

## SCIENTIFIC OPINION

### Marine biotoxins in shellfish – Saxitoxin group<sup>1</sup>

#### Scientific Opinion of the Panel on Contaminants in the Food Chain

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

Adopted on 25 March 2009

#### PANEL MEMBERS

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#### SUMMARY

Saxitoxin (STX)-group toxins are a group of closely related tetrahydropurines and have been detected in filter-feeding bivalve molluscs such as oysters, mussels, scallops, and clams from various parts of the world. They are mainly produced by dinoflagellates belonging to the genus *Alexandrium*: e.g. *Alexandrium tamarensis*, *A. minutum* (syn. *A. excavata*), *A. catenella*, *A. fraterculus*, *A. fundyense* and *A. cohorticula*. STX-group toxins cause paralytic shellfish poisoning (PSP) in humans, characterised by symptoms varying from a slight tingling sensation or numbness around the lips to fatal respiratory paralysis. In fatal cases respiratory arrest occurs 2 to 12 hours following consumption of shellfish contaminated with STX-group toxins. More than 30 different STX analogues have been identified of which STX, NeoSTX, GTX1 and dc-STX seem to be the most toxic ones.

The toxicological database for STX-group toxins is limited and comprises mostly studies on their acute toxicity following intraperitoneal administration. For monitoring purposes using high performance liquid chromatography (HPLC) techniques toxicity equivalency factors (TEFs) have been applied to express the detected analogues as STX equivalents. Until better information is available the Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) proposes the following TEFs based on acute *i.p.* toxicity in mice: STX = 1, NeoSTX = 1, GTX1 = 1, GTX2 = 0.4, GTX3 = 0.6, GTX4 = 0.7, GTX5 = 0.1, GTX6 = 0.1, C2 = 0.1, C4 = 0.1, dc-STX = 1, dc-NeoSTX = 0.4, dc GTX2 = 0.2, GTX3 = 0.4, and 11-hydroxy-STX = 0.3.

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<sup>1</sup> For citation purposes: Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Saxitoxin Group. *The EFSA Journal* (2009) 1019, 1-76.

Based on available information it can be concluded that the binding of STX-group toxins to voltage-gated sodium channels and the consequent blockade of ion conductance through these channels is the major molecular mechanism of action of this group of toxins on nerves and muscles fibres.

No data on the chronic effects of STX-group toxins in animals or humans were available, so the CONTAM Panel could not establish a tolerable daily intake (TDI). In view of the acute toxicity of STX-group toxins, the CONTAM Panel decided to establish an acute reference dose (ARfD). From the available reports on intoxications in humans, comprising more than 500 individuals, a lowest-observed-adverse-effect-level (LOAEL) in the region of 1.5 µg STX equivalents/kg b.w. could be established. Because many individuals did not suffer adverse reactions at higher intakes it is expected that this LOAEL is close to the threshold for effects in sensitive individuals. Therefore the CONTAM Panel concluded that a factor of 3 was sufficient to move from this LOAEL to an estimated no-observed-adverse-effect level (NOAEL) of 0.5 µg STX equivalents/kg b.w. No additional factor for variation among humans was deemed necessary because the data covered a large number of affected consumers, including sensitive individuals. Thus the CONTAM Panel established an acute reference dose (ARfD) of 0.5 µg STX equivalents/kg b.w.

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

The CONTAM Panel noted that consumption of a 400 g portion of shellfish meat containing STX-group toxins at the current EU limit of 800 µg STX equivalents/kg<sup>2</sup> shellfish meat would result in an intake of 320 µg toxin (equivalent to 5.3 µg/kg b.w. in a 60 kg adult). This intake is considerably higher than the ARfD of 0.5 µg STX equivalents /kg b.w. (equivalent to 30 µg STX equivalents per portion for a 60 kg adult) and is a concern for health.

In order for a 60 kg adult to avoid exceeding the ARfD of 0.5 µg STX equivalents/kg b.w., a 400 g portion of shellfish should not contain more than 30 µg STX equivalents corresponding to 75 µg STX equivalents/kg shellfish meat.

Given the considerable differences in toxin profiles, different number of analogues determined and diverse limits of quantification of analytical methods applied in different European Countries, and the high number of non-quantifiable samples, the CONTAM Panel concluded that there were too many uncertainties for a reliable and representative estimation of dietary exposure to STX-group toxins for EU countries. In addition, the difference in acidic conditions used during the extraction step of the various methods could lead to differences in conversion of STX analogues with low toxicity (low TEF) into STX analogues with high toxicity (high TEF). Therefore the CONTAM Panel could not comment on the risks associated with consumption of shellfish that currently reach the market.

Water loss during household processing (cooking, steaming) of shellfish leads to leaching-out of STX-group toxins from the flesh into the cooking fluid. A reduction in the concentrations

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<sup>2</sup> In the Commission Regulation (EC) No 853/2004 a limit value for paralytic shellfish poison (PSP) of 800 micrograms per kilogram is given. In this opinion the CONTAM Panel adopted this figure as being expressed as µg STX equivalents/kg shellfish meat.

of STX-group toxins of about 40-65 % was observed for lobster hepatopancreas, indicating that more STX-group toxins are leached out during processing than would be expected due to water loss only. It was suggested that the levels of some analogues were more reduced than others due to their lesser adsorption in hepatopancreas matrix components. STX-group toxins are heat stable in shellfish at temperatures relevant for cooking and steaming (about 100°C). Commercial processing such as autoclaving at higher temperatures (115-120°C) may lead to a reduction in the concentration of STX-group toxins in shellfish flesh up to 90 %. This was partly attributed to leaching-out of STX-group toxins, partly to destruction at these high temperatures or to interconversion of STX analogues. The CONTAM Panel concluded, however, that the available information made it difficult to draw firm conclusions on possible interconversion or destruction occurring during commercial processing.

The mouse bioassay (MBA) and the Association of Official Analytical Chemists (AOAC) HPLC method (so-called Lawrence method) are officially prescribed methods in the EU for the detection of STX-group toxins. Both methods have been interlaboratory-validated according to international protocols. They are capable to detect STX-group toxins at the current EU regulatory levels of 800µg STX equivalents/kg shellfish meat. The MBA has a limit of detection of approximately 370 µg STX equivalents/kg shellfish meat. The limit of quantification of the Lawrence method depends on toxin profiles, which may differ in practice. For individual toxins limits of quantification range from 10-80 µg STX equivalents for the different STX-analogues. Stringent reductions of the regulatory limit for STX-group toxins would make it necessary to modify the Lawrence method, so as to reduce its limits of quantification, subsequently followed by re-validation of the revised method, to establish new performance characteristics. In the MBA the extraction of STX-group toxins from shellfish meat is carried out by boiling with hydrochloric acid, whereas in the Lawrence method it is boiling with acetic acid. The CONTAM Panel noted that this difference in extraction conditions may lead to differences in toxin profiles detected and to different results when the analytical data are expressed in STX equivalents/kg. Other methods that have potential to determine STX-group toxins are receptor-based assays, antibody-based methods and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). The biomolecular methods are merely suitable for screening purposes. LC-MS/MS has potential for confirmatory analyses. Neither of these methods has been formally validated yet in interlaboratory studies, following internationally recognised protocols, so their performance characteristics cannot be evaluated and compared with the official methods.

**Key words:** Marine biotoxins, saxitoxin (STX)-group toxins, shellfish, bivalve molluscs, mouse bioassay (MBA), acute reference dose, portion size, methods of analysis, human health, risk assessment.

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## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

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

Based on their chemical structure, the toxins have been classified into eight groups, namely, the azaspiracid (AZA), brevetoxin, cyclic imine, domoic acid (DA), okadaic acid (OA), pectenotoxin (PTX), saxitoxin (STX) and yessotoxin (YTX) groups, as agreed at the Joint FAO/IOC/WHO *ad hoc* Expert Consultation held in 2004<sup>3</sup>. Two additional groups, palytoxins (PITX) and ciguatoxins (CTX), may also be considered. STX and its derivatives cause Paralytic Shellfish Poisoning (PSP), and DA causes Amnesic Shellfish Poisoning (ASP). Diarrhoeic Shellfish Poisoning (DSP) is caused by OA-group toxins (OA and dinophysin toxins (DTX)), and AZA group toxins cause Azaspiracid Shellfish Poisoning (AZP). These toxins can all accumulate in the digestive gland (hepatopancreas) of filter-feeding molluscan shellfish, such as mussels, oysters, cockles, clams and scallops, and pose a health risk to humans if contaminated shellfish are consumed. Marine biotoxin-related illness can range from headaches, vomiting and diarrhoea to neurological problems and in extreme cases can lead to death.

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

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

### 1. Legal framework

In 2004, the purported *EU Hygiene Package* of regulations, bringing together and replacing the existing hygiene regulations for the food sector previously contained in numerous individual vertical Directives was published. In Annex II Section VII Chapter V (2) to Regulation 853/2004/EC<sup>4</sup>, are established maximum levels for ASP, PSP and DSP toxins. Annex III of Commission Regulation No 2074/2005/EC<sup>5</sup> of 5 December 2005 lays down the recognised testing methods for detecting marine biotoxins. Annex II Chapter II (14) to

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<sup>3</sup> [ftp://ftp.fao.org/esn/food/biotoxin\\_report\\_en.pdf](ftp://ftp.fao.org/esn/food/biotoxin_report_en.pdf)

<sup>4</sup> Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. OJ L 139, 30.4.2004, p. 55–205.

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



Regulation (EC) 854/2004<sup>6</sup>, 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<sup>7</sup> makes provision for laws, regulations and administrative provisions for the protection of animals used for experimental and other scientific purposes. This includes the use of live vertebrate animals as part of testing strategies and programmes to detect identify and quantify marine biotoxins. Indeed, the scope of Article 3 of the Directive includes the use of animals for the safety testing of food, and the avoidance of illness and disease.

Directive 86/609/EEC sets out the responsibilities that Member States must discharge. As a result of this use of prescriptive language, Member States have no discretion or flexibility, and most of the provisions of the Directive must be applied in all cases. It is clear that Member States have to ensure that: the number of animals used for experimental and other scientific purposes is reduced to the justifiable minimum; that such animals are adequately cared for; and that no unnecessary or avoidable pain, suffering, distress or lasting harm are caused in the course of such animal use.

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

This latter requirement necessitates the use of minimum severity protocols, including appropriate observation schedules, and the use of the earliest humane endpoints that prevent further suffering, once it is clear that the scientific objective has been achieved, that the scientific objective cannot be achieved, or that the suffering is more than can be justified as part of the test procedure. The 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<sup>5</sup> specifies a mouse bioassay (MBA) for the determination of paralytic shellfish poisoning toxins (PSP) and a MBA or the rat bioassay

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<sup>6</sup> Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. OJ L 139, 30.4.2004, p. 206–320.

<sup>7</sup> Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animal used for experimental and other scientific purposes. OJ L 358, 18.12.1986, p. 1–28.

(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<sup>4</sup> 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<sup>8</sup> 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.

#### 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)<sup>9</sup> for the AZA, OA, STX, DA, and YTX-group toxins, respectively (summarized in the Table 1). The Expert Consultation considered that the database for the cyclic imines, 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

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<sup>8</sup> 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 azaspiracids, respectively.

<sup>9</sup> 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).



additional toxicological data in order to perform more accurate risk assessments and to facilitate validation of toxin detection methods in shellfish.

**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 <sup>9</sup>	Derived Guidance Level/ Max Level based on consumption of 100g (1), 250g (2) and 380g (3)	Limit Value currently implemented in EU legislation
<b>AZA</b>	0.4 (1)	10 (H)	0.04 µg/kg 2.4 µg/adult <sup>a)</sup>	0.024 mg/kg SM (1) 0.0096 mg/kg SM (2) 0.0063 mg/kg SM (3)	0.16 mg/kg SM
<b>BTX</b>			N/A		
<b>Cyclic Imines</b>			N/A		
<b>DA</b>	1,000 (1)	10 (H)	100 µg/kg 6 mg/adult <sup>a)</sup>	60 mg/kg SM (1) 24 mg/kg SM (2) 16 mg/kg SM (3)	20 mg/kg SM
<b>OA</b>	1 (1)	3 (H)	0.33 µg/kg 20 µg/adult <sup>a)</sup>	0.2 mg/kg SM (1) 0.08 mg/kg SM (2) 0.05 mg/kg SM (3)	0.16 mg/kg SM
<b>PTX</b>			N/A		
<b>STX</b>	2 (1)	3 (H)	0.7 µg/kg 42 µg/adult <sup>a)</sup>	0.42 mg/kg SM (1) 0.17 mg/kg SM (2) 0.11 mg/kg SM (3)	0.8 mg/kg SM
<b>YTX</b>	5,000 (2)	100 (A)	50 µg/kg 3 mg/adult <sup>a)</sup>	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

<sup>a)</sup> 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<sup>10</sup> or III<sup>11</sup> 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

<sup>10</sup> A Type II method is the 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.

<sup>11</sup> 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.

group will be extremely difficult. Thus the implementation of a marker compound concept and the use of functional assays should be explored.

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

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

In considering their weaknesses and merits, the meeting noted that the various 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.

**ACKNOWLEDGEMENTS**

EFSA wishes to thank the working group members Jan Alexander, Tore Aune, Diane Benford, Luis Botana, Gerhard Heinemeyer, Philipp Hess, Sophie Kryz, Peter Fürst, Angelika Preiss-Weigert, Gian Paolo Rossini, Hans van Egmond, Rolaf van Leeuwen, and Philippe Verger.

## ASSESSMENT

### 1. Introduction

Saxitoxin (STX)-group toxins are marine biotoxins causing paralytic shellfish poisoning (PSP) in humans. Symptoms of human PSP intoxication vary from a slight tingling sensation or numbness around the lips to fatal respiratory paralysis. Fatal respiratory paralysis occurs 2 to 12 hours following consumption of shellfish contaminated with STX-group toxins.

STX-group toxins are a group of closely related tetrahydropurines. They are mainly produced by dinoflagellates belonging to the genus *Alexandrium*: e.g. *Alexandrium tamarenis*, *A. minutum* (syn. *A. excavata*), *A. catenella*, *A. fraterculus*, *A. fundyense* and *A. cohorticula*. Also other dinoflagellates such as *Pyrodinium bahamense* and *Gymnodinium catenatum* have been identified as sources of STX-group toxins (FAO, 2004). Shellfish feeding on these algae can accumulate the toxins, but the shellfish itself is rather resistant to the harmful effects.

STX-producing algae species occur worldwide, both in tropical and moderate climate zones. In Europe they occur alongside the Atlantic coast and the North Sea from Norway to Portugal, but also in the Mediterranean. Other locations are Turkey and Egypt, the north-east coast of Canada and the USA, the Gulf of Mexico, the Pacific coast of Central America, East Asia, Australia and New Zealand.

There is also an immobile form of dinoflagellates, the resting cysts. These cysts sink to the bottom of the sea where they over-winter (FAO, 2004). When favourable growth conditions occur the cysts germinate and inoculate the water with swimming cells that can then bloom. It is not predictable when such a bloom of dinoflagellates will happen. Climatic and environmental conditions such as changes in water salinity, water temperature, increased nutrient content and sunlight can trigger germination of the cysts to a vegetative stage enabling rapid reproduction of the algae. In addition, also hydrographical conditions may play an important role. In particular, the presence of a thermocline, an upper layer of seawater which does not mix with the underlying water layers, is very important for algae growth.

The nitrogen:phosphate (N:P) ratio is expected to have a marked influence on the production of toxin during a bloom. Several studies have reported the effect of differences in N:P ratio on the growth of *Alexandrium* spp. and their toxin content (Béchemin *et al.*, 1999; John and Flynn, 2000). Nitrogen restriction reduced population growth and toxin production, whereas phosphorus restriction reduced population growth but enhanced toxin production (FAO, 2004).

The toxicity of the dinoflagellates is due to a mixture of STX analogues, the composition of which differs per algae species and/or per region of occurrence. The toxin profile of *A. minutum* in New Zealand for instance is predominantly dependent on different proportions of STX, neosaxitoxin (neoSTX), gonyautoxins (GTX1, GTX2, and GTX4) (see Figures 1 and 2). This profile is, however, different from those observed in the same algae species elsewhere in the world (MacKenzie and Berkett, 1997).

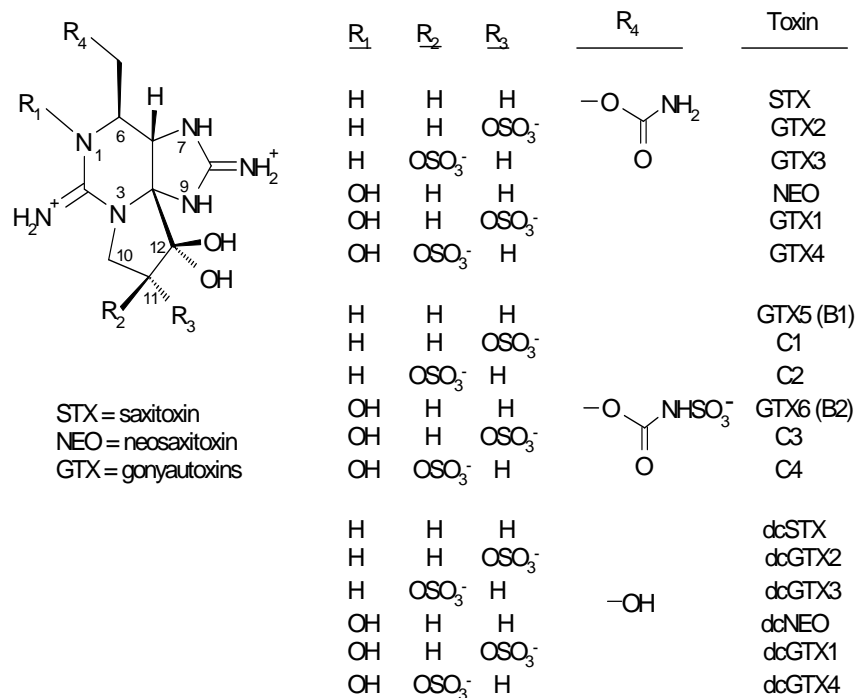
### 2. Chemical characteristics

STX-group toxins are closely related water-soluble tetrahydropurine compounds. They are produced mainly by dinoflagellates belonging to the genus *Alexandrium*, but have also been identified in some cyanobacteria which may occur in fresh and brackish waters. More than 30 STX analogues, mainly from marine dinoflagellates and shellfish that feed on toxic algae, have been identified (FAO, 2004; Dell'Aversano *et al.*, 2004; Dell'Aversano *et al.*, 2008).

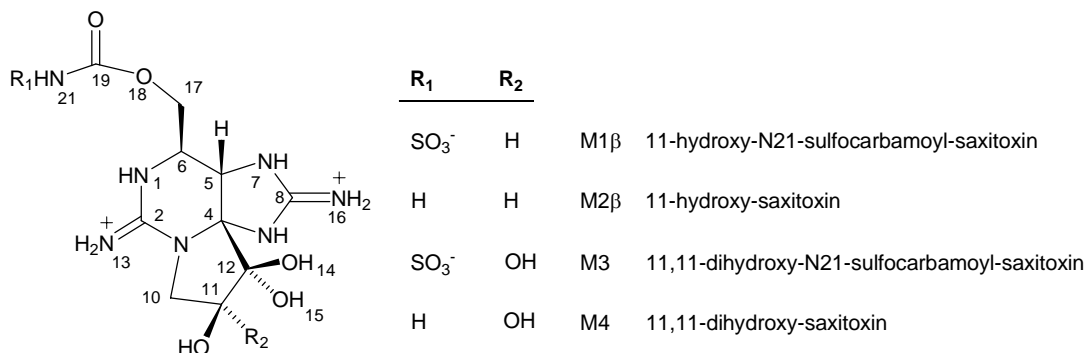
The STX-group toxins make up seven subgroups. Data on toxicity and occurrence in shellfish are available for most of the analogues in the following four subgroups (Figures 1 and 2):

- I) carbamate (STX, neosaxitoxin (NeoSTX) and gonyautoxins (GTX1-4))
- II) N-sulfo-carbamoyl (GTX5-6, C1-4)
- III) decarbamoyl (dc-) (dcSTX, dc-NeoSTX, dcGTX1-4)
- IV) hydroxylated saxitoxins (M1-4)

STX was the first toxin of the STX-group toxins that was isolated from the Alaska butterclam (*Saxidomus giganteus*) and hence the trivial name STX was given.



**Figure 1.** Chemical structures of subgroups I-III saxitoxin (STX) analogues. Indicated  $R_4$  substituents apply for each component in the various subgroups.



**Figure 2.** Chemical structures of hydroxylated saxitoxin (STX) analogues (subgroup IV).

STX-group toxins are prone to various conversions depending on the pH. They are heat stable at acidic pH (with the exception of the N-sulfo-carbamoyl components), but are unstable and easily oxidised under alkaline conditions (Mons *et al.*, 1998). When heated at low pH (approximately 2-4), the toxins with the N-sulfo-carbamoyl moiety as a side chain may be partially converted to the corresponding carbamate toxins through hydrolysis. These conversions take place when STX-group toxins are boiled with strong acid (e.g. HCl solutions) and result into a change of STX analogues with a low toxicity (e.g. GTX5) into ones with a higher toxicity (e.g. STX). For information on the toxicity of the respective analogues see chapters 10.2.1.1 and 10.3. However, in experiments with modelled conditions for the human stomach, the conversions were not found to be significant (FAO, 2004; van Egmond *et al.*, 2007). Conversion does not take place in weak acids (e.g. acetic acid solutions, with approximately pH 3-4), even with boiling (Van Egmond, personal communication).

### 3. Regulatory status

For the control of the STX-group toxins in the European Union (EU), Commission Regulation (EC) No 853/2004<sup>4</sup>, provides details in section VII: “Live bivalve molluscs”, chapters II and IV. Chapter II: “Hygiene requirements for the production and harvesting of live bivalve molluscs. A. Requirements for production areas” states: “*Food business operators may place live molluscs collected from production areas on the market for direct human consumption only, if they meet the requirements of chapter IV*”. Chapter IV: “Hygiene requirements for purification and dispatch centres. A. Requirements for purification centres” states: “*Food business operators purifying live bivalve molluscs must ensure compliance with the following requirements: They must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits: for paralytic shellfish poison (PSP): 800 micrograms per kilogram*”. This limit corresponds with most limits established in countries outside the EU, although these are often expressed differently: as µg STX equivalents/100 g. In this opinion the CONTAM Panel adopted this figure as being expressed as µg STX equivalents/kg shellfish meat.

In Council Directive 96/77<sup>12</sup> the European Commission authorises Spain, under certain conditions, to harvest bivalve molluscs of the species *Acanthocardia tuberculata* (Mediterranean cockle), in areas where the PSP level in edible parts of those molluscs is more than 80 µg per 100 g, but less than 300 µg per 100 g tissue, if heat treatment (see chapter 5.4) is carried out. Those bivalve molluscs may be intended for human consumption after having undergone, after processing, a test lot by lot to verify that they do not contain a PSP level detectable by the bioassay method.

Commission Regulation (EC) No 2074/2005<sup>5</sup> provides details about the “Recognized testing methods for detecting marine biotoxins”. Annex III, Chapter I of this regulation deals with STX-group detection methods. This chapter has been amended by Commission Regulation (EC) No 1664/2006<sup>13</sup> to read:

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<sup>12</sup> European Commission, 1997. Council Directive 96/77 EEC of 18 January 1996 establishing the conditions for the harvesting and processing of certain bivalves from areas where paralytic shellfish poison exceeds the limit laid down by the Council Directive 91/492/EEC. OJ L 15, 46-47.

<sup>13</sup> Commission Regulation (EC) N° 1664/2006. Commission Regulation of 6 November 2006 amending Regulation (EC) N° 2074.2005 as regards implementing measures for certain products of animal origin intended for human consumption and repealing certain implementing measures. OJ L L320, 18.11.2006, p.13-45.



## **Chapter 1. Paralytic Shellfish Poison (PSP) detection method**

1. *The paralytic shellfish poison (PSP) content of edible parts of molluscs (the whole body or any edible part separately) must be detected in accordance with the biological testing method or any other internationally recognized method. The so-called Lawrence method may also be used as an alternative method for the detection of these toxins, as published in AOAC Official Methods 2005.06 (Paralytic Shellfish Poisoning Toxins in Shellfish).*

2. *If the results are challenged, the reference method shall be the biological method.*

3. *Points 1 and 2 will be reviewed in light of the successful completion of the harmonization of the implementing steps of the Lawrence method by the Community Reference Laboratory for marine biotoxins.*

The EU legislative limit is not expressed in STX equivalents, but the various STX-group toxins exhibit different toxicities. In addition to that, neither specific biological methods nor criteria of acceptability of alternative methods are mentioned in the Regulation. Experts of the EU National Reference Laboratories on Marine Biotoxins have agreed, however, that the biological testing method mentioned in Commission Regulation (EC) No 1664/2006 should be the mouse bioassay, performed as described by the Association of Official Analytical Chemists (AOAC) International (AOAC, 2005).

In conclusion the EU legislation for STX-group toxins requires the use of the mouse bioassay but also allows alternatives, provided these have been validated according to an internationally agreed protocol. Currently AOAC method 2005.06 fulfils to this requirement, and can therefore be used.

### **4. Methods of analysis**

Several published methods exist for the detection of the STX-group toxins in plankton and bivalves. Of these, the mouse bioassay is still applied widely despite growing concern with respect to the use of mammalian assays for reasons of animal welfare. Biochemical and chemical methods are also available, however only one, a liquid chromatography method with pre-column derivatization and fluorescence detection (LC-FLD) (AOAC method 2005.06) has been successfully validated in a collaborative study according to the harmonized protocol of ISO/IUPAC/AOAC (Horwitz, 1995). In attempts to advance, develop and validate non-animal methods, research is being undertaken by a number of groups worldwide.

Information on methods that are currently being used or are in the process of being developed and have the potential for use in a regulatory setting is provided below. For a more general overview of other methods, see the Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs (FAO/IOC/WHO, 2004) and the review paper by Hess *et al.* (2006).

#### **4.1. Supply of appropriate reference material**

Currently, certified reference calibrants are provided for the following STX analogues and mixtures by National Research Council Canada (NRCC)<sup>14</sup> :

Saxitoxin dihydrochloride

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<sup>14</sup> [www.nrc-cnrc.gc.ca](http://www.nrc-cnrc.gc.ca)

Decarbamoylsaxitoxin  
Neosaxitoxin  
Decarbamoylneosaxitoxin  
Gonyautoxin-1 and -4  
Gonyautoxin-2 and -3  
Gonyautoxin-5 (B1)  
Decarbamoylgonyautoxin-2 and -3  
N-sulfocarbamoylgonyautoxin-2 and -3

The preparation of further below listed analogues is planned:

N-sulfocarbamoylgonyautoxin-1 and -4  
Decarbamoylgonyautoxin-1 and -4  
Gonyautoxin-6 (B2)

Certified reference material (CRM) for mussel tissue (see below) has been prepared and packaged; certification is expected in 2009:

Mussel tissue CRM for STX-group toxins

Certified lyophilised mussel reference material is available from the Institute for Reference Materials and Measurements (IRMM)<sup>15</sup>.

## 4.2. Mammalian bioassay

Regulation (EC) No. 2074/2005 prescribes the use of the mouse bioassay as reference method for the detection of the STX-group toxins. This test, which has been formally validated in an interlaboratory study by AOAC International in 1959, is described below.

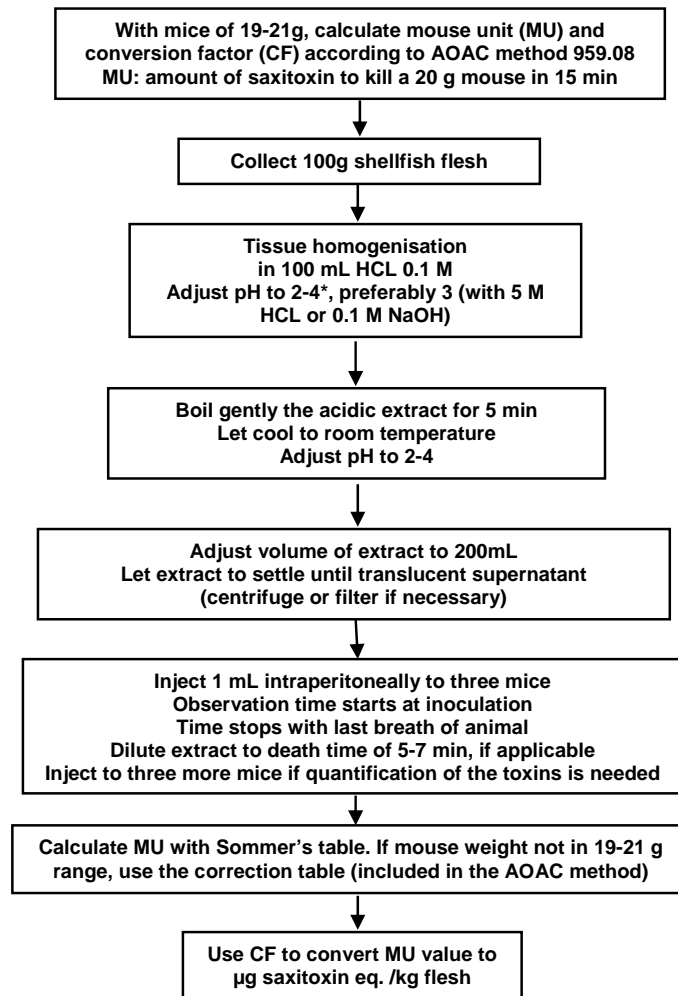
### 4.2.1. Mouse bioassay

Originally designed by Sommer and Meyer (1937), of all the mouse bioassays (MBA) carried out for marine toxins, the one for STX-group toxins is the best in terms of accuracy and speed (AOAC, 2000a). The method allows quantification of the STX-group toxins. It must be previously calibrated with standards, to set the relationship between death time and amount of toxin, and define the mouse unit (MU)<sup>16</sup> (Figures 3 and 4).

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<sup>15</sup> <http://irmm.jrc.ec.europa.eu>

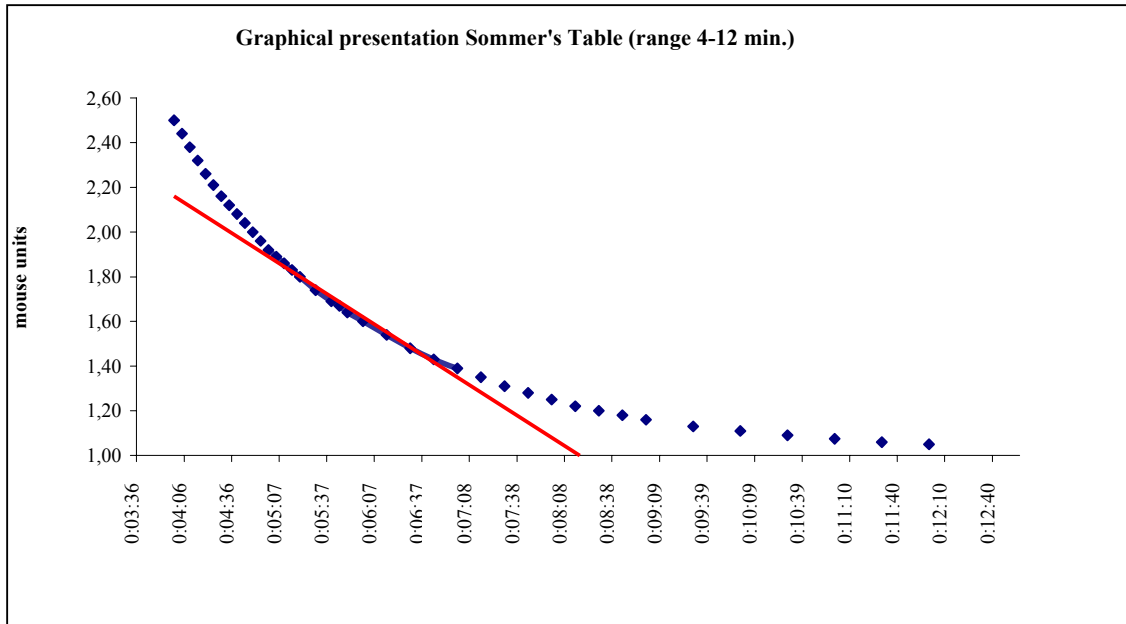
<sup>16</sup> Definition of Mouse Unit (MU) associated with the MBA for STX group toxins: A mouse unit (MU) is defined as the minimum amount needed to cause the death of an 18 to 22 g white mouse in 15 minutes (Shimizu, 1984).



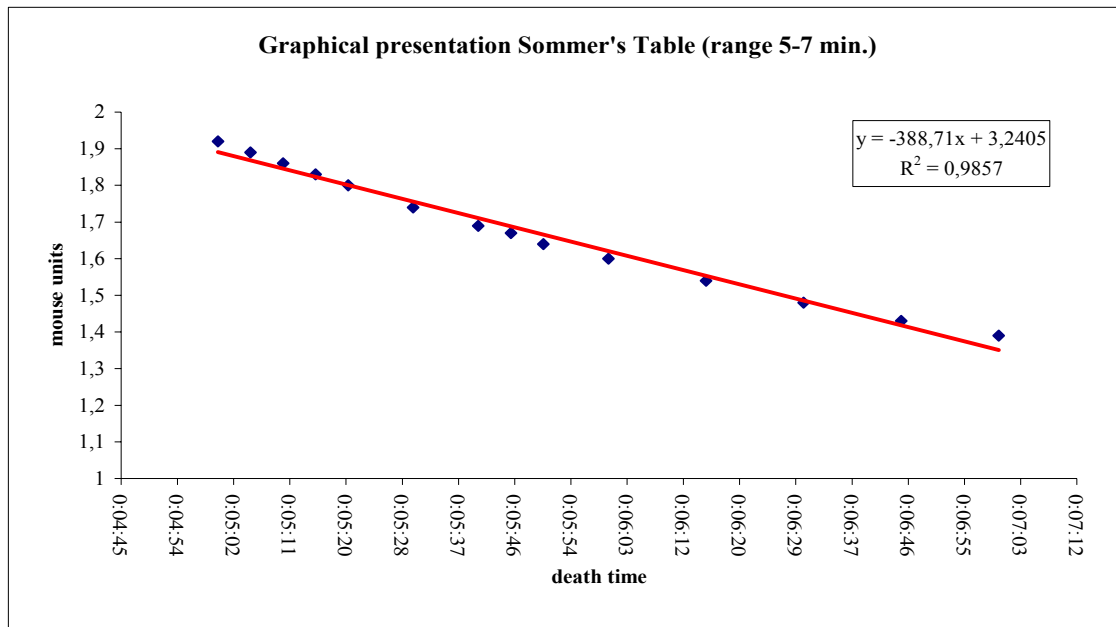
\*AOAC protocol does not specifically indicate this pH range, but National Reference Laboratory/ Community Reference Laboratory (NRL/CRL) network on Marine Biotoxins recommends it to reduce pH effect on toxicity values due to different conversion rates of toxins to more toxic analogues during extraction.

**Figure 3.** AOAC method 959.08 for sample preparation and extraction methods of shellfish flesh for the mouse bioassay (MBA).

As seen in Figure 4, where the Sommer's table is graphically depicted, the relationship is not linear. However, within the applied observation time range of 5 to 7 minutes (see Figure 5) the relationship is near to linear and hence the concentration of STX-group toxins in the analysed sample can be determined. This method has been approved by AOAC International as an official method, and the observation time is less than 10 min (AOAC method 959.08). The detection limit for the bioassay is about 370 µg STX equivalents/kg flesh, which is close to the 800 µg/kg set by the current legal limit. Therefore, a significant reduction of the regulatory limit would result in the MBA not being applicable any more. A further issue with this assay is the ethical problem of animal use which is against Directive 86/609<sup>7</sup>.



**Figure 4.** The relationship between death time of the mouse and amount of toxin expressed as mouse units in the time range of 4-12 minutes (Sommer's table in graphic form).



**Figure 5.** The linear relationship between death time and amount of *i.p.* toxin expressed as mouse units in the measuring time range of 5-7 minutes. (Excerpt of Sommer's table in graphic form (see Figure 4 above))

The extraction of this water soluble group of toxins is performed with hydrochloric acid (HCl), and boiling of the extract. Under these conditions some of the STX-group toxins are (partly) converted to more toxic forms and therefore result in an overestimation of the toxicity equivalence, depending on the toxin profile (Vale *et al.*, 2008).

The conversion rate may be influenced by the pH of the extract, which is prescribed to be <4.5 in the AOAC procedure, but which may vary in shellfish extracts. In order to harmonize this potential pitfall, the European National Reference Laboratory (NRL) network agreed to use a pH 3 when the acidic heat extraction step is performed. However, this is not sufficient to prevent conversions from taking place. In the MBA, test portions are extracted with HCl and boiling. This is the step in the analysis where conversions from one analogue into another largely take place. In the AOAC procedure the pH is adjusted to pH 2-4 after the extraction step. Standardising the pH at about 3 at this stage helps to reduce variability between test results of different laboratories, but does not prevent conversions, because they may have already taken place, thus leading to overestimation of toxicity in the MBA.

Several factors can also modify the results of the mouse bioassay (e.g. cations, mice strain, sex, age, weight, general state of health, diet, stress) (Park *et al.*, 1986).

The main advantages of the mouse bioassay for STX-group toxins are:

- it is very quick;
- it provides a measure of total toxicity based on the biological response of the animals to the toxins;
- it allows quantification using the Sommer table (see AOAC method 959.08);
- it does not require complex analytical equipment.

The main disadvantages of the mouse bioassay for STX-group toxins are:

- it cannot be automated;
- it requires specialised animal facilities and expertise;
- high variability in results between laboratories due to e.g. specific animal characteristics;
- results may differ several fold if different extraction pHs are used;
- the boiling step with HCl during extraction may result in overestimation of the toxicity, depending on the toxin profile;
- prone to some interferences (metal salts);
- the injection volume of one mL exceeds good practice guidelines (< 0.5mL) intended to minimise stress to mice.
- the MBA is considered undesirable for ethical reasons; however, it could be used with sedated animals to avoid suffering.

### 4.3. Biomolecular methods

Biomolecular methods for STX-group toxins are based on three different strategies, use of receptors, use of cytotoxicity characteristics and use of antibodies. It is important to highlight the fact that none of the following methods has been collaboratively validated yet.

#### 4.3.1. Receptors

The receptor to STX-group toxins has been clearly identified as a reversible sodium channel blocker acting specifically on site I (Catterall and Morrow, 1978), but this effect seems not to be the only one in different biological systems (Llewellyn, 2006). The use of preparations of

the sodium channels, from several sources, provided in vitro methods to quantify this toxin group. Radioactive methods were developed with purified brain homogenates (Davio and Fontelo, 1984), semipurified brain homogenates (Vieytes *et al.*, 1993) and synaptoneuroosomes (Doucette *et al.*, 2000). Fluorescent methods were developed using neuroblastoma cells as the receptor container with the membrane potential dye sensor oxonol (Louzao *et al.*, 2001; Vale *et al.*, 2008) and high throughput (Louzao *et al.*, 2003; Louzao *et al.*, 2004), and a different approach with rhodamine and synaptoneuroosomes (Nicholson *et al.*, 2002). A patch clamp single channel-selective method was reported as a screening method (Vélez *et al.*, 2001) and has been used for some time as an official method in Chile. These methods are the best to provide toxicity-equivalent results, but they need further refinement to be used for screening.

The method of Vieytes *et al.* (1993) was further developed by Doucette *et al.* (1997) and made available in a radioactivity-labelled isotope [<sup>3</sup>H] microplate format (Ruberu *et al.*, 2003). The use of the microtiter plate format, in conjunction with microplate scintillation counting, makes the assay suitable for use in a high throughput regulatory setting. The method has undergone an extensive single laboratory validation study, an interlaboratory calibration exercise with 5 laboratories, a comparison study with high-performance liquid chromatography (HPLC) (Lawrence method, AOAC Official Method 2005.06), and a comparison study with the mouse assay. Based on the study reports from 2008, the method has been approved for full AOAC collaborative study, expected to take place in 2009 (Van Egmond, personal communication).

The main advantages of receptor-based assays are:

- they provide toxicity equivalent results;
- they are suitable for high throughput analyses.

The main disadvantages of receptor-based assays are:

- the most advanced assays require the use of radioactivity-labelled isotopes;
- methods have not (yet) been validated in interlaboratory studies;
- they do not provide any information on the toxin profile.

#### 4.3.2. Cytotoxicity assays

Cytotoxicity assays for STX-group toxins are based on the combined effect they exert in the presence of veratridine and ouabain on neuroblastoma cells (Kogure *et al.*, 1988; Gallacher and Birkbeck, 1992; Manger *et al.*, 1993). Pre-treatment of cells with the sodium channel opener veratridine causes sodium entrance into the cells, and the presence of ouabain, which inhibits Na<sup>+</sup>,K<sup>+</sup>ATPase, leads to overall sodium imbalance, causing neuroblastoma cell death. The presence of STX-group toxins, which inhibits sodium entrance, prevents the cytotoxic response in a dose-dependent fashion, allowing the quantification of STX-group toxins in relevant samples by counting morphologically normal cells. Jellett *et al.* (1992) have modified this bioassay to improve its speed and convenience by eliminating the need to count individual cells to determine the STX equivalents. Instead, they have employed a microplate reader for automated determinations of absorption of crystal violet from neuroblastoma cells. In principle the neuroblastoma cell assay could be a good alternative to the mouse bioassay for testing for STX-group toxins. However, the procedure developed by Jellett *et al.* (1992) did not yield satisfactory results when it was tested in an AOAC International collaborative study in 1999. This has led to discontinuation of the application of this method in practice.



The main advantage of cytotoxicity assays is:

- they provide toxicity equivalent results.

The main disadvantages of cytotoxicity assays are:

- facilities are needed for maintenance and handling of cell cultures;
- interlaboratory performance characteristics were not acceptable, as shown from a collaborative study ;
- they do not provide any information on the toxin profile.

#### 4.3.3. Antibodies

Although antibodies are very sensitive, their main problem to detect the STX-group toxins is their lack of good cross reactivity to all the members of the group. Since the differences in toxicity among this group could be very high, it is difficult to match toxicity with toxin levels as quantified by the antibody. There are several technological approaches, none of them being fully satisfactory at this time: Enzyme-Linked Immunosorbent Assay (ELISA) (Chu and Fan, 1985; Usleber *et al.*, 1995), radioimmunoassay (Carlson *et al.*, 1984), lateral immunoflow assay (Jellett *et al.*, 2002), and Surface Plasmon Resonance (SPR) biosensor technology (Campbell *et al.*, 2007; Fonfría *et al.*, 2007). Ongoing prevalidation studies, in the context of the VI<sup>th</sup> FP project BIOCOP, show that SPR biosensor is a promising technology for the screening of this toxin group; however, cross reactivity and possibly matrix effects remain issues.

The main advantages of antibody-based methods are:

- they are very sensitive;
- they are fast, easy to use, and can be applied to screen many samples at any time for further confirmatory analysis.

The main disadvantages of antibody-based methods are:

- the accuracy is questionable when mixtures of analogues are being analyzed which is most often the case;
- they do not provide any information on the toxin profile.

#### 4.4. Chemical methods

STX-group toxins do not have chromophores that would allow their ultraviolet (UV) or fluorescence detection. Therefore, an alkaline oxidation step leading to products that can be measured by fluorescence detection was developed (Bates and Rapoport, 1975). This method determines the total fluorescence of the shellfish extract.

Due to the great range in the relative toxicity (STX: 1 to C1: 0.006) (Oshima, 1995b) and to the different behaviour in fluorescence after oxidation, it is desirable to determine all STX analogues separately, for which HPLC or electrophoresis could be applied. The total toxicity can be calculated by addition, after converting analytical results for individual toxins into

STX equivalents by applying the toxicity equivalency factors (TEFs) (see 10.3). Techniques based on liquid chromatography combined with post- or pre-column oxidation followed by fluorescence detection were developed.

#### 4.4.1. Precolumn methods versus postcolumn methods

During oxidation STX-group toxins produce up to three products and in some cases different toxins form the same oxidation products.

In postcolumn methodology the toxins are first separated and then oxidized allowing the unambiguous identification. However, these methods demand additional post-column equipment.

In precolumn methodology the reaction products of the toxins are chromatographically separated after oxidation. The identification and quantification of the toxins is elaborate.

Both methodologies are time-consuming but have limits of detection low enough to cover the current EU legal limit for STX-group toxins.

#### 4.4.2. Standardised HPLC methods

##### 4.4.2.1. CEN 14526 (precursor HPLC-method)

The precursor oxidation method described by Lawrence and Menard (1991) was used by five laboratories in an interlaboratory study for the certification of STX and dcSTX in lyophilized mussel tissue (Van Egmond *et al.*, 1998). This method was subsequently standardised as CEN method 14526.

This method was successfully applied in a series of proficiency studies on STX-group toxins, carried out in the Netherlands (Van Egmond *et al.*, 2004) (with 4 laboratories and the toxins STX and dcSTX) and in a validation study with minor modifications (extraction only with acetic acid, the optional extraction with HCl was not offered; no purification by solid phase extraction (SPE); the amount of sample and extraction volume were proportionally reduced) in Germany in 2003 (§64-LFBG L 12.03/04-1 with 10 laboratories and the toxins STX, GTX5 and GTX2,3) at national level.

Currently the European Committee for Standardisation (CEN) is working to replace CEN standard 14526 by the current Lawrence method (AOAC Official Method 2005.06.).

The main advantages of the precursor HPLC-method (CEN standard 14526) are:

- it is highly specific and sensitive;
- it can screen and measure the STX-group toxins individually, provided two different oxidation reagents are used ;
- it gives information on the profile of STX-group-toxins in samples;
- it can be automated;
- it performed well in a (limited) interlaboratory study.

The main disadvantages of the precursor HPLC-method (CEN standard 14526) are:

- applying the method requires highly trained personnel;

- due to the overlapping of oxidation products of different STX-group toxins the identification and quantification of the toxins is elaborate;
- it was only validated for two STX analogues;
- only those toxins can be quantified, for which calibrants are available;
- the total toxicity has to be calculated using toxicity equivalency factors.

#### 4.4.2.2. AOAC Official Method 2005.06 (precolumn method)

The method was described by Lawrence and Menard (1991) and was evaluated in 2002 in an international collaborative study involving 18 laboratories (Lawrence *et al.*, 2004, 2005). It was successfully validated for the toxins GTX1 and GTX4 together, GTX2 and GTX3 together, C1 and C2 together, C3 and C4 together, NeoSTX, STX, dcSTX and GTX5. The method was adopted as AOAC Official Method 2005.06. The CODEX Committee on Fish and Fishery Products accepted according to ALINORM 08/3118, Appendix III, this method for the determination of STX-group toxins in the draft standard for live and raw bivalve molluscs (at step 8 of the procedure). Method AOAC 2005.06 (so called Lawrence method) may be officially used in the EU as an alternative to the mouse bioassay, for the determination of STX-group toxins. The extraction of the STX-group toxins is performed with acetic acid. In contrast to the extraction conditions of the mouse assay (see 4.2.), acetic acid extraction does not lead to conversion of some of the STX-group toxins into more toxic forms. Therefore results obtained with the Lawrence method reflect the actual toxin profile in the sample.

An interlaboratory exercise has been organized by the Community Reference Laboratory (CRL) to evaluate the “fitness for purpose” of the Lawrence method for the official control of STX-group toxins in the EU laboratories (Botana *et al.*, 2007). These results were satisfactory, but emphasised the need for trained staff and for the availability of standards.

The main advantages of the precolumn HPLC-method (AOAC Official Method 2005.06) are:

- it is highly specific and sensitive;
- it can screen and measure the STX-group toxins individually, provided two different oxidation reagents and a fractionation on a COOH cartridge are used;
- it gives information on the true profile of STX-group toxins in samples due to acetic acid being used as the extraction solvent;
- it can be automated;
- it has been successfully validated for 12 STX analogues; validation for other STX analogues is in progress.

The main disadvantages of the precolumn HPLC-method (AOAC Official Method 2005.06) are:

- applying the method requires highly trained personnel;
- due to the overlapping of oxidation products of different STX analogues the identification and quantification of the toxins is complex and elaborate
- only those toxins can be quantified, for which calibrants are available;

- the method has not been validated for all existing STX analogues;
- the total toxicity has to be calculated using toxicity equivalency factors.

#### 4.4.2.3. CEN 14194 (postcolumn HPLC-method)

A project to develop shellfish reference materials for STX and dcSTX was carried out by the Community Bureau of Reference (BCR) (1993-1997) (Van Egmond *et al.*, 1998). The laboratories were asked to analyse the samples with the method of their choice (LC-methods with pre- or postcolumn oxidation). Two of the methods used in this certification study have shown good performance characteristics and were consequently selected for standardisation by the European Committee for Standardization (CEN) (CEN methods CEN 14194 and CEN 14526).

The postcolumn oxidation method described by Franco and Fernández-Villa (1993) was standardised as CEN method 14194. From the above mentioned study the results of three laboratories were selected to demonstrate the validation only for STX and dcSTX.

The main advantages of the postcolumn HPLC-method (CEN 14194) are:

- it is highly specific and sensitive;
- it can screen and measure the STX-group toxins individually, provided different chromatographic runs with ionic pair reagents are used;
- it gives information on STX-group toxins in samples;
- it can be automated;
- it performed well in a (limited) interlaboratory study.

The main disadvantages of the postcolumn HPLC-method (CEN 14194) are:

- it requires costly post column equipment and highly trained personnel;
- different chromatographic runs are necessary;
- only those toxins can be quantified, for which calibrants are available;
- it was only validated for two STX analogues;
- the total toxicity has to be calculated using toxicity equivalency factors.

#### 4.4.3. Further methods

##### 4.4.3.1. LC-MS/MS

Hydrophilic interaction liquid chromatography (HILIC), a valuable tool for the separation of polar compounds, in combination with liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) has been used for determination of STX-group toxins (Dell'Aversano *et al.*, 2005). However, the broad chromatographic peaks and the low intensities of the characteristic molecular ions lead to relatively high limits of detection (LODs) and a limited reproducibility in quantification, which restrict the use of LC-MS/MS to determine STX-group toxins. Using the extraction and cleaning method according to the AOAC method 2005.06 the LOD for STX was up to 100 µg STX diHCl equivalents/kg (German NRL, personal communication). Improvements have been recently made on extract

preparation for STX-group toxins allowing pre-concentration and, therefore, resulting in lower detection limits (e.g. STX: 23-42 µg STX diHCl equivalents/kg) (Sayfritz *et al.*, 2008).

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

- it is highly specific;
- it can screen and measure the STX-group toxins individually, without the need for derivatization;
- it gives information on the profile of STX-group toxins in samples;
- it can be automated.

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

- it requires costly equipment and highly trained personnel;
- extraction procedures with concentration steps have to be developed to lower the LODs;
- only those toxins can be quantified, for which calibrants are available;
- it has not been validated in interlaboratory studies;
- the total toxicity has to be calculated using toxicity equivalency factors.

#### 4.4.3.2. Electrophoresis

Taking advantage of the overall positive charge of STX-group toxins at acidic pHs, electrophoretic procedures for the separation of analogues based on their different mobilities in an electric field have been developed (Thibault *et al.*, 1991). Capillary electrophoresis has been mostly used in this case, taking advantage of its particularly high power of resolution, and coupling the electrophoretic separation with either UV or MS detection of resolved compounds. Because of the small volume of samples that can be loaded onto the capillary electrophoretic systems, the procedure has been used for samples of marine biota rather than measurement of STX-group toxin contamination in field samples (Gago-Martínez *et al.*, 1996). Presently detection limits appear to be too high to be of use in monitoring programmes (FAO, 2004).

#### 4.4.4. Proficiency tests

The Food Analysis Performance Assessment Scheme (FAPAS<sup>®17</sup>) organized a pilot study on the determination of STX-group toxins in freeze-dried mussel in 2003 (Earnshaw, 2003). 15 laboratories took part in this exercise and 7 of them applied HPLC-methods. The results for STX and dcSTX varied considerably. Those laboratories that found (sometimes strongly) positive values for STX, all used HCl with boiling in the extraction step (as is also used in the mouse bioassay). In contrast, laboratories that applied acetic acid without boiling in the extraction step found negligible amounts or no STX at all. The reason for this is that HCl extraction with boiling leads to partial hydrolysis of certain STX-group toxins, leading to

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<sup>17</sup> <http://www.fapas.com>

conversion of some STX-group toxins into more toxic analogues (e.g. GTX5 is converted into STX). Acetic acid is a much milder extraction solvent which leaves the toxin profile of the sample essentially intact. The sample used in the FAPAS study was a reference material from BCR, characterised and certified to contain no STX. But it contained GTX5, which led to transition into STX upon boiling with HCl in the extraction step (Van Egmond *et al.*, 1998).

#### 4.5. Summary of methods

The MBA is the reference method prescribed in EU legislation for the determination of STX-group toxins, whereas the Lawrence method (AOAC official method 2005.06) may be officially used in the EU as an alternative to the MBA. Both methods have been formally validated in AOAC interlaboratory validation studies, and both methods are capable to determine STX-group toxins at the current regulatory limit of 800 µg STX equivalents/kg shellfish. There are, however, concerns and limitations with the use and comparability of these official methods and alternatives under development.

Council Directive 86/609/EEC<sup>7</sup> states that Member States may not permit the use of live animals in procedures that may cause pain, suffering distress or lasting harm if another scientific satisfactory method of obtaining the result sought and not entailing the use of live animals is reasonable and practically available.

In the MBA HCl with boiling is used in the extraction step, whereas in the Lawrence method, acetic acid without boiling is used in the extraction step. These different extraction conditions may potentially lead to differences in toxin profiles detected. Due to the (strongly) different TEFs of the various STX analogues, different results may be obtained when these analytical data are expressed in STX equivalents. It should be noted, however, that different HPLC methods with different acidic conditions are used in practice (See chapter 5, Table 3).

Other methods involve techniques such as LC/MS-MS, antibody-based sensors and receptor-based assays. None of these methods have been interlaboratory-validated yet according to internationally accepted protocols, so that their performance characteristics cannot be evaluated and compared yet with the official methods. For the sensor- and receptor-based methods, such validation studies are in preparation.

### 5. Occurrence of STX-group toxins

#### 5.1. Data collection

Following a request by the European Food Safety Authority (EFSA) for data on STX-group toxins France, Germany, Italy, Norway, Portugal, Spain and the United Kingdom (UK) provided data on the occurrence of STX-group toxins in shellfish. A total of 20248 analytical results were submitted. The number of analyses presented by the countries is considerably different from one country to another. Table 2 shows a summary of the number of data submitted by each country including purpose of testing, analytical method applied, limit of detection (LOD) and limit of quantification (LOQ) of the method.



**Table 2.** Data submissions from European Countries for STX-group toxins in the period from 2000 to 2008.

Country	Year(s) of harvesting	Number of samples	Purpose of testing <sup>a)</sup>	Method of testing	LOD <sup>d)</sup> (µg/kg)	LOQ <sup>d)</sup> (µg/kg)
France	2000-2008	1882	pre-MC	MBA		280-402
		290	post-MC			
Germany <sup>b)</sup>	2005-2007	445	post-MC	HPLC-FLD	b)	-
Italy	2001-2008	7046	pre-MC	MBA		350
Norway <sup>c)</sup>	2006-2008	1122	pre-MC	HPLC-FLD	c)	-
Portugal <sup>e)</sup>	2005-2007	2619	pre-MC	HPLC-FLD	e)	-
Spain	2000-2008	2401	pre-MC	MBA		320-400
		271	post-MC			
United Kingdom	2004-2008	4172	pre-MC	MBA		380
<b>Total</b>	<b>2000-2008</b>	<b>20248</b>				

Pre/post-MC = pre-market/post-market control, LOD = limit of detection, LOQ = limit of quantification

<sup>a)</sup> PreMC are samples collected at the place of origin, before or during harvesting; PostMC are samples collected at the place of sale or along the distribution chain.

<sup>b)</sup> Germany reported not detected individual analogues as “<value”. These values were used as LOQs in the calculations.

<sup>c)</sup> Norway reported the LODs for the individual measured analogues. These were used in the calculations.

<sup>d)</sup> For MBA method only one reporting threshold is given (not specified whether LOD or LOQ; it was assumed to be LOQ); relatively small variations are observed between different laboratories.

<sup>e)</sup> Portugal neither reported LOD/LOQ for the sum of STX-group toxins nor for each analogue.

The submissions covered samples collected and tested during years 2000 to 2008 with quantitative MBA, and years 2005 and 2008 with high-performance liquid chromatography-fluorescence detection (HPLC-FLD), and included pre- and post-market control samples (pre-MC and post-MC).

Pre-MC samples which are the samples taken before harvesting for further processing or direct marketing as prescribed in the respective EU legislation, comprised 19242 results. Post-MC samples, which are taken from the market, collected at stores and supermarkets, comprised 1006 results. The dataset from France comprised 1882 pre-MC and 290 post-MC results. Spain submitted 2401 pre-MC and 271 post-MC results. The 445 data from Germany were all post-MC, whereas the data submitted by Italy (7046), Norway (1122), Portugal (2619) and UK (4172) were all from pre-MC samples. The analytical method was also variable between datasets, the different countries having applied either MBA or HPLC-FLD (in the two variations with pre- and post column oxidation). These analytical methods are presented in Table 3 in more detail.

**Table 3.** Methods used for analysing the occurrence data on STX-group toxins in different European Countries. These methods do not necessarily reflect the present situation of the methods used in the laboratories.

Country	Year(s) of harvesting	Method of analysis	Extraction method details		Reference of the method
			Extraction solution (pH)	Heating step	
Portugal	2005-2007	<b>HPLC-FLD</b> (pre-column oxidation): in house validated version of the Lawrence method (AOAC2005.06)	HCl 0.1M; pH 2.5-3.5 (preferably around 3) but no pH control before and after boiling	Boiling (100°C) for 5 minutes	Vale and Taleb, (2005) Jellett <i>et al.</i> (2002) Lawrence <i>et al.</i> (2005)
Norway	2006-2008	<b>HPLC-FLD</b> (post-column oxidation): Oshima method with slight modifications as published in Asp <i>et al.</i> 2004	pH adjustment to 3 +/-0.5 (same extraction method as used for MBA samples)	Boiling (100°C) for 5 minutes	Asp <i>et al.</i> (2004) Oshima (1995b)
Germany	2005-2007	<b>HPLC-FLD</b> (pre-column oxidation): Lawrence method (AOAC2005.06) without SPE	1 % HOAc	Boiling (100°C) for 5 minutes	Lawrence <i>et al.</i> (2005)
		<b>HPLC-FLD</b> (post-column oxidation): Diener <i>et al.</i> 2006 + STX-group toxin extraction from Lawrence method (AOAC2005.06)	1 % HOAc	Boiling (100°C) for 5 minutes	Diener <i>et al.</i> (2006) Lawrence <i>et al.</i> (2005)
Italy	2001-2008 (only MBA data is included in the actual data set)	<b>MBA:</b> Decreto Ministeriale M6/05/2002 based on AOAC 959.08	0.25 N HCl pH: 2-2.5	Boiling (100°C) for 5 minutes	Decreto Ministeriale (2002) AOAC (2000b)
UK	2004-2008 (only MBA data is included in the actual data set)	<b>HPLC-FLD:</b> (pre-column oxidation): Lawrence method (AOAC 2005.06)	1 % HOAc	Boiling (100°C) for 5 minutes	Lawrence <i>et al.</i> (2005)
		<b>HPLC-FLD:</b> (pre-column oxidation): Lawrence method (AOAC 2005.06) + extraction as for the MBA method	0.1N HCl, pH is checked to ensure it is between 2-4	Boiling (100°C) for 5 minutes	Lawrence <i>et al.</i> (2005) AOAC (2000b)
		<b>MBA:</b> AOAC 959.08	0.1 N HCl pH: 2-4 (preferably 3)	Boiling (100°C) for 5 minutes	AOAC (2000b)
Spain	2008-2008	<b>MBA:</b> AOAC 959.08	0.1 N HCl pH: 2.5-4 (preferably 3)	Boiling (100°C) for 5 minutes	AOAC (2000b), modified
France	2000-2008	<b>MBA:</b> AOAC 959.08	0.1 N HCl pH: 2.5-4 (preferably 3), pH adjustment before and after boiling	Boiling (100°C) for 5 minutes	AOAC (2000b), modified

Recognising the need to compare only homogeneous datasets it was decided to keep the data obtained by different analytical methods as well as pre- and post-MC data separate. The pre-MC data were regarded as the best suited for occurrence calculations, because the origin of the data is defined, and the occurrence is not influenced by previous screening analyses. Portuguese data were used to give an overview of the results in the different countries only and not used in the occurrence calculations due to the uncertainties in LOD.

The MBA method measures the total STX-group toxins expressed as STX equivalents, without differentiating between analogues. In contrast, HPLC methods measure a range of analogues. The type and number of the analogues considered depend on the toxin profile dominating in each area (FAO/IOC/WHO, 2004) and on the availability of calibrants. If the HPLC method with pre-column oxidation is applied, some analogues may coelute resulting in a single unresolved analytical peak. In this case, as a conservative approach, the detected peak is attributed to the most toxic analogue contributing to it.

For the MBA method the limit of detection/quantification ranged between 280 and 402 µg/kg STX equivalents. For the HPLC-based methods LODs have been defined separately for each analogue. Due to a continuous improvement of the analytical techniques and equipment LODs have significantly decreased during the observation period. Presently in most laboratories, applying HPLC-FLD based method the LOD of individual toxins varies between 1 and 200 µg/kg (Table 4).

Following an agreement between the NRLs, the countries using HPLC-FLD as a screening method applied toxicity equivalency factors (TEFs) based on the intraperitoneal (*i.p.*) toxicity data of Oshima (Oshima, 1995b; Oshima *et al.*, 2004) to convert the quantities measured for each analogue into STX equivalents (µg/kg of STX dihydrochloride, which is usually considered the reference molecule for STX equivalents). These TEFs, based on molar amounts of toxins, are reported in Table 4, together with the molecular weights applied to convert them into weight-based factors.

**Table 4.** STX analogues analysed in Germany, Norway and Portugal by HPLC with LODs (or LOD ranges) for each analogue (expressed as µg/kg shellfish meat), with the toxicity equivalency factors (TEFs) and molecular weights applied in the reported data.

Toxin	LODs for analogues analysed			TEFs <sup>a)</sup>	Molecular weight (g/mol)
	Germany	Norway	Portugal		
STX-di HCl	-	-	-	-	372.2
STX	5-100	8.8	-	1.00	299.3
GTX 1	25-150	13	nr	0.99	411.4
GTX 2	10-20	1	nr	0.36	395.4
GTX 3	2-10	1.3	nr	0.64	395.4
GTX 4	20-200	17	nr	0.73	411.4
GTX 1,4	20-200 <sup>b)</sup>	-	-	-	-
GTX 2,3	5-150 <sup>b) c)</sup>	-	-	-	-
GTX 5 (B1)	10-75	-	nr	0.06	379.4
GTX 6 (B2)	-	-	nr	0.06	395.4
dc-GTX 2	2-10	-	nr	0.15	352.3
dc-GTX 3	1-75	-	nr	0.38	352.3
dc-GTX 2,3	20*	-	-	-	-
C1	-	-	nr	-	-
C2 (GTX 8)	-	-	nr	0.10	475.4
C3	-	-	nr	-	-
C4	-	-	nr	0.06	491.4
NeoSTX	15-90	39	-	0.92	315.3
dc-STX	5-50	3.5	nr	0.51	256.3
dc-NeoSTX (GTX 7)	-	-	nr	-	-

nr = not reported, - = not analysed

<sup>a)</sup> TEFs calculated based on relative potency data by Oshima *et al.* (2004)

<sup>b)</sup> GTX 1,4, GTX 2,3, dc-GTX 2,3 are unresolved analogues reported in the German data set when a pre-column oxidation of the toxins is applied before the HPLC analysis. In the case of the post-column oxidation HPLC method the results were also reported as the sum of the STX-group toxins.

<sup>c)</sup> The difference in the LODs between GTX-2, GTX-3 and the GTX-2,3 mixture derives from the two different analytical methods applied; the two individual analogues are determined with post-column oxidation whereas GTX-2,3 is determined with pre-column oxidation. The two methods have different LODs. Actually the very high LODs for GTX-2,3 are from samples analysed only with pre-column, without comparison with post-column.

## 5.2. Statistical description of STX-group toxins in shellfish

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

For imputing values reported below LOD or below LOQ the “bounding” approach was applied, which consists of attributing particular values inside the range of their possible variability. The Lower Bound (LB) is obtained by assigning a value of zero (minimum possible value) to all the samples reported as <LOD or <LOQ. The Upper Bound (UB) is obtained by assigning the value of LOD to values reported as <LOD and LOQ to values reported as <LOQ (maximum possible value). Also a Medium Bound or Middle Bound (MB) approach has been used. It consists of assigning the value of LOD/2 to values reported as <LOD and LOQ/2 to values reported as <LOQ (values half-way between lower and upper bound).

A “cumulative” LOD for STX-group toxins measured by HPLC, expressed as µg STX equivalents/kg, may be calculated as the weighted sum of the LODs of the single analogues (or groups of analogues), taking into account the differences in their relative toxicity (see

chapter 10.3). This calculated “cumulative” LOD of the method is therefore heavily influenced by the type and number of analogues measured. Presently, depending on the toxins profile and the available equipment, the cumulative LOD is expected to be in the range 80-350 µg STX equivalents/kg. The HPLC-based methods, if further optimised, have the potential to allow the detection of toxin levels lower than those detected by MBA.

High LOD values in the presence of a high proportion of non-detected results tend to result in apparently high median and/or mean values, because of the influence of the values below LOD, which are substituted with the values of LOD (Upper Bound) or LOD/2 (Medium Bound).

In some cases the Upper Bound approach is likely to significantly overestimate the median and mean. The Lower Bound on the other hand underestimates the non-reported values, since at least a part of them is expected not to be zero. Therefore, the Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) decided to compare in the tables the upper and lower bound values (sensitivity analysis). The two values represent the range of variability of each statistical descriptor as a function of the approach chosen to attribute a numerical value to results reported as “non-detected”.

Table 5 provides an overview of the descriptive statistics of the data, grouping them by analytical method and country. Samples without reported results were assigned upper- and lower bound values. When the statistical descriptors in the two approaches are the same the value is given, otherwise the lower-upper bound range is reported. For Portugal only lower bound values were assigned since the LOD is unknown.

**Table 5.** Statistics of relevant data of STX-group toxins in shellfish sampled in the years 2000-2008, provided by European countries.

Analytical method/Country	N	Median	Mean	P95	Maximum	% of samples not quantified	% of values >800 µg STX eq./kg shellfish meat
		LB/UB	LB/UB	LB/UB	m		
µg STX eq./kg shellfish meat							
<b>Pre-MC samples</b>							
<b>MBA</b>	<b>15501</b>						
France	1882	0/350	102/407	494	7360	89.8	2.7
Italy <sup>a)</sup>	7046	0/350	3/352	0/350	2355	99.5	0.1
Spain	2401	0/400	1050/1251	4500	40800	54.8	26.8
UK <sup>a)</sup>	4172	0/380	24/389	0/380	4130	96.3	0.6
<b>HPLC-FLD</b>	<b>3471</b>						
Norway	1122	13/89	249/313	1011	24678	45.7	6.1
Portugal <sup>b)</sup>	2619	0	1004	5248	67616	63.0	14.2
<b>Post-MC samples</b>							
<b>MBA</b>	<b>561</b>						
France	290					100	
Spain	271					100	
<b>HPLC-FLD</b>	<b>445</b>						
Germany <sup>a)</sup>	445	0/218	14/206	71/345	694/984	84.0	0.2

N = number of samples, STX eq. = STX equivalents

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

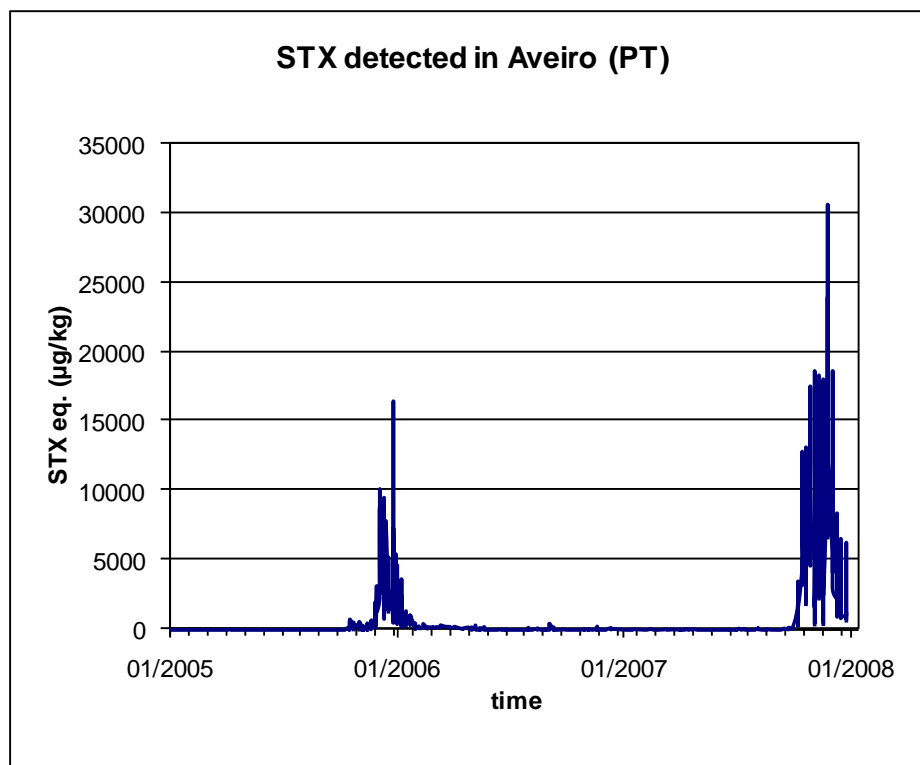
<sup>a)</sup> When the level of contamination is very low or the percentage of non detected results is very high, median, mean, P95 and in some cases even the maximum values (in case of HPLC with many analogues, some of which non-detected) are strongly influenced by the choice of upper or lower bound approach.

<sup>b)</sup> Lower bound values since the LOD is unknown

The percentage of pre-MC MBA samples with not quantified values vary to a large extent, depending on country and year of harvesting, and ranges from 54.8 % for Spain to 99.5 % for Italy. For pre-MC HPLC-FLD data the percentage of not quantified samples is 63.0 % for Portugal and 45.7 % for Norway. The proportion of pre-MC samples exceeding the EU regulatory limit (800 µg/kg) varies among countries: between 0.1 % (Italy) and 26.8 % (Spain).

Apparently some areas are more affected by STX-group toxins than others. Particularly high levels were reported by Portugal (67616 µg STX equivalents/kg shellfish meat) and Spain (40800 µg STX equivalents/kg shellfish meat). Lower levels are reported by Norway and UK, followed by France and Italy. The data from Germany cannot be compared with the others in terms of geographical distribution of STX-group toxins because the data all refer to post-MC samples of which the origin has not been reported.

Marine biotoxins are known to show a non-homogeneous distribution in terms of time and geographical location (Ciminiello *et al.*, 1999) and the data collected on STX-group toxins confirm this. The occurrence of high levels of STX-group toxins usually is limited in time, even in the geographical areas that are affected by these toxins. This is illustrated in Figure 6, where the occurrence of STX-group toxins is shown as a function of time for the area of Aveiro in Portugal. In a three year time frame only two periods lasting a few months occurred with high levels of STX-group toxins above a baseline of low contamination.



**Figure 6.** STX detection versus time in the years 2005-2007 measured in the area of Aveiro (Portugal).



### 5.3. Difference between shellfish species

Mussels were by far the predominant shellfish product tested, followed by clams, oysters, scallops and others. The statistical descriptors for the different species analysed with MBA in pre-MC samples are summarised in Table 6. HPLC-FLD samples are not reported because the Norwegian data are mostly mussels. Samples without reported values were assigned upper- and lower bound values. When the statistical descriptors in the two approaches are similar the highest value is given, otherwise the lower bound and upper bound values have been reported.

**Table 6.** Statistical descriptors for STX-group toxins occurrence in different shellfish sampled before harvesting. The data reported was obtained by MBA analysis.

Shellfish	N	Total concentration of STX-group toxins µg STX eq. /kg shellfish meat				% of samples not quantified	% of values >800 µg STX eq./kg shellfish meat
		Median LB/UB <sup>a)</sup>	Mean LB/UB <sup>a)</sup>	P95 LB/UB	Maximum reported		
<b>MBA (pre-MC)</b>							
Clams	1895	0/350	167/481	763	13800	86.8	4.9
Cockles	1099	0/380	1076/1277	4480	8430	55.0	36.3
Crabs <sup>b, c)</sup>	39	490	559	- <sup>d)</sup>	1140	0.0	10.3
Gastropods <sup>a)</sup>	11	890	2015	- <sup>d)</sup>	7300	45.5	54.5
Mussels	9287	0/350	125/462	400	40800	94.2	2.1
Oysters	1998	0/380	24/378	0/380	7360	96.6	0.5
Scallops	1087	0/350	73/406	238/400	5990	94.9	2.2
Others	85	0/350	53/371	- <sup>d)</sup>	600	88.2	0.0
<b>All</b>	<b>15501</b>						

N = number of samples, STX eq. = STX equivalents

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

<sup>b)</sup> Currently not regulated.

<sup>c)</sup> Based on brown meat (hepatopancreas) and not on whole flesh.

<sup>d)</sup> Values not calculated due to the low number of samples.

Very high maximum levels were found in mussels and clams, but considering the P95 the highest values are recorded for cockles and gastropods. Overall, the occurrence of STX-group toxins appears to similarly affect all considered species, with somewhat higher presence in cockles. No final conclusions on the contamination of crabs and gastropods can be drawn due to the low number of samples.

### 5.4. Influence of processing

Studies have shown that normal home cooking processes, such as boiling, steaming or pan-frying, can reduce the level of STX-group toxins in shellfish meat, due to the partial leaching-out of the toxins into the cooking liquid (“soup”) (Medcof *et al.*, 1947; Quayle, 1969; Lawrence *et al.*, 1994; Wong *et al.*, 2008). Lawrence *et al.* (1994) investigated the effects of cooking on the concentration of STX-group toxins in lobster hepatopancreas and found a reduction of STX equivalents of 40-65 % after steaming or boiling. The water loss associated with the cooking treatment was reported to range from 33 to 38 %. This indicated that STX-group toxins are leached out from the hepatopancreas during the loss of water. They also noticed that concentrations of GTX<sub>2,3</sub> in hepatopancreas are more reduced than those of STX or neoSTX, probably due to the weaker adsorption of GTX toxins to the hepatopancreas

matrix components. For scallops, Wong *et al.* (2008) observed that about 50 % (range 32 % to 64 %) of the total amount of STX-group toxins (expressed in  $\mu\text{g}$  STX equivalents) were passed from the flesh into the soup after steam cooking for 6 minutes. This reduction was consistent with the weight reduction of about 55 % due to water loss. The study did not indicate significant changes in the profile of STX-group toxins in scallops between raw and steamed samples, with the exception of GTX3 for which the decrease following processing did not only indicate leaching-out with water, but also suggested additional thermal degradation or conversion into other analogues.

The effect of higher temperatures (110°C and higher), as applied for canning and extrusion, have also been studied. Prakash *et al.* (1971) reported that cooking and commercial canning including sterilisation could reduce the level of STX-group toxins in clams and blue mussels by 70-90 %. Berenguer *et al.* (1993) and Vieites *et al.* (1999) investigated the effect of industrial canning processes on the concentration of STX-group toxins in cockles (*Acanthocardia tuberculata* L.) and mussels, respectively. Berenguer *et al.* (1993) observed a reduction in the concentration of STX-group toxins (expressed as  $\mu\text{g}$  STX equivalents/100g) of about 75 % in the final canned product after sterilization (autoclaving at 115°C for 45 minutes). Vieites *et al.* (1999) observed a reduction in the concentration of STX-group toxins in mussel meat ranging from 57-86 %, depending on the process applied (pickling, pH 2.6 or brining, pH 6.5). Both processes involved cooking (97°C for 2 minutes) and sterilization (115°C). Part of the observed reduction was attributed to transfer of STX-group toxins into cooking water and packing medium and part by thermal destruction. The authors suggested that the observation that pickling leads to a slightly smaller reduction in concentration of STX-group toxins in mussel meat was indicative for conversion of sulfo-carbamoyl analogues into more toxic carbamate analogues. However, due to the high variability of the reported results, the Panel considers it difficult to draw firm conclusions from this study. Mons *et al.* (1998), referring to Mizuta *et al.* (1995), pointed out that for oysters the reduction in the amount of STX-group toxins was less, about 20 % after boiling and with an additional reduction of 10 % after autoclaving.

## 6. Considerations on samples reaching the market

In contrast to the other marine biotoxins where residual exposure was calculated based on samples negative in the mouse bioassay, for STX-group toxins this approach was not possible because countries exclusively analyse shellfish samples by either the MBA (which in this case provides quantitative results) or by HPLC-based methods. Therefore, representative data which would allow a comparison between results obtained with the two methods for the same samples are lacking. Consequently, only pre-MC samples analysed with MBA and HPLC-FLD which were in compliance with the present EU regulatory limit were used to indicate the occurrence of STX-group toxins in shellfish presently reaching the market in different countries as shown in Table 7.

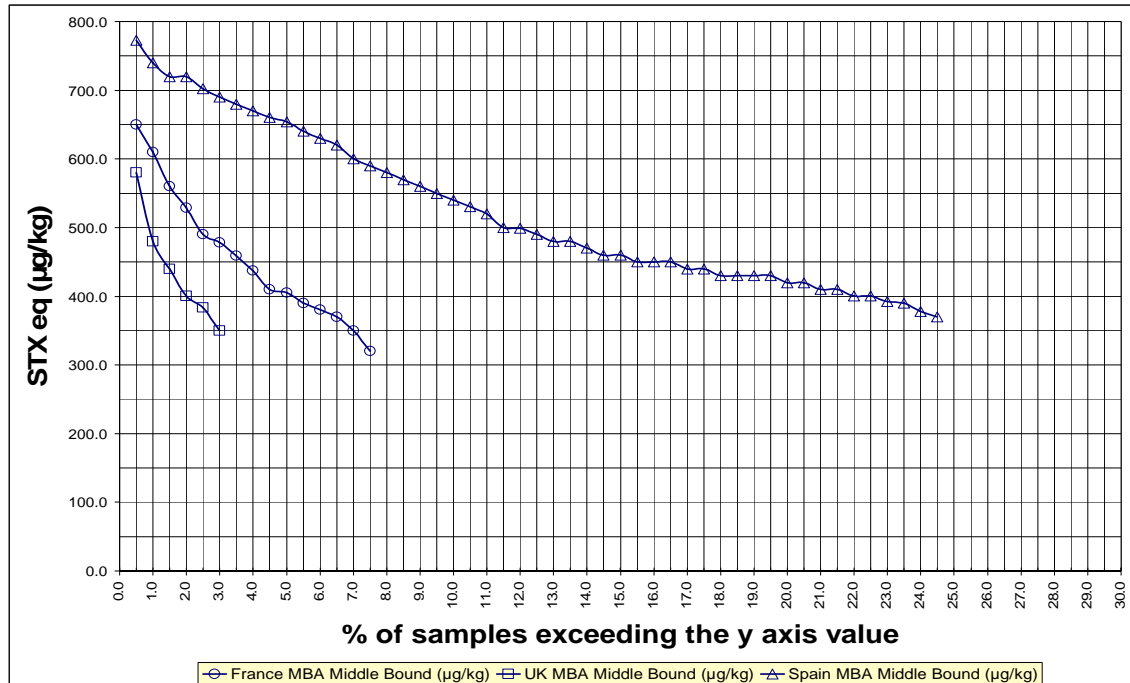
**Table 7.** Statistical descriptors by country for pre-MC samples analysed with HPLC-FLD and MBA, results conforming with the regulatory limit of 800 µg STX equivalents/kg shellfish meat.

Analytical method/ Country	N	Median	Mean	P95	Maximum	% of samples not quantified
		LB/MB/UB <sup>a)</sup>	LB/MB/UB <sup>a)</sup>	LB/MB/UB <sup>a)</sup>		
µg STX eq./kg shellfish meat						
<b>MBA</b>						
France	1832	0/175/350	36/193/350	405	770	92.2
Italy	7036	0/175/350	2/176/351	0/175/350	737	99.7
Spain	1752	0/200/400	131/269/407	654	792	75.1
UK	4146	0/190/380	15/199/383	0/190/380	790	96.9
<b>HPLC-FLD</b>						
Norway	1054	5/44/82	89/122/156	480	797	48.7

N = number of samples, STX eq. = STX equivalents

<sup>a)</sup> When three values are given it indicates the respective lower, medium and upper bound values for samples below the LOD or LOQ. The lower bound is performed substituting 0 to all not detected samples. The medium bound is performed substituting “<LOD” with LOD/2 value and “<LOQ” with LOQ/2 value. The upper bound is performed substituting “<LOD” with LOD value and “<LOQ” with LOQ value. LOD and LOQ are those defined for the specific single analysis.

Figure 7 shows the distribution of analytical values in pre-MC samples analysed with MBA in different countries and compliant with the regulatory limit. The distributions are truncated at the value of the LOQ since values below it are actually unknown. As a result of this truncation data for Italy are not presented in the graph because 99.5 % of samples were below LOQ. The vertical axis represents the total STX equivalents level (µg/kg) whereas the horizontal axis represents the percentage of samples with values above the respective level of STX equivalents. Not-quantified samples were substituted only with Medium Bound values (half the LOQ), to simplify the graph.



**Figure 7.** Distribution of STX equivalents (eq.) values analysed with MBA in different European countries, expressed as percent of values exceeding a given STX equivalent level.

## 7. Human consumption of shellfish

Limited consumption data were available for individual shellfish species across the EU. The EFSA Concise European Food Consumption Database does not yet provide sufficient information since there is no differentiation between meal sizes for fish and other seafood. Therefore, EFSA requested the Member States to provide information on shellfish consumption. Data were submitted by France, Germany, Italy, The Netherlands and UK. A compilation of the data received is presented in Table 8. The mean portion sizes for consumers only ranged between 10 g (France, bivalve molluscs) and 136 g (The Netherlands). The data from Germany, Italy and UK are within this range.

The German national food consumption survey performed by a weighing protocol in the late 1980s indicates a minimum meal size of mussels of 2 g (mainly as an ingredient in dishes), a median of 63 g, a mean of 107 g and a 95<sup>th</sup> percentile of 400 g among mussel consumers. The maximum portion size reported in this study was 1500 g (Adolf *et al.*, 1995). The French Calipso study differentiated mussels from other bivalve molluscs (Leblanc *et al.*, 2006). The maximum portions for mussels (245 g) and other bivalve molluscs (415 g) varied, whereas the mean portions were similar. A survey reported by the United Kingdom indicates a mean shellfish meal size of 114 g and a maximum of 239 g (Henderson *et al.*, 2002). A Dutch study reported a mean portion size of 136 g of shellfish and a maximum of 480 g. These data are for consumers only (Kistemaker *et al.*, 1998). The surveys show a large variation in the percentage of the populations consuming shellfish and it is unclear whether the data are related to cooked or uncooked shellfish.

**Table 8.** Shellfish eating habits in France, Italy, The Netherlands, the UK, and Germany, based on national food consumption surveys.

Country	Study	Number of consumers N (%)	Number of eating occasions for consumers/ year	Mean portion weight (g)	95th percentile (g)	Maximum portion weight (g)	Maximum frequency
France (7 days)	INCA 1999	218/1985 (11 %)	N/A	10			N/A
France (FFQ)	CALIPSO 2004 (bivalve molluscs)	962/997 (96 %)	N/A	32	94	415	N/A
France (FFQ)	CALIPSO 2004 (mussels)	862/997 (86 %)	N/A	22	70	245	N/A
Italy (7 days)	INN-CA 1994-96	212/1981 (11 %)	47	83		1000	4/week
Germany (7 days)	NVS 1985-88	150/23239 (0.6 %)	171	107	400	1500	3/week
UK (7 days)	NDNS 2000-01	212/1631 (13 %)	51	114		239	4/week
The Netherlands (2 days)	DNFCS 1997-98	47/4285 (1.1 %)	39	136	465	480	N/A

FFQ = food frequency questionnaire, 7 days = 7 day diary record, 2 days = 2 day dietary record, N/A = not available  
 INCA= Enquête Individuelle et Nationale sur les Consommations Alimentaires (Volatier, 2000).  
 CALIPSO = Fish and seafood consumption study and biomarker of exposure to trace elements, pollutants and omega 3 (Leblanc *et al.*, 2006)  
 INN-CA = Nationwide Nutritional Survey of Food Behaviour (Turrini *et al.*, 2001)  
 NVS = Nationale Verzehrsstudie (Adolf *et al.*, 1995)  
 NDNS = National Diet and Nutrition Survey (Henderson *et al.*, 2002)  
 DNFCS = Dutch National Food Consumption Survey (Kistemaker *et al.*, 1998)

Because STX-group toxins have acute toxic effects, it is important to identify a high portion size rather than a long term average consumption in order to protect the health of the consumer. In the studies presented in the table above, the maximum reported sizes are in the range of 239 to 1500 g. The Panel noted the highest portion sizes of 1000 g and 1500 g, and considered it likely that the shells were included in these weight estimates. Therefore, the Panel considered the 95<sup>th</sup> percentile as a more realistic estimate of the portion size for high consumers. As shown in Table 8, the 95<sup>th</sup> percentile values range from 70 to 465 g and the Panel chose the figure of 400 g to be used as a high portion size in acute exposure assessments. This is in good agreement with the report of the Joint FAO/IOC/WHO *ad hoc* expert consultation on marine biotoxins (FAO/IOC/WHO, 2004), where 380 g was reported as the highest 97.5<sup>th</sup> percentile portion size for consumers only.

## 8. Exposure assessment

The existing analytical methods and regulatory limits for STX-group toxins lead to a high proportion of non-detected samples. Consequently, the dietary exposure assessment is fully dependent on the approach taken for the non-detected samples. Figures 8a,b,c show the impact of choosing either lower, middle or upper bound on the median, mean and 95th percentile of the estimated dietary exposure based on the results reported by different European countries.

Because monitoring data are based on either a quantitative MBA or on HPLC-based methods (see Chapter 6), occurrence levels in commercial products in the different countries are expected to be below the current EU regulatory limit of 800 µg/kg STX equivalents.

Therefore the exposure for a 60 kg person eating a 400 g portion will maximally be of 5.3  $\mu\text{g}/\text{kg}$  b.w.

The uncertainty of the statistical calculations on exposure based on the lower (LB)-, medium (MB)- or upper (UB)-bound approach with high LOD values and the high number of non detected samples is clearly illustrated in Figures 8a,b,c. The dietary intake of a person eating 400 g of shellfish with a level of occurrence corresponding to the median, mean or P95 of the respective distribution is shown for the five countries, comparing lower-, medium- and upper bound approaches.

Figure 8a

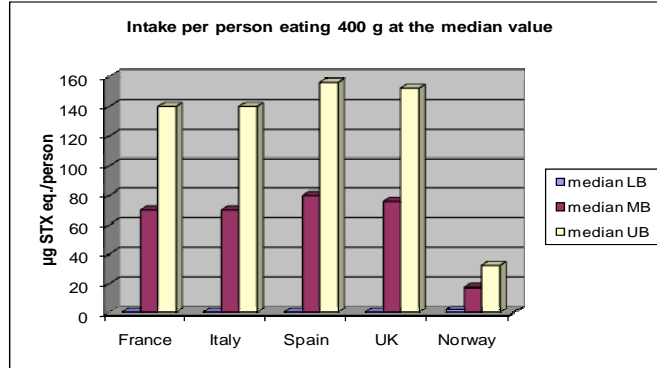


Figure 8b

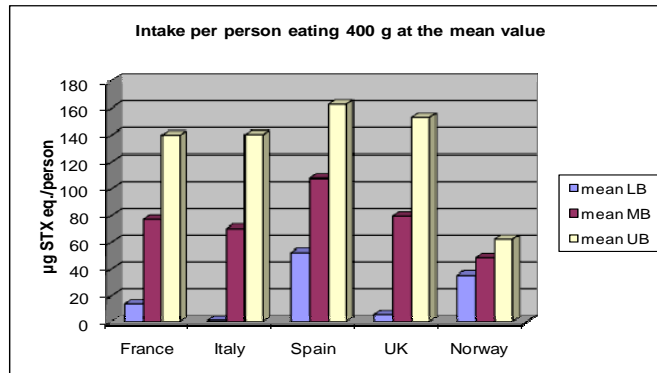
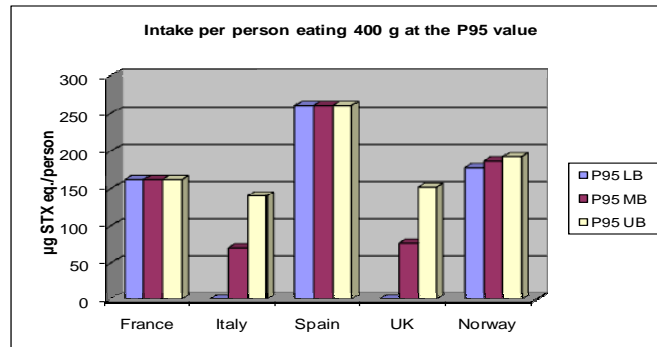


Figure 8c



**Figure 8a, b, c.** Comparison of the effect of adopting lower-, medium- and upper bound approaches in the dietary exposure calculation for a person eating 400 g of shellfish, at different levels of occurrence in different countries.



Quite unusually, the value of the P95 for Italy and the UK are also dependent on the choice of lower-, medium- or upper bound approach because, as it can be seen in Table 7, in these countries the percentage of samples “not quantified” is higher than 95 %. In such cases the value of the P95 is in the range from 0 (lower bound) to the LOQ (upper bound).

As mentioned before in this opinion, the profile of STX analogues varies, both qualitatively and quantitatively, due to differences in the occurrence of these toxins in algae in different regions and over time. In addition, as has been shown in Figures 8 a-c, any exposure estimate for European countries is highly influenced by choosing either lower, middle or upper bound for samples reported as “not quantified”. Therefore, the CONTAM Panel concluded that due to these uncertainties it is not feasible to perform a reliable exposure estimate.

## 9. Toxicokinetics

### 9.1. Absorption

Paraesthesia and numbness around the lips, tongue and mouth which appeared within minutes after eating toxic food indicated local absorption of the toxin through the buccal mucous membranes (Kao, 1993). It is evident from the short onset time reported in many intoxication cases that the STX-group toxins are quickly absorbed and transported in the blood to the other organs including the brain in humans (García *et al.*, 2004). Andrinolo *et al.* (2002b) determined the mechanisms involved in toxin absorption using layers of intestinal epithelial cell lines derived from human and rat and concluded that GTX2 and GTX3 were transported across the epithelium by the paracellular route.

### 9.2. Distribution

There are few data on the distribution of STX-group toxins in the body of human patients, due to the difficulty of analysis as well as obtaining samples. In a post mortem examination of the samples from a victim of toxic coral crab, only trace amounts of toxin were detected in the liver by radioreceptor binding assays, while fairly large amounts were found in the gut contents, blood and urine (Llewellyn *et al.*, 2002). In an intoxication episode in Chile in 2002 (García *et al.*, 2004), two fishermen died 3-4 hours after consumption of 7-9 ribbed mussels (*Aulacomya ater*) containing 8575 µg/100g of STX equivalents (MBA). The STX-group toxin profile from body fluids and tissues from the victims, based on HPLC analysis using post-column derivatisation with fluorescence detection, are shown in Table 9.

**Table 9.** The STX-group toxin profile from body fluids and tissues from the victims, based on HPLC analysis using post-column derivatisation with fluorescence detection (García *et al.*, 2004).

Samples:	STX	neoSTX	dcSTX	GTX-4	GTX-1	GTX-5	GTX-3	GTX-2
	µg/g tissue							
Thyroid glands	2.86	n.d.	n.d.	0.31	2.33	n.d.	n.d.	n.d.
Stomach	14.24	n.d.	n.d.	n.d.	n.d.	0.22	1.04	0.57
Gastric content	39.69	n.d.	n.d.	1.29	1.26	0.10	2.64	1.15
Spleen	0.43	0.22	n.d.	0.23	0.01	0.29	n.d.	n.d.
Liver	0.55	n.d.	0.04	0.34	0.13	0.21	0.01	0.01
Pancreas	8.18	1.30	n.d.	0.10	1.44	n.d.	n.d.	n.d.
Kidney	0.26	n.d.	0.01	0.14	0.07	0.06	0.01	0.05
Adrenal glands	1.52	n.d.	n.d.	0.13	0.25	n.d.	n.d.	n.d.
Fluids:*								
Bile	1.53	0.69	n.d.	0.41	0.09	n.d.	n.d.	n.d.
Cerebrospinal fluid	n.d.	0.77	n.d.	0.02	0.06	n.d.	n.d.	n.d.
Urine	1.80	22.33	n.d.	2.14	0.03	n.d.	n.d.	n.d.
Vitreous humour	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Brain:								
Grey matter	0.65	n.d.	n.d.	0.05	0.65	n.d.	n.d.	n.d.
White matter	0.08	n.d.	n.d.	0.04	0.57	n.d.	n.d.	n.d.
Heart:								
Pericardium	0.37	n.d.	n.d.	0.02	0.65	n.d.	n.d.	n.d.
Myocardium	0.67	n.d.	n.d.	0.05	1.52	n.d.	n.d.	n.d.
Endocardium	n.d.	n.d.	n.d.	0.10	2.25	n.d.	n.d.	n.d.
Papillary muscle	0.63	n.d.	n.d.	0.06	1.03	n.d.	n.d.	n.d.
Aorta	n.d.	n.d.	n.d.	0.28	0.57	n.d.	n.d.	n.d.
Lung	0.75	n.d.	0.06	0.14	0.16	n.d.	0.05	0.03

\*µg/mL, n.d.= not detected

In experiments with cats, STX injected into blood disappeared quickly with a serum half-life of 22 minutes. STX was detected by HPLC analysis in the blood and urine as well as spleen, liver, medulla oblongata and brain (in the order of concentration) (Andrinolo *et al.*, 1999). In another experiment with rats, tritiated saxitoxinol was used ([<sup>3</sup>H]STXOL, saxitoxinol is the reduced analogue of STX), a radioactively stable derivative of STX, showing very low affinity to sodium channels, also showed quick disappearance of toxin from blood (29 minutes serum half-life). One hour after intravenous administration, 5.0, 2.2, 2.2, 1.3, 0.8, 0.2, 0.1 and 0.04 % of total dose of radioactivity was detected in the muscle, liver, kidney, small intestine, large intestine, lung, heart and spleen, respectively (Naseem, 1996).

### 9.3. Biotransformation

There are several reports on the suspected biotransformation of the toxins in the human body, based on the different toxin profiles observed between the causative foods and human biological specimens by HPLC analysis. Gessner *et al.* (1997) found higher proportions of C1, C2 and less GTX2 in the serum and urine, compared to the cooked mussels as the leftover of implicated meal. The gut contents and urine sample of a crab poisoning victim showed higher proportion of STX and less GTX2, 3 in comparison with uneaten crab. Also, GTX1, GTX4 and dcSTX, which were not detected in the crab, were found in the urine (Llewellyn *et al.*, 2002). The authors speculated whether reductive cleavage of 11-hydroxy sulphate (often observed in shellfish during toxin accumulation), oxidation of N-1 (very unusual) and decarbamylation (found in certain clams) took place in the gut as well as in the body. However, toxin identification was rather ambiguous due to many interfering peaks in the chromatograms shown in the paper. In the publication by García *et al.* (2004) referred to above, they describe metabolic transformation of several STX-group toxins in the two fishermen that died 3-4 hours after consumption of 7-9 ribbed mussels (*Aulacomya ater*). The STX-group toxins found in gastric content were STX, GTX4, GTX1, GTX5, GTX3 and GTX2. The STX-group toxin composition in urine and bile showed mainly NeoSTX and GTX4/GTX1 epimers. The NeoSTX was not present in the gastric juice, in a similar way that GTX3/GTX2 were transformed in GTX4/GTX1 epimers. Furthermore, the hydrolysis product of STX, dcSTX, was detected in liver, kidney and lung. The authors concluded that STX-group toxins are metabolically transformed in humans.

When heated at low pH, toxins having a N-sulphocarbamoyl moiety (such as B1 (GTX5), B2 (GTX6), C1-C4)) as a side chain were easily converted to the corresponding carbamate toxins through hydrolysis. Since the reaction resulted in a several fold increase in toxicity (Hall and Reichardt, 1984), this phenomenon has been examined experimentally. B1 (GTX5) was incubated at modelled conditions for the human stomach. The hydrolysis was monitored by the mouse bioassay. After 5 hours incubation at 37°C, in the artificial gastric juice at pH 1.1 about 9 % conversion of toxin was observed. In rat gastric juice at pH 2.2 no conversion was observed (Harada *et al.*, 1984). Similar experiments carried out on C1, C2 using HPLC to monitor conversion showed that 5.5 % of the toxins were converted to GTX2,3 at pH 1.6 and 1.5 % at pH 2.2 after 4 hours incubation (Oshima, unpublished results). These data indicated that any increase in toxicity in the human body due to the hydrolysis of N-sulphocarbamoyl toxins may not be significant.

In addition to the above toxin conversion, the following chemical transformations were reported and often observed during toxin accumulation by shellfish, but they have not been reported in warm-blooded animals (Oshima, 1995a):

- epimerization of 11-hydroxysulfate at neutral pH,
- reduction of N1-OH,

- reductive elimination of 11-hydroxysulfate  
(stable thioether intermediates of this reaction were reported by Sato *et al.*, 2000) ,
- hydrolysis of N-sulfocarbamoyl at neutral pH.

No apparent change was observed in GTX2 and GTX3 incubated with cat liver homogenate (Andrinolo *et al.*, 2002a). Furthermore, when GTX2/3 and C1/2 were incubated with liver enzyme preparations from rats and mice, no transformation of the toxins was detectable (Hong *et al.*, 2003).

#### 9.4. Elimination and bioaccumulation

The detection of high concentration of toxins in the urine of patients indicates that urine is a primary route of human toxin excretion. In patients from Alaska, STX levels of 65-372 nM (corresponding to about 20-120 µg STX equivalents/L) were detected by HPLC analysis in urine, in comparison with 2.8-47 nM (corresponding to about 0.8-14 µg STX equivalents/L) in serum, at acute illness and after acute symptom resolution (Gessner *et al.*, 1997). In the study by García *et al.* (2004), of two fishermen that died in 3-4 hours, STX and NeoSTX were found in urine at concentrations of 1.8 and 22.33 mg/L, respectively, while similar levels in the bile were 1.53 and 0.69 mg/L.

That urine is the main excretion route is supported by the animal experiments in which toxins were administered orally or intravenously. An early study by Prinzmetal *et al.* (1932) reported that 40 % of the toxins were detected by the mouse bioassay in the urine of a dog 2 hours after intravenous injection of crude toxin. From these data, Kao (1993) estimated the half-life of toxin elimination from the body to be in the order of 90 minutes. More recent studies on rats and cats, using more sophisticated methods of toxin analysis, showed slower elimination from the body. Intravenously administered STX in rats, at a dose of 2 µg/kg, was estimated to have a half -life of 17.8 hours by Stafford and Hines (1995). Similar results were observed in cats by HPLC analysis, in which 25 % and 10 % of the administered toxin were excreted in urine within 4 hours when STX was injected at doses of 2.7 µg/kg and 10 µg/kg, respectively (Andrinolo *et al.*, 1999). In the latter report, the authors concluded that glomerular filtration was the main excretion route of STX. In experiments with rats with [<sup>3</sup>H]STXOL, the half-life of toxin elimination from the body was estimated to be of 12.3 hours. Small quantities of non-metabolised STXOL were detected in rat urine up to 144 hours after intravenous administration (Hines *et al.*, 1993, Naseem, 1996). Faecal elimination of STX in non-primates is unlikely, because it was not detected in the bile of cats (Andrinolo *et al.*, 1999) and no radioactivity was recovered in the faeces of rats injected with [<sup>3</sup>H]STXOL (Stafford and Hines, 1995).

## 10. Toxicity data

### 10.1. Mechanistic considerations

It has long been recognized that STX-group toxins act by interfering with voltage-gated sodium channel functioning (Hille, 1966 and 1968; Kao, 1966; Ritchie, 1975). The toxin acts from the exterior of the cells (Hille, 1968), by getting access to the extracellular cavity of the channel and binding to the so-called site 1 of the  $\alpha$ -subunit in the sodium channel (Cestèle and Catterall, 2000). Site 1 is shaped by a short portion (SS2) of the amino acid stretches connecting the S5 and S6 trans-membrane helices in the four domains of the  $\alpha$  subunit of the sodium channel, giving rise to a cavity that accommodates the toxin. STX then forms hydrogen bonds and electrostatic interactions with the side chains of several amino acids (mostly the negatively charged dissociated groups of glutamic acid and aspartic acid) that

participate to the ion selectivity filter of the channel (Cestèle and Catterall, 2000; Shimizu, 2000; Catterall *et al.*, 2007).

The interaction of one STX molecule with the site 1 of the  $\alpha$ -subunit in the sodium channel (Hartshorne and Catterall, 1984) essentially plugs the channel, as originally proposed by Hille (1975), and blocks its ion conductance (Hille, 1968).

The loss of sodium conductance in excitable cells prevents membrane depolarization and the transmission of the action potential, representing the molecular basis of the toxic effects of STX. As a consequence of voltage-gated sodium channel blockade, a progressive loss of neuromuscular function ensues, leading to the reported neurotoxic (paralytic) symptoms that can result in death by asphyxia (section 10.2.1.2).

Because the effects of STX stem from its interaction with voltage-gated sodium channels, the structure of both the STX-group toxin molecules and the amino acid sequence in the SS2 region of ion channels (encompassing site 1) determine the biological responses.

The positively charged guanidinium moiety of STX-group toxins is a major structural determinant for their interaction with site 1 of the  $\alpha$ -subunit in the sodium channel at neutral pH, but other parts of the molecule participate to binding, inasmuch as structural changes in the hydroxyl groups at C<sub>12</sub> and the carbamoyl side chain of STX-group toxins are recognized to affect the binding affinity and/or biological activity of STX analogues (Shimizu, 2000; Llewellyn, 2006; Oshima, 1995b).

On the receptorial side, it is known that nine isoforms of  $\alpha$ -subunits of voltage-gated sodium channels exist (Goldin *et al.*, 2000), with significant, albeit distinct, similarities in amino acid sequences. Thus, different sensitivities among sodium channel systems to STX and its analogues are expected, but their impact on toxicity of this group of toxins remains to be fully clarified.

In more general terms, the significant homologies among channels for different cations, and the high number of STX analogues, could be the basis for a wider array of biological effects of this group of toxins. For instance, the action of STX on potassium and calcium channels has been reported (Wang *et al.*, 2003; Su *et al.*, 2004), but the effective doses in those molecular systems are three-four orders of magnitude higher ( $10^{-6}$ - $10^{-5}$  M) than those affecting voltage-gated sodium channels ( $10^{-10}$ - $10^{-8}$  M), and the toxicological relevance of interaction of STX with ion channels other than the voltage-gated sodium channels remains to be established.

Furthermore, the binding of STX with soluble proteins (saxiphilin and others), has been reported (Llewellyn, 2006), but the functional significance of those interactions remains undetermined.

Based on available information, the Panel concluded that the binding of STX-group toxins to voltage-gated sodium channels and the consequent blockade of ion conductance through the channels is the major molecular mechanism of action of this group of toxins, although the contribution of other molecular events to their effects and toxicity in some biological conditions can not be excluded at the moment.

## 10.2. Effects in laboratory animals

### 10.2.1. Acute toxicity

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

The main adverse effect of STX-group toxins in animal species and humans is neurotoxicity. The *i.p.* lethal dose 50 % (LD<sub>50</sub>) of STX is in the order of 10 µg/kg b.w., which for a 20 g mouse corresponds to 0.2 µg STX. This amount of STX was defined as a mouse unit (MU). Subsequently, using purified STX, Schantz (1986) determined a value of 0.18 µg STX.2HCl for the MU, and this value has frequently been applied in converting concentrations of STX reported in MU into STX equivalents. Prakash *et al.* (1971) noted that the amount of toxin equivalent to one MU depends on the assay technique and strain of mice, specifying that in their laboratory the MU was equivalent to 0.16 µg STX up to May 1966, and thereafter was 0.22 µg STX as a result of changing to a slightly less sensitive strain of mice. Based on an MU value of 0.18 µg STX.2HCl, it can be calculated that the toxicity of STX.2HCl would be 5.5 MU/µg, which equals 2046 MU/µmol.

The acute *i.p.* toxicity of other analogues has also been measured in mice. Usually, reports on the structural elucidation of these analogues also gave information on the toxicity on a weight basis. However, often these values were not accurate because over-drying of the toxins often caused degradation or transformation to other toxins (Genenah and Shimizu, 1981). For a comparison among the large number of analogues known to date, a short description of specific toxicity of major 14 analogues is shown in Table 10 (Oshima, 1995b; Oshima *et al.*, 2004), determined during preparation of HPLC standards. The toxicity of each pure toxin solution was determined using Sommer's table (AOAC method 959.08) for the mouse bioassay, while toxin concentrations on a molar basis were based on the nitrogen content of the solution through combustion analysis, so that the values were given in MU/µmol. The data are close to those presented by Hall *et al.* (1990). It is noteworthy that *i.p.* toxicity to mice and binding affinity were almost parallel for most toxins (Hall *et al.*, 1990).

**Table 10.** Specific toxicities of saxitoxin (STX) analogues (Oshima, 1995b; Oshima *et al.*, 2004).

Toxin	Specific toxicity (MU/µmol)
STX	2483
NeoSTX	2295
GTX1	2468
GTX2	892
GTX3	1584
GTX4	1803
dcSTX	1274
dcGTX2	382 <sup>a)</sup>
dcGTX3	935 <sup>a)</sup>
B1 (GTX5)	160
C1	15
C2	239
C3 <sup>b)</sup>	33
C4 <sup>b)</sup>	143

MU = mouse unit

<sup>a)</sup> Data corrected by Oshima after the 1995b publication (Oshima *et al.*, 2004).

<sup>b)</sup> Estimated by the measurement of GTX1 and GTX4 formed by acid hydrolysis of C3 and C4, respectively.



### 10.2.1.2. Toxicity following intravenous (*i.v.*) administration

Besides the acute lethal *i.p.* toxicity described above, other acute effects on experimental animals were reviewed by Mons *et al.* (1998). These authors described the effects of STX on the respiratory system, myocardium, muscle and nervous tissue (both peripheral and central) in various animal species.

#### Effects on the respiratory system

When STX intoxication occurs, progressive respiratory muscle paralysis leading to respiratory arrest (asphyxiation) is responsible for the fatal outcome. In animals (cat, rabbit) *i.v.* doses of 1-2 µg STX/kg b.w. caused decreased respiratory activity (decline in amplitude and velocity). At a dose of 4-5 µg STX/kg b.w., a strong depression of respiration was observed, which resulted in death unless artificial respiration was provided. At lower doses, respiration may return spontaneously (Mons *et al.*, 1998).

#### Cardiovascular effects

In anaesthetised animals, doses above 1 µg STX/kg b.w. (*i.v.*) can provoke hypotension, with paralysis of muscles already observed at lower dose levels. This cardiovascular effect is seldom observed in human intoxications. It is unclear whether this is a reflection of an effect on the central nervous system, or whether it is more likely the reflection of peripheral effects. There are however uncertainties in a peripheral action. Apart from a direct effect on the muscle tissue, the possibility of an axonal blockade of the sympathetic nervous system cannot be excluded. It is generally accepted that no, or hardly any direct cardiac effects, occur (Mons *et al.*, 1998).

#### Neuromuscular effects

An intravenous dose of 1-2 µg STX in animals (cat, rabbit) causes a fast weakening of muscle contractions; both contractions by direct stimulation as well as contractions by indirect motoneuron stimulation are affected. This dose level induces also a decrease of the action potential-amplitude and a longer latency time in the peripheral nervous tissue. Both motor and sensory neurons are affected. Through the effect on the sensory system the numbness and the proprioceptive loss may be explained, but not the paraesthesia (Mons *et al.*, 1998).

#### Effects on the central nervous system

There are uncertainties about the existence of an effect of the toxins on the central nervous system (Mons *et al.*, 1998). The occurrence of paraesthesia and feeling of lightness in humans are often attributed to a central effect, but effects on the peripheral nervous system may be the cause of these symptoms. Most symptoms can be attributed to peripheral effects.

### 10.2.1.3. Toxicity following oral administration

The LD<sub>50</sub> values of purified STX-dihydrochloride to mice by the different routes of administration were determined at Health Canada by Wiberg and Stephenson (1960). As shown in Table 11, the oral toxicity was 1/25<sup>th</sup> of the intraperitoneal toxicity.



**Table 11.** Acute toxicity of STX in mice by different routes of administration (Wiberg and Stephenson, 1960).

Route	LD <sub>50</sub> in µg/kg body weight
oral	260-263
intravenous	2.4-3.4
intraperitoneal	9.0-11.6

For a comparison of susceptibility among different animals, only one report is available. The oral LD<sub>50</sub> values for several species of warm-blooded animals were examined by McFarren *et al.* (1960) as shown in Table 12. Not much difference was observed among the mammals tested.

**Table 12.** Oral toxicities (LD<sub>50</sub> in µg/kg b.w.) of STX in various species.

Animals	Mons <i>et al.</i> (1998)*	McFarren <i>et al.</i> (1960)
mouse	260-263	420
rat	192-212	212
monkey	277-800	400-800
cat	254-280	280
rabbit	181-200	200
dog	180-200	200
guinea pig	128-135	128
pigeon	91-100	100

\*With reference to Krogh (1983) and Shimizu (1978).

Andrinolo *et al.* (2002a) reported that an oral dose of 35 µg/kg of GTX 2,3 was lethal to cats. Comparing this observation with the oral LD<sub>50</sub> for STX cited in Table 12 suggests that GTX analogues might be more toxic than STX via the oral route in cats, but based on the limited information available and the lack of direct comparative testing with STX, it is not possible to draw definite conclusions on this.

### Tolerance development

Prior exposure to non-lethal doses of STX-group toxins seems to lower the susceptibility of rats to lethal doses of these toxins. In a study using Sprague-Dawley rats (sex not indicated), the oral LD<sub>50</sub> value for the purified extract containing STX-group toxins was determined (McFarren *et al.*, 1960). One group of rats was given a non-lethal dose of about one-third of the LD<sub>50</sub>, 14 days before the test. The LD<sub>50</sub> for the pre-treated rats was about 50 % higher than that for untreated rats. This finding corroborates the fact noted by Prakash *et al.* (1971) that fishermen who habitually eat shellfish containing low levels of STX-group toxins may be less susceptible for the toxic effects of these toxins.

### 10.3. Relative potency of analogues

The relative toxicity of STX analogues has been studied in MBAs (Genenah and Shimizu, 1981; Koehn *et al.*, 1982; Oshima, 2004; Schantz, 1986; Sullivan *et al.*, 1983, 1985; Wichman *et al.*, 1981; Vale *et al.*, 2008) and *in vitro* (Vale *et al.*, 2008). The results are shown in Table 13. Although overall the values seem to be similar, there are some discrepancies. The MU has been calculated as 0.18 µg STX (Schantz, 1986), 0.16 µg STX and 0.22 µg STX (Prakash, 1971) 0.261 µg STX (Botana *et al.*, 1996) and 0.274 µg STX (Vale *et al.*, 2008), which leads to differences in specific activities for STX-group toxins cited in the literature. Most of these studies were conducted with non certified standards. A recent work evaluated the relative toxicity with NRC certified standards (Vale *et al.*, 2008).

Although the potency is generally similar in the different reports, the toxicity of dc-STX, seems to be underestimated in Oshima's work compared to that of Vale *et al.* (2008). Similarly, the potency of the GTX1,4 reference material seems to indicate that the toxicity of GTX1 was overestimated in earlier reports (see Table 13).

**Table 13.** Relative potencies of STX-group toxins.

Toxin	Specific toxicity of STX-group toxins (potency relative to STX)								
	1 MBA (MU/ μmol)	2 MBA (MU/ μmol)	3 MBA (MU/ μmol)	4 MBA (MU/ μmol)	5 MBA (MU/ μmol)	6 MBA (MU/ μmol)	7 * MBA (MU/ μmol)	8 ** MBA (MU/ μmol)	8 *** IC <sub>50</sub> (μM)
STX	2400 (1.0)		2045 (1.0)	1667 (1.0)	2100 (1.0)	2045 (1.0)	2483 (1.0)	1360 (1.0)	0.0051 (1.0)
NeoSTX	2900 (1.2)		1038 (0.5)	1563 (0.9)			2295 (0.9)	1328 (1.0)	0.0062 (0.8)
GTX 1			1638 (0.8)				2468 (1.0)		
GTX 2		1040	793 (0.4)				892 (0.4)		
GTX 3		1480	2234 (1.1)				1584 (0.6)		
GTX 4			673 (0.3)				1803 (0.7)		
GTX1,4 (75, 25 %)								994 (0.7)	0.0095 (0.5)
GTX2,3 (75, 25 %)								824 (0.6)	0.0136 (0.4)
GTX 5 (B1)	150 (0.1)		354 (0.2)				160 (0.1)		0.052 (0.1)
GTX 6 (B2)	180 (0.1)								
C1 (epi-GTX 8)		17		28 (0.02)			15 (0.0)		
C2 (GTX 8)		238		286 (0.17)			239 (0.1)		
C3						8 (0.0)	33 (0.01)		
C4						57 (0.0)	143 (0.1)		
dc-STX				847 (0.5)	900 (0.4)	1220 (0.6)	1274 (0.5)	1355 (1.0)	0.0063 (0.8)
dc-NeoSTX (GTX 7)					900 (0.4)				0.0105 (0.5)
dc-GTX 1					950 (0.5)				
dc-GTX 2					380 (0.2)	530 (0.3)	382 (0.2)		
dc-GTX 3					380 (0.2)	990 (0.5)	935 (0.4)		
dc-GTX 4					950 (0.5)				
dcGTX2,3 (78, 22 %)								259 (0.2)	0.0228 (0.2)
11α-hydroxy- STX				943 (0.6)					
11 β-hydroxy- STX				787 (0.7)					

MBA = mouse bioassay, IC<sub>50</sub> = Inhibitory concentration - the concentration of a substance that reduces the effect by 50 %

\* STX.2HCl, \*\* All results obtained with certified reference standards, and STX results originally obtained with acetate salt,

\*\*\* *In vitro* results obtained with certified reference standards in isolated rat neurons (7 day cerebellar granule cells (CGC)).

(1) Koehn *et al.* (1982)

(5) Sullivan *et al.* (1983; 1985)

(2) Wichmann *et al.* (1981)

(6) Sullivan *et al.* (1985) as cited in Franco and Fernández (1993), excluding the data that are clearly replicated

(3) Genenah and Shimizu (1981)

(7) Oshima *et al.* (2004)

(4) Schantz (1986)

(8) Vale *et al.* (2008), recalculated in MU/μmol for EFSA by the authors

Based on an evaluation of the relative potencies presented in Table 13, and giving greater weight to more recent data and to those derived using certified reference material, the CONTAM Panel proposed TEF values for STX-group toxins as shown in Table 14. For most analogues the TEFs are comparable with those resulting from the Oshima data (Oshima 1995b; Oshima *et al.*, 2004) as presented in Table 10. For dc-STX, however, the CONTAM Panel concluded that recent information based on pure reference material indicated a TEF of 1.0, rather than a value of about 0.5 that could be calculated from the Oshima data. In addition, TEFs were not proposed for the reference material containing mixtures of toxins (GTX1,4, GTX 2,3 and dc-GTX 2,3) because they would not apply to material containing different proportions of the analogues.

**Table 14.** Toxicity equivalency factors (TEFs) of STX-group toxins proposed by the CONTAM Panel (to be applied on a molar basis).

Toxin	Proposed TEFs
STX	1.0
NeoSTX	1.0
GTX 1	1.0
GTX 2	0.4
GTX 3	0.6
GTX 4	0.7
GTX 5 (B1)	0.1
GTX 6 (B2)	0.1
C2 (GTX 8)	0.1
C4	0.1
dc-STX	1.0
dc-NeoSTX (GTX 7)	0.4
dc-GTX 2	0.2
dc-GTX 3	0.4
11-hydroxy-STX	0.3

The TEFs as given in Table 14 suggest a high level of precision. It should be noted that this is not due to the confidence the Panel has in the physiological basis of the TEFs, but merely the result of the fact that the Panel did not want to deviate, if not necessary, too far from the TEFs currently applied in the analytical methods for the detection of STX-group toxins.

#### 10.4. Impact of the use of the TEFs proposed by the Panel to the statistical descriptors

The impact of applying the new TEFs proposed by the Panel to the statistical descriptors is shown comparatively with the TEFs based on the Oshima data (Oshima, 1995b; Oshima *et al.*, 2004) for the German and Norwegian datasets, considering the samples either from the market or from the monitoring studies performed for fulfilling the present EU regulatory limits (Table 15). The Norwegian statistical descriptors show very limited sensitivity to the change of TEFs; for Germany the maximum is strongly influenced whereas median, mean and P95 are not. The observed effect appears therefore to depend on the toxin profile, mainly in relation to the proportion of the dc-STX analogue. The overall impact of the new TEFs is expected to be limited, except for situations where dc-STX is present in significant amounts.

**Table 15.** Comparison of statistical descriptors for samples analysed with HPLC-FLD conforming to the regulatory limit, calculated with the toxicity equivalency factors (TEFs) derived from Oshima and the new TEFs proposed by the CONTAM Panel.

Analytical method/ Country	N	Median	Mean	P95	Maximum
		LB/MB/UB <sup>a)</sup>	LB/MB/UB <sup>a)</sup>	LB/MB/UB <sup>a)</sup>	LB/MB/UB <sup>a)</sup>
<b>HPLC-FLD</b>					
Norway OT	1,054	5/44/82	89/122/156	480	797
Norway NT	1,054	5/47/87	90/126/163	498	830
Germany OT	445	0/150/218	14/110/206	71/182/345	694/839/984
Germany NT	445	0/121/194	17/117/217	72/189/359	1,351/1,490/1,629

N = number of samples, STX eq. = STX equivalents, OT = previously adopted TEFs based on Oshima publications, NT = New TEFs proposed by the Panel

<sup>a)</sup> When three values are given it indicates the respective lower, medium and upper bound values for samples below the LOD or LOQ. The lower bound is performed substituting 0 to all not detected samples. The medium bound is performed substituting “<LOD” with LOD/2 value and “<LOQ” with LOQ/2 value. The upper bound is performed substituting “<LOD” with LOD value and “<LOQ” with LOQ value. LOD and LOQ are those defined for the specific single analysis.

## 11. Observations in humans

Historical accounts of PSP in humans date back to at least the eighteenth century, when a group of seamen developed symptoms after eating mussels off the British Columbia coast (Fortuine, 2007). Since the 1940s cases of PSP have been reported in many countries, including Norway (Langeland *et al.* 1984), the UK (McCullum *et al.*, 1968), Canada (Prakash *et al.*, 1971; Tennant *et al.*, 1955), North America (Gessner *et al.*, 1997; Gessner and Middaugh, 1995), Chile (García *et al.*, 2005), South Africa (Popkiss *et al.*, 1979) Japan and Indonesia (Kao, 1993). This chapter focuses on reports that provide quantitative information on the amount of toxins consumed.

Symptoms of PSP have been categorised as mild, moderately severe and extremely severe (Prakash *et al.* 1971). Mild symptoms include a tingling sensation or numbness around the lips gradually spreading to the face and neck, a prickly sensation in fingertips and toes, headache, dizziness and nausea. Moderately severe symptoms are incoherent speech, progression of prickly sensation to arms and legs, stiffness and non-coordination of limbs, general weakness and feeling of lightness; slight respiratory difficulty and rapid pulse plus backache as a late symptom (FAO/IOC/WHO, 2004). In extremely severe cases, symptoms include muscular paralysis, pronounced respiratory difficulty and a choking sensation.

In fatal cases, death is caused by respiratory paralysis in the absence of artificial respiration. Patients who survive PSP for 24 hours, with or without mechanical intervention, have a high probability of a full and rapid recovery. As medical intervention can influence the outcome of extremely severe poisoning, the presence or absence of such treatment in particular cases may affect estimates of lethal doses.

There are a number of uncertainties in the human case reports, predominantly relating to estimates of exposure. While leftovers from the meal associated with illness were analysed in some incidents, other reports are based on toxin concentrations determined in uncooked shellfish, either from the batch that had been consumed or one that was obtained from the same harvesting area, restaurant or retailer. In some reports such samples were collected on the same day as shellfish involved in the PSP incident, while in others they were collected on a different day.

Further uncertainties relate to estimates of amounts of shellfish consumed and assumptions regarding the weight of edible portions of specific shellfish species. In addition, studies have shown that cooking can reduce the toxicity of STX-group toxin-contaminated shellfish by as

much as 70 % (Prakash *et al.*, 1971), and therefore some reports applied a correction factor to data on toxin levels in raw shellfish. However, the toxins are not completely destroyed by cooking and are, at least in part, leached into the cooking fluids. While the fluids are frequently discarded following steaming, substantial amounts may still be ingested, for example when eating chowder. Therefore the precise influence of particular cooking and eating practices on toxin levels and exposure is uncertain.

In the majority of reports toxin levels were determined by MBA, and hence no information is available on the profile of the STX analogues present. In the earlier reports the MBA results were cited as MU. To aid comparison, these values have been converted to STX equivalents, assuming a conversion factor of 0.18 µg STX equivalents per MU (Schantz, 1986). If this was an underestimate of the toxin content, then the dietary exposures will also have been underestimated. The CONTAM Panel considered this source of uncertainty to be small compared to other aspects. Where specific conversion factors were calculated by the authors, these were used in estimating toxin intakes in cases of human poisonings.

A detailed report from Norway describes an incident that occurred in 1981, in which 8 out of 10 individuals who consumed mussels developed symptoms of PSP (Langeland *et al.*, 1984). Symptoms developed between 5 minutes and 4 hours following shellfish consumption, and lasted from 12 hours to 4 days. MBA analysis of leftover mussels from meals eaten by 5 of the individuals, together with estimation of mussel consumption and measurement of the individuals' body weights, indicated that patients with slight, moderate or severe symptoms had toxin intakes ranging from 10-75, 35-100 and 85-100 MU/kg b.w., respectively. Applying the conversion factor of 0.18 µg STX equivalents per MU, the doses can be calculated as ranging from 1.8-13.5, 6.3-18 and 15.3-18 µg STX equivalents/kg b.w. Two patients who had an estimated intake of 3.6 µg STX equivalents/kg b.w. did not experience any symptoms.

One of the largest reviews of PSP case reports can be found in an unpublished Health Canada report, in which data on over 90 individuals were assessed (Kuiper-Goodman and Todd, 1991). This review predominantly focused on Canadian cases reported between 1970 and 1990, together with information on outbreaks in Canada from 1944-1970 and Guatemala in 1987. Estimated intakes for patients with mild, moderately severe and extremely severe poisoning ranged from 0.7-70, 1.5-150 and 5.6-300 µg STX equivalents/kg b.w., respectively. One patient with moderately severe symptoms had an estimated intake of 0.3 µg STX equivalents/kg b.w., but this was considered a probable outlier. Some individuals did not develop symptoms after apparently consuming doses up to around 63 µg STX equivalents/kg b.w. The authors of this report noted that there were only two cases, both non-fatal, where the STX-group toxin dose was less than 1.4 µg STX equivalents/kg b.w.

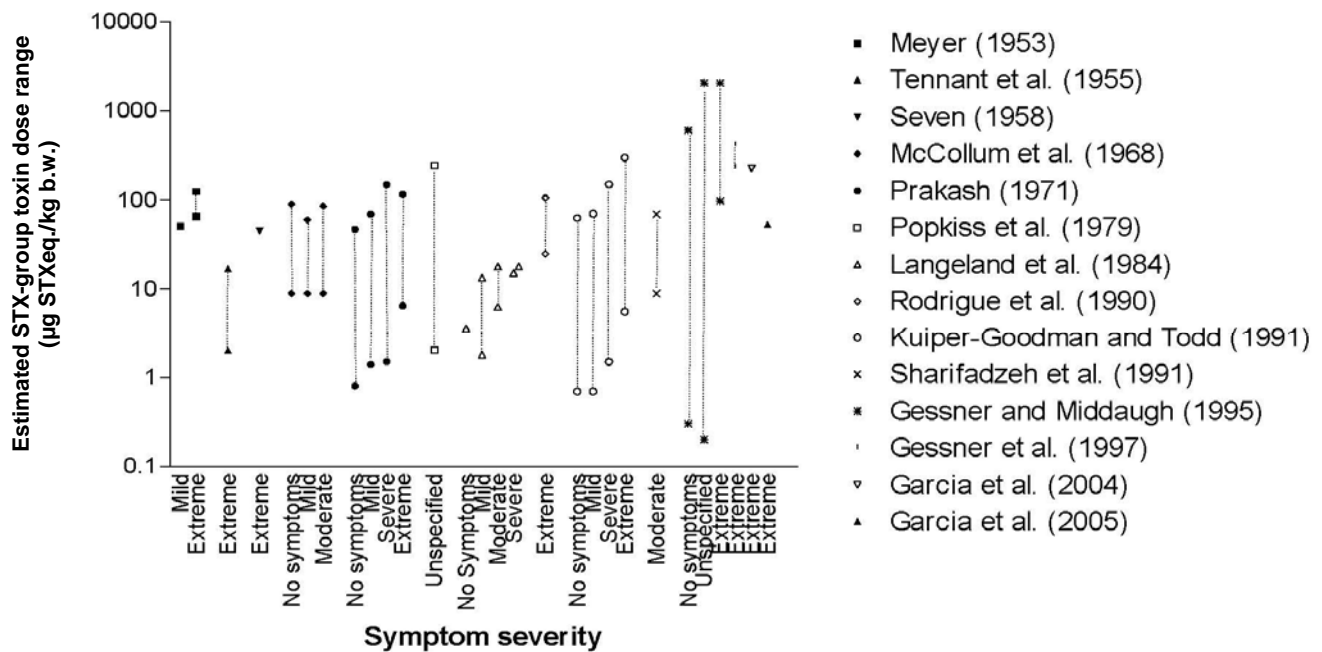
Overall, the case reports from around the world, involving several hundred cases of human illness, indicate a lowest-observed-adverse-effect level (LOAEL) for PSP of around 1.5 µg STX equivalents/kg b.w. (see Table 16). Only two reports have estimated a LOAEL below this. As reported above, the authors of the Health Canada report noted that only two cases (non-fatal) occurred where the estimated dose was less than 1.4 µg STX equivalents/kg b.w. A review of cases that had occurred in Alaska between 1973 and 1992 estimated a LOAEL of 0.2 µg STX equivalents/kg b.w., but the authors of this report acknowledged that there was considerable uncertainty in this estimate (Gessner and Middaugh, 1995).

Doses associated with severe illness generally ranged from 5.6-2058 µg STX equivalents/kg b.w. One study reported a fatality associated with an estimated intake of 1-2 µg STX equivalents/kg b.w. (Llewellyn *et al.*, 2002). This related to an adult male in East Timor who died within three hours of eating a meal of the crab *Zosimus aeneus*, based on measurements of toxin levels in a gut contents pellet obtained at post mortem, but this may have been an

underestimate of the amount consumed since the victim had vomited. Also it was possible that the victim’s meal may have contained other toxins, and therefore the Panel concluded that this was not a reliable estimate of a lethal dose of STX. Among individuals consuming contaminated shellfish who did not develop symptoms, estimated STX-group toxin intakes generally ranged from 0.3 to 90 µg STX equivalents/kg b.w., although one study reported a maximum dose of 610 µg STX equivalents/kg b.w. (Gessner and Middaugh, 1995).

In these reports of human poisoning, there is generally very little information on the precise methodology used for the MBA. If the extraction involved boiling at low pH, the results of the MBA will have overestimated the amount of toxin present in the shellfish, by varying amounts depending on the toxin profile. This could contribute to the wide variation seen in the estimated doses of STX-group toxins associated with human poisoning. The Panel considered it most likely that such over-estimation would apply to the higher estimated intakes, and that the lower end of the range of LOAELs was more likely to be reflect actual intakes. In most cases the actual bodyweights of the poisoned individuals were not recorded and the Panel used a standard value of 60 kg for an adult to calculate the dose of toxin. If the affected individuals weighed more than 60 kg, then the LOAELs would be lower.

The ranges of estimated STX-group toxin intakes associated with different severities observed in incidents of human poisoning are summarised in Figure 9.



**Figure 9.** Estimated STX-group toxin intakes reported in human case reports.



**Table 16.** Summary of STX-group toxin epidemiology data.

Cases	Reported STX-group toxin contamination of shellfish	Shellfish implicated in outbreak(s)	Source country contaminated shellfish	Method used to determine toxin concentration	Toxins detected	Source of sample tested to provide epidemiology data	Sample storage prior to analysis	Assumptions and comments	Dietary intake of STX-group toxins ( $\mu\text{g}$ STX eq./kg b.w.)
3 adults, 2 M and 1 F (Meyer, 1953)	Not specified	Mussels	USA	MBA	ND	Cooked and raw mussels left over from meal	Not specified	Exact number of mussels eaten known by number of shells left after meal  Reported intake in MU/person converted to $\mu\text{g}$ STX eq./kg b.w. using 0.18 conversion factor and assuming 60 kg b.w.	Mild symptoms: 51  Respiratory failure: 66  Fatality: 126
6 adult cases, 2 M and 4 F, and 1 F aged 12 years (Tennant <i>et al.</i> , 1955)	3450-7650 MU/100 g shellfish meat (estimate)	<i>Mya arenaria</i> (soft shell clam)	Canada	MBA	ND	Clam samples collected from implicated beach on days before and after the shellfish associated with the incident were harvested	Not specified	Toxin concentrations in clams associated with illness estimated by interpolation of levels in clams collected before and after incident  Assumed 70 % of toxin lost during cooking  'Probable' toxin intake estimated by authors based on toxin levels in MU and estimated number of clams consumed. Converted to $\mu\text{g}$ STX eq./kg b.w. using 0.18 conversion factor and assuming 60 kg b.w.	2 fatalities and 1 surviving patient: 7-17  Other patients: 2-7

ND = not determined; eq. = STX equivalents, M = male; F = female

Table 16. Continued.

Cases	Reported STX-group toxin contamination of shellfish	Shellfish implicated in outbreak(s)	Source country contaminated shellfish	Method used to determine toxin concentration	Toxins detected	Source of sample tested to provide epidemiology data	Sample storage prior to analysis	Assumptions and comments	Dietary intake of STX-group toxins ( $\mu\text{g}$ STX eq./kg b.w.)
2 adult cases, 1 M and 1 F (Seven, 1958)	2492 MU/100 g shellfish meat	Mussels	USA	MBA	ND	Unconsumed mussels; cooked or uncooked not stated	Not specified	Number of empty shells (60) in the home of patients used to estimate toxin intake  Reported intake in MU/person converted to $\mu\text{g}$ STX eq./kg b.w. using 0.18 conversion factor and assuming 60 kg b.w.	>45
71 cases, age and gender not specified (McCollum <i>et al.</i> , 1968)	Raw: 17500 MU/ 100 g shellfish meat  Cooking for 5 mins reduced levels to 70 %, of which 14 % was in liquor. 20 mins cooking reduced levels to 60 %, of which 32 % was in liquor	Mussels (70 individuals)  Cockles (1 individual)	UK	MBA	ND	Mussels from retailer that supplied 67 cases  Mussels cooked for 20 mins (as retailer had done for 63 cases), or 5 mins as per practise of some individuals at home	Not specified	Assumption that levels in tested samples same as that in mussels consumed by affected individuals.  Intake assessment appears to be based on patient interviews.  Reported intake in MU/person converted to $\mu\text{g}$ STX eq./kg b.w. using 0.18 conversion factor and assuming 60 kg b.w.	No symptoms: 9-90  Mild: 9-60  Moderate: 9-86

Table 16. Continued.

Cases	Reported STX-group toxin contamination of shellfish	Shellfish implicated in outbreak(s)	Source country contaminated shellfish	Method used to determine toxin concentration	Toxins detected	Source of sample tested to provide epidemiology data	Sample storage prior to analysis	Assumptions and comments	Dietary intake of STX-group toxins ( $\mu\text{g}$ STX eq./kg b.w.)
Review data on 49 cases, male and female and including some children, and 82 individuals without symptoms (Prakash <i>et al.</i> , 1971)	Not specified	Clams (65 % cases) Mussels (24 %) Whelks (9 %) Scallops (<1 %)	Canada	MBA	ND	Not specified but is stated that cases occurred in areas where shellfish toxicity was being monitored	Not specified	STX-group toxin intake calculated from data on species, size and number of shellfish consumed, meat yields of shellfish and toxicity data.  When toxins were measured in raw shellfish it was assumed that 70 % was lost during cooking.  Doses calculated assuming 60 kg adult b.w.	No symptoms: 0.8-47  Mild: 1.4-69  Severe: 1.5-150  Extreme: 6.5-117
17 cases, 10 M and 7 F (Popkiss <i>et al.</i> , 79)	Maximum 7283 $\mu\text{g}$ STX eq./100 g shellfish meat	<i>Choromytilus meridionalis</i> (black mussel)	South Africa	MBA	ND	Mussels collected from restaurants or affected coastal sites	Not specified	Assumed toxin levels in tested samples representative of those consumed  Correction factor applied for effect of cooking – 0.3 if cooking fluid discarded, 0.5 if consumed as mussel soup. Uniform weight of mussel assumed  Intake assessment appears to be based on patient interviews  Reported intake in MU/person converted to $\mu\text{g}$ STX eq./kg b.w. using authors' approximate conversion factor of 0.25 and assuming 60 kg b.w.	2-244

Table 16. Continued.

Cases	Reported STX-group toxin contamination of shellfish	Shellfish implicated in outbreak(s)	Source country contaminated shellfish	Method used to determine toxin concentration	Toxins detected	Source of sample tested to provide epidemiology data	Sample storage prior to analysis	Assumptions and comments	Dietary intake of STX-group toxins ( $\mu\text{g}$ STX eq./kg b.w.)
8 cases and 2 unaffected individuals (Langeland <i>et al.</i> , 1984)	7900 MU/100 g shellfish meat	<i>Mytilus edulis</i> (mussel)	Norway	MBA	ND	Steamed mussels left over from meal eaten by 3 ill and 2 non-ill individuals	Frozen	STX-group toxin levels in mussels tested assumed representative of those eaten by other 5 cases.  Method of estimating mussel consumption and weight (g) not specified.  Individuals weighed to determine b.w.  Intake reported in MU/kg b.w. and converted to STX eq. using 0.18 conversion factor	No symptoms: 3.6  Mild: 1.8-13.5  Moderate: 6.3-18  Severe: 15.3-18
187 cases aged from <6 to adult (Rodrigue <i>et al.</i> , 1990)	7500 $\mu\text{g}$ STX eq./100 g clam meat  12.7 MU/ml clam soup	<i>Amphichaena kindermanni</i> (clams)	Guatemala	MBA and HPLC	B1; STX; NeoSTX	Soup obtained from an affected household  Shellfish collected from local beaches	Not specified	Child intake calculated by authors based on estimated consumption of 275 ml soup containing 12.7 MU/ml and 25 kg b.w.  Adult intake calculated based on reported consumption of 30-85 g clam meat containing 7500 $\mu\text{g}$ STX eq./100 g and assuming adult b.w. of 60 kg  Assumed toxin levels in locally sourced shellfish samples after outbreak representative of those consumed	Fatality (child): 25  Fatality (adults): 38-106

Table 16. Continued.

Cases	Reported STX-group toxin contamination of shellfish	Shellfish implicated in outbreak(s)	Source country contaminated shellfish	Method used to determine toxin concentration	Toxins detected	Source of sample tested to provide epidemiology data	Sample storage prior to analysis	Assumptions and comments	Dietary intake of STX-group toxins ( $\mu\text{g}$ STX eq./kg b.w.)
Review data on >90 cases in Canada from 1944-1990 and an outbreak in Guatemala [reported by Rodrigue <i>et al.</i> , (1990)]. Age and gender not specified (Kuiper-Goodman and Todd, 1991)	Not specified	Not specified	Canada and Guatemala	MBA	ND	Not specified	Not specified	Assumptions included: edible portion sizes for shellfish species; number of shellfish consumed; proportion of edible meat for various shellfish species, if number of shellfish consumed unknown literature values used  Unspecified correction factor applied for effects of cooking  Adult and child b.w. of 60 and 25 kg appear to have been assumed	No symptoms: 0.7-63  Mild: 0.7-70  Moderately severe: 1.5-150  Extremely severe: 5.6-300  <b>NB:</b> only 2 cases reported where dose <1.4
6 adult males (Sharifadzeh <i>et al.</i> , 1991)	4280 $\mu\text{g}$ STX eq./100 g shellfish meat	<i>M. edulis</i> (mussel)	USA	Not specified – ‘laboratory examination’	ND	Leftover cooked mussels	Not specified	Patients reported eating 3-4, 4, 4-5, 6, 12 or 18-24 mussels  Intake calculated assuming edible mass of 4 g per mussel and 60 kg b.w.	Moderately severe symptoms: 9-69  <b>NB:</b> severity increased with intake, but all met criteria for moderately severe illness

Table 16. Continued.

Cases	Reported STX-group toxin contamination of shellfish	Shellfish implicated in outbreak(s)	Source country contaminated shellfish	Method used to determine toxin concentration	Toxins detected	Source of sample tested to provide epidemiology data	Sample storage prior to analysis	Assumptions and comments	Dietary intake of STX-group toxins ( $\mu\text{g}$ STX eq./kg b.w.)
Review data on 117 cases in Alaska between 1973-1992 (Gessner and Middaugh, 1995)	Shellfish collected from cases: 39-7750 $\mu\text{g}$ STX eq./100 g shellfish meat	<i>Saxidomus giganteus</i> (butter clams; 58 % of cases) <i>M. edulis</i> or <i>M. californianus</i> (mussels, 22 %) <i>Clinocardium nuttalli</i> (cockles; 13 %) <i>Siliqua patula</i> (razor clams; 2 %) <i>Protothaca staminea</i> (littleneck clams; 2 %)	USA	MBA	ND	Leftover shellfish collected from persons involved in an outbreak, or gathered from beach implicated in outbreak	Not specified	Assumed toxin levels in tested samples representative of those consumed  Uniform weight of mussel assumed.  Method of assessing shellfish consumption not specified  Correction for effects of cooking not reported  Data collection over the 20 year period performed by ‘numerous’ people and not standardised  Estimated dose calculated by assuming 60 kg b.w.	No symptoms: 0.3-610  Symptoms: 0.2-2058  Respiratory arrest: 98-2058

Table 16. Continued.

Cases	Reported STX-group toxin contamination of shellfish	Shellfish implicated in outbreak(s)	Source country contaminated shellfish	Method used to determine toxin concentration	Toxins detected	Source of sample tested to provide epidemiology data	Sample storage prior to analysis	Assumptions and comments	Dietary intake of STX-group toxins (µg STX eq./kg b.w.)
11 cases, 5 M and 6 F aged 13-61 years (Gessner <i>et al.</i> , 1997)	1778-19418 µg STX eq./100 g shellfish meat	<i>M. edulis</i> or <i>M. californianus</i> (mussels)	USA	MBA	STX; GTX1,4; GTX2,3; C1; C2; dcGTX2, 3	Mussels collected from implicated beach within 24h of outbreak (3 cases) or leftover cooked and uncooked mussels (1 case)	Not specified	Conversion of median intake to dose based on 55 kg b.w. B.w. used for other estimates not specified.	Lowest dose: 21 Respiratory arrest: 230-411
2 adult M cases (García <i>et al.</i> (2004)	8575 µg STX eq./100 g shellfish meat	<i>Aulacomya ater</i> (ribbed mussels)	Patagonian fjords, Chile	MBA for mussels, HPLC for body tissues and fluids	ND in shellfish. STX, GTX1-5 in body tissues and fluids	Remaining mussels analysed the day after the incident. Not clear if cooked or raw	On fishing vessel – conditions not specified	Victims ate 7-9 mussels. Assume average mussel weight of 23g, based on Garcia <i>et al.</i> (2005) and average weight of victims was 70kg	Fatality 3-4 hours after ingestion: 225
4 adult M cases (García <i>et al.</i> 2005)	8066 µg STX eq./100 g shellfish meat	<i>Aulacomya ater</i> (ribbed mussels)	Chiloé Island, Chile	HPLC-FLD	GTX2,3	Source and whether cooked or uncooked unspecified	Not specified	Patients each ate two mussels, average Chilean mussel weight of 23 g assumed  Average weight of patients was 70.2 kg	Respiratory failure: 53



## 12. Hazard characterisation

Because of the lack of data relating to repeated oral administration of STX-group toxins in animals or humans, it was not possible to establish a tolerable daily intake (TDI). In view of the acute toxicity of STX-group toxins, the Panel decided to establish an acute reference dose (ARfD). The Panel noted that there were no recent reports of PSP from consumption of shellfish in European countries, but in the absence of a formal reporting system could not discount the possibility that some cases had occurred.

From the available reports of human poisoning, affecting more than 500 individuals, the LOAEL was in the region of 1.5 µg STX equivalents/kg b.w. In these reports many individuals did not suffer adverse reactions at much higher intakes and therefore it is expected that this LOAEL is very close to the threshold for effects in the most sensitive individuals. The Panel applied a factor of 3 to the LOAEL in order to estimate a no-observed-adverse-effect level (NOAEL) of 0.5 µg STX equivalents/kg b.w. No additional factor for variation among humans was required because the data were from reports of a large number of affected consumers, including the most sensitive individuals.

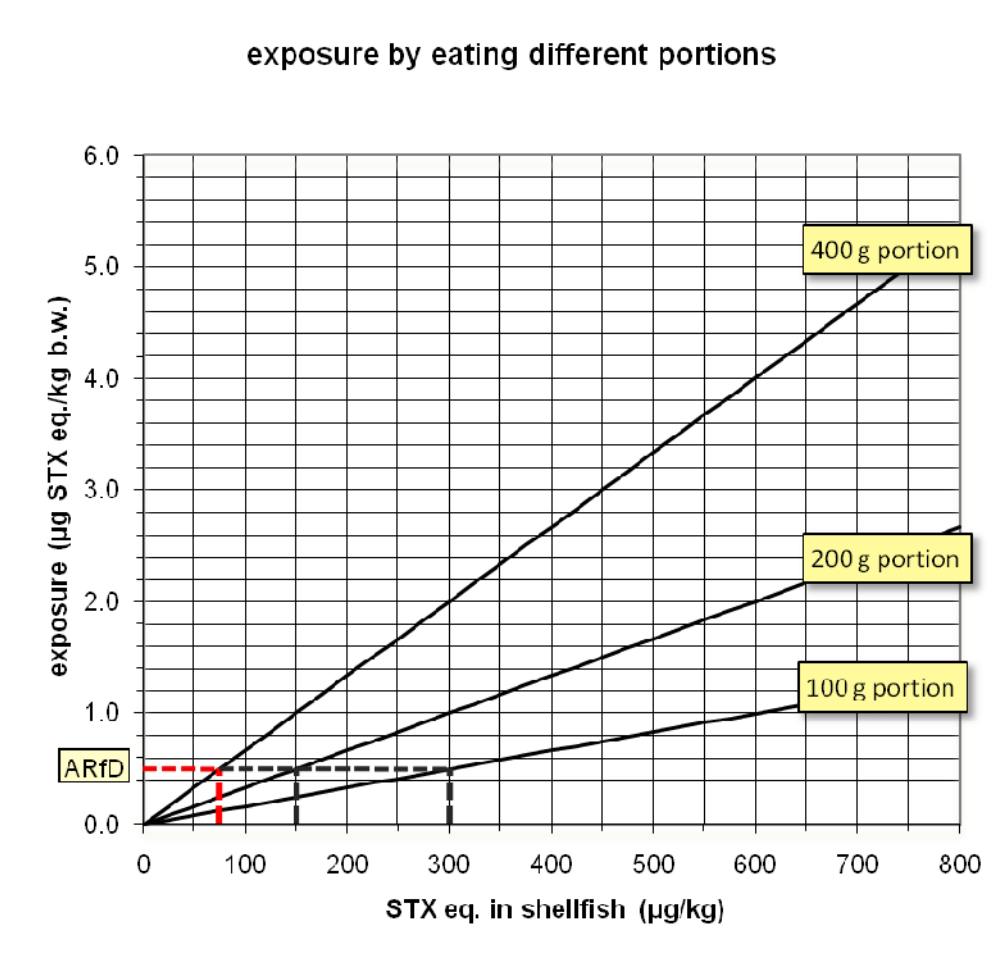
Therefore, the Panel established an acute reference dose of 0.5 µg STX equivalents/kg b.w.

In support of the ARfD, the Panel noted that the oral LD<sub>50</sub> values reported for STX in a range of animal species were in the region of 200 µg STX equivalents/kg b.w. The margin between this dose level and the ARfD of 0.5 µg STX equivalents/kg b.w. is 400, indicating that the ARfD based on data from humans is not over-precautionary.

## 13. Risk characterisation

Because STX-group toxins have acute toxic effects, the Panel concluded that the identification of a high portion size rather than a long term average consumption is of importance to assess the health risk of the consumers. It considered the 95<sup>th</sup> percentile as a realistic estimate of the portion size for high consumers, and identified the figure of 400 g to be used in acute exposure assessments.

Consumption of a 400 g portion of shellfish meat containing STX-group toxins at the current EU limit of 800 µg STX equivalents/kg shellfish meat would result in an intake of 320 µg toxin (equivalent to 5.3 µg/kg b.w. in a 60 kg adult). This intake is considerably higher than the ARfD of 0.5 µg STX equivalents/kg b.w. (equivalent to 30 µg STX equivalents per portion for a 60 kg adult) and is a concern for health for the consumer. The dietary intake of STX-group toxins when consuming a 400 g portion with different levels of contamination with STX-group toxins is shown in Figure 10, indicating also the concentration of STX equivalents/kg shellfish meat associated with a dietary exposure at the level of the ARfD. For comparison also the information for a portion size of 200 and 100 gram is presented.



**Figure 10.** Dietary exposure when eating a portion of 100, 200 or 400 g of shellfish with different levels of STX-group toxins. The concentration associated with a dietary exposure at the ARfD is indicated in red.

In order for a 60 kg adult to avoid exceeding the ARfD of 0.5 µg STX equivalents/kg b.w., a 400 g portion of shellfish should not contain more than 30 µg STX equivalents corresponding to 75 µg STX equivalents/kg shellfish meat.

Given the considerable differences in toxin profiles, different number of analogues determined and the diverse limits of quantification of analytical methods applied in different European Countries and the very high number of non quantifiable samples, the CONTAM Panel concluded that there were too many uncertainties for a reliable and representative estimation of dietary exposure to STX-group toxins for EU countries. Therefore the Panel could not comment on the risks associated with consumption of shellfish that could reach the market.

#### 14. Uncertainty

The evaluation of the inherent uncertainties in the assessment of exposure to STX-group toxins has been performed following the guidance of the Opinion of the Scientific Committee related to Uncertainties in Dietary Exposure Assessment (EFSA, 2006). In addition, the draft report on “Characterizing and Communicating Uncertainty in Exposure Assessment” which is in preparation to be published as WHO/IPCS monograph, has been considered (WHO/IPCS,

2007). According to the guidance provided by the EFSA opinion (2006) the following sources of uncertainties have been considered: assessment objectives, exposure scenario, exposure model, and model input (parameters).

#### 14.1. Assessment objectives

The objectives of the assessment were clearly specified in the terms of reference and the Panel prepared a risk assessment including the derivation of an ARfD and description of the different detection methods.

#### 14.2. Exposure model/scenario

Occurrence data were available from seven European countries either produced by MBA or HPLC-based methods. For the HPLC-based methods, the high number of possible STX-group toxin analogues combined with considerable differences in toxin profiles, different number of analogues determined and the diverse limits of quantification of analytical methods applied in different European countries are a source of uncertainty and impairs the comparability of data. In addition the difference in acidic conditions used during the extraction step hampers a direct comparison of the results obtained by MBA and the different HPLC-based methods.

The existing analytical methods with relatively high limit of detection lead to a very high proportion of not quantified samples. Consequently, a dietary exposure assessment would be fully dependant on the method used to manage the respective left-censored data. This is demonstrated by the fact that not only the median and mean concentrations of STX-group toxins in shellfish for all countries are affected by the choice of lower, medium or upper bound approaches, but for two countries even the 95<sup>th</sup> percentiles resulted in potential daily dietary exposures between 0 and about 150 µg STX equivalents/kg b.w.

Recognising the overall substantial uncertainty in the occurrence data the Panel concluded that a reliable estimate of exposure to STX-group toxins from shellfish currently on the market is not feasible.

It was shown that household processing (cooking, steaming) leads to a reduction of STX-group toxins in shellfish flesh due to leaching-out of these compounds to the cooking fluid (“soup”). As no information on consumption of this “soup” is available this adds to the uncertainty of any exposure estimate.

#### 14.3. Model input (parameters)

Although analytical methodology is assumed to deliver comparable results, the occurrence data were produced with different, non-comparable methods (see above).

TEFs have been used to convert the concentrations of the STX analogues into STX equivalents. However, as pointed out in chapter 10.3, these TEFs are based on limited *i.p.* toxicity data rather than on oral toxicity data and hence their relevance for dietary exposure is unclear.

#### 14.4. Summary of uncertainties

In Table 17 a summary of the uncertainty evaluation is presented, highlighting the main sources of uncertainty and indicating an estimate of whether the respective source of uncertainty might have led to an over- or underestimation of the exposure or the resulting risk.

**Table 17.** Summary of qualitative evaluation of the impact of uncertainties on the risk assessment of the dietary exposure of STX-group toxins.

Sources of uncertainty	Direction
Uncertainty in analytical results	+ <sup>a)</sup>
Extrapolation of occurrence data from a limited number of European countries to Europe as a whole	+
Incomplete database for shellfish consumption in Europe; data only from limited number of Member States and limited data on shellfish species other than mussels	+
Influence of non-detects on exposure estimate	+/-
Consideration of shellfish sampled for pre-market control for systematic dietary estimation of exposure	+
Use of <i>i.p.</i> TEFs for estimating oral intake (STX equivalents)	+/-
Uncertainties with respect to estimated exposure in the intoxication data used for establishing the ARfD	+

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

- = uncertainty with potential to cause under-estimation of exposure/risk

The CONTAM Panel recognised that, as the overall uncertainty in the occurrence data was high, a reliable estimate of exposure to STX-group toxins from shellfish currently on the market is not feasible.

## CONCLUSIONS AND RECOMMENDATIONS

### CONCLUSIONS

#### Hazard identification

- Saxitoxin (STX)-group toxins are produced mainly by dinoflagellates. They are closely related tetrahydropurines. More than 30 STX-group toxins have been identified of which STX, neosaxitoxin (NeoSTX), gonyautoxin-1 (GTX1) and decarbamoyl saxitoxin (dcSTX) seem to be the most toxic.
- STX-group toxins cause Paralytic Shellfish Poisoning (PSP). Symptoms of increasing severity range from mild sensations of tingling or numbness around the lips to incoherent speech, lack of coordination and general weakness, to muscular paralysis and respiratory difficulty. In extreme cases of poisoning, death occurs as a result of respiratory paralysis.
- The main adverse effect of STX-group toxins is neurotoxicity. Binding to voltage-gated sodium channels and the consequent blockade of ion conductance through these channels is considered the major molecular mechanism for their neurotoxicity.
- The limited toxicological information does not allow the setting of robust toxicity equivalency factors (TEFs) for STX analogues for the oral route.
- TEFs based on relative potency data of Oshima *et al.* (2004) for intraperitoneal (*i.p.*) toxicity were applied to the occurrence data reported by the European countries. The CONTAM Panel also took into account more recent data obtained with certified reference calibrants in proposing TEFs.
- Assuming a common mode of action, the toxicity of the STX-group toxins is expressed as the sum of STX equivalents when determined by liquid chromatography techniques. Until better information is available the following factors are adopted, based on acute toxicity following *i.p.* administration to mice: STX = 1, NeoSTX = 1, GTX1 = 1, GTX2 = 0.4, GTX3 = 0.6, GTX4 = 0.7, GTX5 = 0.1, GTX6 = 0.1, C2 =

0.1, C4 = 0.1, dc-STX = 1, dc-NeoSTX = 0.4, dc GTX2 = 0.2, GTX3 = 0.4 and 11-hydroxy-STX = 0.3.

- The data on the chronic effects of STX-group toxins in animals or humans were insufficient for a tolerable daily intake (TDI) to be established.
- In view of the acute toxicity the CONTAM Panel decided to establish an acute reference dose (ARfD). The CONTAM Panel concluded that the lowest-observed-adverse-effect level (LOAEL) for mild symptoms of PSP in humans was in the region of 1.5 µg STX equivalents/kg body weight (b.w.). Since many individuals did not suffer adverse reactions at higher intakes, it is expected that this LOAEL is close to the threshold for effects in the most sensitive individuals. The CONTAM Panel applied a factor of 3 to the LOAEL in order to estimate a no-observed-adverse-effect level (NOAEL).
- The CONTAM Panel established an ARfD of 0.5 µg STX equivalents/kg b.w.

### ***Occurrence/Exposure***

- There is a lack of representative occurrence data for STX-group toxins in different species of shellfish in most European countries.
- The high proportion of not quantified samples is caused by the relatively high limits of detection of the currently applied analytical methods aiming to check for compliance with the current legal limit for PSP toxins. In addition, extreme variations in toxin levels over time and in location may also contribute to this high number of non-detects.
- Consumption data for shellfish are only available for a few Member States. These data do not always distinguish between shellfish species or the type of processing. In addition, different study designs were used in the collection of the consumption data. From these data, the CONTAM Panel identified the figure of 400 g as the high portion size to be used for acute exposure assessments.
- STX-group toxins are heat stable in shellfish at temperatures relevant for cooking and steaming (about 100°C), but leaching-out of STX-group toxins into the cooking fluid may lead to a reduction in concentration in the shellfish flesh.
- Recognising the considerable uncertainties in the occurrence data the CONTAM Panel concluded that a reliable dietary exposure assessment is not feasible for the European population.

### ***Risk characterisation***

- Consumption of a 400 g portion of shellfish containing STX-group toxins at the current European Union (EU) limit of 800 µg STX equivalents/kg shellfish meat would result in a dietary exposure of 320 µg STX equivalents per person (5.3 µg STX equivalents/kg body weight (b.w.)). This is approximately ten times higher than the acute reference dose (ARfD) of 0.5 µg STX equivalents/kg b.w., established by the CONTAM Panel and is a concern for health for the consumer.
- In order for a 60 kg adult to avoid exceeding the ARfD, a 400 g portion of shellfish should not contain more than 30 µg STX equivalents, i.e. 75 µg STX equivalents/kg shellfish meat.

- Because it was not possible to make reliable estimates of dietary exposure to STX-group toxins, the CONTAM Panel could not comment on the risks associated with the consumption of shellfish that currently reaches the market.

### ***Methods of analysis***

- The mouse bioassay (MBA) and the high-performance liquid chromatography (HPLC) method with pre-column derivatization and fluorescence detection (the so-called Lawrence method) can both be used in official analysis to determine STX-group toxins. Both methods have been interlaboratory-validated according to international protocols and are official AOAC methods. They are capable to detect STX-group toxins at the current EU regulatory levels of 800 µg STX equivalents/kg shellfish meat.
- Although the mouse bioassay is considered undesirable for ethical reasons it is the official reference method in case the analytical results are challenged. It has a limit of detection of approximately 370 µg STX equivalents/kg shellfish meat.
- The Lawrence method provides analogue-specific data and is more sensitive than the mouse bioassay.
- In the mouse bioassay boiling with HCl is used in the extraction step, whereas in the Lawrence method, boiling with acetic acid is used. These different extraction conditions may lead to differences in toxin profiles detected and to different results when these analytical data are expressed as STX equivalents.
- Other methods that have potential to determine STX-group toxins are receptor-based assays, antibody-based methods and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). The biomolecular methods are merely suitable for screening purposes. LC-MS/MS has potential for confirmatory analyses. Neither of these methods has been formally validated yet in interlaboratory studies, following internationally recognized protocols.

### **RECOMMENDATIONS (INCL. KNOWLEDGE/DATA GAPS)**

#### ***Hazard identification and characterisation***

- Reporting systems for outbreaks of paralytic shellfish poisoning in Member States should be improved to better reflect the true incidence and to allow efficient follow up of intoxications caused by biotoxins in shellfish species.
- Detailed reports on shellfish consumption and reliable data on toxin content in the event of outbreaks of paralytic shellfish poisoning should be provided in order to reduce uncertainty in the ARfD for STX-group toxins.
- Further toxicological data are needed for the establishment of robust TEFs for the most frequently occurring analogues of STX for the oral route of administration. The assumption of dose additivity should be assessed following exposure to combinations of STX analogues. Milligram amounts of purified STX-group toxins should be produced for this purpose.

#### ***Occurrence/Exposure***

- To improve comparison of occurrence data reported by different European countries at least STX analogues for which TEFs values have been proposed should be analysed.



- Further data on the effect of processing on levels of STX-group toxins in shellfish are needed.
- The database on shellfish consumption should be extended including data on portion size, frequency and different types of shellfish.

### *Methods of analysis*

- Reference calibrants for the most frequently occurring analogues and certified tissue reference materials with relevant compositions and levels of STX-group toxins should be made available.
- Currently applied HPLC methods for official control purposes need to be improved to obtain lower limits of detection.
- Biomolecular methods, as receptor-based assays and antibody-based assays, need to be interlaboratory-tested to derive performance characteristics and to test their suitability to rapidly detect STX-group toxins at the levels of interest.
- LC-MS/MS-based methods should be further elaborated to improve selectivity and sensitivity. Subsequent (interlaboratory) validation studies are needed.

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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
BCR	Community Bureau of Reference
BTX	Brevetoxin
b.w.	Body weight
C1-4	N-sulfo-carbamoyl
CCFPF	Codex Committee for Fish and Fishery Products
CCMAS	Codex Committee on Methods of Analysis and Sampling
CEN	European Committee for Standardization
CF	conversion factor
CGC	Cerebellar granule cells
CONTAM Panel	Panel on Contaminants in the Food chain
CRL	Community Reference Laboratory
CRM	Certified reference material
CTX	Ciguatoxins
dcGTX1-4	Decarbamoyl gonyautoxin 1-4
dc-NeoSTX	Decarbamoyl neosaxitoxin
dcSTX	Decarbamoyl saxitoxin
DA	Domoic acid
DG SANCO	Health and Consumer Protection Directorate General
DSP	Diarrhoeic Shellfish Poisoning
DTX	Dinophysins toxins
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
eq.	Equivalent
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
FAPAS <sup>®</sup>	Food Analysis Performance Assessment Scheme
GTX1-4	gonyautoxins
GTX5-6	N-sulfo-carbamoyl
HCl	hydrochloric acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HPLC-FLD	High-performance liquid chromatography-fluorescence detection
IC <sub>50</sub>	Inhibitory concentration - the concentration of a substance that reduces the effect by 50 %
IOC	Intergovernmental Oceanographic Commission of UNESCO
<i>i.p.</i>	Intraperitoneal
IRMM	Institute for Reference Materials and Measurements
ISO/IUPAC/AOAC	International Organization for Standardization/ International Union of Pure and Applied Chemistry/Association of Analytical Communities
<i>i.v.</i>	intravenous
JMPR	Joint FAO/WHO Meetings on Pesticide Residues
LB	Lower Bound
LC-FLD	Liquid chromatography-fluorescence detection

LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LD <sub>50</sub>	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
M1-4	Hydroxylated saxitoxins
MB	Median/Medium bound
MBA	Mouse bioassay
MS	mass spectroscopy
MU	Mouse Unit: the minimum amount needed to cause the death of an 18 to 22 g white mouse in 15 minutes
NeoSTX	neosaxitoxin
NOAEL	No-observed-adverse-effect level
N:P	nitrogen:phosphate
NRCC	National Research Council Canada
NRL	National Reference Laboratory
OA	Okadaic acid
OJ	Official Journal of the European Union
PITX	Palytoxins
Post-MC	Post-market control
PP2A	Protein phosphatase-PP2A
Pre-MC	Pre-market control
Post-MC	Post-market control
PSP	Paralytic Shellfish Poisoning
PTP	Permeability transition pore
PTX	Pectenotoxin
PTX1	Pectenotoxin 1
PTX2	Pectenotoxin 2
RBA	Rat bioassay
SLV	Single laboratory validation
SM	Shellfish meat
SOP	Standard operating procedure
SPE	Solid Phase Extraction
SPR	Surface Plasmon Resonance
STX	Saxitoxin
TDI	Tolerable daily intake
TEF	Toxicity equivalency factor
UB	Upper Bound
UK	United Kingdom
UNESCO	United Nations Educational, Scientific and Cultural Organization
UV	Ultraviolet
WG	Working group
WHO	World Health Organization
YTX	Yessotoxin
YTX eq.	Yessotoxin equivalents