herring/Kipper/Bloater (Clupea)	41	2	125	120	332	370
Plaice (Pleuronectes)	42	2	129	106	346	400
Sole (Solea; Limanda)	26	2	140	145	315	327
minimum			**75**	**63**	**185**	**265**
maximum			**152**	**154**	**369**	**610**
median			**101**	**97**	**258**	**360**

P97.5: 97.5th percentile of consumption

7. Exposure assessment

The few data on occurrence of CTX-group toxins in fish do not allow any exposure assessment for the European population.

8. Toxicokinetics

No studies specifically addressing absorption of CTX-group toxins have been identified, but absorption of CTX-group toxins from the gastrointestinal (GI) tract is indicated on the basis of toxicity studies following oral administration in mice (Lehane and Lewis, 2000).

The biotransformation of CTX-group toxins in animals is indicated by the observation that one hour after *i.p.* injection of 0.12-2.34 ng C-CTX-1/g b.w., both non-polar and polar CTX-group toxins were detected in blood (Bottein Dechraoui et al., 2005b).

CTX-group toxins could be transferred from the mother to the foetus through the placenta (Pearn et al., 1982; Senecal and Osterloh, 1991; Fenner et al., 1997) and from the mother to her offspring through the milk (Bagnis and Legrand, 1987; Blythe and de Sylva, 1990).

Some data on elimination of CTX-group toxins from the blood are available from investigations carried out in the mouse (Bottein Dechraoui et al., 2005b, 2008). In those studies, the blood concentrations of both C-CTX-1 and P-CTX-1 reached a peak between 30 and 60 minutes after injection, and rapidly decreased in the first 3-4 hours. P-CTX-1 was investigated for three days and persisting low levels were found for that period (Bottein Dechraoui et al., 2008).

9. Toxicity data

9.1. Mechanistic considerations of CTX-group toxins

The voltage-gated sodium channel (Na_V) is the primary molecular target of CTX-group toxins, and their binding to the neurotoxin receptor site 5 of Na_V causes the opening of the ion pore, activation of the sodium channels and sodium entrance into the cells (Boyarsky and Rayner, 1970; Setliff et al., 1971; Bidard et al., 1984; Lombet et al., 1987; Catterall et al. 2007). The increased sodium entrance into the cell leads to membrane depolarisation and functional impairment of excitable cells (Terao, 2000). Secondary responses observed in cells exposed to CTX-group toxins include Ca^{2+} entry into the cell by reverse action of Na^+/Ca^{2+} exchangers (Lewis and Endean, 1986; Molgó et al., 1993) eventually leading to muscular contraction (Lewis and Endean, 1986) and neurotransmitter release in a variety of experimental systems (Bidard et al., 1984; Lewis, 1988; Seino et al., 1988; Molgó et al., 1993). Water entry into cells exposed to CTX-group toxins is another effect that follows sodium influx, leading to cell swelling and cytotoxicity (Mattei et al., 1999).

The altered ion conductance and increased neurotransmitter secretion represent the molecular basis for the CTX induced loss of cell excitability in nervous and muscular systems which may lead to paralysis in animals exposed to CTX-group toxins.

9.2. Effects in laboratory animals

9.2.1. Acute toxicity

Intraperitoneal and oral administration of CTX-group toxins to experimental animals results in a number of acute toxic effects as a result of opening sodium channels in both nervous tissues and muscles.

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

The CTXs are acutely toxic upon *i.p.* administration with LD_{50}-values of 0.25, 2.3 and 0.9 µg/kg b.w. for CTX-1, CTX-2 and CTX-3 (presumably P-CTX-group toxins), respectively (Lewis et al., 1991). For C-CTX-1 LD_{50}-values of 3.7 µg/kg (Dickey, 2008) and 3.6 µg/kg b.w. (Vernoux and Lewis, 1997) have been reported. The main signs of toxicity for P-CTX-1 are hypothermia, piloerection, diarrhoea, lacrymation, hypersalivation, dyspnoea, wobbly upright gait, gasping and terminal convulsions with tail arching and death from respiratory failure. For P-CTX-2 and P-CTX-3 progressive hind limb paralysis is seen in addition. At doses near the LD_{50} the minimum time to death varies from a half to one hour for the three P-CTX toxins.

Upon *i.p.* injection of crude extracts of the initially characterised I-CTX, signs similar to those of P-CTX- and C-CTX-group toxins were observed (the dose of I-CTX was not reported) (Hamilton et al., 2002a).

In a study in male ICR mice weighing 23-26 g (Ito et al., 1996), "semi-pure" P-CTX was given *i.p.* at doses between 1/30 and 2 MU, corresponding to 0.009 to 0.56 µg/kg b.w. (1 MU = 7 ng pure CTX in this experiment). At a dose of 0.22 µg/kg b.w. the weights of the pancreas and liver and to a lesser extent thymus and spleen decreased, but no remarkable pathological changes were seen. Diarrhoea occurred at doses between 0.04 and 0.28 µg/kg b.w. At the higher doses there was an increase in watery stools with a gradual decrease in pH by time. Morphological changes in the colon were recorded by light and scanning electron microscopy at a dose of 0.22 µg/kg b.w. and higher. Bleeding in the mucosa was also observed.

In another study in mice, a second *i.p.* dose of 0.26 µg/kg b.w. P-CTX-1 three days after an initial *i.p.* dose of 0.26 µg/kg b.w. prolonged the hypothermic response and enhanced the reduced activity seen

after the first administration. The prolonged effects were accompanied by an increased serum concentration of P-CTX-1 only during the first hour after the second injection (Bottein Dechraoui et al., 2008).

Effects of single and repeated *i.p.* exposures to CTX (isolated from reef snappers, *Lutjanus bohar* and presumed to be P-CTX-1 by the CONTAM Panel) or P-CTX-4C (identity not specified, a less polar CTX of >99 % purity, isolated from the dinoflagellate *Gambierdiscus toxicus*) at a single dose of 0.7 µg/kg b.w. (Terao et al., 1991) or multiple dose of 0.1 µg/kg b.w. for 15 days (Terao et al., 1992) were examined in male ICR mice four weeks of age weighing 20-23 g. It should be noted that these two publications report both *i.p.* and oral administration studies, the toxicity is described in qualitative terms and the results description does not clearly discriminate between toxicity following *i.p.* and oral administration. 24 Mice received a single dose of 0.7 µg/kg b.w. and groups of three mice were killed at different time points from 10 minutes to 24 hours after treatment (Terao et al., 1991). The mice showed laborious movements and lumbar muscle contraction followed by severe watery diarrhoea that lasted for only 90 minutes, and followed by an apparent recovery after a few hours. Then, suddenly severe dyspnoea and cyanosis appeared and 70 % (this value is unclear as 21 of the 24 mice were killed before 24 hours) of the mice died within 24 hours. The remaining 30 % (unclear value) showed paralysed paws, and in half of them, penis erection and dilated and filled urinary bladder. Dilated heart and lung oedema and marked congestion of the organs were observed. Upon histopathology, necrotic cells were seen in the heart and by electron microscopy, characteristic ultrastructural changes in the heart were rounded mitochondria and marked oedema between myofibrils and other organelles. Degeneration of adrenal medulla and erect penises with cavernous thrombi were observed. The livers were congested with the presence of thrombi and the toxins caused swollen synapses in the smooth muscle layer of the vas deferens. Similar changes were observed in the smooth muscular layer of the small intestine, but despite severe diarrhoea no changes were seen in the mucosal layer.

In a second experiment with the main emphasis on effects on the heart (Terao et al., 1992) male ICR mice were given a single *i.p.* dose of 0.7 µg/kg b.w. of P-CTX-1 or P-CTX-4C and were followed for up to seven months. After 24 hours similar effects as those in the previous experiment were observed (Terao et al., 1991). Apparently some serum and erythrocyte effusions in the interstitium of the heart persisted for a month, which subsequently turned into fibrotic tissue. In this study P-CTX-1 or P-CTX-4C were given *i.p.* to groups of 20 mice in multiple doses of 0.1 µg/kg b.w. for 15 days. Two mice from each group were killed 24 hours after the first injection, and immediately after the last injection, and then monthly up to the seventh month and then at month 14. A single dose of 0.1 µg/kg b.w. of P-CTX-1 did not cause any morphological effect in the heart either seen at macroscopic, microscopic or ultrastructural examination. 15 Repeated doses of 0.1 µg/kg b.w. caused effects of similar severity as those seen after a single dose of 0.7 µg/kg b.w. Within a month after the last dose, the myocytes and capillaries appeared normal whereas bundles of dense collagen in the interstitial spaces persisted at 14 months. No differences in clinical signs or histopathology between P-CTX-1 and P-CTX-4C were observed.

9.2.1.2. Toxicity following oral administration

Male ICR mice weighing 23-26 g were exposed to “semi-pure” CTX (presumed to be P-CTX-1 by the CONTAM Panel), by oral gavage at doses between 2/3 and 2 MU, corresponding to 0.19 to 0.56 µg/kg b.w. (1 MU = 7 ng pure P-CTX-1 in this experiment). The lethal dose and clinical signs were almost the same as those seen following *i.p.* administration, except that diarrhoea only occurred after parenteral administration (Ito et al., 1996).

In the same experiments as described above in 9.2.1.1., effects of oral exposures to P-CTX-1 or P-CTX-4C at a single dose of 0.7 µg/kg b.w. (Terao et al., 1991), or following repeated administration of 0.1 µg/kg b.w. for 15 days (Terao et al., 1992), were examined in male ICR mice. As noted above these two publications did not clearly discriminate between toxicity following *i.p.* and oral administration. The mice receiving a single dose of 0.7 µg/kg b.w. showed similar symptoms and

histopathological changes as those seen after *i.p.* administration (Terao et al., 1991). Atropine pre-treatment prevented the diarrhoea indicating that it was caused by actions on the autonomic nerve system in the intestinal wall. However, atropine did not prevent cardiac injuries. No differences in clinical signs or histopathology between P-CTX-1 and P-CTX-4C were observed.

In a second experiment with the main emphasis on effects on the heart (Terao et al., 1992), groups of male mice were given daily doses of 0.1µg/kg b.w. P-CTX-1 (n=20) or 0.1 µg/kg b.w. P-CTX-4C (n=18) by gastric intubation for 15 days. Two mice from each group were killed 24 hours after the first dose, immediately after the last dose, and then monthly up to the seventh month and then at month 14. A single dose of 0.1 µg/kg b.w. of P-CTX-1 (n=2) did not cause any morphological effects in the heart either seen at macroscopic, microscopic or ultrastructural examination. However, repeated exposures of 0.1 µg/kg b.w. for 15 times caused effects including marked swelling of myocardial and capillary endothelial lining similar to those seen after a single dose of 0.7 µg/kg b.w. Within a month after the last exposure, the myocytes and capillaries appeared normal whereas bundles of dense collagen in the interstitial spaces persisted at 14 months. No differences in clinical signs or histopathology between P-CTX-1 and P-CTX-4C were observed.

9.3. Relative potency of analogues

The different CTX-group toxins share the same molecular receptor of toxicity (neurotoxin receptor site 5 of Na_V) and dose addition is presumed to be the result of simultaneous exposure to multiple analogues. The CONTAM Panel therefore found it appropriate to assign toxicity equivalency factors (TEFs) for the different CTX-group toxins. The CTX-group toxins show different relative receptor affinity, which to a certain extent does accord with relative toxicity as determined by the LD_{50} following *i.p.* administration in mice. The relative *in vivo* toxicity is most likely also a result of i.a. toxicokinetic properties such as lipophilicity. Based on *i.p.* toxicity of the different CTX-group toxins in mice the CONTAM Panel adopted the TEF-values as listed in Table 6.

Table 6: The toxicity equivalency factors (TEFs) adopted by the CONTAM Panel for different CTX-group toxins based on intraperitoneal (*i.p.*) toxicity.

CTX-group toxin	TEF	LD_{50} (*i.p.* in mice) µg/kg b.w.	Reference
P-CTX-1	1	0.25	Lewis et al. (1991); Dickey (2008)
P-CTX-2		2.3	Lewis et al. (1991)
	0.3	0.9	Lewis (2001)
P-CTX-3	0.3	0.9	Lewis et al. (1991)
P-CTX-3C		1.3	Satake et al. (1993b)
	0.2	2	Lewis (2001)
2,3-dihydroxy P-CTX-3C	0.1	1.8	Satake et al. (1998)
51-hydroxy P-CTX-3C	1	0.27	Satake et al. (1998)
P-CTX-4A	0.1	2	Satake et al. (1997)
P-CTX-4B	0.05	4	Murata et al. (1990); Satake et al. (1997)
C-CTX-1	0.1	3.6	Vernoux and Lewis (1997); Lewis et al. (1998)
C-CTX-2	0.3	1	Vernoux and Lewis (1997)

b.w.: body weight.

Until better information is available the TEFs adopted by the CONTAM Panel should be applied to express individual analogues identified with quantitative detection methods as P-CTX-1 equivalents.

10. Observations in humans

CFP is estimated to affect 10 000 to 50 000 people worldwide per year and this might represent only a fraction of actual cases. The Centres for Disease Control and Prevention estimate that only 2-10 % of ciguatera cases are reported (CDC, 2006). CFP is characterised by gastrointestinal, neurological and cardiovascular disturbances. In severe cases, the symptoms may begin as soon as 30 minutes after ingestion of contaminated fish, while in milder cases they may be delayed for 24 to 48 hours. Gastrointestinal symptoms, including vomiting, diarrhoea, nausea and abdominal pain, occur in greater than 50 % of cases, typically in the early stages. Neurological symptoms, including tingling of the lips, hand and feet, reversal of temperature sensation and severe localised itching of the skin, occur in greater than 70 % of cases. These symptoms and profound fatigue (90 % of cases) can occur throughout the illness and may persist for weeks to months or even years. Muscle (>80 %), joint (>70 %) and tooth ache (>30 %) occur to varying extents. Severe cases can involve hypotension with bradycardia, respiratory difficulties and paralysis, but death is uncommon. It has been speculated that the toxicity of CTX-group toxins to fish, which could limit the accumulation of these toxins in live fish, might be the reason for the few reported cases of human fatalities (Lewis, 1992).

The cardiovascular symptoms of CFP usually resolve within five days of onset (De Fouw et al., 2001; FAO, 2004). Mood disorders including depression and anxiety occur less frequently (De Fouw et al., 2001; FAO, 2004). In addition, hallucinatory symptoms (lack of coordination, loss of equilibrium, hallucinations, mental depression and nightmares) have been reported in 16 % of cases in the Indian Ocean area (Quod and Turquet, 1996).

Symptoms may recur during periods of stress, such as exercise, weight loss, or excessive alcohol consumption (Lehane, 2000). Individuals who have previously suffered from CFP experience a recurrence of symptoms after eating fish that do not cause symptoms in other persons (De Fouw et al., 2001). This phenomenon has been referred to as sensitisation, but could also reflect accumulation of the toxin. It has also been reported that the toxin can transfer between partners during sexual intercourse, resulting in localised pain and other symptoms in the partner who had not consumed the affected fish (De Fouw et al., 2001).

The nature, duration and severity of symptoms vary between ethnic groups and between the sexes, although it is not clear whether this is due to genetic predisposition, different eating preferences, or different toxin profiles in different regions or type of fish. In the Pacific, men are more likely to experience diarrhoea and abdominal pain, whereas women more often report arthralgia and myalgia. Whilst cases have spanned all ages, there appear to be more in males than females, and more in the 30-49 year age group (Lehane and Lewis, 2000; FAO, 2004). In addition the duration of symptoms and the outcome are influenced by the availability of treatment, such as mannitol infusion.

A small number of reported cases have involved pregnant women. A pregnant woman ate fish from the Great Barrier Reef two days before the expected delivery. She and other family members who also ate the same fish experienced characteristic gastrointestinal and neurological symptoms and presence of CTX-group toxins was confirmed by MBA. The pregnant woman reported tumultuous foetal movements and an intermittent foetal "shivering" which continued for about 18 hours then decreased over the ensuing 24 hours. The infant exhibited left-sided facial palsy and possible myotonia of the hands at birth but was reported to be normal at six weeks (Pearn et al., 1982). A 20-year old woman at the 16th week of her second pregnancy, suffered severe gastrointestinal and neurological symptoms of CFP, and increased foetal movements commencing four hours after consumption of barracuda. The increased foetal movements persisted for only a few hours, whereas many of the mother's symptoms persisted for several weeks. Her baby was normal at birth and during 10 months of follow-up. A range of tests on the fish (including immunoassay and MBA) indicated a CTX-group toxin or related toxin content >1 µg/kg fish tissue (Senecal and Osterloh, 1991). A 33-year old woman (11 weeks pregnant) in Queensland, Australia, ate about 500 g of coral trout and became distressed, with vomiting and dehydration within 60-90 minutes, and was treated with mannitol. On day seven she presented with very slight burning sensation of the hands and mouth and mild itch. At birth (39 weeks), the infant had

mild respiratory distress at three hours and was diagnosed as having persistent pulmonary hypertension, for which he was treated. He had no residual symptoms at a two-month follow-up. A toxin content of 1.3 µg/kg in the fish was estimated by MBA (Fenner et al., 1997).

Diagnosis of CFP is based on the clinical signs and symptoms, and despite the very large number of reported cases, there are few in which the concentrations of CTX-group toxins were measured and exposure to the toxins estimated. In some cases toxin concentrations were reported in MUs, in which case it provides a measure of total toxicity. Based on the *i.p.* lethality of P-CTX-1 in the mouse (35 µg/kg b.w.) (Yasumoto, 2001), 1 MU has been reported to be equivalent to 7 µg P-CTX-1 (Oshiro et al., 2009).

A number of publications (Lewis and Sellin, 1992; 1993; Lewis, 1994; Hokama et al., 1998a; Lewis et al., 1999; Lehane, 2000; Lehane and Lewis, 2000) note that most cases of CFP in the Pacific involved the consumption of fish containing the equivalent of 0.1-5 µg P-CTX-1/kg of fish flesh. This observation is largely based on papers reporting on development of analytical methods using stored fish that had been reported to be associated with human illness, with the concentration of toxin quantified by MBA, and is supported by more recent reports.

A 42-year old male experienced mainly neurological symptoms of CFP after consumption of fish containing 0.3 µg P-CTX-1/kg of flesh, determined using a sodium channel-specific mouse neuroblastoma assay. He reported having consumed a "large portion" for dinner the evening before developing symptoms and more fish for lunch the following day. His wife and two children ate smaller portions and experienced no ill-effects (Arnett and Lim, 2007).

Oshiro et al. (2009) reviewed 33 outbreaks involving 103 patients reported in Japan between 1997 and 2006. The toxin content of the leftover fish, as determined by MBAs, ranged from 0.025 to above 0.8 MU/g shellfish meat (equivalent to 0.175 - >5.6 µg P-CTX-1/kg).

An otherwise healthy 87-year old, man (88 kg) died six days after eating sawtooth barracuda in Brisbane Australia. His wife and son-in-law also ate the fish and became ill, but made full recoveries. Analysis of the fish revealed concentrations of 1.1 µg P-CTX-1 equivalents/kg fish flesh by brevetoxin binding assay and 5.6, 7.9 and 1.4 µg/kg for P-CTX-1, P-CTX-2 and P-CTX-3, respectively by LC-MS/MS. A sample of the victim's liver was found to contain 0.14 µg P-CTX-1 equivalents/kg by brevetoxin binding assay but insufficient tissue was available for analysis by LC-MS/MS. Assuming the three individuals ate equal portions of the total estimated amount of fish (~1 kg), the authors estimated that a combination of ~3.5, ~5 and ~1 µg P-CTX-1, P-CTX-2 and P-CTX-3 respectively, was apparently lethal for the older male (Hamilton et al., 2009). Applying the TEFs identified in Table 6 this combination corresponds to 4.3 µg P-CTX-1 equivalents, which would have resulted in an intake of about 50 ng P-CTX-1 equivalents b.w. for the 88 kg victim.

C-CTX-1 has been reported to be less toxic than P-CTX-1 (FAO, 2004). However there are reports of outbreaks of CFP associated with concentrations of 0.6 µg C-CTX-1/kg of fish flesh, determined by sodium channel-specific mouse neuroblastoma assay (CDC, 2009), 1 µg C-CTX-1/kg of fish flesh, determined by LC-MS/MS (Perez-Arellano et al., 2005), 20 µg C-CTX-1/kg of fish flesh, determined by a brevetoxin competitive displacement assay (Poli et al., 1997), indicating a similar range to that seen for P-CTX-1.

In the Fenner et al. (1997) report, fish was consumed by a family of four including the pregnant mother described above, all of whom experienced symptoms of CFP. The father ate about 1000 g, the mother ate about 500 g and the two children (a boy of 4 years and girl of 6 years) each ate "a small piece of fish". Symptoms were most severe in the father, and minor in the children. Based on the toxin content of 1.3 µg/kg in the fish flesh, determined by MBA, Lehane (1999) estimated dietary exposure levels at 19 ng/kg b.w. for the father (assuming b.w. of 70 kg) and 11 ng/kg b.w. for the mother (assuming b.w. of 60 kg). This led Lehane (1999) to conclude that 10 ng/kg b.w. would be expected to be definitely toxic in most people. There were no estimates of the amounts of fish consumed in the

other case reports. Lehane (1999) noted that since the mild CFP had been associated with consumption of fish containing as little as 0.1 µg/kg P-CTX-1, the lowest dose of P-CTX-1 that might be expected to be toxic in an adult would be 50 ng, or about 1 ng/kg b.w. (assuming consumption of 500 g fish by a 50 kg individual) and this exposure might cause about two people out of 10 to be sick.

11. Hazard characterisation

There are very few oral studies in experimental animals and there are no long term studies that would allow establishing a tolerable daily intake (TDI). In a limited study in mice a single oral dose of 0.1 µg/kg b.w. of P-CTX-1 caused no clinical or histopathological changes whereas an oral dose of 0.7 µg/kg b.w. caused severe toxicity in the heart, adrenal gland and penis. Repeated oral doses of 0.1 µg/kg b.w. also caused severe toxicity (Terao et al., 1991, 1992).

Based on case reports in humans it appears that consumption of a single meal of fish containing about 1 µg/kg of P-CTX-1 produced clear toxic symptoms. The lowest P-CTX-1 concentration in fish associated with mild toxicity in humans was estimated to be 0.1 µg/kg.

In view of the acute toxicity of CTX-group toxins the CONTAM Panel considered establishing an acute reference dose (ARfD). However, due to the very limited quantitative data both in experimental animals as well as related to human intoxications, the CONTAM Panel concluded that the establishment of an oral ARfD was not possible. In addition, it concluded that an ARfD may also not be adequately protective to humans exposed several times to CTX-group toxins even when incidents occurred months apart.

The CONTAM Panel noted that a number of publications state that cases of CFP in the Pacific mostly occur following the consumption of fish containing the equivalent of 0.1-5 µg P-CTX-1/kg of fish flesh. In line with the approach of FAO (2004), the CONTAM Panel applied an uncertainty factor of 10 to the lowest concentration 0.1 µg equivalents of P-CTX-1/kg in fish associated with mild symptoms to indicate a concentration of 0.01 µg equivalents of P-CTX-1/kg of fish, which is expected not to exert effects in sensitive individuals. In many of the cases of CFP the MBA or a biomolecular method, was used as the analytical method, which would also detect other potent CTX-group toxins that might co-occur with P-CTX-1. Therefore the CONTAM Panel concluded that this concentration value should be taken as 0.01 µg P-CTX-1 equivalents/kg fish, to cover all CTX-group toxins that could be present in fish.

12. Risk characterisation

CTX-group toxins have only been found incidentally in fish in Europe. Because of the very limited occurrence data, the CONTAM Panel could not comment on the risk associated with the exposure to CTX-group toxins in fish that could reach the European market.

13. Uncertainty

The few data on occurrence of CTX-group toxins in fish do not allow any exposure assessment for the European population. In addition, there are limited animal toxicity data, and doses related to clinical signs and symptoms following CFP in humans that are not well defined. Therefore, the CONTAM Panel concluded that the overall uncertainty is large and a detailed consideration of the various potential sources of uncertainty is not meaningful.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

General

- Ciguatoxin (CTX)-group toxins are lipid-soluble polyether compounds. They are classified as Pacific (P), Caribbean (C) and Indian Ocean (I) CTX-group toxins.
- CTX-group toxins occur in fish as a result of biotransformation of precursor gambiertoxins produced by the benthic dinoflagellate *Gambierdiscus toxicus*.
- CTX-group toxins cause ciguatera fish poisoning (CFP), which is characterised by gastrointestinal (e.g. vomiting, diarrhoea, nausea), neurological (e.g. tingling, itching) and cardiovascular (e.g. hypotension, bradycardia) effects.
- Other toxins such as gambiertoxin and maitotoxin have also been isolated from *G. toxicus* and associated with CFP.

Methods of analysis

- The mouse bioassay (MBA) has been widely used to detect CTX-group toxins in fish. For reasons of animal welfare there is growing concern with respect to its use. The MBA has poor specificity and insufficient detection capability and is therefore not considered an appropriate detection method for CTX-group toxins.
- In vitro (cytotoxicity and receptor binding) assays provide sufficient detection capability, and can detect all active analogues of CTX-group toxins, but they also do not provide information on toxin profiles.
- Immunochemical methods, mostly enzyme-linked immunosorbent assays (ELISA), are fast and easy to use, but the use of antibodies to region-specific CTX-group toxins makes them unsuitable to detect toxins from other regions. In addition, they do not provide information on the toxin profile, and they do not allow reliable quantification.
- Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods allow specific detection of individual analogues of P-, C- and I-CTX-group toxins and they would be of value for the quantification in fish extracts, subject to further development.
- None of the current methods of analysis to determine CTX-group toxins in fish has been formally validated.

Occurrence/Exposure

- Recently CTX-group toxins were identified for the first time in fish in Europe.
- There are very few occurrence data on CTX-group toxins in fish in Europe. These data do not allow any exposure assessment for the European population.

Hazard identification and characterisation

- The CTX-group toxins cause acute toxicity by binding to voltage-gated sodium channels resulting in activation and sodium influx into cells.

- Until better information is available the Panel on contaminants in the food chain (CONTAM Panel) adopted the following toxicity equivalency factors (TEFs) for CTX-group toxins based on their acute intraperitoneal LD_{50} in mice: P-CTX-1 = 1, P-CTX-2 = 0.3, P-CTX-3 = 0.3, P-CTX-3C = 0.2, 2,3-dihydroxy P-CTX-3C = 0.1, 51-hydroxy P-CTX-3C = 1, P-CTX-4A = 0.1, P-CTX-4B = 0.05, C-CTX-1 = 0.1 and C-CTX-2 = 0.3. These TEFs should be applied to express individual analogues identified with quantitative detection methods as P-CTX-1 equivalents.

- In view of the acute toxicity of CTX-group toxins the CONTAM Panel considered establishing an acute reference dose (ARfD). However, due to the very limited quantitative data both in experimental animals as well as related to human intoxications, the CONTAM Panel concluded that the establishment of an oral ARfD was not possible.

- The CONTAM Panel applied an uncertainty factor of 10 to the lowest concentration of 0.1 µg equivalents of P-CTX-1/kg in fish associated with mild symptoms to indicate a concentration of 0.01 µg equivalents of P-CTX-1/kg of fish, which is not expected to exert effects in sensitive individuals. This concentration should be taken as 0.01 µg P-CTX-1 equivalents/kg fish, to cover all CTX-group toxins that could be present in fish.

Risk characterisation

- Because of the very limited occurrence data, the CONTAM Panel could not comment on the risk associated with the exposure to CTX-group toxins in fish that could reach the European market.

RECOMMENDATIONS

Methods of analysis

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

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

Occurrence/Exposure

- More information is needed on occurrence of the CTX-group toxins, gambierol and maitotoxins in fish and other seafood.

- Due to the high acute toxicity of CTX-group toxins and their emerging occurrence, appropriate strategies to protect human health need to be developed.

Hazard identification and characterisation

- Further information is needed to better characterise the oral toxicity of CTX-group toxins and their relative potencies.

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ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
ARfD	Acute reference dose
ASP	Amnesic Shellfish Poisoning
AZA	Azaspiracid
AZP	Azaspiracid Shellfish Poisoning
BTX	Brevetoxin
b.w.	Body weight
CCFFP	Codex Committee for Fish and Fishery Products
CCMAS	Codex Committee on Methods of Analysis and Sampling
C-CTX	Carribean ciguatoxin
CDC	Centers for Disease Control and Prevention
CFP	Ciguatera fish poisoning
CI	Cyclic imine
CONTAM Panel	Panel on Contaminants in the Food chain
CRL	Community Reference Laboratory
CTX	Ciguatoxin
DA	Domoic acid
DG SANCO	Health and Consumer Protection Directorate General
DSP	Diarrhoeic Shellfish Poisoning
DTX	Dinophysis toxins
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EFSA	European Food Safety Authority
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FAO/IOC/WHO	Food and Agriculture Organization of the United Nations/ Intergovernmental
Oceanographic	Commission of UNESCO/World Health Organization
FLD	fluorescence detection
FSANZ	Food Standards Australia New Zealand
GI	Gastrointestinal
GTX-4A	Gambiertoxin-4A
GTX-4B	Gambiertoxin-4B
HPLC	High-performance liquid chromatography
HPLC-FLD	High-performance liquid chromatography-fluorescence detection
I-CTX	Indian Ocean ciguatoxin
IOC	Intergovernmental Oceanographic Commission of UNESCO
i.p.	Intraperitoneal
JMPR	Joint FAO/WHO Meetings on Pesticide Residues
LC	Liquid chromatography
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
MAB	Monoclonal antibody
MBA	Mouse bioassay

MHWL	Ministry of Health, Welfare, and Labour
MIA	Membrane immunobead assay
MS	Mass spectrometry
MTS	3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan
MU	Mouse unit
Na	Sodium
Na_V	Voltage-gated sodium channel
NOAA	National Oceanic Atmospheric Administration laboratories
NOAEL	No-observed-adverse-effect level
OA	Okadaic acid
OH	Hydroxy
OJ	Official Journal of the European Union
P95	95^{th} percentile
P97.5	97.5^{th} percentile
P-CTX	Pacific ciguatoxin
PlTX	Palytoxins
PMS	Phenazine methosulfate
PSP	Paralytic shellfish poisoning
PTX	Pectenotoxin
RBA	Rat bioassay
RIA	Radioimmunoassay
SEARO	Regional Office for South-East Asia
SM	Shellfish meat
SPE	Solid Phase Extraction
STX	Saxitoxin
T	Time of death, in hours
TDI	Tolerable daily intake
TEF	Toxicity equivalency factor
UK	United Kingdom
UNESCO	United Nations Educational, Scientific and Cultural Organization
US FDA	U.S. Food and Drug Administration
UV	Ultraviolet
WG	Working group
WHO	World Health Organization
WHO/SEARO	World Health Organization/Regional Office for South-East Asia
YTX	Yessotoxin