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SCIENTIFIC OPINION

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

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

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ABSTRACT

The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) assessed the risks to human health related to the consumption of brevetoxin-(BTX) group toxins in shellfish and fish. They are marine biotoxins which can accumulate in shellfish and fish. BTX-group toxins are primarily produced by the dinoflagellate *Karenia brevis* and cause neurologic shellfish poisoning (NSP). Symptoms and signs of NSP include e.g. nausea, vomiting, diarrhoea, parasthesia, cramps, bronchoconstriction, paralysis, seizures and coma. To date BTX-group toxins have not been reported in shellfish or fish from Europe and currently there are no regulatory limits for BTX-group toxins in shellfish or fish in Europe. The toxicological database for BTX-group toxins is limited, comprising mostly acute toxicity studies. In view of the acute toxicity and the lack of chronic toxicity data for BTX-group toxins, the CONTAM Panel considered that an acute reference dose (ARfD) should be established but due to the lack of data this was not possible. There is some evidence that BTX-2 forms DNA adducts. This raises concern about its potential carcinogenicity and consequential long term effects. Due to the lack of occurrence data on shellfish or fish in Europe, the limited data on acute toxicity and the lack of data on chronic toxicity, the CONTAM Panel could not comment on the risk associated with the BTX-group toxins in shellfish and fish that could reach the European market. The mouse bioassay (MBA) has traditionally been used to detect BTX-group toxins. However, due to poor specificity and ethical concerns it is not considered an appropriate method. *In vitro* and immunoassays have been developed as alternative, but they need further development. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods would be of value for the quantification of BTX-group toxins, but certified reference materials are needed to allow further method development and (interlaboratory) validation.

KEY WORDS

Marine biotoxins, brevetoxin (BTX)-group toxins, shellfish, fish, acute reference dose, methods of analysis, human health, risk assessment.

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SUMMARY

Brevetoxin-(BTX) group toxins are marine biotoxins which can accumulate in shellfish and fish. They are primarily produced by a dinoflagellate *Karenia brevis*. BTX-group toxins are lipid-soluble cyclic polyether compounds, which are grouped in two types of chemical structures (A and B), based on their backbones. A number of BTX-group toxins have been identified. BTX-2 (type B) is reported to be the most abundant BTX-group toxin in *K. brevis*. BTX-1 (type A) and BTX-2 are considered to be the parent toxins from which other BTX-group toxins derive. BTX-group toxins are metabolised in shellfish and fish and several metabolites of BTX-group toxins have been characterised. Consumers of contaminated shellfish and fish are thus primarily exposed to BTX-group toxin metabolites rather than parent algal BTX-group toxins.

BTX-group toxins cause neurologic (neurotoxic) shellfish poisoning (NSP). Symptoms and signs of NSP include e.g. nausea, vomiting, diarrhoea, parasthesia, cramps, bronchoconstriction, paralysis, seizures and coma. They typically occur within 30 minutes to 3 hours of consuming contaminated shellfish and last for a few days. Persistent symptoms and fatalities have not been reported. Dermal or inhalation exposure can result in irritant effects. NSP appears to be limited to the Gulf of Mexico, the east coast of the United States of America (U.S.A.), and the New Zealand Hauraki Gulf region.

To date BTX-group toxins have not been reported in shellfish or fish from Europe. However, the discovery of new BTX-group toxin producing algae and the apparent trend towards expansion of algal bloom distribution, suggest that BTX-group toxins could also emerge in Europe. Currently there are no regulatory limits for BTX-group toxins in shellfish or fish in Europe.

The toxicological database for BTX-group toxins is limited and comprises only studies on their acute toxicity following intravenous (*i.v.*), intraperitoneal (*i.p.*) and oral administration. BTX-group toxins bind to the voltage-gated sodium channels in cell membrane thereby causing depolarization of neuronal and muscle cell membranes.

There are several indications of clastogenic activity (chromosomal aberrations and DNA damage) of BTX-group toxins *in vitro*. BTX-2 also induced DNA damage *in vivo*. Neither BTX-2 nor BTX-6 was mutagenic in a reverse mutation assays, but there is evidence that BTX-2 forms DNA adducts in isolated rat lung cells treated *in vitro* and in lung tissue following intratracheal administration to rats. These observations raise concern about potential carcinogenicity of BTX-2 and its consequential long term effects.

There are no long term studies on BTX-group toxins in experimental animals that would allow establishing a tolerable daily intake (TDI). In view of the acute toxicity of BTX-group toxins the Panel on Contaminants in the Food Chain (CONTAM Panel) considered that an acute reference dose (ARfD) should be established for BTX-group toxins. However, due to the limited quantitative data both in experimental animals and related to human intoxications, the CONTAM Panel concluded that the establishment of an oral ARfD was not possible.

Due to the lack of occurrence data on shellfish or fish in Europe, the limited data on acute toxicity and the lack of data on chronic toxicity, the CONTAM Panel could not comment on the risk associated with the BTX-group toxins in shellfish and fish that could reach the European market.

The mouse bioassay (MBA) has traditionally been used to detect BTX-group toxins in shellfish. However, for reasons of animal welfare there is a growing concern with respect to its use. The MBA has shown poor specificity and some of the BTX-group toxins are not efficiently extracted by the standard method. Therefore it is not considered an appropriate detection method for BTX-group toxins. Alternative assays such as *in vitro* and immunoassays have shown to be able to detect BTX-group toxins in shellfish and fish extracts. Although like the MBA they do not provide information on toxin profiles, they could be further developed to be applied as screening methods for BTX-group toxins. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods allow specific

detection of individual BTX-group toxins and they would be of value for their quantification in shellfish and fish. None of the current methods of analysis to determine BTX-group toxins in shellfish and fish has been formally validated in interlaboratory studies. The CONTAM Panel noted that certified reference materials for toxicologically relevant BTX-group toxins need to be provided to allow further method development and (interlaboratory) validation.

TABLE OF CONTENTS

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, p. 55-205.
[6] OJ L 338, 22.12.2005, p. 27-59.
[7] OJ L 139, 30.4.2004, p. 206-320.
[8] OJ L 358, 18.12.1986, p. 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 rat bioassay (RBA) for lipophilic marine biotoxins. Alternative test methods can be applied if they are validated following an internationally recognised protocol and provide an equivalent level of public health protection.

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

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

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

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

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

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

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

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

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

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

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

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

SM: shellfish meat; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level; N/A: not available; EU: European Union; (a): Person with 60 kg body weight (b.w.)

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

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

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

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

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

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

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

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

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

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

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

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

ASSESSMENT

1. Introduction

Brevetoxin-(BTX) group toxins are marine biotoxins which can accumulate in shellfish and fish. They are primarily produced by a dinoflagellate *Karenia brevis* (formerly called *Gymnodinium breve* and *Ptychodiscus brevis*) first identified in the Gulf of Mexico in 1947 (Gunter et al., 1947; Davis, 1948). A number of BTX-group toxins have been isolated and identified. Based on their molecular backbone structures they are grouped into types A and B (Figure 1). BTX-2 (or PbTx-2)[13] (type B) is reported to be the most abundant BTX-group toxin in *K. brevis* (Landsberg, 2002; Plakas and Dickey, 2010). BTX-1 (or PbTx-1)[13] (type A) and BTX-2 are considered to be the parent toxins from which other BTX-group toxins derive (Baden et al., 2005). BTX-group toxins are metabolised in shellfish and fish, and several metabolites of BTX-group toxins have been isolated and characterised (Ishida et al., 1995, 1996, 2004a; Murata et al., 1998; Morohashi et al., 1995; 1999; Dickey et al., 1999; Plakas et al., 2002; Wang et al., 2004; Abraham et al., 2006). Some other algae species (*Chattonella antiqua, Chattonella marina, Fibrocapsa japonica, Heterosigma akashiwo*) have also been reported to produce BTX-like toxins (FAO, 2004).

BTX-group toxins cause neurologic (neurotoxic) shellfish poisoning (NSP), which is characterised by mainly neurological and gastrointestinal effects. The symptoms and signs include e.g. nausea, vomiting, diarrhoea, parasthesia, cramps, bronchoconstriction, paralysis, seizures and coma (FAO/IOC/WHO, 2004; Watkins et al., 2008). NSP appears to be limited to the Gulf of Mexico, the east coast of the United States of America (U.S.A.), and the New Zealand Hauraki Gulf region. However, the discovery of new BTX-group toxin producing algae and the apparent trend towards expansion of algal bloom distribution suggest that BTX-group toxins are emerging in other regions in the world (FAO/IOC/WHO, 2004). To date BTX-group toxins have not been reported in shellfish or fish from Europe. There are indications that also other marine biotoxins such as gambiertoxin, tetrodotoxin, maitotoxin, gliotoxin and peptaibols are emerging in either shellfish or fish in other regions in the world including Europe (Grovel et al., 2003; Poirier et al., 2007; Cassinay, 2008; Kerzaon et al., 2008; Otero et al., 2010). It has been reported that BTX-group toxins can cause significant mortalities in fish, sea birds and marine mammals (Flewelling et al., 2005; Plakas and Dickey, 2010; Shen et al., 2010).

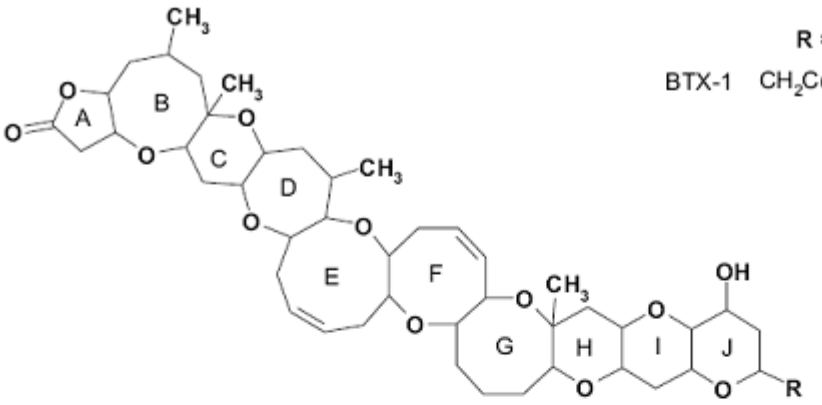
2. Chemical characteristics

BTX-group toxins are lipid-soluble cyclic polyether compounds. Based on their molecular backbone structures two types of BTX-group toxins can be differentiated, named type A and type B, sometimes also called type 1 and type 2, respectively. The molecular structure consists of 10 to 11 transfused rings (Figure 1) (Baden, 1989; FAO/IOC/WHO, 2004).

Several BTX-group toxins and metabolites of BTX-group toxins in algae, shellfish and fish have been isolated and characterised (Shimizu et al., 1986; Ishida et al. 1995, 1996, 2004a,b; Morohashi et al. 1995, 1999; Murata et al., 1998; Dickey et al., 1999; Plakas et al., 2002; Wang et al. 2004, Abraham et al., 2006; Fire et al., 2008). BTX-1 and BTX-2 are considered to be the parent toxins from which other BTX-group toxins derive (Baden et al., 2005). BTX-group toxins are metabolised in shellfish, in particular BTX-2 (Abraham et al., 2008; Plakas and Dickey, 2010). Consumers of contaminated

[13] In the older literature BTX-group toxins are abbreviated as PbTx, while most of their metabolites are abbreviated as BTX. In the more recent literature BTX is also used for PbTx (Abraham et al., 2008; Plakas and Dickey, 2010).

shellfish and fish are thus primarily exposed to BTX-group toxin metabolites rather than parent algal BTX-group toxins.



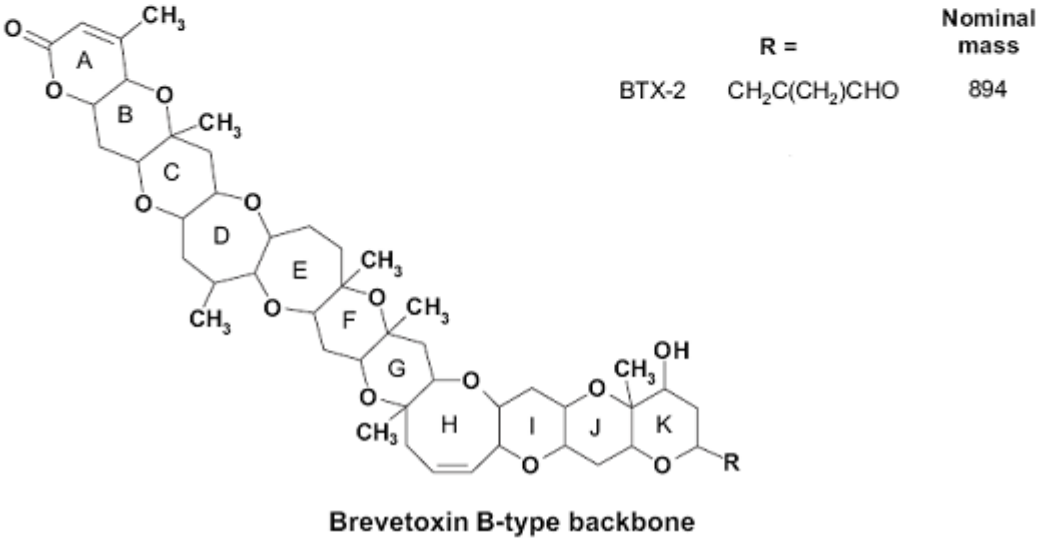


Figure 1: Chemical structures of BTX-group toxins (A- and B-type backbone structures) (from Abraham et al., 2008).

The major metabolites of BTX-2 identified in shellfish include e.g. BTX-3 (Radwan and Ramsdell, 2006), BTX-B1 (Ishida et al., 1995), BTX-B2 (Murata et al., 1998), S-desoxy-BTX-B2 (Plakas et al., 2002), BTX-B3 (Morohashi et al., 1995), BTX-B4 (Morohashi et al., 1999) and BTX-B5 (Ishida et al., 2004b) (Table 2). Probable metabolites of BTX-1 in oysters have been reported by Plakas et al. (2002) and Wang et al. (2004).

Table 2: Chemical forms of the major metabolites of BTX-2 (type B).

Abbreviation	Chemical form
BTX-3	Reduced form of BTX-2
BTX-6	H-ring epoxide of BTX-2
BTX-9	α-methylene reduced BTX-3
BTX-B1	Taurine conjugate of oxidized BTX-2
BTX-B2	Cysteine sulfoxide conjugate of BTX-2
S-desoxy-BTX-B2	S-desoxy form of BTX-B2
BTX-B3	Fatty acid of the opened D-ring of BTX-2 backbone with oxidation of the aldehyde terminus
BTX-B4	N-myristoyl and N-palmitoyl conjugates of the cysteine sulfoxide moiety of BTX-B2
BTX-B5	Oxidised form of the terminal aldehyde of BTX-2

BTX-group toxins are stable compounds, resistant to heat and steam autoclaving (Poli, 1988). Both acid and alkaline hydrolyses can lead to reversible lactone ring opening of the BTX-molecule and alkaline hydrolysis proceeds faster than acid hydrolysis (Hua and Cole, 1999).

3. Regulatory status

Currently there are no regulations on BTX-group toxins in shellfish or fish in Europe. However, some countries in other regions of the world have set action levels or maximum levels for BTX-group toxins in shellfish. In the U.S.A. the action level is 20 mouse units (MUs)/100 g (0.8 mg BTX-2 equivalents/kg shellfish) (U.S. FDA, 2001). In New Zealand and Australia the maximum level for BTX-group toxins is 20 MUs/100 g, but the BTX analogue is not specified (NZFSA, 2006; FSANZ, 2010).

4. Methods of analysis

Mouse bioassay (MBA), cytotoxicity assays, receptor binding assays, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISA) and liquid chromatography-tandem mass spectrometric (LC-MS/MS) methods have been used for the determination of BTX-group toxins in algae, shellfish and fish.

4.1. Supply of appropriate reference material

Certified reference standards are commercially available for BTX-2 and BTX-3. However, the analysis of shellfish and fish for BTX-group toxins is complicated by the fact that suitable certified reference materials are not commercially available.

4.2. Mouse bioassay

MBA has been historically used for detecting BTX-group toxins in shellfish. In the standard method (APHA, 1970) the BTX-group toxins are extracted from shellfish with diethyl ether. The results are reported as MUs per 100 g shellfish meat. One MU has been defined as that amount of crude toxin (injected intraperitoneally (*i.p.*)) that will kill 50 % of the mice (20 g weight) in 930 minutes. One MU is approximately 4 µg BTX-2 equivalents. Several studies have demonstrated that diethyl ether does not efficiently extract some of the BTX-group toxins (e.g. cysteine conjugates) (Dickey et al., 1999; Naar et al., 2002; Nozawa et al., 2003).

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:

- diethyl ether is not an efficient extraction solvent for some of the BTX-group toxins;
- no specific information is provided on individual BTX-group toxins;
- it cannot be automated;
- it requires specialised animal facilities and expertise;
- the inherent variability in results between laboratories due to e.g. specific animal characteristics (strain, sex, age, weight, general state of health, diet, stress);
- it has not been validated;
- in many countries the use of the MBA is considered undesirable for ethical reasons.

4.3. *In vitro* assays

4.3.1. Cytotoxicity assays

Several cytotoxicity assays using several endpoints and detection methods have been developed for the determination of BTX-group toxins in shellfish (Manger et al., 1993, 1994, 1995; Trainer et al., 1995; Fairey et al., 1997; Dickey et al., 1999; Louzao et al., 2004; Bottein Dechraoui et al., 2007; Dechraoui Bottein et al., 2010). The cytotoxicity assay is based on the action of BTX-group toxins on voltage-gated sodium channels. In the most typical cytotoxicity assay neuroblastoma cells are pre-treated with veratridine to promote transition of voltage-gated sodium channels to the open state and ouabain (Na^+K^+ ATPase inhibitor) to block the sodium-potassium pump activity. BTX-group toxins bind to veratridine-activated voltage-gated sodium channels and increase the ion flux. The cell viability is measured by using the metabolic reduction of a tetrasodium dye (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium (MTT)) to its formazan product (Manger et al., 1993, 1994; Plakas and Dickey, 2010). Manger et al. (1993) reported a limit of detection (LOD) for BTX-1 of 0.25 mg/kg shellfish. Dickey et al. (2004) reported that results of the cytotoxicity assay were highly variable in an interlaboratory study.

4.3.2. Receptor binding assay

The receptor binding assay is based on the affinity of BTX-group toxins for the sodium channel receptor. In the receptor binding assay most often used, BTX-group toxins are determined in shellfish and fish extract based on binding competition between radioactive ^{3}H-BTX-3 and native BTX-group toxins for receptor sites. Isolated membrane preparations obtained from excitable tissues or whole-cell preparations are used (Van Dolah et al., 1994; Trainer and Poli, 2000; Bottein Dechraoui et al., 2007; Fire et al., 2008). The affinity of cysteine conjugates (BTX-B2, S-desoxy-BTX-B2) for the sodium channel receptor has been shown to be 3-10 times less than BTX-3 (Poli et al., 2000; Bottein Dechraoui et al., 2007). The limit of quantification (LOQ) of the receptor binding assay has been reported to be 30 µg BTX-3 equivalents/kg oyster homogenate (FAO/IOC/WHO, 2004).

The main advantages of *in vitro* assays are:

- some endpoints are related to mode of action of the BTX-group toxins;
- the use of microplate format enables multiple samples to be analysed in a single run.

The main disadvantages of *in vitro* assays are:

- they do not provide any information on the toxin profile;
- they are prone to interferences from other substances acting on sodium channels;
- facilities are needed for maintenance and handling of cell cultures and/or radioactive materials;
- they have not been validated.

4.4. Immunoassays

Both RIAs and ELISAs have been applied to detect BTX-group toxins in shellfish and fish (Baden et al., 1988; Levine and Shimizu, 1992; Baden et al. 1995; Poli et al., 1995; Naar et al., 1998, 2002; Trainer and Poli, 2000; Poli et al., 2007; Fire et al., 2008; Plakas et al., 2008, Zhou et al., 2010). Naar et al. (2002) developed a competitive ELISA to measure BTX-group toxins in shellfish. Goat anti-BTX antibodies were used, with a high specificity for type-B BTX-group toxins. An LOD of 25 µg/kg for BTX-group toxins in spiked oysters was reported (Naar et al., 2002). For the competitive chemiluminescence-based ELISA, a lower LOD for BTX-group toxins in shellfish (1 µg/kg) was reported (Poli et al., 2007). The applied antibody had also cross-reactivity for cysteine conjugates of BTX-2 (S-desoxy-BTX-B2 and BTX-B2) (Plakas et al., 2004; Bottein Dechraoui et al., 2007; Maucher et al., 2007). Recently Zhou et al. (2010) reported a new competitive ELISA for BTX-toxins based on bovine serum albumin and human gamma globulin, and Zhou et al. (2009) reported a one-step immunochromatographic assay using a colloidal gold-labelled monoclonal antibody probe for the detection of BTX-group toxins in shellfish.

The main advantages of immunoassays are:

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

The main disadvantages of immunoassays are:

- use of radioactive materials in radioimmunoassay;
- they do not provide any information on the toxin profile;
- due to cross-reactivities positive results need to be confirmed;
- they have not been validated.

4.5. LC-MS/MS

LC-MS/MS methods have been extensively used for the characterisation and determination of BTX-group toxins in shellfish, fish and algae (Hua et al., 1995; Dickey et al., 1999; Hua and Cole, 2000; Plakas et al., 2002, 2004; Nozawa et al. 2003; Wang et al., 2004; Dickey et al., 2004; Ishida et al., 2004b,c, 2006; Pierce et al., 2006; Fire et al., 2008; Plakas et al., 2008; Wang and Cole, 2009). Dickey et al. (1999) and Hua and Cole (2000) used LC-MS/MS with electrospray ionisation (ESI) mode to determine BTX-group toxins in shellfish. Ishida et al. (2004b,c, 2006) applied LC-MS/MS with ESI in positive and negative ion modes using selected reaction monitoring (SRM) for determination of major shellfish metabolites of BTX-group toxins. They reported LOQs to be at a sub µg/kg shellfish level. Nozawa et al. (2003) reported LODs of 0.4 µg/kg for BTX-B1 and 2 µg/kg for BTX-3 in shellfish. Ishida et al. (2004a) reported LOQs of 2, 0.2, 0.4 and 0.4 µg/kg for BTX-2, BTX-3, BTX-B5 and BTX-B1 in shellfish, respectively.

In an interlaboratory study, Dickey et al. (2004) tested the performance of LC-MS/MS. In practice, all LC-MS/MS methods easily measured BTX-3 in spiked samples at levels at least one order of magnitude below the U.S.A. action level of 20 MUs/100 g or 0.8 mg BTX-2 equivalents/kg shellfish (U.S. FDA, 2001).

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

- very specific and thus superior to be used as confirmatory methods;
- they can screen and measure BTX-group toxins individually.

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

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

4.6. Proficiency tests

There are no ongoing proficiency tests for BTX-group toxins.

4.7. Summary of methods

The MBA has traditionally been used to detect BTX-group toxins in shellfish but for reasons of animal welfare there is growing concern with respect to its use and it has shown poor specificity as some BTX-group toxins are not efficiently extracted by the standard method. *In vitro* and immunoassays have been shown to be able to detect BTX-group toxins in shellfish and fish extract. However, like the MBA they cannot provide specific information about toxin profiles. Recent studies have focussed on the development of LC-MS/MS methods for the detection and quantification of BTX-group toxins. LC-MS/MS methods would be of value for the determination of BTX-group toxins, pending optimisation of these methods for application to shellfish and fish extracts, their (interlaboratory) validation and the development of standards and reference materials.

5. Occurrence of BTX-group toxins

So far, no NSP outbreaks in humans or occurrence of BTX-group toxins in shellfish or fish have been reported in Europe. However, the discovery of new BTX-group toxin producing algae and the apparent trend towards expansion of algal bloom distribution (FAO/IOC/WHO, 2004), suggest that BTX-group toxins could also emerge in Europe. The occurrence experienced in other areas may therefore provide some information with respect to possible contamination of shellfish and fish with BTX-group toxins.

In areas of the world affected by NSP, measurements of BTX-group toxins in shellfish or fish have been performed. For example, the concentrations of BTX-group toxins in shellfish have been reported to range from 880 to 49 000 µg BTX-2 equivalents/kg, using the conversion of one MU being 4 µg BTX-2 equivalents (BTX-group toxins in contaminated shellfish have been mainly reported in MUs/100 g) (Cummins et al., 1971; Tester and Fowler, 1990; Steidinger et al., 1998; Pierce et al., 2004; Naar et al., 2007; Watkins et al., 2008). Naar et al. (2007) reported results from analyses performed in Florida. Concentrations of BTX-group toxins in fish measured by competitive ELISA using a BTX-3 standard, varied from 580 to 6 000 µg BTX-3 equivalents/kg. The Panel on Contaminants in the Food Chain (CONTAM Panel) noted, however, that the number of BTX-group toxins reported depends on the analytical methods used and the available standards.

5.1. Influence of processing

There are no data to draw conclusions on the influence of processing on the levels of BTX-group toxins in shellfish or fish.

6. Exposure assessment

Due to the lack of occurrence data, exposure to BTX-group toxins can not be estimated for the European population.

7. Toxicokinetics

Toxicokinetic studies for BTX-group toxins are limited to BTX-2 and BTX-3. Following *i.p.* administration of BTX-2 and BTX-3 to rats peak blood levels were reached after one hour, being about three times higher for BTX-2 then for BTX-3. BTX-2 is excreted within 24 hours largely in urine, in the form of a cysteine conjugate (Radwan et al., 2005). BTX-3 intravenously administered to rats is cleared from the circulation within one minute and eliminated within 24 hours primarily through biliary excretion in the faeces (Poli et al., 1990). Following oral administration, BTX-3 is rapidly and substantially absorbed and widely distributed to all organs with the highest concentration found in the liver. Elimination is approximately equally distributed between urine and faeces (Cattet and Geraci, 1993; FAO/IOC/WHO, 2004).

Both in mice and human plasma, BTX-3 binds to lipoproteins and particularly to high-density lipoproteins (HDLs) (Woofter et al., 2005, 2007). In urine specimens from patients diagnosed with NSP a number of metabolites including BTX-3, methylsulfoxy-BTX-3, 27-epoxyBTX-3, cysteine conjugates, reduced BTX-B5 were identified using LC-MS/MS. Several minor hydrolysis metabolites of BTX-1 were also identified (Abraham et al., 2008).

Because inhalation can also be an important route of exposure leading to NSP, the toxicokinetics of ^{3}H-BTX-3 administered by intratracheal instillation in male F344/Crl BR rats and male CBA/CaJ mice was investigated. In rats, 80 % of the intratracheal dose was rapidly absorbed from the lung to the blood and distributed to all tissues. The majority of the BTX-3 was cleared rapidly from lung, liver, and kidneys with approximately 20 % of the initial concentration in each organ retained for 7 days (Benson et al., 1999). In mice, the elimination half-life ranged from approximately 28 hours for fat, heart, intestines, kidneys, liver, and muscle to approximately 90 hours for brain and testes. Approximately 90 % of excretion occurred within 96 hours, with 11 % and 64 % excreted in urine and feces, respectively (Tibbetts et al., 2006).

8. Toxicity data

The potency of BTX-group toxins depends on two factors: the affinity of the toxin for its target and the efficacy of that binding to elicit a response in target cells (Ramsdell, 2008).

8.1. Mechanistic considerations

BTX-group toxins bind with high affinity to receptor site 5 of the voltage-gated sodium channels (Na_V) in cell walls (Baden et al., 2005). Binding leads to the activation of Na_V, uncontrolled Na^+ influx into cells and depolarization of neuronal and muscle cell membranes (Watkins et al., 2008). In primary cultures of rat cerebellar granule neurons the activation of receptor site 5 of Na_V, by BTX-group toxins leads to acute neural injury and cell death through an increase in intracellular Ca^{2+} with a 2-fold larger response for BTX-1 compared to BTX-2 or BTX-3 (Le Page et al., 2003). Evidence suggests that BTX-group toxins can affect mammalian cortical synaptosomes and neuromuscular preparations and possibly mast cells. All these effects have been associated with substantial and persistent membrane depolarisation (FAO/IOC/WHO, 2004). Respiratory problems, associated with the inhalation of aerosolised BTX-group toxins, are also due to the opening of Na_Vs on nerve cell membranes by these toxins (FAO/IOC/WHO, 2004).

In rat brain membrane preparations and in HEK cells expressing skeletal muscle or cardiac Na_Vs BTX-B2 and desoxyBTX-B2 showed a 8-16-fold lower affinity than BTX-3. In neuroblastoma cells, their potency was 3- and 8-fold lower than BTX-3, respectively (Bottein Dechraoui et al., 2007). BTX-group toxin activation of Na_V has also been shown in immune cells leading to other biological responses such as cell proliferation, gene transcription, cytokine production and apoptosis (Murrell and Gibson, in press).

8.2. Effects in laboratory animals

8.2.1. Acute toxicity

BTX-group toxins induce central depression of respiratory and cardiac function, spontaneous repeated dose-dependent muscular contractions resulting in fasciculations, twitching or leaping, a precipitous dose-dependent depression in respiratory rate, a bronchoconstrictor response that is central and peripheral in origin, and copious rhinorrhea (Baden, 1989).

8.2.1.1. Toxicity following intraperitoneal (*i.p.*) and intravenous (*i.v.*) administration

Acute toxicity data on BTX-group toxins and metabolites are limited and are presented in Table 3. No lethal dose (LD_{50}) data are available for BTX-1. Acute toxicity in mice for BTX-2 and BTX-3 was first reported by Baden and Mende (1982) with 24-hour LD_{50} of 200 and 170 mg/kg body weight (b.w.), respectively. Selwood et al. (2008) reported an *i.p.* LD_{50} for BTX-3 of 250 mg/kg b.w. The minimum LD_{50} of BTX-B1 administered *i.p.* in mice was 50 mg/kg b.w. (Ishida et al., 1995). The reported LD_{50} for BTX-B2 was 306 mg/kg b.w. (Murata et al., 1998). Using semi-synthetic standards, Selwood et al. (2008) reported LD_{50} values of 400, 250 and 211 mg/kg b.w. for BTX-B2, S-desoxy-BTX-B2 and for BTX-3, respectively. BTX-B3 was not toxic at doses up to 300 mg/kg *i.p.* in mice whereas the minimum lethal doses for BTX-B4 and BTX-B5 were 100 and 300-500 mg/kg b.w., respectively (Morohashi et al., 1995; Ishida et al., 2004b).

Table 3: Acute toxicity of BTX-group toxins after intravenous (*i.v.*) and intraperitoneal (*i.p.*) administration in mice (adapted from Plakas and Dickey (2010)).

Compound	Route	Toxicity endpoint µg/kg b.w.	Reference
BTX-2	*i.v.*	LD_{50} 200	Baden and Mende (1982)
BTX-3	*i.v.*	LD_{50} 94	Baden and Mende (1982)
BTX-2	*i.p.* 24 hours	LD_{50} 200	Baden and Mende (1982)
BTX-3	*i.p.* 24 hours	LD_{50} 170	Baden and Mende (1982)
BTX-3	*i.p.* 24 hours	LD_{50} 250	Selwood et al. (2008)
BTX-B2	*i.p.* 24 hours	LD_{50} 400	Selwood et al. (2008)
S-deoxy-BTX-B2	*i.p.* 24 hours	LD_{50} 211	Selwood et al. (2008)
BTX-B1	*i.p.* <2 hours	MLD 50	Selwood et al. (2008)
BTX-B2	*i.p.* <1 hour	MLD 306	Murata et al. (1998)
BTX-B3	*i.p.* 24 hours	MLD >300[(a)]	Morohashi et al. (1995)
BTX-B4	*i.p.* 6-24 hours	MLD 100	Morohashi et al. (1999)
BTX-B5	*i.p.*[(b)]	MLD 300-500	Ishida et al. (2004b)

i.v.: intravenous; *i.p.*: intraperitoneal; LD_{50}: dose lethal dose resulting in 50 % death of the mice, MLD: minimum lethal dose; (a): no death at this dose; (b): time to death not reported

Signs of intoxication after *i.p.* injection in mice have been described for BTX-B2, BTX-3 and S-desoxy-BTX-B2 and are similar for these compounds. At lethal *i.p.* doses, signs included: immobility after 15 minutes, respiration paralysis, exophthalmia and rapid flicking movements of the hind legs immediately before death. No macroscopic lesions were observed at necropsy. At sublethal doses, fast abdominal breathing was noticed directly after injection followed by a precipitate drop of the respiration rate, limbs appeared to be completely paralysed, but movement was regained after 3-5 hours. No gross pathological abnormalities were recorded at the end of the 7-day observation period (Baden, 1989; Selwood et al., 2008). After *i.v.* administration toxic signs and death were observed almost instantly.

8.2.1.2. Toxicity following oral administration

LD_{50} values after oral administration in mice are only available for BTX-2 (6600 mg/kg b.w.) and BTX-3 (520 mg/kg b.w.). In contrast to *i.v*. and *i.p*. administration where toxic signs including death occurred almost instantly, after oral administration they occurred approximately after 5 hours. The oral potency of BTX-2 is more than one order of magnitude lower than that of BTX-3 (Baden and Mende, 1982). The authors suggest that the difference in oral toxicity between BTX-2 and BTX-3 might be due to differences in their rate of absorption rather than first pass metabolism of BTX-2 in the liver.

8.3. Relative potency of analogues

Based on *i.p.* toxicity BTX-2, BTX-3, BTX-B2 and S-deoxy-BTX-B2 appear to have similar toxic potencies. Other BTX-group toxin analogues could not be evaluated due to lack of adequate data. The CONTAM Panel noted, however, that based on the limited oral toxicity data it appears that the toxicity of BTX-3 is about 10 fold higher than that of BTX-2.

8.4. Genotoxicity

Results of several studies indicated the clastogenic activity of BTX-group toxins *in vitro*. BTX-2 induced chromosomal aberrations in Chinese hamster ovary cells (Sayer et al., 2006). DNA-damage (strand breaks) was found with the comet assay after incubation of human lymphocytes with BTX-2, BTX-3 and BTX-9 (Sayer et al., 2005). DNA damage was also found in Jurkat E6-1 cells following incubation with BTX-2, BTX-3 and BTX-6 (Murell and Gibson, 2009). Leighfield et al. (2009) reported that BTX-2 induced DNA-damage in liver cells of rats following intratracheal exposure, indicating that BTX-2 is also clastogenic *in vivo*.

BTX-2 has been shown to form DNA adducts with cytidine in rat lung fibroblasts *in vitro* and with adenosine and guanosine in lung cells after intratracheal exposure of rats. No adducts were detected *in vitro* and *in vivo* following exposure to BTX-2 epoxide (BTX-6) (Radwan and Ramsdell, 2008). Neither BTX-2 nor BTX-6 was mutagenic in a reverse mutation assay (Ames test) with TA-98 and TA-100, in the presence or absence of metabolic activation (Leighfield et al., 2009).

9. Observations in humans

BTX-group toxins cause NSP, which is characterised by mainly neurological and gastrointestinal effects. The symptoms and signs include nausea, vomiting, diarrhoea, chills, sweats, reversal of temperature sensation, hypotension, arrhythmia, parasthesia of the lips, face and extremities, cramps, bronchoconstriction, paralysis, seizures and coma. They typically occur within 30 minutes to 3 hours of consuming contaminated shellfish and last for a few days. Reports of persistent symptoms or mortality have not been identified. Dermal or inhalation exposure can result in irritant effects (FAO/IOC/WHO, 2004; Watkins et al., 2008). Most outbreaks of NSP have occurred in the coastal states around the Gulf of Mexico and in New Zealand. In most outbreaks there is no information on the concentration of BTX-group toxins present in contaminated shellfish, and a no-observed effect level in humans has not been defined. During an outbreak in North Carolina in 1987, 48 of 85 persons who had eaten oysters developed symptoms of NSP, with the number of cases increasing with the number of oysters consumed (Morris et al., 1991). Samples of oyster from two meals eaten by four affected persons were analysed by MBA and found to contain 35 and 60 MUs BTX-group toxins. Samples of oysters harvested from the same general areas also tested positive for BTX-group toxins in the MBA (mean 62 MUs, range 48-170 MUs). The amount of oyster flesh containing this amount of toxins appears to have been 100 g, although this is not clearly stated by Morris et al. (1991). Based on the 35 and 60 MUs BTX-group toxins in the left-over meals and assuming the amounts relate to 100 g shellfish tissue, and that 12 oysters weighing 10 g were consumed, Gessner (2000) estimated a low toxic dose of 42-72 MUs per person. This is equivalent to 168 to 288 µg BTX-2, corresponding to 2.8-4.8 µg/kg b.w. for a 60 kg person. In a few cases in Florida in 2006, toxin levels were reported to

be in excess of 20 mg/kg shellfish, which is far above the U.S. FDA action level of 0.8 mg/kg shellfish (20 MUs/100 g) BTX-2 equivalent (U.S. FDA, 2001; Watkins et al., 2008).

10. 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 view of the acute toxicity of BTX-group toxins the CONTAM Panel considered establishing an acute reference dose (ARfD). Data on oral toxicity in experimental animals are very limited. Limited quantitative data on human intoxications suggest that effects can be encountered at dietary exposure in the range of 2.8-4.8 µg BTX-2/kg b.w. This is similar to the 2-3 µg BTX equivalents/kg b.w. estimated by FAO/IOC/WHO (2004) to be toxic in humans. No information, however, is available on doses not causing toxicity, and it is uncertain whether the estimated BTX intake actually represents the dose experienced by the affected individuals. Therefore, the CONTAM Panel concluded that the available data do not allow the establishment of an oral ARfD for BTX-group toxins.

The CONTAM Panel noted that there are several indications of clastogenic activity (chromosomal aberrations and DNA damage) of BTX-group toxins *in vitro*. BTX-2 also induced DNA damage *in vivo*. Neither BTX-2 nor BTX-6 was mutagenic in a reverse mutation assays, but there is evidence that BTX-2 forms DNA adducts in isolated rat lung cells treated *in vitro* and in lung tissue following intratracheal administration to rats. These observations raise concern about potential carcinogenicity of BTX-2 and its consequential long term effects.

11. Risk characterisation

Due to the lack of occurrence data on shellfish or fish in Europe, the limited data on acute toxicity and the lack of data on chronic toxicity, the CONTAM Panel could not comment on the risk associated with the BTX-group toxins in shellfish and fish that could reach the European market.

12. Uncertainty

There are no data on occurrence of BTX-group toxins in shellfish or fish in Europe and therefore an exposure assessment for the European population was not possible. In addition, there are limited animal toxicity data, and there are insufficient quantitative data on human illness attributed to BTX-group toxins. 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

- BTX-group toxins are produced primarily by the dinoflagellate *Karenia brevis*.
- BTX-group toxins are lipid-soluble cyclic polyether compounds, which are grouped in two types of chemical structures (A and B), based on their backbones.
- BTX-1 (type A) and BTX-2 (type B) are considered to be the parent toxins in algae. In shellfish and fish other BTX-group toxins are derived by metabolism and can accumulate.

- BTX-group toxins cause neurologic (neurotoxic) shellfish poisoning (NSP), which is characterised by mainly neurological and gastrointestinal effects.

Methods of analysis

- The mouse bioassay (MBA) has traditionally been used to detect BTX-group toxins. For reasons of animal welfare and poor specificity the MBA is not considered an appropriate detection method for BTX-group toxins.

- *In vitro* and immunoassays have been shown to be able to detect BTX-group toxins in shellfish and fish extracts. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods allow specific determination of individual BTX-group toxins. They are of particular value for the quantification of BTX-group toxins in shellfish and fish, subject to further development, and the availability of certified reference materials.

- None of the current methods of analysis to determine BTX-group toxins in shellfish or fish has been formally validated in interlaboratory studies.

Occurrence/Exposure

- To date BTX-group toxins have not been reported in shellfish or fish from Europe.

- There is no information available on the influence of processing on BTX-group toxin levels in shellfish or fish.

Hazard identification and characterisation

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

- There is evidence that BTX-2 forms DNA adducts in isolated rat lung cells *in vitro* and in lung tissue following intratracheal administration to rats. This raises concern about its potential carcinogenicity and consequential long term effects.

- There are no long term studies on BTX-group toxins in experimental animals that would allow establishing a tolerable daily intake (TDI).

- In view of the acute toxicity of BTX-group toxins the CONTAM Panel considered that an acute reference dose (ARfD) should be established for the BTX-group toxins. However, due to the limited quantitative data both in experimental animals and related to human intoxications, the CONTAM Panel concluded that the establishment of an oral ARfD was not possible.

Risk characterisation

- The discovery of new BTX-group toxin producing algae and the apparent trend towards expansion of algal bloom distribution, suggest that BTX-group toxins could also emerge in Europe.

- Due to the lack of occurrence data on shellfish or fish in Europe, the limited data on acute toxicity and the lack of data on chronic toxicity, the CONTAM Panel could not comment on the risk associated with the BTX-group toxins in shellfish and fish that could reach the European market.

RECOMMENDATIONS

Methods of analysis

- Certified reference materials for toxicologically relevant BTX-group toxins need to be provided to allow further method development, method validation and reliable application of analytical methodology in control programmes.

- Methods other than the MBA, in particular *in vitro* and immunoassays for screening and LC-MS/MS methods for confirmation, should be further developed and optimised for BTX-group toxins in shellfish and fish. Subsequent (interlaboratory) validation studies are needed.

Occurrence/Exposure

- The potential emergence of BTX-group toxin producing algae should be monitored and analysis of BTX-group toxins in shellfish and fish should be considered in Europe.

Hazard identification and characterisation

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

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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	Brevetoxin
b.w.	Body weight
CCFFP	Codex Committee for Fish and Fishery Products
CCMAS	Codex Committee on Methods of Analysis and Sampling
CI	Cyclic imine
CONTAM Panel	Panel on Contaminants in the Food chain
CRL	Community Reference Laboratory
CTX	Ciguatoxins
DA	Domoic acid
DG SANCO	Directorate General for Health and Consumers
DSP	Diarrhoeic Shellfish Poisoning
DTX	Dinophysis toxins
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
EU	European Union
FAO/IOC/WHO	Food and Agriculture Organization of the United Nations/ Intergovernmental Oceanographic Commission of UNESCO/World Health Organization
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
i.p.	Intraperitoneal
i.v.	Intravenous
JMPR	Joint FAO/WHO Meetings on Pesticide Residues
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LD_{50}	Lethal dose
LOAEL	Lowest-observed-adverse-effect level
LOD	Limit of detection
LOQ	Limit of quantification
MBA	Mouse bioassay
MLD	Minimum lethal dose
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium
MU	Mouse unit
N/A	Not available
Na_V	Voltage-gated sodium channel
NOAEL	No-observed-adverse-effect level
NSP	Neurologic shellfish poisoning
OA	Okadaic acid
OJ	Official Journal of the European Union
PlTX	Palytoxins
PSP	Paralytic shellfish poisoning
PTX	Pectenotoxin
RBA	Rat bioassay
RIA	Radioimmunoassay
SM	Shellfish meat

SRM	Selected reaction monitoring
STX	Saxitoxin
TDI	Tolerable daily intake
U.S.A.	United States of America
WG	Working group
YTX	Yessotoxin