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SCIENTIFIC OPINION

Marine biotoxins in shellfish – Saxitoxin group[1]

Scientific Opinion of the Panel on Contaminants in the Food Chain

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

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PANEL MEMBERS

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

SUMMARY

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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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[2] 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

[2] 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[3]. Two additional groups, palytoxins (PlTX) and ciguatoxins (CTX), may also be considered. STX and its derivatives cause Paralytic Shellfish Poisoning (PSP), and DA causes Amnesic Shellfish Poisoning (ASP). Diarrhoeic Shellfish Poisoning (DSP) is caused by OA-group toxins (OA and dinophysis toxins (DTX)), and AZA group toxins cause Azaspiracid Shellfish Poisoning (AZP). These toxins can all accumulate in the digestive gland (hepatopancreas) of filter-feeding molluscan shellfish, such as mussels, oysters, cockles, clams and scallops, and pose a health risk to humans if contaminated shellfish are consumed. Marine biotoxin-related illness can range from headaches, vomiting and diarrhoea to neurological problems and in extreme cases can lead to death.

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

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

1. Legal framework

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

[3] ftp://ftp.fao.org/es/esn/food/biotoxin_report_en.pdf

[4] 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.

[5] 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[6], 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[7] 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[5] specifies a mouse bioassay (MBA) for the determination of paralytic shellfish poisoning toxins (PSP) and a MBA or the rat bioassay

[6] 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.

[7] 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[4] 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[8] 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)[9] 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

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

[9] 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[9]	Derived Guidance Level/ Max Level based on consumption of 100g (1), 250g (2) and 380g (3)	Limit Value currently implemented in EU legislation
AZA	0.4 (1)	10 (H)	0.04 µg/kg 2.4 µg/adult [a)]	0.024 mg/kg SM (1) 0.0096 mg/kg SM (2) 0.0063 mg/kg SM (3)	0.16 mg/kg SM
BTX			N/A		
Cyclic Imines			N/A		
DA	1,000 (1)	10 (H)	100 µg/kg 6 mg/adult [a)]	60 mg/kg SM (1) 24 mg/kg SM (2) 16 mg/kg SM (3)	20 mg/kg SM
OA	1 (1)	3 (H)	0.33 µg/kg 20 µg/adult [a)]	0.2 mg/kg SM (1) 0.08 mg/kg SM (2) 0.05 mg/kg SM (3)	0.16 mg/kg SM
PTX			N/A		
STX	2 (1)	3 (H)	0.7 µg/kg 42 µg/adult [a)]	0.42 mg/kg SM (1) 0.17 mg/kg SM (2) 0.11 mg/kg SM (3)	0.8 mg/kg SM
YTX	5,000 (2)	100 (A)	50 µg/kg 3 mg/adult [a)]	30 mg/kg SM (1) 12 mg/kg SM (2) 8 mg/kg SM (3)	1 mg/kg SM

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

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

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

Test methods for the eight toxin groups were reviewed and recommendations for Codex purposes made. Mouse bioassays are widely used for shellfish testing but for technical and ethical reasons it is highly desirable to move to new technologies which can meet Codex requirements more adequately. Most currently available methods do not meet fully the strict criteria for Codex type II[10] or III[11] 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

[10] 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.

[11] 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.

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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 tamarensis*, *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):

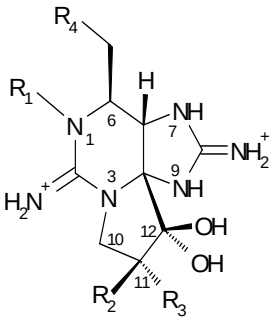
I) carbamate (STX, neosaxitoxin (NeoSTX) and gonyautoxins (GTX1-4))

II) N-sulfo-carbamoyl (GTX5-6, C1-4)

III) decarbamoyl (dc-) (dcSTX, dc-NeoSTX, dcGTX1-4)

IV) hydroxylated saxitoxins (M1-4)

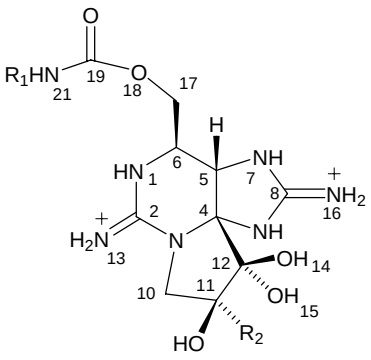
STX was the first toxin of the STX-group toxins that was isolated from the Alaska butterclam (*Saxidomus giganteus*) and hence the trivial name STX was given.



STX = saxitoxin
NEO = neosaxitoxin
GTX = gonyautoxins

R_1	R_2	R_3	R_4	Toxin
H	H	H	$-O-C(=O)-NH_2$	STX
H	H	OSO_3^-		GTX2
H	OSO_3^-	H		GTX3
OH	H	H		NEO
OH	H	OSO_3^-		GTX1
OH	OSO_3^-	H		GTX4
H	H	H		GTX5 (B1)
H	H	OSO_3^-		C1
H	OSO_3^-	H		C2
OH	H	H	$-O-C(=O)-NHSO_3^-$	GTX6 (B2)
OH	H	OSO_3^-		C3
OH	OSO_3^-	H		C4
H	H	H		dcSTX
H	H	OSO_3^-		dcGTX2
H	OSO_3^-	H	$-OH$	dcGTX3
OH	H	H		dcNEO
OH	H	OSO_3^-		dcGTX1
OH	OSO_3^-	H		dcGTX4

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



R_1	R_2		
SO_3^-	H	M1β	11-hydroxy-N21-sulfocarbamoyl-saxitoxin
H	H	M2β	11-hydroxy-saxitoxin
SO_3^-	OH	M3	11,11-dihydroxy-N21-sulfocarbamoyl-saxitoxin
H	OH	M4	11,11-dihydroxy-saxitoxin

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[4], 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[12] 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[5] 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[13] to read:

[12] 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.

[13] Commission Regulation (EC) Nº 1664/2006. Commission Regulation of 6 November 2006 amending Regulation (EC) Nº 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)[14]:

Saxitoxin dihydrochloride

[14] 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)[15].

4.2. Mammalian bioassay

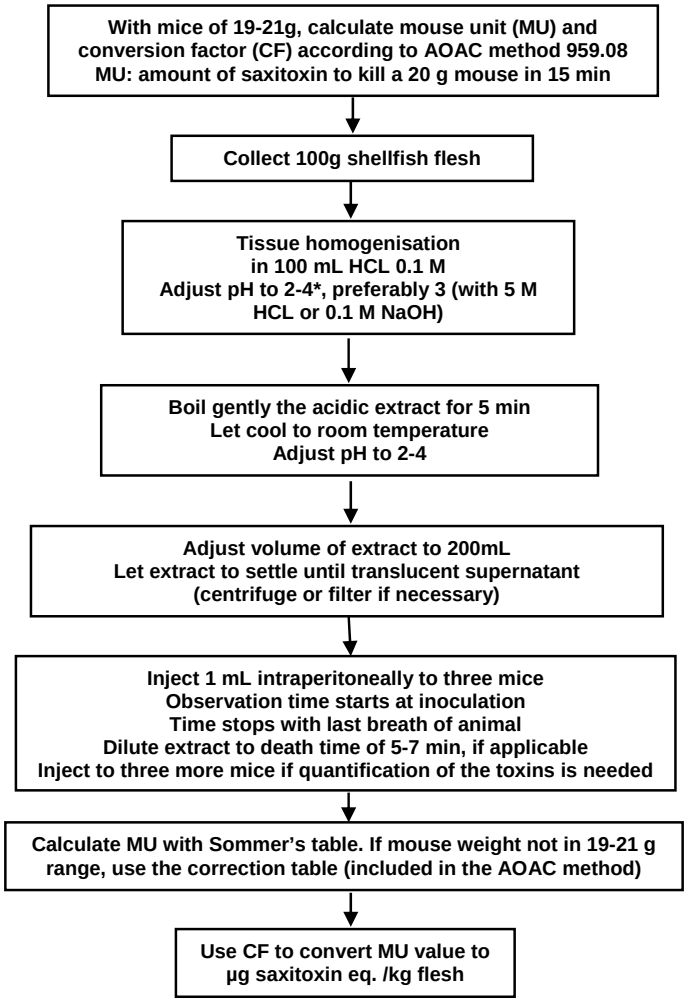
Regulation (EC) No. 2074/2005 prescribes the use of the mouse bioassay as reference method for the detection of the STX-group toxins. This test, which has been formally validated in an interlaboratory study by AOAC International in 1959, is described below.

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)[16] (Figures 3 and 4).

[15] http://irmm.jrc.ec.europa.eu

[16] 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[7].

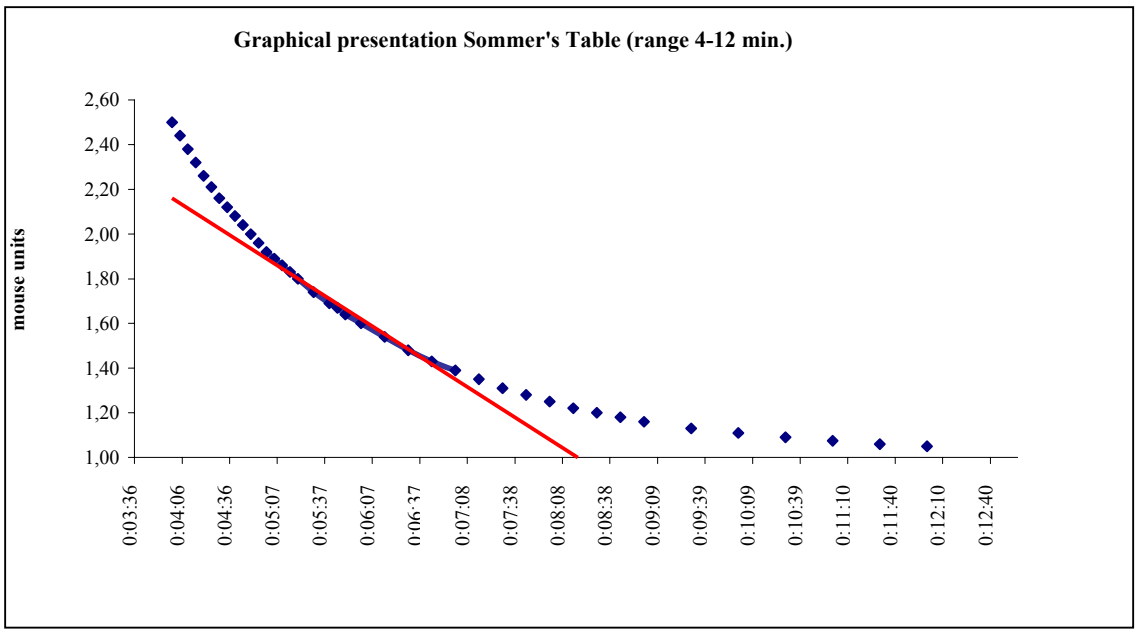


Figure 4. The relationship between death time of the mouse and amount of toxin expressed as mouse units in the time range of 4-12 minutes (Sommer's table in graphic form).

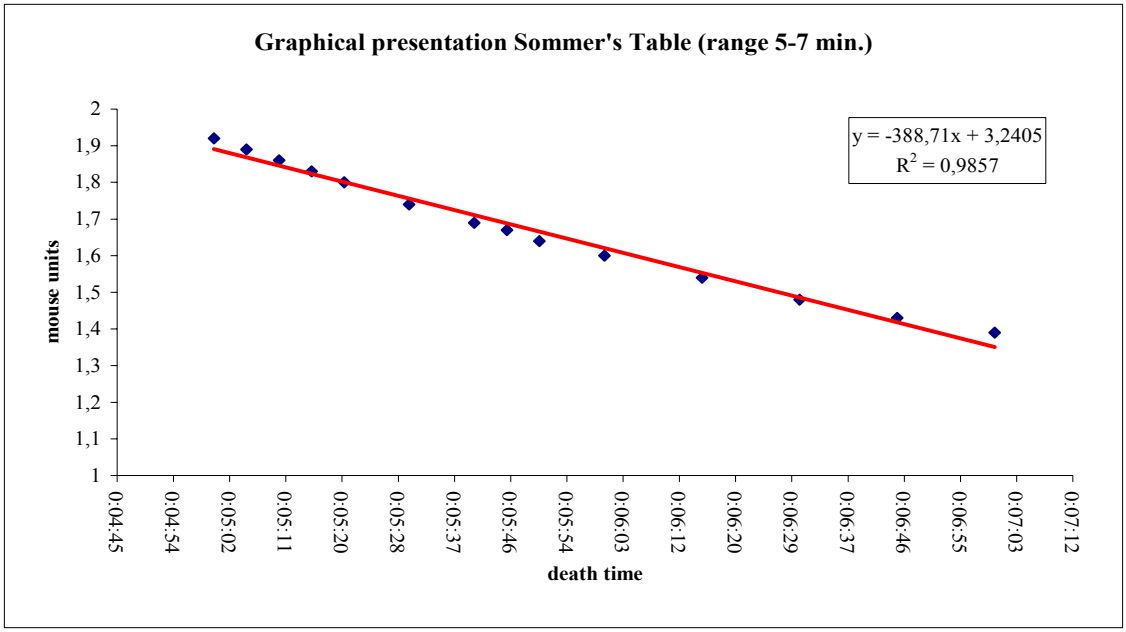


Figure 5. The linear relationship between death time and amount of *i.p.* toxin expressed as mouse units in the measuring time range of 5-7 minutes. (Excerpt of Sommer's table in graphic form (see Figure 4 above))

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 synaptoneurosomes (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 synaptoneurosomes (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 [^{3}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^+,K^+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 VIth 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 (precolumn HPLC-method)

The precolumn 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 precolumn 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 precolumn 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®[17]) 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

[17] 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[7] 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 [a)]	Method of testing	LOD [d)] (µg/kg)	LOQ [d)] (µg/kg)
France	2000-2008	1882	pre-MC	MBA		280-402
		290	post-MC			
Germany [b)]	2005-2007	445	post-MC	HPLC-FLD	b)	-
Italy	2001-2008	7046	pre-MC	MBA		350
Norway [c)]	2006-2008	1122	pre-MC	HPLC-FLD	c)	-
Portugal [e)]	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
Total	**2000-2008**	**20248**				

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

[a)] PreMC are samples collected at the place of origin, before or during harvesting; PostMC are samples collected at the place of sale or along the distribution chain.

[b)] Germany reported not detected individual analogues as "<value". These values were used as LOQs in the calculations.

[c)] Norway reported the LODs for the individual measured analogues. These were used in the calculations.

[d)] 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.

[e)] 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	**HPLC-FLD** (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 *et al*. (2002) Lawrence *et al*. (2005)
Norway	2006-2008	**HPLC-FLD** (post-column oxidation): Oshima method with slight modifications as published in Asp *et al*.2004	pH adjustment to 3 +/-0.5 (same extraction method as used for MBA samples)	Boiling (100°C) for 5 minutes	Asp *et al*. (2004) Oshima (1995b)
Germany	2005-2007	**HPLC-FLD** (pre-column oxidation): Lawrence method (AOAC2005.06) without SPE	1 % HOAc	Boiling (100°C) for 5 minutes	Lawrence *et al*. (2005)
		HPLC-FLD (post-column oxidation): Diener *et al*. 2006 + STX-group toxin extraction from Lawrence method (AOAC2005.06)	1 % HOAc	Boiling (100°C) for 5 minutes	Diener *et al*. (2006) Lawrence *et al*. (2005)
Italy	2001-2008 (only MBA data is included in the actual data set)	**MBA**: 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)	**HPLC-FLD**: (pre-column oxidation): Lawrence method (AOAC 2005.06)	1 % HOAc	Boiling (100°C) for 5 minutes	Lawrence *et al*. (2005)
		HPLC-FLD: (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 *et al*. (2005) AOAC (2000b)
		MBA: AOAC 959.08	0.1 N HCl pH: 2-4 (preferably 3)	Boiling (100°C) for 5 minutes	AOAC (2000b)
Spain	2008-2008	**MBA**: 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	**MBA**: 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[a)]	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[b)]	-	-	-	-
GTX 2,3	5-150[b) c)]	-	-	-	-
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

[a)] TEFs calculated based on relative potency data by Oshima *et al.* (2004)

[b)] 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.

[c)] 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

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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
CCFFP	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	Dinophysis toxins
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
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®	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_{50}	Inhibitory concentration - the concentration of a substance that reduces the effect by 50 %
IOC	Intergovernmental Oceanographic Commission of UNESCO
i.p.	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.v.	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_{50}	Lethal dose – the dose required to kill half the members of a tested animal population
LOAEL	Lowest-observed-adverse-effect level
LOD	Limit of detection
LOQ	Limit of quantification
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
PlTX	Palytoxins
Post-MC	Post-market control
PP2A	Protein phosphatise-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