JOINT FAO/WHO

TECHNICAL PAPER

Toxicity Equivalency Factors for Marine Biotoxins Associated with Bivalve Molluscs

Food and Agriculture Organization of the United Nations

World Health Organization

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DECLARATIONS OF INTEREST

All participants in the Joint FAO/WHO Expert Meeting on Toxicity Equivalency Factors for Marine Biotoxins completed a Declaration of Interest form in advance of the meeting. None were considered to present any potential conflict of interest.

Abbreviations and acronyms

AhR	aryl hydrocarbon receptor
AMPA	alphaamino-5-methyl-3-hydroxyisoxazolone-4-propionate
AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
ARfD	acute reference dose
ASP	amnesic shellfish poisoning
AZA	azaspiracid
AZP	azaspiracid poisoning
BTX	brevetoxin
b.w.	body weight
CAC	Codex Alimentarius Commission
CCFFP	Codex Committee on Fish and Fishery Products
CCMAS	Codex Committee for Methods of Analysis and Sampling
CEN	Comité Européen de Normalisation (European Committee for Standardization)
CID	Collusion induced dissociation
CONTAM Panel	Panel on Contaminants in the Food chain (EFSA)
DA	domoic acid
dcGTX1–4	Decarbamoyl gonyautoxin 1–4
dc-NeoSTX	Decarbamoyl neosaxitoxin
dcSTX	Decarbamoyl saxitoxin

DSP	diarrhetic shellfish poisoning
DTX	dinophysistoxin
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FL	fluorescence method
GTX	gonyautoxin
HCl	hydrochloric acid
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
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
i.p.	intraperitoneal
i.v.	intravenous
LC	liquid chromatography
LC-MS	liquid chromatography with mass spectrometry detection
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LC-UV	liquid chromatography with ultraviolet detection
LD_{50}	median lethal dose
LOAEL	lowest observable adverse effect level
MBA	mouse bioassay
MLD	minimum lethal dose
MS	mass spectrometry
MU	Mouse Unit
NeoSTX	neosaxitoxin
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NOAEL	no observable adverse effect level
NSP	neurotoxic shellfish poisoning

NSSP	National Shellfish Sanitation Program
OA	okadaic acid
PBDD	polybrominated dibenzo-p-dioxin
PBDF	polybrominated dibenzofuran
PCB	polychlorinated biphenyle
PCDF	polychlorinated dibenzofuran
PSP	paralytic shellfish poisoning
RBA	receptor-binding assay
RfD	reference dose
REP	relative effect potency
STX	saxitoxin
TDI	tolerable daily intake
TEF	toxicity equivalence factor
TFA	Trifuoroacetic acid
US-EPA	United States Environmental Protection Agency
UV	ultraviolet
WHO	World Health Organization

Preparation and purpose of this document

BACKGROUND

On the basis of the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs (FAO/IOC/WHO, 2004), the Codex Committee on Fish and Fishery Products (CCFFP) has developed the Standard for Live and Raw Bivalve Molluscs (CODEXSTAN 292–2008, rev.2015). The Standard includes the following provisions for biotoxins:

Name of biotoxin group	Maximum level per kg of mollusc flesh
Saxitoxin (STX) group	≤0.8 mg (2HCL) of saxitoxin equivalent
Okadaic acid (OA) group	< 0.16 mg of okadaic acid equivalent
Domoic acid (DA) group	< 20 mg domoic acid
Brevetoxin (BTX) group	< 200 mouse units or equivalent
Azaspiracid (AZA) group	< 0.16 mg

Each of these biotoxin groups includes several analogues and the limit in the Codex standard is expressed as the level of a reference toxin.

The 34th Session of Codex Committee on Methods of Analysis and Sampling (CCMAS) encouraged CCFFP to elaborate Toxicity Equivalency Factors (TEFs) for all biotoxins listed in the standard and to establish appropriate sampling plans. The 33rd Session of CCFFP developed performance criteria for reference and confirmatory methods for marine biotoxins included in the standard. However, the CCFFP agreed that it was premature to include TEFs as part of the standard and requested FAO/WHO to provide scientific advice in this area.

FAO/WHO EXPERT MEETING ON TEFS FOR MARINE BIOTOXINS

Following the request from CCFFP, FAO/WHO agreed to develop a technical document presenting the status of science of marine biotoxins associated with bivalve molluscs, toxin analogues and their biological activity. Professor Luis Botana (University of Compostela, Spain) and Dr Philipp Hess (IFREMER, France) prepared the first draft consisting of two major sections (a) chemistry of biotoxins and methods of analysis, and (b) TEFs for biotoxins. The draft was circulated and reviewed by a group of experts including Dr Rex Munday (AgResearch Ltd, New Zealand), Dr Nathalie Arnich (ANSES, France), Dr Stacey Degrasse (United States Food and Drug Administration), Dr Mark Feeley (Health Canada), Dr Toshiyuki Suzuki (National Research Institute of Fisheries Science, Japan) and Professor Martin Van den Berg (Utrecht University, Netherlands). The technical paper was discussed and finalized by th above Expert Group during the Joint FAO/WHO Expert Meeting on Toxicity Equivalency Factors for Marine Biotoxins held in Rome on 22–24 February 2016. The meeting was chaired by Professor Martin Van den Berg and Luis Botana was the rapporteur.

Definitions of terms used

Acute reference dose (ARfD) — The estimate of the amount of a substance in food and/or drinking water, expressed on a body weight basis, that can be ingested in a period of 24 hours or less without appreciable health risk to the consumer. It is derived on the basis of all known facts at the time of evaluation.

Analogues — In chemistry, a structural analogue, also known as a chemical analogue or simply an analogue, is a compound having a structure similar to that of another one, but differing from it in respect of a certain component. It can differ in one or more atoms, functional groups or substructures, which are replaced with other atoms, groups or substructures. A structural analogue can be imagined to be formed, at least theoretically, from the other compound.

Apoptosis — Active process of programmed cell death requiring metabolic energy, often characterized by fragmentation of DNA, and without associated inflammation.

Bioavailability — For food additives, contaminants and pesticide residues, a term referring to the proportion of a substance that reaches the systemic circulation unchanged after a particular route of administration.

Cardiotoxic — Causing damage to the heart.

Certified Reference Material/Certified Calibrant — A certified reference material or a certified calibrant is a matrix or a calibrant with a documentation issued by an ISO 34 (soon to be ISO 17034) accredited unit, authorized by an accreditation body, that provides one or more specified property values with associated uncertainties and traceability, using valid procedures. Generally, amount and stability are the commonly certified parameters.

Congener — One of two or more substances related to each other by origin, structure or function.

Contaminant — Any substance not intentionally added to food or feed for food producing animals, which is present in such food or feed as a result of the production (including operations carried out in crop husbandry, animal husbandry and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food or feed, or as a result of environmental contamination. The term does not include insect fragments, rodent hairs and other extraneous matter .

Cytotoxic — Causing damage to cell structure or function.

Dose — Total amount of an agent administered to, taken up by or absorbed by an organism, system or (sub)population.

Dose-response — Relationship between the amount of an agent administered to, take up by or absorbed by an organism, system or (sub)population and the change developed in that organism, system or (sub)population in reaction to the agent.

Epimerization — Interconversion of epimers.

Epimers — Diastereoisomers that have the opposite configuration at only one of two or more tetrahedral stereogenic centres present in the respective molecular entities.

Exposure — Concentration or amount of a particular agent that reaches a target organism, system or (sub)population in a specific frequency for a defined duration.

Extract — The separated phase (often – but not necessarily – organic) that contains the material extracted from the other phase.

Gavage — Administration by intragastric intubation.

Hazard — A biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect

Hepatotoxic — Causing damage to liver cells.

Hydrophilic — Water loving. The capacity of a molecular entity or of a substituent to interact with polar solvents, in particular with water, or with other polar groups.

Intraperitoneal (i.p.) — Into the peritoneum (body cavity).

Intravenous (i.v.) — Into or inside the vein.

in vitro — In glass, referring to a study in the laboratory usually involving isolated organ, tissue, cell or biochemical systems.

in vivo — In the living body, referring to a study performed on a living system.

Isomer — One of several species (or molecular entities) that have the same atomic composition (molecular formula) but different line formulae or different stereochemical formulae and hence different physical and/or chemical properties.

Lethality — May refer to the minimum lethal dose or a dose that killed an unspecified number of animals.

Lipophilic — Fat-loving. Applied to molecular entities (or parts of molecular entities) having a tendency to dissolve in fat-like (e.g. hydrocarbon) solvents.

Lowest observed adverse effect level (LOAEL) — Lowest concentration or amount of a substance, found by experiment or observation, that causes an adverse alteration of morphology, functional capacity, growth, development or lifespan of the target organism distinguishable from normal (control) organisms of the same species and strain under the same defined conditions of exposure.

Mechanism of action — The specific biochemical interaction through which a substance produces an effect on a living organism or in a biochemical system.

Median — The midpoint value obtained by ranking all values from highest to lowest and choosing the value in the middle. The median divides a population into two equal halves.

Median lethal dose (LD_{50}) — The dose that kills half the population of animals tested.

Metabolism — The entire physical and chemical processes involved in the maintenance and reproduction of life in which nutrients are broken down to generate energy and to give simpler molecules which by themselves may be used to form more complex molecules.

Metabolite — Any intermediate or product resulting from metabolism.

Minimum lethal dose (MLD) — Lowest amount of a substance that, when introduced into the body, may cause death to individual species of test animals under a defined set of conditions.

Mode of action — A biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. A mode of action describes key cytological and biochemical events – that is, those that are both measurable and necessary to the observed effect – in a logical framework.

No observed adverse effect level (NOAEL) — Greatest concentration or amount of a substance, found by experiment or observation, that causes no adverse alteration of morphology, functional capacity, growth, development or lifespan of the target organism distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure.

Pharmacokinetics — Description of the fate of drugs in the body, including a mathematical account of their absorption, distribution, metabolism and excretion.

Potency — Pharmacological parameter that defines the amount of a compound required for a certain effect.

Relative effect potency — Value of potency compared to an amount of a reference compound that shares the same mechanism of action (defined as the biochemical and signalling interaction between a drug and its target).

Relative toxicity — Value of toxicity compared to an amount of a reference compound that shares the same mechanism of toxicity (consequence of the same mechanism and mode of action).

Reference dose — An estimate of the daily exposure dose that is likely to be without deleterious effect even if continued exposure occurs over a lifetime.

Reference material — Material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process or in examination of nominal properties.

Risk Assessment — A scientifically based process consisting of the following steps: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization.

Risk Characterization — The qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment.

Tolerable daily intake (TDI) — An estimate of the amount of a substance in food and/or drinking water, normally expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk to the consumer. It is derived on the basis of all the known facts at the time of the evaluation. Analogous to acceptable daily intake, the term tolerable is used for agents such as contaminants that are not deliberately added to foods.

Toxicity — The potential of a substance to cause injury (adverse reaction) to a living organism.

Toxicity equivalency factor (TEF) — The toxicity ratio of a compound from a chemical group that shares the same mode of action of a reference compound in the same group. The toxicity of the congener is expressed as a fraction of the toxicity of the reference compound in terms of potency, which is a pharmacological parameter that defines the amount of compound required for a certain effect.

Validated Method — An analytical method which has been subjected to a multi-laboratory study for accuracy, precision, reproducibility performance and ruggedness. Concise written procedures for sample selection, preparation, and quantitative analysis are provided for inter-laboratory quality assurance and consistency of results, on which an appropriate regulatory method of analysis can be established.

Validation — Process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose. Different parties define "reliability" as establishing the reproducibility of the outcome of the approach, method, process or assessment over time. "Relevance" is defined as establishing the meaningfulness and usefulness of the approach, method, process or assessment for the defined purpose.

Executive summary

Currently, the Codex Standard for Live and Raw Bivalve Molluscs (CODEX STAN 292–2008, rev.2015) includes the following provisions for biotoxins:

Name of biotoxin group	Maximum level per kg of mollusc flesh
Saxitoxin (STX) group	≤ 0.8 mg (2HCL) of saxitoxin equivalent
Okadaic acid (OA) group	< 0.16 mg of okadaic acid equivalent
Domoic acid (DA) group	< 20 mg domoic acid
Brevetoxin (BTX) group	< 200 mouse units or equivalent
Azaspiracid (AZA) group	< 0.16 mg

Each of these biotoxin groups includes several analogues, and the limit in the Codex standard is expressed as level of a reference toxin for each group. There is the need to know the relative toxicity of each of the analogues, so that the total toxicity of the material in the shellfish extract can be estimated. This requires the determination of Toxicity Equivalency Factors (TEFs), defined as the *toxicity ratio of a compound from a chemical group that shares the same mode of action of a reference compound in the same group*. The toxicity of the analogue is expressed as a fraction of the toxicity of the reference compound.

The 34th Session of the Codex Committee on Fish and Fishery Products (CCFFP) requested FAO/WHO to provide scientific advice on TEFs for biotoxins included in the standard. FAO/WHO set up an Expert Group to develop a technical paper providing scientific explanations and recommendations on TEFs. This technical paper was discussed and finalized by the Expert Group during the Joint FAO/WHO Expert Meeting on Toxicity Equivalency Factors for Marine Biotoxins, held in Rome on 22–24 February 2016.

CRITERIA USED FOR DETERMINING TEFS

The Expert Group developed an approach to be used for the development of TEFs within each group of biotoxins. Studies by oral administration, both for toxicity and bioavailability, were considered to be most relevant for updating TEFs. In cases where such data were not available, other toxicity data were considered provided the analogues cause similar symptoms and the mechanism of action could be linked to the symptoms. For the establishment of TEFs, data were considered in the following order of importance:

1. Data from human cases (outbreaks);
2. oral LD_{50} in animals;
3. i.p. LD_{50} in animals;
4. Mouse bioassay;
5. *In vitro* data.

BIVALVE-ASSOCIATED TOXINS

Based on their chemical structure, the toxins included in the Codex standard are classified into five groups, namely, the saxitoxin (STX), okadaic acid (OA), domoic acid (DA), brevetoxin (BTX) and azaspiracid (AZA) groups. An additional toxin group was also considered, tetrodotoxin (TTX), due to its emergence in shellfish. The six toxin groups are also known for the four syndromes they cause: (i) paralytic shellfish poisoning, i.e. PSP (STX and TTX groups), (ii) diarrhetic shellfish poisoning, i.e. DSP (OA and AZA groups) and (iii) amnesic shellfish poisoning, i.e. ASP (DA group) and (iv) neurotoxic shellfish poisoning, i.e. NSP (BTX group). Other biotoxins, such as yessotoxin, pectenotoxin, palytoxin and cyclic imines were briefly discussed.

Saxitoxin and analogues: More than 50 analogues have been reported and at least 18 have toxicological relevance. This group of toxins is separated into carbamoyl analogues, N-sulphocarbamoyl analogues, and decarbamoyl analogues. The different groups of analogues have different relative toxicities, and it is possible that some compounds transform into others, at acidic pH, with an increase in toxicity. Some of these conversions may take place naturally at the acidic pH of the stomach. At the same time, transformation into a different set of toxins could take place at slightly alkaline pH.

Okadaic acid and analogues: Okadaic acid is a polyether toxin and dinophysistoxin–1 (DTX1) and DTX2 are the structural analogues of this toxin. Fatty acid ester analogues of these toxins are referred to as DTX3. Esters of allylic diols with the carboxylic acid at C1 of OA and DTXs have been reported and these esters were named DTX4, –5 etc.

Domoic acid and analogues: Domoic acid is a cyclic amino acid and although several isomers of DA, epi-domoic acid and isodomoic acids, have been reported, so far only DA and epi-DA have been shown to be of toxicological relevance. DA transforms into epi-DA through long-term storage and epimerisation is accelerated with warming.

Azaspiracid and analogues: Azaspiracids are polyether toxins with a unique spiral ring assembly, a cyclic amine, or aza group. Over 30 AZA analogues have been identified in phytoplankton and shellfish. AZAs have been named by numbering in the chronological order of their detection or postulation. AZA1–10, 26, 33–34, 36–37 have been isolated and fully characterized. Shellfish are known to transform AZAs by two different types of reactions: hydroxylation and carboxylation.

Brevetoxin group: Brevetoxins are cyclic polyether toxins grouped based on their backbone structure into group A type and group B type with the former having ten transfused ether rings and the latter eleven. Side chains account for a number of analogues. Brevetoxins are extensively metabolized in shellfish by oxidation, reduction, hydrolysis and conjugation, which may lead to formation of further analogues.

Tetrodotoxin and analogues: Tetrodotoxins (TTXs) are traditionally known as the main toxins found in puffer fish and a range of other animals. TTXs are also frequently found in other marine animals, including some species of gobies, octopuses, sea stars, crabs, bivalves and gastropods. Bacteria such as *Vibrio* spp., e.g. *Vibrio alginolyticus*, also produce TTXs, although the source of these toxins in marine animals has not yet been conclusively identified. About 30 known analogues of TTX have been described. Profiles in shellfish included TTX, 4-epi-TTX, 4,9-anhydro-TTX and 5,6,11-tri-deoxy-TTX.

METHODS FOR DETECTION OF BIVALVE ASSOCIATED BIOTOXINS

Saxitoxin and analogues: The mouse bioassay (MBA) for detection of paralytic shellfish toxins has been formally validated in an interlaboratory trial and a standardized AOAC method is available. The test does not provide analogue-specific data but gives a result in equivalents of STX in European Union and United States of America protocols or in equivalents of dc-STX in Japan.

The receptor-binding assay (RBA) using tritiated STX is also an assay that gives a sum value for STX-equivalents. This test also has undergone formal interlaboratory validation and has reached a good level of acceptance in some countries. Analogue-specific methods for analysis of STXs are based on separation by liquid chromatography and fluorescence or mass spectrometric detection, and a number of protocols have been validated. However, these methods require several analytical runs per sample in the case of complex natural toxin profiles.

Okadaic acid, azaspiracid and their analogues: OA and AZA and their analogues may all be detected by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). This analogue-specific methodology has now replaced the lipophilic MBA in some countries, although for practical reasons the MBA continues to be used in many countries. It should be noted, however, that the MBA has never been formally validated for lipophilic marine biotoxins. For the OA-group of toxins, there is an enzyme-based assay, based on the inhibition of phosphoprotein-phosphatase 2a (PP2a). This assay has also been recently validated and is accepted in some countries. This assay will provide a sum of OA-equivalent toxicity present in a sample.

Domoic acid: Initial efforts in method validation focused on using the extraction protocol for the PSP MBA, but this method has been superseded by the knowledge that DA is not very stable in strongly acidic conditions. Hence, methods using an extraction protocol based on aqueous methanol with HPLC-UV detection are now generally preferred and such methods have undergone collaborative trials for validation. As DA is a relatively simple compound with one major analogue and a single epimer, an ELISA has also been developed and a collaborative trial permitted interlaboratory validation and formal standardization as an AOAC method.

Brevetoxins: The MBA is widely used for detection of BTX and the Codex standard also specifies the limit in mouse units. Chemical methods like LC-MS have been studied, but the challenge is that purified standards are not available for many of the analogues. Other methods that are in various stages of validation include radioimmunoassay, ELISA, neuroblastoma assay and receptor binding assay. None of these detect individual analogues but indicate overall toxicity.

TOXICITY EQUIVALENCY FACTORS (TEFS) FOR SPECIFIC BIOTOXINS

Saxitoxin, okadaic acid, azaspiracid and analogues

The TEFs agreed by the Expert Group and the rationale for the decisions are indicated in the following table.

Compound	Recommended TEF	Rationale
Saxotoxin and analogues		
Saxitoxin	1.0	
NeoSTX	2.0	Both oral studies support higher toxicity than STX. A value of 2.0 is recommended and supported by some Na channel *in vitro* results (range of relative potency 0.7–3.7).
GTX1	1.0	No new data1
GTX2	0.4	No new data
GTX3	0.6	No new data
GTX4	0.7	No new data
GTX5	0.1	Relative potency values from oral LD_{50} studies suggest a lower TEF than from LD_{50} ip. 0.1 TEF also in agreement with original Oshima TEF. As with NeoSTX, a number of *in vitro* Na channel assays also support a TEF of 0.1.
GTX6	0.05	New oral data show lower than 0.1.
C1	0.01	No new data (rounded up)
C2	0.1	No new data
C3	0.01	No new data
C4	0.1	No new data
dcSTX	0.5	From recent oral data (more weight on oral data, also supported by ip toxicity data)
dcNeoSTX	0.2	From recent oral data (more weight on oral data, also supported by *in vitro* data)
dcGTX2	0.2	No new data
dcGTX3	0.4	No new data
Okadaic acid and analogues		
OA	1.0	

Compound	Recommended TEF	Rationale
DTX1	1.0	In the case of the OA group of toxins, several case reports of human intoxication are available, and these reports are analogue specific. As outlined in the recent risk assessment by EFSA, a human poisoning event in Japan with DTX1 as main analogue suggested a LOAEL of 48 ug DTX1 per person. This dose is smilar to that reported to induce toxic effects in poisoning events in Sweden, Norway, UK and Portugal. Thus, it is not surprising that the currently used TEF of 1.0 in some countries appears protective for public health. Still, it should be noted that multiple *in vitro* studies suggest that the intrinsic potency of DTX1 could be higher than that of OA. However, large uncertainty is associated with these studies (a factor of approximately a 5-fold difference between results depending on the cell line used). Therefore, the recommended TEF of 1.0 for DTX1 should be verified in future studies to corroborate the observations in humans, and also through more controlled oral toxicity studies in vivo (in animals).
DTX2	0.5	Consistent among the different assays; based on acute oral, i.p. and *in vitro* toxicity in mice, DTX2 is on average half as toxic as DTX1. This value is also supported by the various *in vitro* data.
DTX3		No TEF is recommended since DTX3 esters per se are non-toxic. They may however be hydrolysed after ingestion to release the parent compound(s) (OA, DTX1, DTX2) in the gastrointestinal tract and more information on this possibility is required
Azaspiracid and analogues		
AZA 1	1.0	
AZA 2	0.7	Based on recent oral data (also consistent with recent i.p. data)
AZA 3	0.5	Based on recent oral data (also consistent with recent i.p. data)
AZA 4		Cannot determine TEF due to lack of data.
AZA 5		Cannot determine TEF due to lack of data.
AZA 6	0.7	No oral data; based on recent i.p. data.

[1] In the case of saxitoxin analogues, for which no oral toxicity data were available, TEFs recommended are based on intra-peritoneal toxicity data of Oshima (Appendix I).

Domoic acid

Domoic acid's analogues, isodomoic acids D, E and F, are found in shellfish, but they have no toxicological significance. DA transforms to epi-DA in storage or by ultraviolet light, and in general DA and epi-DA are considered as one toxin and expressed as DA. Hence, no TEFs are necessary for this group.

DATA GAPS AND FURTHER CONSIDERATIONS FOR RESEARCH

The Expert Group identified several data gaps and areas for further research, including:

- Lack of information on pharmacokinetics for all groups of marine biotoxins.
- Limited information on oral toxicity of analogues for all groups of marine biotoxins.
- Need to establish TEFs for TTX analogues found in bivalves.
- Need for comparative studies on the oral toxicity of TTX congeners.
- Lack of information on chronic toxicity of DA, OA and AZA groups.

RECOMMENDATIONS FOR RISK MANAGERS

- The Expert Group recommended that the proposed TEF values be reviewed and re-evaluated in a 5 to 10-year time-frame, reflecting new data that might be available. This process could be facilitated through the establishment of a database on relative potency studies and related data at FAO/WHO.
- In view of evolving geographical distribution of biotoxins, the Expert Group recommended that national toxin monitoring programmes be updated and expanded to include new and emerging toxins. Also, monitoring should occur during shellfish production.
- The Expert Group recommended that the analytical methods and monitoring strategies be strengthened and expanded to include the different range of biotoxin analogues.
- The Expert Group recommended that if a method cannot separate some of the analogues, the highest TEF should be used to provide maximum consumer protection.

1

The need for and concept of Toxicity Equivalency Factors

1.1 THE NEED FOR TOXICITY EQUIVALENCY FACTORS

Shellfish can be contaminated with toxic substances derived from marine microalgae. In order to protect consumers from poisoning by these toxins, regulatory authorities have defined limits as to the maximum amount of such toxins in shellfish intended for human consumption, and analyses must be conducted in order to ensure that such limits are not exceeded.

Several groups of shellfish toxins have been described, such as the paralytic, diarrhetic and amnesic toxins. Within each group, several structurally-related toxins may be present that contribute to the overall toxic potential.

In the past, testing has largely relied on the mouse bioassay (MBA). This involves injecting an extract of a sample of shellfish into the peritoneum of a group of mice, and recording the time that the mice take to die. The test gives an estimate of the total toxicity of the extract, i.e. the sum of the toxicities of the various congeners present in the sample. The MBA, however, has raised ethical concerns. It is widely accepted that the use of animals should be reduced, refined or replaced as far as is practicable. Furthermore, this route of administration is not relevant to the human situation, and the MBA is an **assay,** not a toxicological parameter. When comparisons have been made, it has been shown that there is no correlation between acute toxicity and the results obtained in the MBA.

Over the last few years, it has proved possible to isolate pure samples of shellfish toxins for use as analytical standards. The development of new analytical techniques, particularly LC-MS, allows individual concentrations of toxin congeners in a shellfish extract to be determined with accuracy and precision.

For risk assessment and management, however, knowledge of the amount of toxin congeners in the shellfish is not sufficient. There is also the need to know the relative toxicity of each of the congeners, so that the total toxicity of the material in the extract can be estimated. This requires the determination of **Toxicity Equivalency Factors (TEFs)**, defined as the *toxicity ratio of a compound from a chemical group that shares the same mode of action of a reference compound in the same group* (Van den Berg *et al.,* 2006; Botana *et al.*, 2010). The toxicity of the congener is expressed as a fraction of the toxicity of the reference compound in terms of potency, which is a pharmacological parameter that defines the amount of compound required for a certain effect.

1.2 TEF: USE OF THE CONCEPT

In general, risk assessment of complex mixtures is done on the basis of individual compounds. However, there are many situations where humans are exposed to a combination of compounds that exhibit a similar mechanism of action, e.g. dioxin-like compounds and organophosphate pesticides. Based on this common mechanism of action, the toxicity equivalency concept has been developed during the last decades to support risk assessment of specific complex mixtures.

From a methodological point of view the total toxicity equivalent (TEQ) is defined by the sum of the concentrations of each compound multiplied by individual TEF values, which provides an estimate of the total toxicological or biological activity relative to a reference compound. Independent of the nature of the compounds used with a TEF concept, the major prerequisite for using it is based on additivity and similar mechanisms or modes of action.

At present, the TEF concept has been most thoroughly developed for the dioxin-like compounds. Since the early 1990s, the World Health Organization (WHO) regularly organized expert meetings to develop and harmonize TEFs for halogenated dioxins, polychlorinated dibenzodioxins (PCDDs), polybrominated dibenzo-p-dioxin (PBDDs) and dioxin-like compounds – polychlorinated dibenzofurans (PCDFs), polybrominated dibenzofurans (PBDFs) and polychlorinated biphenyls (PCBs) – at an international level, thereby giving recommendations to national regulatory authorities (Van den Berg *et al.*, 1998, 2005, 2014).

In the case of dioxin-like compounds, the TEFs have undergone changes over the years, based on emerging toxicological data and knowledge about the mechanism of action. Nevertheless, the criteria to include a compound in the TEF concept of dioxin-like compounds have remained the same and these specifically include:

- show a structural relationship to the PCDDs and PCDFs;
- bind to the aryl hydrocarbon receptor (AhR);
- elicit AhR-mediated biochemical and toxic responses; and
- be persistent and accumulate in the food chain.

In 2010, the United States Environmental Protection Agency (US-EPA) added that "the Ah receptor mediates most if not all of the biologic and toxic effects of tetrachlorodibenzodioxin (TCDD) and the DLCs" (dioxin-like compounds) (Agency, 2010). In the European regulation for dioxin-like compounds (Commission Regulation (EC) No 1881/2006), TEF values range from 1 to 0.00003 (a factor of 33 333 between the lowest and the highest values). It should be noted that the TEF concept has not been applied to polycyclic aromatic hydrocarbons (PAHs) by the European Food Safety Authority (EFSA) for the sum of 4 congeners (EFSA, 2008d).

"The CONTAM Panel explored whether a toxicity equivalency factor (TEF) approach in the risk characterization of the PAH mixtures in food could be applied and concluded that the TEF approach is not scientifically valid because of the lack of data from oral carcinogenicity studies on individual PAHs, their different modes of action and the evidence of poor predictivity of the carcinogenic potency of PAH mixtures based on the currently proposed TEF values".

The principles for risk characterization of chemicals in food have been reviewed by FAO and WHO in Environmental Health Criteria, where chapter 7 specifically addresses the use of TEFs (FAO/WHO, 2009).

In addition, the assignment of WHO TEF values for dioxin-like compounds was done in either increments of 0.01, 0.05, 0.1, etc. (van den Berg *et al.*, 1998) or a half order-of-magnitude increments on a logarithmic scale of 0.03, 0.1, 0.3, etc. (van den Berg *et al.*, 2005). Although the most recent WHO re-evaluations of TEF values using the range of relative effect potencies (REPs) derived from individual studies initially, the final decision on a TEF value for an individual isomer or congener was based on expert judgment, for which the type of study most relevant for human risk assessment was given more weight. Because, in time, new emerging toxicological data of dioxin-like compounds became available, regular changes in a TEF value were made. In view of this dynamic character of TEF values for dioxin-like compounds, it was decided to define these as "interim" values for risk assessment purposes.

1.3. Deriving TEFs

The most common potency parameter used is the median lethal dose (LD_{50}), which is the dose that kills half the population of animals tested, and this is clearly the most appropriate parameter for some toxins, such as the paralytic shellfish toxins, that are known to cause death in humans. With other toxins, however, the LD_{50} may not be relevant. For example, no deaths have been reported after consumption of azaspiracids or the diarrhetic shellfish toxins. The effects seen in humans are gastrointestinal, involving nausea, vomiting, diarrhoea and stomach cramps. Although rodents are unable to vomit, they do suffer diarrhoea when dosed orally with these substances, and TEFs based on relative diarrhoeagenic activity could be considered.

In the past, it was difficult to obtain enough pure toxin congeners in order to conduct acute toxicity studies in animals. Furthermore, some of the early studies were carried out with toxins for which the concentration and stability was not properly assessed. Many of the toxicological results reported only the "minimum lethal dose (MLD)" or "lethality" of a compound, rather than the defined parameter of the LD_{50}. Without information on the doses used to identify the MLD, such results cannot be compared with LD_{50} values. "Lethality" is an ambiguous term that could indicate an MLD or a dose that killed an unspecified number of animals in a group, and is therefore useless for making comparisons.

Several approaches toward the determination of TEFs have been taken. However, because of the shortage of test material, early studies focussed on toxicity by intraperitoneal (i.p.) injection, which is inappropriate for the definition of TEFs, since the natural route of exposure is oral ingestion (Botana, 2012). At the same time, for biotoxins with similar bioavailability, i.p. administration can still provide useful toxicity comparison information. Materials dosed by i.p. injection are rapidly and extensively absorbed from the peritoneal cavity, whereas the gastrointestinal tract is designed to minimize absorption of many harmful substances. I.p. injection will therefore usually give an unrealistically high estimate of the toxicity of compounds that require absorption for expression of their harmful effects. At the same time, if a toxin were to be metabolized to a more toxic analogue in the gastrointestinal tract, then the i.p. route may give a low estimate (as may happen with neosaxitoxin, which is more toxic than saxitoxin). It should be noted that no correlation exists between i.p. LD_{50} values and those following oral administration (Table 1).

TABLE 1. Comparison of LD_{50} values of toxins by i.p. injection versus gavage

Compound	Ratio of toxicity by gavage to toxicity by i.p. injection
Palytoxin	708
GTX-1&4	110
Neosaxitoxin	79
Pinnatoxin E	49
Saxitoxin	43
Spirolide A	15
13-Desmethyl spirolide C	23
Gymnodimine	8
Pinnatoxin F	2

Sources: Munday, 2006; Munday *et al.*, 2004; 2012a, 2012b, 2013; Selwood *et al.*, 2010.

TEFs have also been estimated by comparing toxicity to cultured cells *in vitro*. Considerable effort has been made toward developing *in vitro* tests to replace in vivo acute toxicity evaluations, as undertaken by The Interagency Coordinating Committee on the Validation of Alternative Methods in the United States of America and the European Centre for the Validation of Alternative Methods. It has been concluded, however, that at the present time, no *in vitro* test method is sufficiently accurate to replace animals for regulatory hazard classification purposes (ICCVAM, 2006a, b). There may, however, be an exception with marine toxins as the mechanism by which the toxins cause death is known, as discussed below for the paralytic shellfish poisons. Cell-based assays may, in the future, also be appropriate for other toxins when the target site and mode of action have been unambiguously identified.

Since toxins are ingested by humans, toxicity by oral administration is the most relevant parameter for determining TEFs, but even here, care must be taken. Because of the semi-solid content of the rodent stomach, material administered by gavage can flow around the material and rapidly enter the absorptive area of the duodenum, rather than mixing with the food in the stomach as occurs with the liquid stomach contents of humans. Such mixing does occur when materials are dosed acutely to rodents by voluntary consumption. While training of animals is required to ensure that they eat small quantities of food containing the toxin within seconds, this technique is the most relevant to the human situation (Munday, 2014). It is only recently that sufficient amounts of certified material have become available for such studies to be conducted.

At present, TEFs for shellfish toxins are based on acute effects. Very little information on the chronic toxicity of these substances is available. Acute toxicity studies provide information on the "Acute Reference Dose" (ARfD) of a compound for humans, defined as:

"An estimate of the amount of a substance in food and/or drinking water, normally expressed on a milligram per kilogram basis, that can be ingested in a period of 24 hours or less without appreciable health risk to the consumer."

Repeated doses at low levels may also be toxic, and for risk assessment of certain marine toxins, particularly those that are slowly eliminated from the body and may therefore accumulate after repeated exposure, an estimate of the "Tolerable Daily Intake" (TDI), defined as "An estimate of the amount of a substance in food and/or drinking water, normally expressed on a milligram per kilogram body weight basis, that can be ingested daily over a lifetime without appreciable health risk to the consumer" would be valuable. Estimates of the TDI require studies in mice continuously fed the toxin over a period of weeks.

Although the TEF concept is rather simple, for marine toxins it is very complex to implement, for several reasons:

- It has been difficult to obtain the required quantities of the compound to do acute oral toxicity studies in animals. In the past, many of the studies were carried out with toxins or extracts for which the concentration and stability were not properly assessed, and this is a major source of variation in reported toxicological studies.
- The death of an animal does not necessarily show the real risk in humans of a compound that may be ingested in low doses or chronically. Many of the toxicological results only provide the concentration that causes the death of mice, and this value is often not as the LD_{50} but as a lethal dose (see above), so that variation in the way the experiments are conducted and in the way that the results are expressed makes it very difficult to compare data.
- The death of an animal is the end point of a mechanism of action over activated, and with lower doses the toxic effects are, in many cases, quite different.
- The mechanism of action of a toxic compound may involve several targets, and those leading to the death of the animal may not be the targets responsible for the toxic symptoms observed in humans.
- If the compounds target several receptors, the sensitivity of each receptor can be different, and this would cause a progressive appearance of symptoms as each of the different targets are being activated.

- In many cases, not all targets are known. For this reason, it is imperative to know the mechanism of action of the compound and the implications, at a systemic level, of the modification of the target.
- The target of the compound may show very different effects in different systems or organs and, on many occasions, the physiology of the specific target in the organ is not well understood.

Given the complexity of the subject, the most appropriate TEF is the one that identifies the potency of each congener of the same toxic group (analogues sharing the same mode of action) in the specific organ or system where the toxicity is reported, relative to a defined compound. In general, this is difficult to obtain. Finally, it is important to bear in mind that TEFs are needed only for those scenarios in which humans are exposed to a mixture of related compounds, i.e. with the same toxicological profile but different toxic potency, that can be detected together, in order to assess the total toxicity of the mixture.

Given the complexity of the derivation of a TEF for each desired analogue in a toxin group, and due to the scarcity of solid information at the time, the Working Group on Marine Biotoxins, which was part of the Contaminants Panel of the European Food Safety Authority (EFSA), defined TEFs for most of the marine toxin groups based on the i.p. acute toxicity of each analogue (EFSA, 2008c). These TEFs are biased in some cases because they take into account neither the oral bioavailability nor the local effect of some toxin groups, but at least they provided a value to be used as a reference. However, *TEF values should not be used unless there is data to prove that the compounds share the same mode of action and that this mode of action explains the symptoms observed in humans.* Clearly, as more mechanistic information is obtained with each of the toxin groups, an update on the use of TEFs becomes appropriate.

It is now time to engage in such a reflection at an international level to develop a common view. The question is: Is it possible at present to apply the TEF concept to marine biotoxins included in the Codex standard (CODEX STAN 292–2008) In the following sections, the criteria used for assigning TEFs, the values agreed during the meeting and the rationale have been explained.

1.3.1 Criteria for assigning TEFs

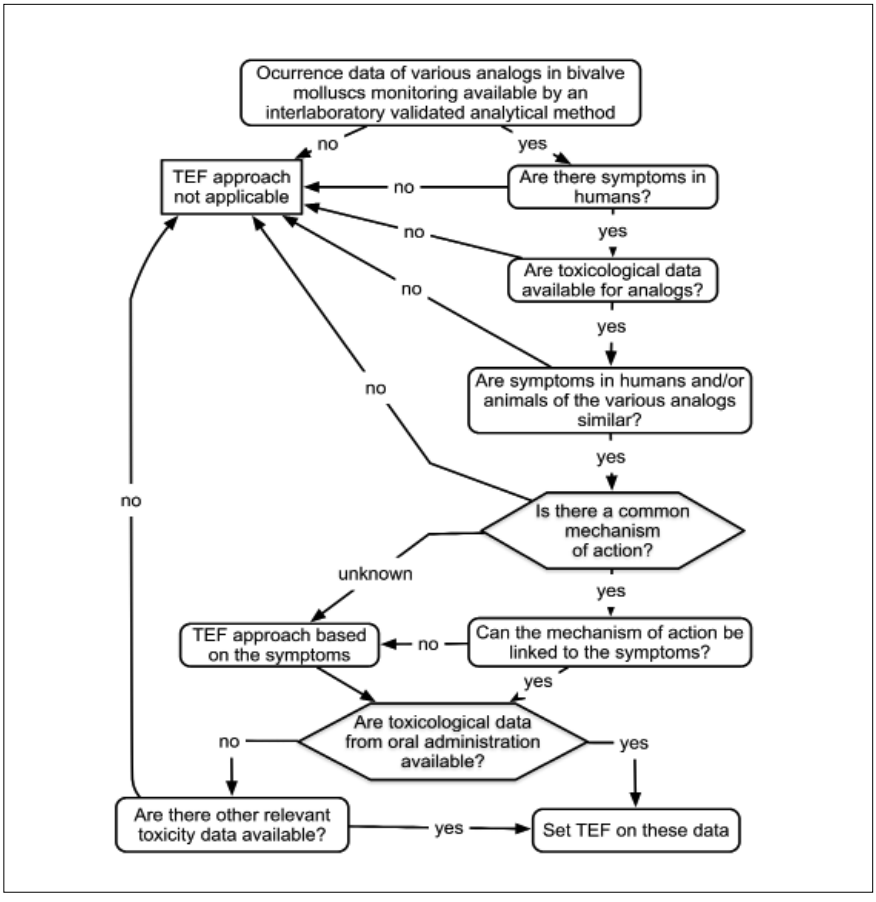
The Expert Group discussed the criteria for using different data for assigning TEFs. They conclude:

- Results of studies in which certified reference materials were not used or where no quality control of the toxins was taken must be considered with caution. Some commercial supplies may provide quantification errors that invalidate calculations of the lethal doses (Crespo *et al.*, 2015).

- Studies by oral administration are most relevant.
- In order to permit evaluation by *in vitro* studies, it is essential to define the mechanism of action responsible for the toxic effect, or the different targets responsible for each toxic effect.
- With some toxins, sub-chronic toxicity studies, involving dietary administration of the material to mice for 28 or 90 days, following Organisation for Economic Co-operation and Development (OECD) Guidelines (OECD 1998a, 1998b) are required in order to assess possible harmful effects after repeated exposure.

Based on the above criteria, a scheme was used to decide whether TEFs could be assigned during the review by the Expert Group (Figure 1).

FIGURE 1. Schema to decide whether TEF assignment was applicable in the frame of the Expert Group



2

Chemistry and detection methods for biotoxins

2.1 CHEMICAL NATURE AND MOLECULAR STRUCTURE OF ANALOGUES

Shellfish toxins, a subgroup of marine toxins, are mainly produced by micro-organisms (bacteria, cyanobacteria and micro-algae). Based on their chemical structure, the toxins included in *Codex Standard 292–2008* can be classified into five groups, namely, the saxitoxin (STX), okadaic acid (OA), domoic acid (DA), brevetoxin (BTX), and azaspiracid (AZA) groups. An additional toxin group will also be considered in Section 4.2.1, tetrodotoxin (TTX), due to its emergence in shellfish. The five toxin groups are also known for the four syndromes they cause: (i) paralytic shellfish poisoning (PSP) (STX and TTX groups); (ii) diarrhetic shellfish poisoning (DSP) (AZA and OA groups); and (iii) amnesic shellfish poisoning (ASP) (DA group) and neurotoxic shellfish poisoning ie NSP (BTX). While TTXs have not been specifically mentioned in the Codex standard, they have the same mode of action as STXs (for which analogue-specific methods are under discussion) and belong to the group of paralytic shellfish toxins. They also have been recently detected in bivalves (Turner *et al.*, 2015b; Vlamis *et al.*, 2015). This section briefly describes the chemical nature of the molecules involved and their structures.

The toxins of these five groups are molecules with a range from around 200 to 900 Da, and the structures can be classified according to various chemical classes (Table 2).

TABLE 2. Overview of characteristics of key toxins, including five groups in Codex Standard 292–2008

Toxin	Syndrome	Chemical class	Formula	Molar weight g/mol	UV [nm]	Lipophilicity
Saxitoxin[1]	PSP	tetrahydro-purine alkaloid	$C_{10}H_{17}N_7O_4$	299	n/a(2)	hydrophilic
Okadaic acid[1]	DSP	polyether, spiro-keto ring assembly	$C_{44}H_{68}O_{13}$	804	n/a	lipophilic
Domoic acid[1]	ASP	cyclic amino acid, 3 carboxy groups	$C_{15}H_{21}NO_6$	311	242	hydrophilic
Brevetoxin[1]	NSP	Polyether with contiguously fused rings	$C_{49}H_{70}O_{13}$	867	n/a	lipophilic
Azaspiracid[1]	DSP[4]	polyether, second amine, 3-spiro-ring assembly	$C_{47}H_{71}NO_{12}$	841	n/a	lipophilic
Tetrodotoxin	PSP[3]	Guanidinum-derivative of penta-hydroxylated 2,4-dioxaadmantane	$C_{11}H_{17}N_3O_8$	319	n/a	hydrophilic

Notes: [1] in Codex standard. [2] n/a = no specific absorption above 210 nm. [3] TTX is not classified as PSP, but included here in this group based on observed symptoms. [4] AZA differs chemically from Okadaic acid but produces the same symptom.

2.1.1 Saxitoxin and analogues

Saxitoxin (STX)-group toxins are mainly produced by dinoflagellates belonging to the genus *Alexandrium,* e.g. *A. 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, as well as cyanobacteria of the genera *Anabaena, Cylindrospermopsis, Aphanizomenon, Planktothrix and Lyngbia* (Wiese *et al.*, 2010).

This group of toxin analogues has STX as the reference compound, and they share a common structure of tetrahydropurine. They are soluble in water and thermostable at acidic pH; at alkaline pH they are quickly degraded (Kodama and Sato, 2008). More than 50 compounds have been reported (Table 3) (Dell'Aversano *et al.*, 2008; Wiese *et al.*, 2010) and at least 18 have toxicological relevance (Figure 2). This group of toxins is separated into carbamoyl analogues, (STX, neoSTX and gonyautoxins (GTX) 1 to 4), N-sulphocarbamoyl analogues (gonyautoxins 5 and 6, toxins C 1 to 4), and decarbamoyl analogues (dcSTX, dcNeoSTX and dcGTX 1 to 4). The different groups of analogues have different relative toxicities, and it is possible that some compounds transform into others, at acidic pH, with an increase in toxicity: GTX5, GTX6, C1, C2, C3, C4 will transform at acidic pH with heat into STX, NeoSTX, GTX2, GTX3, GTX1 and GTX4, respectively (Vale *et al.*, 2008b). Some of these conversions may take place naturally at the acidic pH of the stomach

(Harada, Oshima and Yasumoto, 1984). At the same time, transformation into a different set of toxins at slightly alkaline pH (7.4–7.5) is also possible with heat: GTX5, GTX6, C1, C2, C3, C4 will transform into dcSTX, dcNeoSTX, dcGTX2, dcGTX3, dcGTX1 and dcGTX4, respectively. The toxicity of some of these toxins may vary, since they epimerise until they reach thermodynamic equilibrium and that depends on the storage conditions, i.e. GTX2, 3 reach an equilibrium ratio of 2.7; GTX1 4 of 3.4; C1 2 of 3.5; and dcGTX2, 3 of 4.4.

STX analogues do not exhibit a strong ultraviolet (UV) absorbance or fluorescence. They are typically stable to heat treatment up to 100°C. Different acid and base treatments will lead to various transformations. In particular, all C11-epimeric pairs (e.g. GTX2 and 3 or GTX1 and 4) will interconvert and equilibrate to a constant ratio at high pH. Similarly, carbamoyl- and sulphocarbamoyl-derivatives will convert to decarbamoyl-analogues through cleavage of the carbamoyl-ester group at high pH (e.g. C1 to dc-GTX2, and C2 to dc-GTX3). Under acidic conditions, the carbamoylester is relatively stable but the sulphate ester will be cleaved to convert sulphocarbamoyl groups into carbamoyl groups (e.g. C1 to GTX2, and C2 to GTX3). These transformations may only occur partially when shellfish tissues, human tissues or fluids contaminated with STXs are exposed to these conditions, as biological tissues typically buffer the pH. Since conversion reactions can result in a several-fold increase in toxicity, a potential danger from these toxins was suggested (Hall and Reichardt, 1984). To examine this phenomenon experimentally, B1 (GTX5) was incubated at conditions simulating the human stomach and analysed by the mouse bioassay. After 5 h incubation at 37°C, a two-fold increase of toxicity, corresponding to 9 percent conversion of toxin, was observed in the artificial gastric juice at pH 1.1 and no apparent increase of toxicity was observed in rat gastric juice at pH 2.2 (Harada, Oshima and Yasumoto, 1984).

The marine organisms most often affected are mussels, oysters and clams, but also puffer fish and marine snails (e.g. abalone) have been reported to accumulate dangerous concentrations (Pitcher *et al.*, 2001; Harwood *et al.*, 2014). The hydrophilic character of the compounds may partially explain the relatively rapid depuration of these toxins from mussels. This rapid depuration complicates the regulatory surveillance for these toxins, which is therefore most often complemented by observations of the algae responsible for *in situ* production.

FIGURE 2. Most commonly reported saxitoxin analogues of toxicological relevance

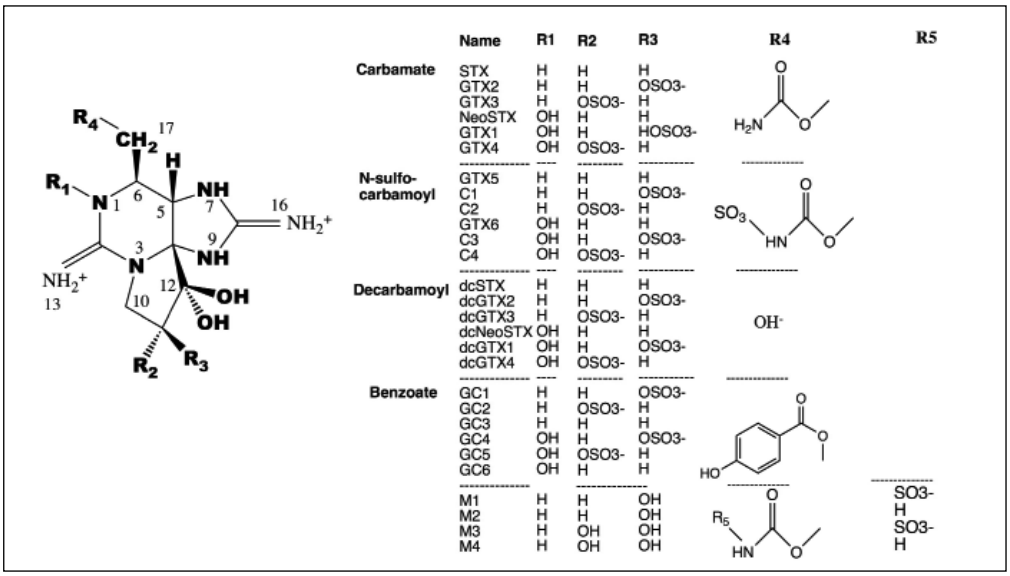


TABLE 3. STX and analogues reported from phytoplankton and shellfish organisms

Name	Abbreviation	Molecular formula	Molar mass (g/mol)	Biological origin
Saxitoxin	STX	$C_{10}H_{17}N_7O_4$	299,1	Phytoplankton
Neosaxitoxin	NEO	$C_{10}H_{17}N_7O_5$	315,1	Phytoplankton
21-Sulfo-11a-hydroxysaxitoxin sulfate	C1, 2	$C_{10}H_{17}N_7O_{11}S_2$	475,0	Phytoplankton
21-Sulfo-11a-hydroxyneosaxitoxin sulfate	C3, 4	$C_{10}H_{17}N_7O_{12}S_2$	491,0	Phytoplankton
Deoxydecarbamoyl Gonyautoxin-2	doGTX2, 3	$C_9H_{16}N_6O_6S$	336,1	Phytoplankton
Decarbamoyl GTX-1,4	dcGTX1, 4	$C_9H_{16}N_6O_8S$	368,1	Phytoplankton
Decarbamoyl GTX-2,3	dcGTX2, 3	$C_9H_{16}N_6O_7S$	352,1	Phytoplankton
Gonyautoxin-2,3	GTX2	$C_{10}H_{17}N_7O_8S$	395,1	Phytoplankton
Gonyautoxin-1,4	GTX1	$C_{10}H_{17}N_7O_9S$	411,1	Phytoplankton
Gonyautoxin-5	GTX5 (B1)	$C_{10}H_{17}N_7O_7S$	379,1	Phytoplankton
Gonyautoxin-6	GTX6 (B2)	$C_{10}H_{17}N_7O_8S$	395,1	Phytoplankton
12-hydroxy-deoxy-GTX4	deoxy-GTX4 12ol	$C_{10}H_{17}N_7O_8S$	395,1	Phytoplankton
Deoxydecarbamoyl-saxitoxin	doSTX	$C_9H_{16}N6O_2$	240,1	Phytoplankton
Decarbamoyl-saxitoxin	dcSTX	$C_9H_{16}N_6O_3$	256,1	Phytoplankton
Decarbamoyl-neosaxitoxin	dcNEO	$C_9H_{16}N_6O_4$	272,1	Phytoplankton
Gymnodinium catenatum toxin 1	GC1	$C_{16}H_{20}N_6O_9S$	472,1	Phytoplankton
Gymnodinium catenatum toxin 2	GC2	$C_{16}H_{20}N_6O_9S$	472,1	Phytoplankton
Gymnodinium catenatum toxin 3	GC3	$C_{16}H_{20}N_6O_5$	376,1	Phytoplankton
Gymnodinium catenatum toxin 4	GC4	$C_{16}H_{20}N_6O_{10}S$	488,1	Phytoplankton

Name	Abbreviation	Molecular formula	Molar mass (g/mol)	Biological origin
Gymnodinium catenatum toxin 5	GC5	$C^{16}H_{20}N_6O_{10}S$	488,1	Phytoplankton
Gymnodinium catenatum toxin 6	GC6	$C_{16}H_{20}N_6O_6$	393,1	Phytoplankton
hydroxy-GC1	GC1a	$C_{10}H_{17}N_7O_8S$	488,1	Phytoplankton
hydroxy-GC2	GC2a	$C_{16}H_{20}N_6O_{10}S$	488,1	Phytoplankton
hydroxy-GC3	GC3a	$C_{16}H_{20}N_6O_{10}S$	392,1	Phytoplankton
hydroxy-GC4	GC4a	$C_{16}H_{20}N_6O_6$	504,1	Phytoplankton
hydroxy-GC5	GC5a	$C_{16}H_{20}N_6O_{11}S$	504,1	Phytoplankton
hydroxy-GC6	GC6a	$C_{16}H_{20}N_6O_{11}S$	409,1	Phytoplankton
sulfo-GC1	GC1b	$C_{16}H_{20}N_6O_{11}S_2$	536,1	Phytoplankton
sulfo-GC2	GC2b	$C_{16}H_{20}N_6O_{11}S_2$	536,1	Phytoplankton
sulfo-GC3	GC3b	$C_{16}H_{20}N_6O_7S$	440,1	Phytoplankton
sulfo-GC4	GC4b	$C_{16}H_{20}N_6O_{12}S_2$	552,1	Phytoplankton
sulfo-GC5	GC5b	$C_{16}H_{20}N_6O_{12}S_2$	552,1	Phytoplankton
sulfo-GC6	GC6b	$C_{16}H_{20}N_6O_8S$	457,1	Phytoplankton
Lyngbia wollei toxin 1	LWTX1	$C_{11}H_{18}N_6O_7S$	378,1	Phytoplankton
Lyngbia wollei toxin 2	LWTX2	$C_{11}H_{18}N_6O_8S$	394,1	Phytoplankton
Lyngbia wollei toxin 3	LWTX3	$C_{11}H_{18}N_6O_8S$	394,1	Phytoplankton
Lyngbia wollei toxin 4	LWTX4	$C_9H_{16}N_6O_2$	240,1	Phytoplankton
Lyngbia wollei toxin 5	LWTX5	$C_{11}H_{18}N_6O_4$	298,1	Phytoplankton
Lyngbia wollei toxin 6	LWTX6	$C_{11}H_{18}N_6O_3$	282,1	Phytoplankton
shellfish metabolite 1	M1α	$C_{10}H_{17}N_7O_8S$	395,1	Shellfish
Shellfish metabolite 1	M1β	$C_{10}H_{17}N_7O_8S$	395,1	Shellfish
Shellfish metabolite 2	M2α	$C_{10}H_{17}N_7O_5$	315,1	Shellfish
Shellfish metabolite 2	M2β	$C_{10}H_{17}N_7O_5$	315,1	Shellfish
Shellfish metabolite 3	M3	$C_{10}H_{17}N_7O_9S$	411,1	Shellfish
Shellfish metabolite 4	M4	$C_{10}H_{17}N_7O_6$	331,1	Shellfish
Shellfish metabolite 5	M5	$C_{10}H_{17}N_7O_8S$	395,1	Shellfish
Shellfish metabolite 6	M6	$C_{10}H_{17}N_7O_5$	315,1	Shellfish
Shellfish metabolite 8	M8	$C_{10}H_{17}N_7O_6$	331,1	Shellfish
Shellfish metabolite 10	M10	$C_{10}H_{17}N_7O_7S$	347,1	Shellfish
11-saxitoxinethanoic acid	SEA	$C_{12}H_{16}N_7O_6$	431,1	Shellfish

Source: Adapted from Wiese *et al.*, 2010.

2.1.2 Okadaic acid and analogues

Okadaic acid (OA) was originally isolated from the sponge *Halichondria okadaii* (Tachibana *et al.*, 1981) but was later identified as a shellfish contaminant following a series of poisoning events in 1976 (Yasumoto, Oshima and Yamaguchi, 1978) (Figure 3). Subsequently, it was clearly demonstrated that diarrhetic shellfish poisoning was associated with blooms of *Dinophysis fortii*, a dinoflagellate from which OA and an analogue of OA, dinophysistoxin–1 (DTX1) were isolated (Yasumoto *et al.*, 1980). The same class of compounds was promptly discovered to

be the causative agents of diarrhetic shellfish poisoning in Europe (Kumagai *et al.*, 1986). Dinophysistoxin–2 (DTX2) was discovered to be the third main analogue Dinophysistoxin–2 (DTX2) was discovered to be the third main analogue (Hu *et al.*, 1992b), explaining diarrhetic activity found in Irish mussels. OA and DTXs are produced by a variety of different dinoflagellates from the *Dinophysis* and *Prorocentrum* genera, including *D. acuta* and *D. acuminata*, as well as *P. lima* and *P. belizeanum*. Although the toxins of the OA group have been mainly reported from Japan and Europe, it has recently been shown that *Dinophysis* in the Gulf of Mexico may also produce DSP under appropriate environmental conditions (Swanson *et al.*, 2010). Also Chinese and South American *Dinophysis* strains have been shown to produce okadaic acid (Fux *et al.*, 2011; Mafra, Tavares and Schramm, 2014; Li *et al.*, 2015) and shellfish from all over the world have been shown to be contaminated with these toxins (Zhao *et al.*, 1993; Suzuki et al., 2004; Villar-Gonzalez *et al.*, 2007; Li *et al.*, 2012). Therefore, a global distribution of these toxins is now widely accepted.

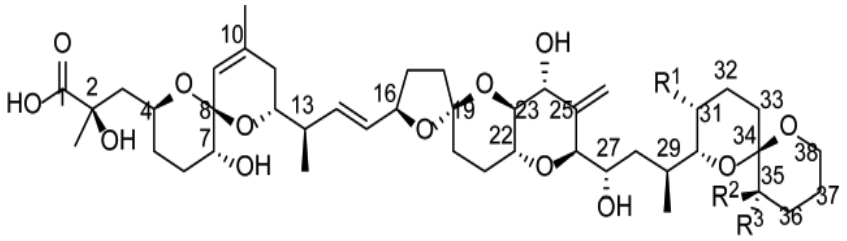
Chemically, OA is one of the many polyether toxins among phycotoxins. Its structure is characterized by a carboxylic acid group and three spiro-keto ring assemblies, one of which connects a five with a six-membered ring (Figure 3). OA, DTX1 and DTX2 withstand a wide pH range from mildly acidic to strongly basic, e.g. no degradation is found for up to 40 minutes at 76°C in 0.3 molar methanolic NaOH solution. Treatment with strong mineral acids, e.g. HCl, leads to rapid degradation: OA and DTX1 are completely destroyed within 20 min at 76°C in 0.3 molar methanolic HCl, even in the presence of shellfish matrix in the extract (Yasumoto *et al.*, 1985). However, without the addition of acid, the compounds are stable to heat. Also, recent work on stomach simulation experiments in the laboratory suggests that the food itself has a buffering capacity on the acid and the toxins may not be destroyed significantly in the gastric juice. In normal cooking procedures the toxins are not destroyed, although the coagulation of proteins in shellfish tissues may lead to redistribution within the organs of shellfish and some toxins may be released into the cooking fluids (McCarron, Kilcoyne and Hess, 2008; Blanco *et al.*, 2015).

Different types of esters of OA and DTXs have been reported. Initially, the palmitoyl ester of DTX1 was isolated and named DTX3 (Yasumoto *et al.*, 1985) with other ester analogues shown to be present; subsequently, all fatty acid esters of variable chain length of OA, DTX1 and DTX2 have been referred to as DTX3 (in a simplifying fashion). In micro-algae (so far mainly *P. lima* and *P. belizeanum*), esters of allylic diols with the carboxylic acid at C1 of OA and DTXs have been reported (Yasumoto *et al.*, 1989; Hu *et al.*, 1992a, 1995a); these esters were named DTX4, DTX5, etc. When the algae enter shellfish through natural filter-feeding, it

is believed that these esters are rapidly degraded (Vale, 2007). The shellfish then further metabolize OA and its analogues to form esters of OA and DTXs with fatty acids (at the C7-OH group); these esters were initially identified for DTX1 as shellfish derivatives (Yasumoto *et al.*, 1985) and their toxicity has been described to be similar to the parent compounds, although the onset appears later in the i.p. mouse model (Yanagi *et al.*, 1989). A further fatty acid ester of DTX1 at the C27-OH group has been reported in a sponge (Britton *et al.*, 2003), and most recently, Torgersen *et al.* (2008b) also reported mixed esters of diols (at the C1 carboxyl-end) and fatty acids (at the C7-OH position) in shellfish, suggesting that partial degradation and simultaneous metabolism may co-occur during digestion of algae by shellfish.

The multitude of compounds potentially present in shellfish (free toxins, diol esters and their derivatives, fatty acids and mixtures of diol- and fatty acid esters) complicates the determination of the complete toxin content in shellfish samples. This complexity has added to the difficulties in estimating the potency of these toxins and evaluation of the risk they present. The ester-bond has not shown any degradation in long-term stability studies, although fatty acids have been reported to oxidise easily if they contain double bonds. All of the esters discussed above (either at the C1-carboxy or at the C7-OH) group are quantitatively cleaved through treatment with strong base, e.g. 0.3 molar methanolic NaOH at 76°C for 10 to 40 minutes. This characteristic, in combination with the stability of the parent compounds (OA, DTX1 and DTX2) to base treatment, has been extensively used to quantify the equivalent of the parent compound present in any given shellfish sample (Lee *et al.*, 1989).

FIGURE 3. Okadaic acid and analogues dinophysistoxins–1 and –2



		R^1	R^2	R^3	31	34	35
1	Okadaic acid	CH_3	H	H	*R*	*S*	-
2	DTX-1	CH_3	CH_3	H	*R*	*R*	*R*
3	DTX-2	H	H	CH_3	-	*R*	*S*

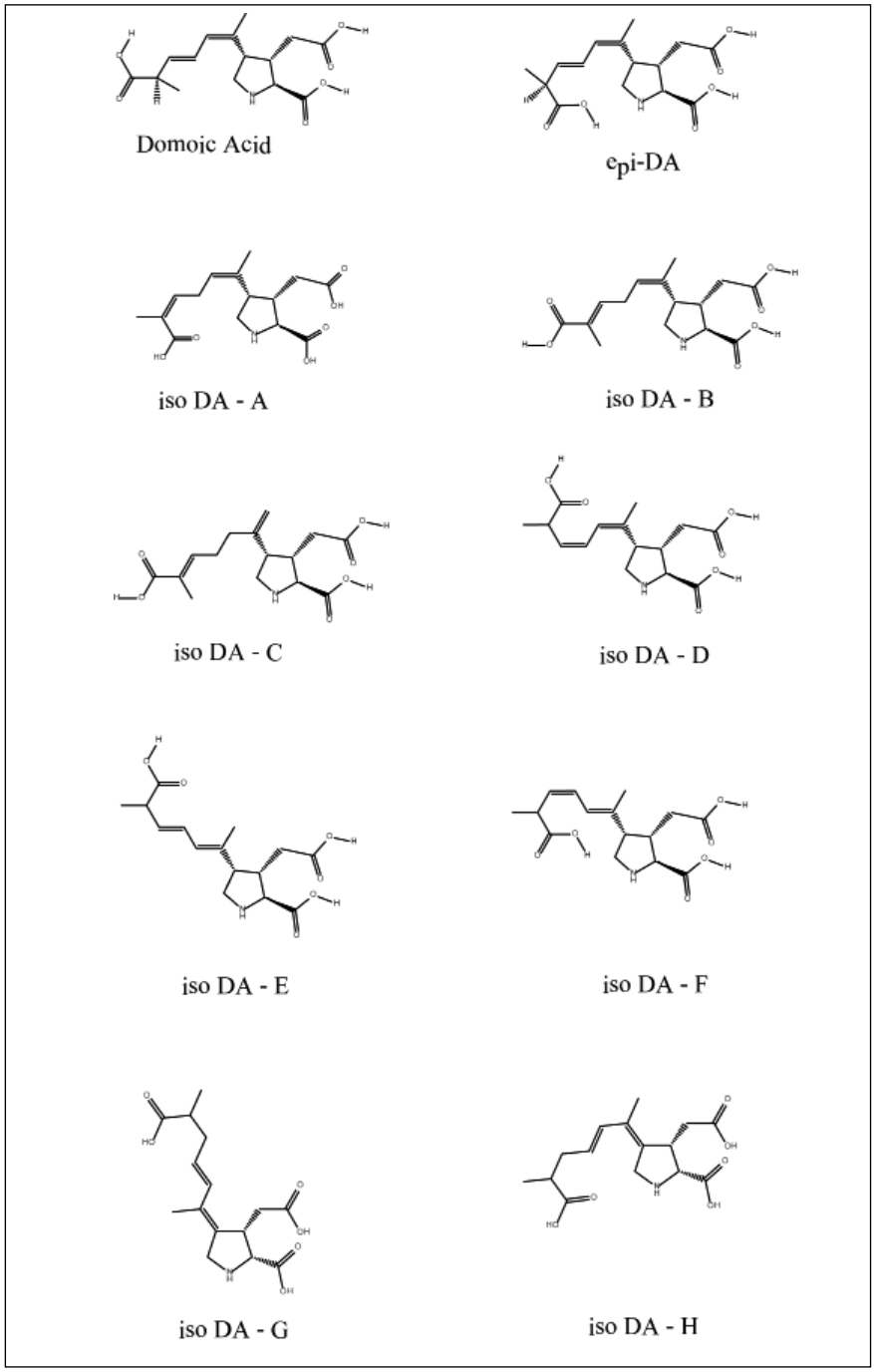
2.1.3 Domoic acid and analogues

Domoic acid (DA) is a cyclic amino acid (311 Da) with three carboxylic acid groups which are responsible for its water solubility and its high polarity (Quilliam *et al.*, 1989d) (Figure 4). DA has been known for a long time to be the active constituent of the marine red algae of the genus *Chondria* and this has also been recently confirmed by culture experiments of *Chondria armata* (Daigo, 1959; Jiang *et al.*, 2014). The production of DA in the genus *Pseudo-nitzschia* is widely known since the 1987 incident of ASP in Canada (Quilliam, and Wright, 1989a). Its widespread occurrence throughout the genus *Pseudo-nitzschia* has recently been reviewed (Trainer *et al.*, 2012). Since the earlier studies, DA has now also been detected in other diatom species, including *Nitzschia navis-varingica* (Romero *et al.*, 2011) and *Nitzschia bizertensis* (Smida *et al.*, 2014).

Structurally, DA is very similar to another known toxin, kainic acid. For the three carboxylic acids the acid constant values (pKa values) are 2.10–4.97 and for the cyclic amino group, 9.82 (Piñeiro *et al.*, 1999), and hence DA can exist in different charged states depending on pH (Jeffery *et al.,* 2004). Although several isomers of DA epi-domoic acid (epi-DA) (domoic acid C5'-diastereomer) and isodomoic acids A, B, C, D, E, F, G and H (iso-DA A–H)) have been reported (Maeda *et al.*, 1986, 1987; Wright *et al.*, 1990a; Zaman *et al.*, 1997; Holland *et al.*, 2005), so far only DA and epi-DA have been shown to be of toxicological relevance (Ramsdell, 2007). DA transforms into epi-DA through long-term storage (Quilliam *et al.*, 1989b, Quilliam, Xie and Hardstaff.1995) and degrades and transforms to epi-DA and isodomoic acids through exposure to ultra violet light (Wright *et al.*, 1990a; Djaoued, Balaji and Priya, 2007). Epimerization is also accelerated with warming (Quilliam, 2003). It has been suggested that DA may be a biosynthetic product, which is subsequently converted to isodomoic acids in suitable environmental conditions (Wright *et al.*, 1990b).

Due to the conjugated double bond in the aliphatic side chain, DA absorbs ultra violet (UV) light and is light-sensitive. The conjugated double bond is also the cause of radical-mediated oxidative metabolism. DA does not degrade at ambient temperature or when it is exposed to light in sterile saline solution (Johannessen, 2000), but in acidic conditions DA has been shown to decompose. At pH 3 a loss of 50 percent of DA was observed in one week (Quilliam *et al.*, 1989b). As a contaminant in shellfish tissues, DA is heat stable and cooking does not typically destroy the toxin. Its stability under various conditions has been studied, and storage of raw or autoclaved tissues only resulted in approximately 50 percent degradation of the toxin after 5 months (McCarron, Burrell and Hess, 2007b). DA rapidly degrades under the influence of gamma irradiation, either in solution or in shellfish tissue, but can be stabilized in shellfish tissue by freeze-drying the tissue (McCarron, Emteborg and Hess, 2007a; McCarron *et al.*, 2007c).

FIGURE 4. Domoic acid and its isomers



Nota bene: isomers A – C do not have conjugated double bonds and are not easily detected using HPLC-UV.

2.1.4 Azaspiracids and analogues

Azaspiracids (AZAs) are marine algal toxins produced by the dinoflagellate genera *Azadinium* (Krock *et al.*, 2009; Tillmann *et al.*, 2009) and *Amphidoma* (Tillmann *et al.*, 2012, 2014). This class of toxins was first identified in the 1990s following an outbreak of human illness in the Netherlands associated with consumption of contaminated mussels from Killary Harbour, Ireland (McMahon *et al.*, 1996). Although the symptoms were typical of DSP toxins, i.e. okadaic acid (OA) and dinophysistoxins (DTX), the levels of DSP toxins in these mussels were well below the regulatory level. Subsequently, it was established that the shellfish were contaminated with a novel marine toxin, originally named "Killary-toxin" or KT–3 (Satake *et al.*, 1998a). Shortly thereafter, the toxin was renamed as azaspiracid (AZA) to more appropriately reflect its chemical structure: a cyclic amine, or aza group, with a tri-spiro- assembly and carboxylic acid group (Satake *et al.*, 1998a, b).

To date, over 30 AZA analogues have been identified in phytoplankton and shellfish (Satake *et al.*, 1998b; Ofuji *et al.*, 1999, 2001; Lehane *et al.*, 2002; James *et al.*, 2003a; Rehmann, Hess and Quilliam, 2008; McCarron *et al.*, 2009; Jauffrais *et al.*, 2012a; Hess *et al.*, 2014).

Over the last 15 years, AZAs have been reported in shellfish from many coastal regions of Western Europe (James *et al.*, 2001, 2002; Braña Magdalena *et al.*, 2003; Furey *et al.*, 2003; Hess *et al.*, 2007b; Amzil *et al.*, 2008; Twiner *et al.*, 2008b), Northern Africa (Taleb *et al.*, 2006; Elgarch *et al.*, 2008), South America (Alvarez *et al.*, 2010) and North America (M. Quilliam, pers. comm.; A. Robertson, pers. comm.).

In addition, AZAs have been found in Japanese sponges (Ueoka *et al.*, 2009) and Scandinavian crabs (Torgersen *et al.*, 2008a). Not surprisingly, the global distribution of AZAs appears to correspond to the apparent wide spread occurrence of *Azadinium* (Tillmann *et al.*, 2010, 2011, 2014; Akselman and Negri, 2012). Empirical evidence is now available that unambiguously demonstrates the accumulation of AZAs in shellfish via direct feeding on AZA-producing *A. spinosum* (Salas *et al.*, 2011; Jauffrais *et al.*, 2012a).

Whereas extensive study of this toxin class has been historically constrained by limited availability of purified material, these restraints are now less of an impediment due to advances in isolation and purification of AZAs from naturally contaminated shellfish (Kilcoyne *et al.*, 2012) and the identification of the toxigenic organism *A. spinosum* coupled with its mass culture in bioreactors (Jauffrais *et al.*, 2012c). As such, certified reference materials of naturally produced AZA1–3 are now commercially available (Perez *et al.*, 2010). Although not yet realized for

commercial purposes, limits on toxin supply may be further mitigated by advances in the organic total synthesis of AZA1 (Nicolaou *et al.*, 2004a, 2004b) and AZA3 (C. Forsyth, pers. comm.). Accessibility to purified AZAs has led to rapid progress with respect to understanding AZA toxicology over the last few years.

AZAs have sporadically been detected in plankton (James *et al.*, 2003b; Krock *et al.*, 2008) and sea water (Rundberget *et al.*, 2007; Fux, Buré and Hess, 2009), but no progenitor of these toxins could be assigned until 2007 (Krock *et al.*, 2009), when a small dinoflagellate, later named *Azadinium spinosum* (Krock *et al.*, 2009), was unambiguously identified as an AZA1 and –2 producing organism (ca. 20 and 7 fg cell^{-1}, respectively). Later, additional AZAs with molecular masses of 715 Da (ca. 7 fg cell-1) and 816 Da (AZA33 and –34) have been found in environmental samples and cultures of *A. spinosum* (Kilcoyne *et al.*, 2014b) (Table 4).

TABLE 4. Structural variants of AZAs, their protonated masses, fragment type and origin

	Protonated mass $[M+H]^+$	Type	Origin	Status
AZA1	842.5	362	*A. spinosum*	phycotoxin
37-*epi*-AZA1	842.5	362	*A. spinosum*	artefact
AZA2	856.5	362	*A. spinosum*	phycotoxin
AZA3	828.5	362	shellfish	metabolite
AZA4	844.5	362	shellfish	metabolite
AZA5	844.5	362	shellfish	metabolite
AZA6	842.5	362	shellfish	metabolite
AZA7	858.5	362	shellfish	metabolite
AZA8	858.5	362	shellfish	metabolite
AZA9	858.5	362	shellfish	metabolite
AZA10	858.5	362	shellfish	metabolite
AZA11	872.5	362	shellfish	metabolite
AZA12	872.5	362	shellfish	metabolite
AZA13	860.5	362	shellfish	metabolite
AZA14	874.5	362	shellfish	metabolite
AZA15	874.5	362	shellfish	metabolite
AZA16	888.5	362	shellfish	metabolite
AZA17	872.5	362	shellfish	metabolite
AZA19	886.5	362	shellfish	metabolite
AZA21	888.5	362	shellfish	metabolite
AZA23	902.5	362	shellfish	metabolite
AZA26	824.5	362	shellfish	metabolite
AZA28	838.5	362	shellfish	metabolite
AZA29	842.5	362	shellfish	artefact
AZA30	856.5	362	*A. spinosum*	artefact
AZA32	870.5	362	*A. spinosum*	artefact

	Protonated mass $[M+H]^+$	Type	Origin	Status
AZA33	716.5	362	*A. spinosum*	phycotoxin
AZA34	816.5	362	*A. spinosum*	phycotoxin
AZA35	830.5	362	*A. spinosum*	phycotoxin
AZA36	846.5	348	*A. poporum*	phycotoxin
AZA37	858.5	348	*A. poporum*	phycotoxin
AZA38	830.5	348	*A. languida*	phycotoxin
AZA39	816.5	348	*A. languida*	phycotoxin
AZA40	842.5	348	*A. poporum*	phycotoxin
AZA41	854.5	360	*A. poporum*	phycotoxin

Notes: AZAs highlighted in grey have been isolated and fully characterized (LC-MS and NMR).

Recently, two strains of *Azadinium poporum*, a species previously reported to be non-toxigenic (Tillmann *et al.*, 2011; Potvin *et al.*, 2012), were proven to be the producers of two previously unknown AZAs (Krock *et al.*, 2012). AZA37 from a North Sea isolate of *A. poporum* (Tillmann *et al.*, 2011) with a molecular mass of 845 Da (ca. 10 fg cell^{-1}) was determined as 39-desmethyl–7,8-dihydro-3-hydroxy-AZA1 by nuclear magnetic resonance (NMR) spectroscopy (Table 4) (Krock *et al.*, 2015). The other strain of *A. poporum*, from Shiwha Bay, Republic of Korea (Potvin *et al.*, 2012) produced AZA36 with a molecular mass of 857 Da (ca. 2 fg cell-1), which was determined as 39-desmethyl-3-hydroxy-AZA2 (Table 4). Both *A. poporum*-derived AZAs have a 3-hydroxy substitution and a 39-desmethyl moiety in common.

Whereas the 3-hydroxy function is also found in shellfish metabolites of AZA1 and –2, e.g. AZA4 and –9 (Kilcoyne *et al.*, 2015), the 39-desmethyl moiety is unique to a new class of dinoflagellate AZAs. This new class of 39-desmethyl AZAs is easily recognized in tandem mass spectrometry by a characteristic m/z 348 fragment, whereas all other AZAs have a m/z 362 fragment. Two additional AZAs with m/z 348 fragments and molecular masses of 815 Da and 829 Da (ca. 11 and 6 fg cell-1 respectively) 18 AZAs were also identified in a strain of *Amphidoma languida* (Tillmann *et al.*, 2012).

AZAs were also detected in isolates of *A. poporum* from Chinese coastal waters (Gu *et al.*, 2013). Whereas one strain did not produce any AZAs, three other strains produced exclusively AZA2 at cell quotas ranging from 1.8 to 23 fg cell-1. In addition, new AZAs with the m/z 348 fragment were detected in a fifth strain, which also produced AZA36 (1.4 fg cell-1). In contrast to the Korean strains, this Chinese strain produced AZAs (with the m/z 348 fragment) with molecular masses of 919 and 927 Da (ca. 0.02 and 0.14 fg cell-1, respectively). A sixth strain of *A. poporum* from China produced an AZA with a molecular mass of 871 Da

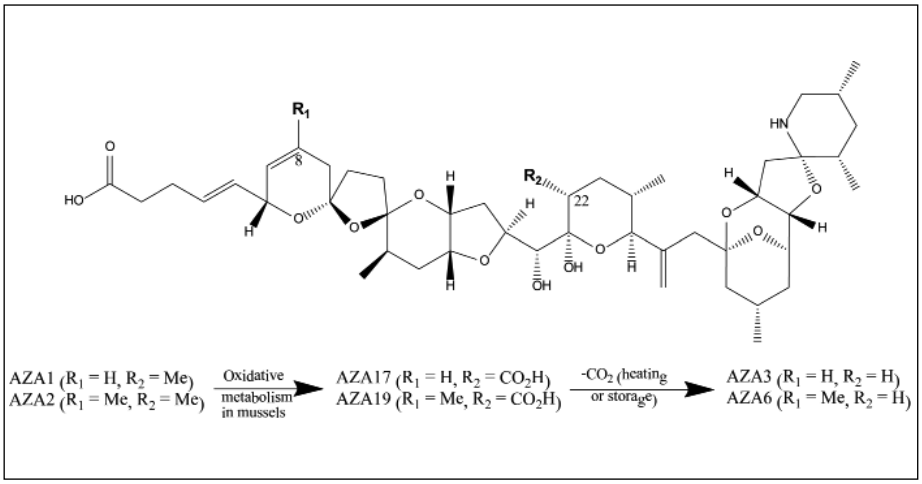
(0.9 to 1.9 fg cell-1) that was tentatively identified as AZA11 by comparison of retention times and Collusion induced dissociation (CID) spectra (Gu *et al.*, 2013). Whereas 3-hydroxylated AZAs, such as AZA36 and AZA37, seem to be biosynthesized by several strains of *A. poporum*, AZA11 may be the first case of an AZA being independently produced by dinoflagellate biosynthesis as well as through shellfish metabolic activity.

It is likely that several more analogues of this toxin group will be reported soon, as there are not yet many strains or species isolated from most regions. For instance, *Azadinium dexteroporum*, a new species recently isolated from the Mediterranean Sea (Italy) has also been reported to produce some novel analogues which have not yet been fully characterized (Percopo *et al.*, 2013).

As the exact nomenclature of AZAs, according to the rules of the International Union of Pure and Applied Chemistry (IUPAC), are long and complicated, AZAs have been named by numbering in the chronological order of their detection or postulation. All AZAs up to AZA23 were originally identified in or postulated from shellfish, however, AZA1 and –2 are of dinoflagellate origin, whereas AZA3 to AZA23 have not been detected in planktonic samples and have been shown to be shellfish metabolites, with the exception of AZA11 (Rehmann, Hess and Quilliam, 2008; Gu *et al.*, 2013).

Shellfish are known to transform AZAs by two different types of reactions: (1) hydroxylation at C3 and C23, and (2) carboxylation and subsequent decarboxylation (James *et al.*, 2003a; Rehmann, Hess and Quilliam, 2008; McCarron *et al.*, 2009; Kittler *et al.*, 2010; O'Driscoll *et al.*, 2011; Jauffrais *et al.*, 2012b). Recent investigations with feeding experiments (Salas *et al.*, 2011; Jauffrais *et al.*, 2012a) revealed that blue mussels (*M. edulis*) metabolize AZAs quickly (Figure 5). AZA17 and –19 were the most abundant metabolites of AZA1 and –2, respectively, suggesting that carboxylation of the methyl group at C22 is a preferred metabolic pathway (Jauffrais *et al.*, 2012b). Hydroxylation and decarboxylation seem to be secondary degradation routes (McCarron *et al.*, 2009).

FIGURE 5. Conversion of algal metabolites into shellfish metabolites



Note: Currently only AZA1, AZA2 and AZA3 are regulated in some legislative frameworks. AZA17, AZA19 and AZA6 are also relevant for assessing public safety of a shellfish sample.

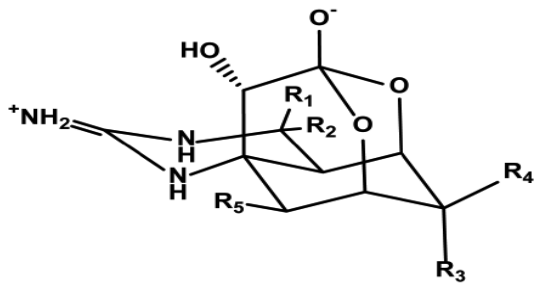
Hydroxylations of AZAs by shellfish metabolism occur at C3 on the carboxylic acid side chain to form 3-hydroxy-AZAs (e.g. AZA7, –11) as well as at C23 at the E-ring of the molecule, resulting in 23-hydroxy-AZAs (AZA8, –12) (Table 4). Furthermore, the methyl group at C22 can be oxidized to 22-carboxy-AZAs (AZA17, –19), which are subsequently decarboxylated to form the 22-desmethyl-AZAs (AZA3, –6) (McCarron *et al.*, 2009; O'Driscoll *et al.*, 2011). AZA 22-decarboxylation may be a shellfish metabolic activity, but this reaction occurs rapidly during heating of shellfish meat and slowly in extracts stored at ambient temperature (McCarron *et al.*, 2009). In addition, combinations of these processes are possible, to produce many of the remaining AZAs (AZA4, –5, –9, –10, –13, –14, –15, –16, –21 and –23). Some of the other AZAs originally detected without structural elucidation (AZA25, –29, –30 and –32) 23 AZAs were later identified as extraction artefacts (AZA29, –30 and –32) (Jauffrais *et al.*, 2012a). In contrast to shellfish metabolism, phase I metabolites of AZA1 using rat liver microsomes (S9-mix) included an oxidation of the F-ring of the molecule, which is not observed in shellfish metabolites. Glucuronides were found as the only phase II metabolites of AZA1, and via precursor ion experiments it could be proven that glucuronic acid is bound to AZA1 at C1 via an ester linkage (Kittler *et al.*, 2010). One study reported on binding of AZAs to proteins (Nzoughet *et al.*, 2008) found that AZAs in mussel hepatopancreas bind to as yet unidentified proteins with molecular masses of 21.8 and 45.3 kDa.

2.1.5 Tetrodotoxin and analogues

Tetrodotoxins (TTXs) are traditionally known as the main toxins found in

puffer fish, and pure TTX was first isolated as a crystalline prism from puffer fish by Yokoo (1950). TTX is also frequently found in other marine animals, including some species of gobies, octopuses, sea stars, crabs and gastropods (Hashimoto, Noguchi and Watabe, 1990; McNabb *et al.*, 2010). Recent reports also confirmed tetrodotoxins in European shellfish in both the English Channel and the Mediterranean Sea (Turner *et al.*, 2015a; Vlamis *et al.*, 2015). TTXs are also produced by bacteria that reside within blue-ringed octopuses (Hwang *et al.*, 1989b). The most common bacteria associated with TTX production are *Vibrio* spp., with *Vibrio alginolyticus* being the most common species (Hwang *et al.*, 1989a; McNabb *et al.*, 2010). although the source of these toxins in marine animals has not yet been conclusively identified. TTX is of low molecular weight and positively charged in neutral solutions. The toxin is odourless and heat stable but unstable at pH levels above 8.5 and below 3.0. A recent review gives an overview of the chemistry and structures (Figure 6) of the 30 known analogues of TTX (Bane *et al.*, 2014).

FIGURE 6. Most common TXX analogues in aquatic organisms



	R_1	R_2	R_3	R_4	R_5
TTX	H	OH	OH	CH_2OH	OH
4-epi-TTX	OH	H	OH	CH_2OH	OH
6-epi-TTX	H	OH	CH_2OH	OH	OH
11-deoxy-TTX	H	OH	OH	CH_3	OH
6,11-dideoxy-TTX	H	OH	H	CH_3	OH
8,11-dideoxy-TTX	H	OH	OH	CH_3	H
11-oxo-TTX	H	OH	OH	$CH(OH)_2$	OH
11-nor-TTX-6,6-diol	H	OH	OH	OH	OH
11-nor-TTX-6(R)-ol	H	OH	H	OH	OH
11-nor-TTX-6(S)-ol	H	OH	OH	H	OH
TTX-8-O-hemisuccinate	H	OH	OH	CH_2OH	$OOC(CH_2)2COO^-$
TTX-11-carboxylic acid	H	OH	OH	COO^-	OH

Source: Structure adapted from Nishikawa and Isobe ,2013: analogues from Bane *et al.*, 2014.

Profiles in the shellfish included TTX, 4-epi-TTX, 4,9-anhydro-TTX and 5,6,11-tri-deoxy-TTX.

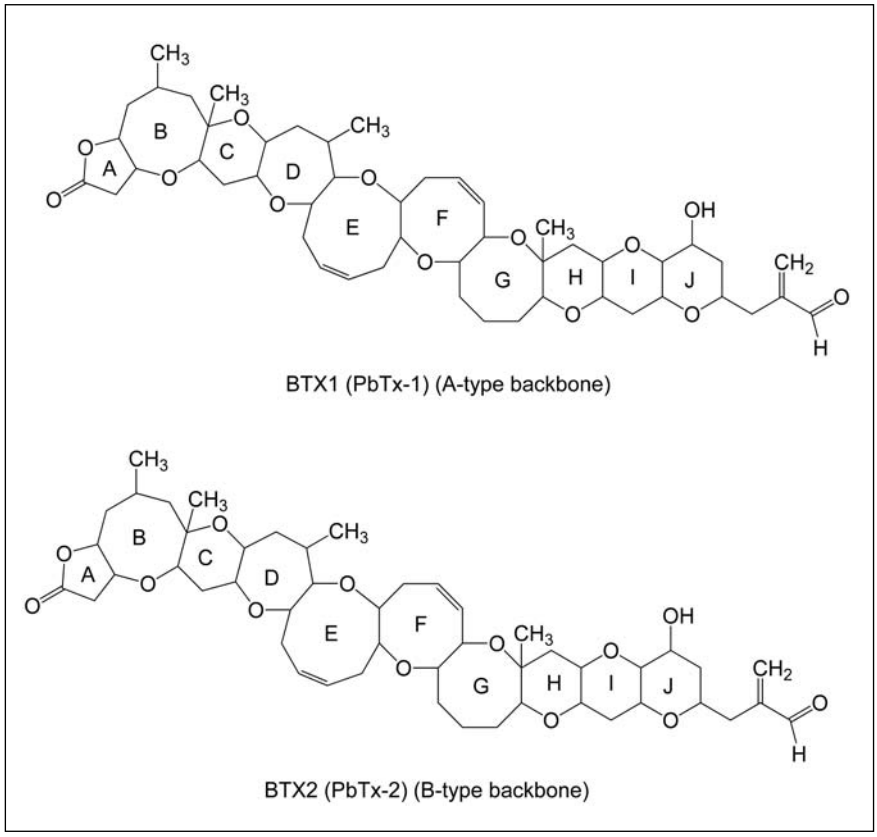
2.1.6 Brevetoxin and analogues

A group of lipophilic cyclic polyether natural products comprise the brevetoxins (BTXs). These toxins are produced by certain species of dinoflagellates belonging to the genus *Karenia*, notably *K. brevis* (Lin *et al.,* 1981). Recent evidence indicates that BTX-like analogues are also produced by some raphidophytes, including C*hattonella marina* (Haque and Onoue, 2002; Band-Schmidt *et al.,* 2012), *C. antiqua* (Haque and Onoue, 2002) and *Fibrocapsa japonica* (de Boer *et al.,* 2009; Band-Schmidt *et al.,* 2012). Brevetoxins, along with their metabolites, are responsible for neurotoxic shellfish poisoning (NSP) through oral exposure when contaminated shellfish are consumed (Poli *et al.,* 2000; Watkins *et al.,* 2008).

Brevetoxins are tasteless, odourless, and heat-stable. These cyclic polyethers (Figure 7) as isolated from *K. brevis*, fall into two structural backbone types: A-type and B-type (Lin *et al.* 1981; Shimizu *et al.* 1986). PBX1 is an A-type analogue, which has ten trans-fused ether rings; PBX2, a B-type analogue, has eleven trans-fused ether rings. PBX7 and PBX10 are also A-types; PBX3, PBX5, PBX6, PBX8, and PBX9 are B-type. PBX1 is the most potent of the BTXs, whereas PBX2 is the most abundant analogue produced by *K. brevis*. While PBX1 and PBX2 have the same side chains, variations in side chains (tail regions) account for additional BTX analogues. Baden *et al.* (2005) hypothesized that PxTx1 and PBX2 are the parent analogues from which the others are derived. Additional analogues are products of reduction, oxidation and hydrolysis.

Following an NSP outbreak in New Zealand in 1992–1993, it was found that BTXs are extensively metabolized in shellfish (Ishida *et al.,* 1995). Subsequent investigations in the United States of America found the same (Dickey *et al.,* 1999; Poli *et al.,* 2000). Oxidation, reduction, hydrolysis and conjugation are the pathways that lead to BTX metabolites. Metabolism largely occurs via modifications of the α,β–unsaturated aldehyde side chains of PBX1 and PBX2 (Plakas and Dickey, 2010). These metabolites vary in polarity and hydrophobicity, contributing to species-specific differences in assimilation in, and elimination from, shellfish tissues.

FIGURE 7. Chemical structures of brevetoxins PBX1 (A-type) and PBX2 (B-type)



Source: Plakas and Dickey, 2010.

2.2 Detection methods

Many different detection methods exist for marine biotoxins (Botana *et al.*, 2014a). However, this review focuses on the methods that have undergone collaborative validation trials, as these are the most commonly accepted methods across trade blocks (Table 5).

TABLE 5. Overview of detection methods for marine biotoxins, validated for bivalve mollusc matrices through collaborative trials

Toxin group	Method-type	Specificity [y/n](1)	Validation reference	EU	US	JP
STX	MBA	n	AOAC-OMA 959.08	√	√	√[5]
	HPLC-FLD, precox	y	AOAC-OMA 2005.06	√		
	HPLC-FLD, postcox	y	AOAC-OMA 2011.02		√	
	RBA	n	AOAC-OMA 2011.27		√[4]	
OA	LC-MS/MS	y	EURL (2015)	√	√[3]	√[3]
	LC-MS/MS	y	These *et al.*, 2011			
	LC-MS/MS	y	van den Top *et al.*, 2011			
	PP2a	n	Smienk *et al.*, 2013	√		
DA	ELISA	n	AOAC-OMA 2006.02	√		
	HPLC-UV	y	pre-EN14176[7]	√	√	√
AZA	LC-MS/MS	y	EURL (2015)	√		
	LC-MS/MS	y	These *et al.*, 2011			
	LC-MS/MS	y	van den Top *et al.*, 2011			
PBX	MBA					
TTX	MBA[2]	n	AOAC-OMA 959.08			√[6]
	RBA[2]	n	AOAC-OMA 2011.27			

Notes: [1] y = the method gives analogue-specific results; n = the method does not give analogue-specific results.

[2] the method has not been validated for this analogue group but should perform as for STXs as the mode of action is exactly the same.

[3] modified from EURL-validated method, with only OA group quantified, separately validated.

[4] approved as mouse replacement for mussels, and also screening clams and scallops. Oyster data under review.

[5] adapted protocol using dc-STX as calibrant.

[6] an adapted protocol is used for confirmation of food poisoning events.

[7] method has undergone interlab trial and is undergoing normalization.

√ denotes acceptance of the method in major trade blocks (EU = European Union); US = United States of America; JP = Japan.

2.2.1 Saxitoxins

As STXs are the most lethal group of algal toxins, mouse bioassay was developed relatively early (Sommer and Meyer, 1937a). The PSP mouse bioassay (MBA) was formally validated in an interlaboratory trial and led to a standardized AOAC method (OMA 959.08) (McFarren, 1959). Despite some ethical and technical issues (Hess *et al.*, 2006), the test is rapid, quantitative and has relatively few interferences. The test is thus still used in many countries (DeGrasse and Martinez-Diaz, 2012; Hess, 2012; Suzuki and Watanabe, 2012)(Table 5). The MBA does not provide analogue-specific data but gives a result in equivalents of STX (in European Union and United States of America protocols) or in equivalents of dc-STX (in Japan).

The receptor-binding assay (RBA) using tritiated (i.e. radio-labelled) STX is also an assay that gives a sum value for STX-equivalents (van Dolah *et al.*, 1995; Doucette *et al.*, 1997). After successful validation in a single laboratory (Van Dolah *et al.*, 2009), the test has also undergone formal interlaboratory validation (Van Dolah *et al.*, 2012) and has reached a good level of acceptance in the United States of America (Table 5), and some other countries. In the European Union, a restriction for the use of radiolabelled material typically prevents use of the assay since other methods that do not require the use of radioactive material are available.

The above two methods (MBA and RBA) are based on the mode of action of STXs and can thus also be used for TTXs as the latter share their mode of action with STXs. However, it should be noted that the assay has not been formally validated for this compound group.

Analogue-specific methods for STXs are based on their fluorescence after oxidation and were developed in the 1990s (Lawrence *et al.*, 1991b; Oshima, 1995). Subsequently, these methods have been standardized and have undergone single laboratory and collaborative trials for formal validation (Lawrence, Niedzwiadek and Menard, 2004; Van de Riet *et al.*, 2009, 2011; Ben-Gigirey, Rodriguez-Velasco and Gago-Martinez, 2012a; Ben-Gigirey *et al.*, 2012b). The protocols differ slightly in that one approach uses an oxidation step prior to chromatographic separation of analogues while the other approach separates compounds first and oxidises them after separation on a HPLC column. However, both methods have in common that they are analogue-specific, are not suitable for the detection of TTXs and require several injections per sample in the case of complex natural toxin profiles.

2.2.2 Okadaic acid and Azaspiracid and their analogues

OA and AZA and their analogues may all be detected by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Three major validation exercises with collaborative trials have recently been undertaken to achieve

widespread acceptance of this methodology (These *et al.*, 2011; van den Top *et al.*, 2011; Brana-Magdalena *et al.*, 2014).

This analogue-specific methodology has now replaced the lipophilic MBA in Europe as a routine method for testing for lipophilic toxins (EU, 2011). MBAs according to different protocols developed by Yasumoto, Oshima and Yamaguchi (1978c) or Yasumoto *et al.* (1984) are still being used in other parts of the world for practical reasons. However, the MBA assay has never been formally validated for lipophilic marine biotoxins.

For the OA group of toxins only, there is also an enzyme-based assay available, based on the inhibition of phosphoprotein-phosphatase 2a (PP2a). This assay has also been recently validated and is accepted in Europe for the OA-group toxins (Smienk *et al.*, 2013). While the LC-MS/MS methodology provides analogue-specific information, i.e. concentrations per analogue, the PP2a-assay provides a sum of OA-equivalent material present in a sample.

As mentioned earlier, the chemical resistance of OA and its two major analogues DTX1 and DTX2 towards strong bases allows for conversion of the various ester derivatives to these three analogues for ease of analysis. This methodological approach appears justified by the fact that the esters could also be converted in the human digestive system, and the esters appear to have similar toxicity as the parent compounds. The base hydrolysis step should therefore be added for any of the methods, LC-MS/MS or PP2a assay.

2.2.3 Domoic acid and analogues

While initial efforts in method validation focused on using the extraction protocol for the PSP MBA (Lawrence *et al.*, 1991a), this method has been superseded by the knowledge that DA is not very stable in strongly acidic conditions. Hence, methods using an extraction protocol based on aqueous methanol with HPLC-UV detection (Quilliam, Xie and Hardstaff, 1995a) are now generally preferred. Such methods have undergone collaborative trials for validation and are currently undergoing standardization in the frame of the European Normalisation Committee (CEN; EN14176).

As the DA group is relatively simple, with only one major analogue and an epimer (DA and epi-DA), an ELISA has also been developed and a collaborative trial (Kleivdal *et al.*, 2007) permitted interlaboratory validation and formal standardization as AOAC method OMA 2006.02. This ELISA has strong approval by stakeholders in the scallop processing industry in Scotland, and the assay is also approved for use in official control in the European Union.

2.2.4 Brevetoxin and analogues

The mouse bioassay (MBA) has been used conventionally for detecting BTXs (APHA, 1970). It is the only method approved in the United States of America under the National Shellfish Sanitation Program (NSSP) for use in making regulatory decisions regarding the harvest of bivalves due to BTXs (FDA, 2013). The current CODEX Standard for BTXs also involves the use of MBA. The MBA involves the intraperitoneal administration of the sample extract into mice. The time of death of the animals is correlated with the BTX content of the sample. Results are reported in mouse units (MU) per 100 g shellfish tissue, a MU being defined as the amount of crude extract that would kill 50% of the test animals in 930 minutes. The MBA has been criticized not only for its use of animals, but also because of assay variability, lack of specificity, and the limitations of the MBA extraction to account for all potential BTX analogues and metabolites (e.g. Dickey *et al.* 1999; Plakas and Dickey., 2010). One major advantage of the MBA is that it has demonstrated its efficacy in public health protection. The action level for NSP toxins established by the United States Food and Drug Administration (US FDA) is 20 MU per 100 g tissue or 0.8 ppm PBX2 equivalents (FDA. 2011). When toxicity exceeds this level as determined by the MBA, growing areas are closed to the harvest of bivalve molluscs.

While the MBA and a radioimmunoassay were used to investigate the NSP outbreak in New Zealand in 1993 (Temple, 1995), an internally validated liquid chromatography mass spectrometry (LC-MS) method is now used for monitoring BTXs in New Zealand (McNabb *et al.* 2012). An advantage of the LC-MS method is that it allows for the identification of individual BTX analogues and metabolites. However, the limitation regarding an extraction procedure that recovers all relevant forms also applies. Another challenge is that standards for all of the BTX analogues and metabolites are not readily available. Further, if applying the action level of 0.8 ppm PBX2 equivalents established in the United States of America, a level of 0.8 mg/kg is used, yet TEFs are needed to present the results in PBX2 equivalents.

Additional methods for the determination of BTXs have been developed and are in various stages of validation and use. These include a neuroblastoma assay (Manger *et al.,* 1993), enzyme linked immunoassays (ELISAs) (Naar *et al.,* 2002), and a receptor binding assay (Trainer and Poli, 2000). None of these methods detects individual BTX analogues or metabolites, but are indicative of overall toxicity.

3

Toxicity Equivalency Factors (TEFs) for specific biotoxin groups

CONSIDERATIONS FOR THE ESTABLISHMENT OF TEFS

The following approach was taken by the Expert Group when considering the establishment of TEFs:

- Data consideration in order of importance/relevance
  1. Data from human cases (outbreaks)
  2. oral LD_{50} /toxicity data in animals
  3. ip LD_{50} /toxicity data in animals
  4. Mouse bioassay
  5. *In vitro* data
- when TEFs are below 0.1, an increment of 0.05 is used .
- TEF should not be established for combinations of different analogues because they will be dealt with by individual TEFs.

3.1 Saxitoxin and analogues

In 1937, Sommer and Mayer reported a quantitative assay for STX, based on the dose-death time relationship in mice dosed intraperitoneally with this substance. They noted that the dose-death time curve was relatively linear between 5 and 7 minutes, and the assay, which is now an approved AOAC method (Hungerford, 1995), involves determining the amount of STX required to cause death in this time interval. Although initially set up for STX, it is now used for estimating the toxicity of mixtures of STX analogues expressed as the specific activity (Mouse

Units per micromole). This assumes that the dose-death time curves for all the analogues are identical, (Munday *et al.,* 2013), calling into question the validity of this assay for comparing analogues.

The MBA has been widely used for comparing the toxicity of STX analogues. Again, this depends on the assumption that the dose-death time relationship is the same for all analogues. It was, however, used when only small amounts of material were available, and this assay has been widely used for estimating TEFs (Oshima, 1995).

The relative potencies determined by the MBA are shown in Table 6.

TABLE 6. Relative potency of STX derivatives as indicated by the MBA

Compound	Relative specific activity in the MBA	Relative LD_{50} by i.p. injection[1]
Saxitoxin	1.0	1.00
NeoSTX	0.50, 0.75[2], 0.90, 0.90, 1.0, 1.16[1], 1.2	3.12
GTX-1	0.80, 1.0	
GTX-4	0.30, 0.70	
GTX-1&4	0.70, 1.02[1], 0.65[2]	1.90
GTX-2	0.40, 0.40	
GTX-3	0.60, 1.1	
GTX-2&3	0.60, 0.60[1], 0.52[2]	0.757
GTX-5	0.10, 0.10, 0.20	0.222
GTX-6	0.10	0.122
C-1	0.02, 0.00	
C-2	0.10, 0.17	
C-3	0.0, 0.01	
C-4	0.0, 0.10	
dcSTX	0.40,0.48[3], 0.50, 0.50, 0.60, 0.64[1], 1.0, 1.02[2]	0.785
dcNeoSTX	0.40, 0.020[4]	0.058
dc-GTX-1	0.5	
dc-GTX-2	0.20, 0.20, 0.30	
dc-GTX-3	0.20, 0.40, 0.50	
dc-GTX-4	0.50	
dc-GTX-2&3	0.20, 0.19[2]	0.695
11α-Hydroxy-STX	0.60	
11β-hydroxy-STX	0.70	

Sources: [1] Munday *et al.*, 2013; [2] Vale *et al.*, 2008b; [3] Suzuki and Machii, 2014; [4] Munday, MS in preparation.
All other data are from Table 13 of the 2009 ESFA report on saxitoxin group toxins (EFSA, 2009b).

As seen in Table 6, there are wide variations in estimates of the relative potencies. While such differences most likely reflect the use of impure compounds, this cannot

be the whole answer, since, for example, the estimates for NeoSTX reported in references (Munday *et al.,* 2013) and (Vale *et al.*, 2008b) are significantly different, even though both were conducted using certified materials. There are also errors in the relative potencies given in the EFSA report (EFSA, 2009b). The figure of 0.40 given for dcNeoSTX was referenced to Sullivan *et al.,* (1983, 1985). However, these authors did not conduct a MBA. They assumed that the specific activity of dcNeoSTX was the same as that of dcSTX. The actual figure for the specific activity of dcNeoSTX, measured on a pure sample of this substance, is 0.020. The figures given for the isomeric 11-hydroxysaxitoxins are also incorrect. The reference given for these data in Table 13 of the EFSA report is Schantz (1986). The material tested by Schantz was not, however, the isomers of 11-hydroxySTX but the isomers of 11-hydroxySTX sulphate, better known as GTX–2 and GTX–3.

It should also be noted that there are limitations to interpreting the potential impact to human health from the specific activities of the STX analogues determined in the mouse bioassay in relation to the measured endpoint (fatality). As shown in Table 6, while there is a correlation between the relative specific activity and relative toxicity by intraperitoneal injection with some saxitoxin derivatives, with others, particularly NeoSTX, GTX–1&4 and dcGTX–2&3, there is no correlation. This is attributable to differences in the dose-death time relationship (Munday *et al.*, 2013).

Because the toxic effect of the analogues was originally obtained with an i.p. injection, Munday *et al.*, (2013) re-evaluated, with certified materials, the relative potencies for some of the toxins using oral administration (gavage or feeding) of the toxins. The results provided similar results to the i.p. administration, although the relative potency was different for dcSTX (0.37 by feeding, 0.46 by gavage, versus 0.64 by i.p. route), and similar to the value obtained in an i.p. study of STX versus dcSTX specifically performed to validate dcSTX for bioassay, with a relative potency for dcSTX of 0.48 (Suzuki and Machii, 2014). Another toxin that provided a rather different value was NeoSTX, with a relative potency of 1.7 by gavage, 2.54 by feeding and 1.16 by i.p. injection (Munday *et al.*, 2013).

The shortage of materials also led to the development of *in vitro* methods for comparing the effects of STX with those of its congeners. Although STX has been reported to potentially target many receptors, specially sodium, potassium and calcium channels (Su *et al.*, 2004; Llewellyn, 2006a; Zakon, 2012; Cusick and Sayler, 2013), there is strong evidence that STX and derivatives exert their toxic effects in animals by binding to the voltage-gated sodium channel (Na_v) (Payandeh *et al.*, 2011). This channel contains one alpha subunit and one to three small beta subunits. There are 9 alpha subunits of the Na_v channel (Na_v 1 to 9) (Wingerd, Vetter and Lewis, 2012), and originally they were divided into tetrodotoxin

(TTX)-sensitive (Na_v 1, 2, 3 and 7) and TTX insensitive. The alpha subunits contain 4 homologous domains, each with 6 hydrophobic transmembrane segments. There are 6 binding sites identified that are the target for many toxins, including several phycotoxin groups. Site 1 is the receptor for TTX and STXs; site 5 is the receptor for ciguatoxins and BTXs (Hartshorne and Catterall, 1981). The major molecular mechanism of toxicity of both TTX and STX is to block the channel pore with a ratio 1:1, hence inhibiting the conductance of the channel and the transmission of electrical action potentials generated by the influx of sodium ions into the cell. This mechanism is responsible for muscle paralysis, potentially leading to paralysis of the diaphragm and death.

With the improved availability of pure and certified PSP materials, a more defined list of TEFs was proposed by EFSA, based on the study of the effect of certified toxins on sodium channels of neuronal cultures by means of fluorescent dyes sensitive to membrane potential (Vale *et al.,* 2008b; EFSA, 2009b). Because this study did not cover the same toxins studied by Oshima (1995), the EFSA list provided a combined set of results that were basically similar, with the exception of dcSTX (reported now as more toxic) and GTX1–4 as Oshima provided a higher value for GTX1, and all the values reported by Oshima were with pure GTX1 to 4. These TEF values were later re-evaluated by means of a more precise electrophysiological patch clamp recording method in primary cultured neurons, and the ratio results obtained were equivalent, but with a slightly lower toxicity for GTX1–4 (Perez *et al.*, 2011) and a potency of NeoSTX of 1.02, similar to STX (Perez *et al.,* 2011).

Since the mechanism of toxicity of these substances was known to involve voltage-gated sodium channels, attention was focused on these channels, with ever increasing levels of sophistication. Since each Na_v channel subtype has different affinities for STX and derivatives, an automated electrophysiology platform was used to determine the relative potencies of the analogues in human Na_v subtypes 1.1 to 1.7, transfected in HEK–293 cells (Alonso *et al.*, 2016). This information indicates direct binding in transfected human receptors. The results show that the subtype that best matches the results from previous works is obtained with Na_v subtypes 1.2 and specially 1.6, although the toxicity of the epimer mixture GTX1–4 is higher than previously reported (relative potency of 1.4 for Na_v 1.6).

Again, there are wide variations among the different assays. The results with various methodologies are summarized in Table 7. Comparing the relative potencies based on *in vitro* studies on sodium channels with those based on median lethal doses by consumption in mice, it is interesting to note that assays involving the frog sciatic nerve (method 3) and the rat muscleplasma membrane (method 5) indicate a relatively high activity of NeoSTX, in accord with the acute toxicity data in mice.

TABLE 7. Relative toxicities of STX and derivatives in mice (MBA) and relative reactivities toward sodium channels *in vitro*

Compound	[9]	Relative activity toward sodium channels *in vitro* by type of assay method[1]								
		[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8, Na_v1,6]	[8, Na_v1,2]
STX	1.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
neoSTX	2.54/1.7	–	0.69	4.5	1.0	3.6/3.7	0.82	1.02	1.2	2.0
GTX 1		–	–	–	–	0.28	–	–		
GTX 1&4	0.936/0.739	–	0.98	–	–	–	0.53	0.50	1.4	0.54
GTX 2		0.2		0.22		0.15/0.16				
GTX 2&3	0.572/0.533	–	0.32	–	–	–	0.38	0.28	0.15	0.4
GTX 3		0.42	–	1.4	–	0.96				
GTX 4		–	–	–	–					
GTX 5	0.064/0.05	–	0.031/0.039	–	–	0.024	0.09	0.09	0.11	0.01
GTX 6	< 0.017/0.038	–	–	–	–	–				
dcSTX	0.368/0.457	–	0.097/0.29	–	0.2	0.44	0.84	1.00	0.96	0.25
dcNeoSTX	0.224/0.216	–	–	–	0.004	–	0.48	0.44	0.25	0.1
dcGTX2,3	0.108/0.167								0.02	0.05
C1	–	–	–	–	–	0.0017/0.0028				
C2		–	–	–	–	0.029				
C1,2	0.043/0.034								0.09	0.01
C3	–	–	–	–	–	0.002				

Key to assay methods: [1] Relative blockade of sodium channels in the squid giant axon (Kao *et al.*, 1985b).
[2] Relative binding to sodium receptors of the rat cerebral cortex (Usup *et al.*, 2004; Llewellyn, 2006b).
[3] Relative blockade of impulses in frog sciatic nerve (Strichartz, 1984).
[4] Relative blockade of sodium current in frog skeletal muscle fibre (Kao *et al.*, 1982; Yang *et al.*, 1992b).
[5] Relative blockade of sodium channels from rat muscle plasma membrane (Moczydlowski *et al.*, 1984; Guo *et al.*, 1987).
[6] Blockade of veratridine-induced changes in membrane potential in cultured neurons (Vale *et al.*, 2008b).
[7] Sodium currents voltage-dependent inhibition in primary cultures of cerebellar neurons (Perez *et al.*, 2011).
[8] High-throughput electrophysiology system, in cells stably transfected with specific subunits of sodium channels (Alonso *et al.*, 2016).
[9] Relative toxicity by voluntary feeding/gavage (Munday *et al.*, 2013).

3.1.1 Toxicity of epimers and influence of hydrolysis

Some of the toxins are epimers due to the possible double position on the radical 11-hydroxysulfate. Therefore the pairs GTX1,4, GTX2,3, C3,4 and C1,2 are epimers. This process of 11-hydroxysulfate epimerization is spontaneous and the thermodynamic equilibrium is achieved at a higher pH above acidic stability. The ratios, depending on the conditions (temperature, ionic strength, pH), may vary between 2 and 4.5 (Hall *et al.*, 1990; Reeves *et al.*, 2003). For this reason, it is difficult to implement a TEF for the mixture of epimers because the ratio may be different, depending on the source of the standard. The ratio of GTX2,3 and GTX1,4 in Munday *et al.* (2013) was 2.2 and 4.06, respectively, while in Vale *et al.* (2008b) it was 3 for both mixtures. The supply of pure epimers is difficult to obtain because of the spontaneous process of epimerization, therefore it is useful to have the TEF of the mixture, even with a range of results, as there are no two mixtures with the exact same proportion, and the toxicity of both epimers, especially GTX1 and GTX4, is very different, GTX1 being more potent. Although the TEF is to be applied to an analytical result, this possible conversion of epimers may modify the final calculated toxicity of a sample, depending on the storage conditions.

Another important source of possible toxicity change is the modification of the toxicity of a sample after hydrolysis by boiling at acidic pH, which may cause a 10-fold increase in the toxicity of a sample by converting C toxins into GTXs (Vale *et al.*, 2008b). Even though the ratio value of the toxicity of each compound is the same, the final toxicity of a sample may change notably. For this reason, **the application of a TEF has to be done with a thorough understanding of the chemical profile of the sample, after all possible conversions have taken place.**

3.1.2 Conclusion

Although the absolute LD_{50} values of STX and its analogues by gavage and by voluntary consumption were different, the relative toxicities by these routes, as indicated by the TEFs, were similar. The EFSA TEFs for GTX–1&4, GTX–2&3 and C1,2 are consistent with those determined by oral administration. In contrast, and based on oral toxicity to mice, the TEF for NeoSTX was significantly higher than that proposed by EFSA, while the EFSA TEFs for GTX–5, GTX–6, dcSTX, dcNeoSTX were lower.

There are two toxins that require further clarification: dcSTX was recently reported by some authors to be less toxic than STX, with a TEF of 0.8 (Vale *et al.*, 2008b), 0.64 (Munday *et al.*, 2013), 0.478 (Suzuki and Machii. 2014) and 0.37 (Suzuki and Machii, 2014), although the TEF adopted by EFSA was 1. With regard to dcSTX, the application of EFSA TEFs in samples with high levels of dcSTX, such as surf clams, results in a significant improvement in the agreement between HPLC and

the mouse assay (Turner *et al.*, 2011). Another toxin that requires clarification is NeoSTX, as the range of toxicity values is very large, from 1 (EFSA 2009b, Alonso *et al.*, 2016) to 2.54 (Munday *et al.*, 2013). If TEFs were to be based on oral toxicity, the EFSA TEFs for GTX–5, GTX–6, dcSTX, dcNeoSTX should be revised downwards, while the TEF for NeoSTX should be increased. It is interesting to note that there is a better match between the results obtained with Na_v subtype 1.2 (Alonso *et al.*, 2016) and the results obtained with oral administration to mice (Munday *et al.*, 2013). The TEFs recommended by the Expert Group are indicated in Table 8.

Table 8. TEFs recommended by the Expert Group

Compound	Oshima Relative Toxicity values (MU/ µmole)	Mouse LD_{50} (i.p.)	TEF based on LD_{50} by gavage	TEF based on LD_{50} by voluntary consumption	EFSA proposed TEF	Recommended TEF	Rationale
Saxitoxin	1	1.00	1.00	1.00	1.0	1.0	
NeoSTX	0.92	3.12	1.70	2.54	1.0	2.0	Both oral studies support higher toxicity than STX. A value of 2.0 is recommended and supported by some Na channel *in vitro* results (range of RP 0.7–3.7).
GTX1	0.99				1.0	1.0	No new data[1]
GTX2	0.36				0.4	0.4	No new data
GTX3	0.64				0.6	0.6	No new data
GTX4	0.73				0.7	0.7	No new data
GTX5	0.064	0.222	0.063	0.050	0.1	0.1	Relative potency values from oral LD_{50} studies suggest a lower TEF than from LD_{50} i.p. 0.1 TEF also in agreement with original Oshima TEF. As with NeoSTX, a number of *in vitro* Na channel assays also support a TEF of 0.1.
GTX6		0.122	0.038		0.1	0.05	New oral data show lower than 0.1.
C1	0.006					0.01	No new data (rounded up)
C2	0.096				0.1	0.1	No new data
C3	0.013					0.01	No new data
C4	0.058				0.1	0.1	No new data
dcSTX	0.51	0.785	0.457	0.368	1.0	0.5	From recent Oral data (more weight on oral data, also supported by i.p. toxicity data)
dcNeoSTX		0.058	0.216	0.224	0.4	0.2	From recent Oral data (more weight on oral data, also supported by *in vitro* data)
dcGTX2	0.15				0.2	0.2	No new data
dcGTX3	0.38				0.4	0.4	No new data

[1]In the case of saxitoxin analogues, for which no oral toxicity data were available, TEFs recommended are based on intraperitoneal toxicity data of Oshima (Appendix I).

3.2 Okadaic acid and analogues

OA and DTXs are polyether compounds that exist in the algae that produce them (*Dinophysis, Prorocentrum)* (Reguera *et al.*, 2008) as unesterified compounds (OA, DTX1, DTX2) and as esters (diol esters and DTX4, –5 and –6), and in shellfish also as DTX3, which is a mixture of esters of several fatty acids with acyl moieties of C14 to C22 and with up to 6 unsaturated bonds, the most common being palmitic esters of OA, DTX1 or DTX2 (Yanagi *et al.*, 1989).

From a toxicological point of view, the most relevant compounds, due to their known involvement in toxic episodes, are OA, DTX1, DTX2 and the DTX3 ester forms. Although DTX3 esters *per se* are non toxic, they may be hydrolysed after ingestion to release the parent compound(s) (OA, DTX1, DTX2) in the gastrointestinal tract.

The historical record of intoxications caused by these toxins has many inconsistencies most likely due to variation due to the quantification errors caused by the use of non-certified or non-quality-controlled materials, and the poor traceability of the intoxications (EFSA, 2008c). Many cases of human intoxication have been reported; however, an acute reference dose (ARfD) of 0.3 μg/kg body weight (b.w.) was proposed in 2007 by the EFSA Panel on marine toxins, based on effects in consumers (EFSA, 2008c). This ARfD is consistent with the provisional value of 0.33 μg/kg b.w. set in 2004 by the Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs (CODEX, 2006). Both panels concluded that no tolerable daily intake could be established because of the lack of data on the chronic toxicity of OA.

A well known effect of OA and analogues is the inhibition of cytosolic phosphatases, especially protein-phosphatase 2A (PP2A) as the main target (ID_{50} 1.2 nM) and, as secondary targets, PP1 (ID50 315 nM) and PP2B (ID50 4530 nM) (Takai *et al.*, 1987; Bialojan and Takai, 1988). There is no evidence of a toxic mechanism linked to any other type of phosphatase. These toxins are lethal in rodents above a certain value, although large ranges in estimates of toxicity have been reported, again attributable to the lack of use of certified materials that guarantee defined quantities. In general, 200 μg/kg b.w. body weight is a lethal i.p. dose in mice (Munday, 2014), with some variations in the time to death, depending on the strain (Suzuki, 2012).

A recent review of poisoning events suggests that esters of OA and DTXs have very similar toxicity to the parent compounds in human poisoning (EFSA, 2008c). OA and DTX1 are considered to be of approximately equal toxicity when injected intraperitoneally into mice, while DTX2 has been reported to have only 50 to 60 percent of the toxicity of OA (Aune *et al.*, 2007), both by i.p. injection into mice

and by *in vitro* assessment of their inhibitory character towards phosphoprotein phosphatases.

3.2.1 Acute toxicity

These toxins are lipophilic and they have good oral bioavailability due to their lipophilic nature. There are several studies of the toxic effect of OA and analogues in the intestine, the greatest damaging effects being seen after i.p. administration of OA, for which the lethal dose is consistently around 200 µg/kg b.w. (Aune *et al.*, 2007; Munday, 2014). There is very limited information on the toxicity of OA analogues (Table 9). DTX1 is reported to be more potent than OA, with a lethal dose of 160 µg/kg b.w. (Tubaro *et al.*, 2008a) for DTX1 versus 200 µg/kg b.w. for OA (Ogino *et al.*, 1997; Tubaro *et al.*, 2008a). DTX2 is 40 percent less potent than OA, as determined by i.p. toxicity in mice (352 µg/kg b.w. for DTX2 and 204 for OA) (Aune *et al.*, 2007). Even though the i.p. route of administration is interesting for the purpose of toxicological studies, there is no clear agreement about its use for the definition of a TEF, as the natural route is oral administration (Botana, 2012), although, as stated above, for biotoxins with similar bioavailability, i.p. administration can still provide useful toxicity comparison information.

TABLE 9. Acute toxicities of OA and its analogues by i.p. injection

Compound	Parameter	Acute toxicity (µg/kg b.w.) and source
OA	LD_{50}	192 (Tachibana *et al.*, 1981)
OA	LD_{50}	200 (pers. comm. T. Yasumoto, 1991)
OA	No death	200 (Ito and Terao, 1994)
OA	LD_{50}	204 (Aune *et al.*, 2012)
OA	LD_{50}	210 (Dickey *et al.*, 1990)
OA	LD_{50}	225 (Tubaro *et al.*, 2003)
OA	LD_{40} to LD_{100}	mean 227, range 216–242 (Suzuki, 2012)
OA	LD_{100}	375 (Ito and Terao, 1994)
DTX1	MLD	160 (Murata *et al.*, 1982; Yasumoto and Murato, 1990)
DTX1	LD_{50}	160 (pers. comm. T. Yasumoto, 1991; Dominguez *et al.*, 2010)
DTX1	LD_{100}	375 (Ito and Terao, 1994)
DTX2	LD_{50}	352 (Aune *et al.*, 2007)
DTX3	LD_{100}	375 (Ito and Terao, 1994)
DTX3	MLD	500 (Yasumoto *et al.*,1985)
DTX4	LD_{50}	610 (Hu *et al.*,1995a)

From these data, it would appear that DTX1 is slightly more toxic than OA, although it must be noted that the toxicity parameter for the former compound was an MLD, not an LD_{50}. DTX2, –3 and –4 were less toxic than OA when administered by this route.

There are several reports of oral administration of OA and analogues, but only few of them report concentrations checked against a certified material (Aune *et al.*, 2012; Vieira *et al.*, 2013). This may explain the large discrepancy in reported lethal doses, ranging from 2 000 µg/kg b.w. (Tubaro *et al.*, 2003), to 400 µg/kg b.w. (Ito *et al.*, 2002a), and to death with 575 µg/kg b.w. and no death with 610 µg/kg b.w. in two different experiments in the same report (Le Hegarat *et al.*, 2006). An oral dose of 1 000 µg/kg b.w. in rats was not lethal, again with a commercial supply of OA that was not quality controlled (Berven *et al.*, 2001). Those oral studies where the concentration has been checked against certified standards show a consistent lethal dose (LD_{50}) of 880 µg/kg b.w. in 18–22 g NMRI (Naval Medical Research Institute) female mice (Aune *et al.*, 2012) and 1 000 µg/kg b.w. in 18–23 g CD–1 (Cluster Differentiation 1) female mice (Vieira *et al.*, 2013). Even though mouse sex and strain show some differences, in general this is not a key factor for dose variability (Suzuki, 2012, 2013). A study of susceptibility of different mouse strains to OA (Suzuki, 2012) found that, depending on the strain, 40 to 100 percent of the mice died after an i.p. injection of 4 µg OA (mean 227 µg/kg b.w., range 216–242 µg/kg b.w.). No difference with regard to the sex of the animal was observed (Suzuki, 2013).

The oral administration of DTX1 shows similar toxic effects to i.p. administration, with fluid accumulation being observed with 0.4 and 0.32 µg/mouse for OA and DTX1, respectively (Tubaro *et al.*, 2008a). The lethal dose of DTX1 after oral administration was reported to be above 750 µg/kg b.w. (no death) and below 300 µg/kg b.w. (all animals dead) in fasted animals (Ogino, Kumagai and Yasumoto, 1997; Munday, 2014). According to Ogino, Kumagai and Yasumoto (1997), the oral LD_{50} in mice is around 300 µg/kg b.w. However, in other studies, death was not reported in mice or rats given DTX1 orally at 750 mg/kg b.w (Terao *et al.*, 1993; Ito and Terao, 1994).

No published report about the oral toxicity of DTX2 is available, although a work not yet published (Louzao *et al,* manuscript in preparation, an up-and-down protocol using certified DTX2) has concluded that the oral LD_{50} is 2 150 µg/kg b.w. (death at 24 h, mice fasted for 12 h, administration by gavage); the same study concluded that the LD100 is 3 000 µg/kg b.w., all animals showing death before 5 h.

The minimum toxic dose of DTX3 (no death) was reported after an oral administration of 750 μg/kg b.w. (Ito and Terao, 1994). Such toxicity is probably related to hydrolysis in the digestive system, hence it is more reliable to refer the toxicity to the toxin obtained after hydrolysis (AO, DTX1 or DTX2). DTX3 actually represents a mixture of 7-O-acyl ester derivatives of OA, DTX1 and DTX2. DTX3 is formed as metabolites of free OA, DTX1 and DTX2 in bivalves that have consumed toxic dinoflagellates. During analysis by LC-MS/MS techniques, an alkaline hydrolysis step during extraction yields the parental toxins and therefore no TEF specific to DTX3 was considered necessary by the Expert Group.

The toxicity of DTX1 by gavage appears to be higher than that of OA, but with the variability in published values for the latter compound, an estimate of TEFs based on oral toxicity is not possible. A recent study on the cardiotoxic effects of OA (20 μg/kg b.w.) and DTX1 (16 μg/kg b.w.) in rats shows no cardiotoxic effects of these compounds in acute experiments as assessed either by the electrocardiogram or by biomarkers (Ferreiro *et al.*, 2015).

3.2.2 Toxicity of OA and analogues to cells in vitro

The toxicities of OA, DTX1 and DTX2 have been recently evaluated in a number of cell lines *in vitro* (See Table 10).

TABLE 10. Relative toxicities of OA, DTX1 and DTX2 in cells *in vitro*

Compound	Relative toxicity in the specified cell line					
	SH-SY5Y (1)	Neuro-2a (1)	NG108-15 (1)	MCF-7 (1)	Caco-2 (2)	HT29-MTX (2)
OA	1.0	1.0	1.0	1.0	1.0	1.0
DTX1	4.4	2.1	2.4	3.8	2.2	3.4
DTX2		0.52	0.52	0.73	0.47	0.35

Sources: (1) Louzao *et al.*, 2015. (2) Ferron *et al.*, 2014.

The end points used in the sources for Table 10 were: transepithelial electrical resistance and cell viability assessed by the trypan blue exclusion assay in SH-SY5Y (Louzao *et al.*, 2015); colorimetric [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium], MTT assay in Neuro–2a (Solino, Sureda and Diogene, 2015); NG108–15 (Solino, Sureda and Diogene, 2015); MCF–7 (Solino, Sureda and Diogene, 2015); and uptake of neutral red in Caco–2 (Ferron *et al.*, 2014) and HT29-MTX (Ferron *et al.*, 2014).

There is remarkable consistency among the cell lines tested, with DTX1 showing a 2–4 fold greater activity than OA, and DTX2 showing less toxicity than OA, by a factor of between 0.35 and 0.73.

Taking the averages of these figures, the relative potencies based on cytotoxicity for OA, DTX1 and DTX2 would be 1.0, 3.1 and 0.52 respectively.

3.2.3 Diarrhetic effect

The main effect of OA and analogues, which was used as a tag to the group, is diarrhoea, hence the common designation of this group of compounds as diarrhetic shellfish poisons or DSP after the first description of the intoxication by Yasumoto in 1978 (Yasumoto, Oshima and Yamaguchi, 1978a, b).

The association of diarrhoea with phosphatase inhibitors is not established. There are several non-marine substances that are also potent phosphatase inhibitors, such as microcystins, nodularins, tautomycin, calyculin A or cantharidin (MacKintosh and Klumpp, 1990; Laidley, Cohen and Casida, 1997; Craig and Holmes, 2000). Their effect is mostly hepatotoxicity (Craig and Holmes, 2000), while the most evident short-term effect of OA is diarrhoea. The diarrhoea caused by OA and DTXs has been proposed to be caused by an increase in intestinal epithelial paracellular permeability (Tripuraneni *et al.*, 1997) as indicated by transepithelial ion and mannitol fluxes in human intestinal epithelial T_{84} cell monolayers. The purity of the OA employed in this study was not, however, checked against a certified standard. This study proposed that OA does not stimulate intestinal secretion directly, but increases paracellular permeability. The fact that other phosphatase inhibitors did not show this same diarrhetic effect suggest that other factors are involved in the mechanism of toxicity of OA and DTXs.

There is a no clear relationship between reported effects of OA and DTXs on the small intestine in the presence of diarrhetic symptoms and the doses tested, probably attributable to uncertainties on the final amount of OA used as mentioned above. The same group reported damage to the intestinal mucosa in one study (Tubaro *et al.*, 2003) but none in another study, although similar doses were used (Tubaro *et al.*, 2004). Other studies reported damage to the mucosa with 750 µg/kg b.w. (Wang *et al.*, 2012), or with very low concentrations of 75–250 µg/kg b.w., the lethal oral dose in this case being 400 µg/kg b.w. (Ito *et al.*, 2002a).

Other studies in fasted and fed animals report little or no damage to the intestinal mucosa at a dose of 880 µg/kg b.w. (Aune *et al.*, 2012) and none at a dose of 1 000 µg/kg b.w. (Tubaro *et al.*, 2004; Vieira *et al.*, 2013). Although no damage to the mucosa was observed at these doses, severe diarrhoea was induced, and OA was detected in several organs, such as the liver and kidneys (Vieira *et al.*, 2013). Doses that do not induce damage to the intestinal mucosa and other organs (stomach, lungs, kidney, brain) do induce alterations in the liver and a decrease of its antioxidant and detoxifying capacity (Vieira *et al.*, 2013).

NMR structural studies of DTX1 and DTX2 obtained from natural sources showed that DTX1 has an equatorial 35-methyl group and that DTX2 has an axial 35-methyl group. Molecular modelling studies indicated that an axial 35-methyl could exhibit unfavorable interactions in the PP2A binding site, and this has been proposed as the reason for the reduced toxicity of DTX2. Concerning PP1, results suggest that the inhibitory potency of DTX2 should be similar to those of OA and DTX1 (Larsen *et al.*, 2007; Huhn *et al.*, 2009).

Compared with OA and DTX1, DTX3 investigated as 7-O-palmitoyl-OA and 7-O-palmitoyl-DTX1 were found to be weak inhibitors of PP2A and PP1 (Nishiwaki *et al.*, 1990; Takai *et al.*, 1992a, b). It is concluded that the toxic potency of the OA-toxins group is highly associated with the presence of the free carboxylic acid group. Therefore, OA-group toxins where the carboxylic acid is acylated are assumed to be less toxic. However, hydrolysis to free the corresponding unesterified parent toxins (OA, DTX1 or DTX2) will most likely be a rate limiting step for exerting the toxic effects (EFSA, 2008c).

For the sulphate derivatives and OA-diol esters, although they demonstrate weak potential to inhibit PP1 and PP2A *in vitro, it is presumed that, similar to DTX3, they can be hydrolysed to free the parent toxin in vivo* (Doucet, Ross and Quilliam, 2007; Torgersen *et al.*, 2008b).

The inhibitory effect of OA and DTXs on phosphatases *in vitro, especially PP1 over PP2A shows a ratio of 4800 for OA and 8700 for DTX1* (Bialojan and Takai, 1988; Takai *et al.*, 1992b). Therefore, OA is 4800 times more effective on PP2A than on PP1 (Ki OA for PP2A is 30 pM, for PP1 is 145 nM), and DTX1 is 8 700 times more effective on PP2A (Ki DTX1 for PP2A is 19 pM; for PP1 it is 165 nM) (Takai *et al.*, 1992b). Therefore, the inhibition equivalent factor of DTX1 to OA for PP2A is 0.63, which means that DTX1 is about 37 percent more potent than OA according to PP2A phosphatase inhibition. In contrast, DTX2 is 47 percent less potent than OA, with an IC_{50} value in PP2A of 5.94 ng/ml for DTX2 and 2.81 ng/ml for OA (Huhn *et al.*, 2009; Pang *et al.*, 2011).[1]

1 DTX1 inhibited PP1 as potently as OA (Holmes *et al.*, 1990; Takai *et al.*, 1992b). With respect to PP2A, conflicting results have been found in the literature when using recombinant or stabilized phosphatases for the purposes of biochemical detection. The reason of these results is the enzyme quaternary structure that can change during purification, and differs between different tissues and sources (Rubiolo *et al.*, 2012, 2013). The use of the whole enzyme, the catalytic subunit, and the type of isoform may greatly modify the results (Rubiolo *et al.*, 2012, 2013). DTX1 has been reported to be less potent than OA by a factor of 3 (Holmes *et al.*, 1990), 1.6 (Mountfort, Suzuki and Truman, 2001), 1.33 (Smienk *et al.*, 2012) or 1.1 (Ikehara *et al.*, 2010). In contrast, DTX1 was also reported to be more potent than OA by a factor of 1.6 (Takai *et al.*, 1992b) or 2.4 (using PP2A from mussel) (Rivas *et al.*, 2000). There is a report of inhibition equivalency factors (IEFs) of 1.1 and 0.9 for DTX1 compared with OA in recombinant and wild PP2A (Garibo *et al.*, 2013). DTX–2 exerts a lower inhibitory potency toward PP2A compared with OA and DTX1, with a ratio of OA/DTX–2 ranging from 0.4 to 0.6 (Aune *et al.*, 2007; Pang *et al.*, 2011; Garibo *et al.*, 2013), although a similar potency has also been reported (Smienk *et al.*, 2012).

The relative potencies based on PP2A inhibition for OA, DTX1 and DTX2 would be 1.0, 1.6 and 0.5 respectively.

3.2.4 Intestinal mucosa effect

Although the effects of these toxins on protein phosphatase are well established, the *in vivo* diarrhetic effects do not relate directly to PP inhibition. Several lines of evidence support this conclusion. The effect of DSP toxins is cell line-dependent, and the inhibitory potency on phosphatases does not match their effect in some cell types, i.e. OA and DTX2 are equipotent as cytotoxic compounds in hepatic Clone 9 cells, but OA is more potent than DTX2 in HepG2 cells, with IC_{50} (77 and 124 nM for OA and DTX2, respectively) that match the effect of PP2A (Rubiolo *et al.*, 2011). A similar observation was earlier reported in cerebellar neurons, DTX2 being less potent than OA (Perez-Gomez *et al.*, 2004). The toxicity of DTX4 in mice is 0.3-fold the value of OA (Hu *et al.*, 1995a), but the effect on PP2A is 0.002-fold (Hu *et al.*, 1995b; Munday, 2013). As discussed by Munday (2013), there is no direct demonstration that links an alteration of paracellular permeability due to alterations of tight junction integrity with phosphatase inhibition by OA or DTXs. Methyl okadaate, which does not inhibit PP2A or PP1, has a higher potency than OA to disrupt F-actin (Espina *et al.*, 2010), which is a common effect for OA and analogues (Vale and Bontana, 2008a). Therefore, there is no clear link between increased paracellular permeability and phosphatase inhibition. Also, the effect of these toxins on the small intestine involves changes in at least 58 proteins, showing that the process is very complex (Wang *et al.*, 2012). The threshold for organ damage, at a microscopic level has been proposed to be 500 µg/kg b.w. by oral administration, with 99 percent of the toxin being absorbed after one hour (Vieira *et al.*, 2013).

A recent article proposed a new theory for OA effect on the intestine by means of inhibition of the level of neuropeptide Y (Louzao *et al.*, 2015). Although the work has been done in an *in vitro* Caco–2 model, it suggests a physiological reason for OA to induce diarrhoea, as neuropeptide Y plays a role in protecting against diarrhoea by inhibition of intestinal motility and water and electrolyte secretion (Holzer, Reichmann and Farzi, 2012). According to this, DSP toxins would cross the intestinal wall to act on enteric neurons, primary afferent neurons, sympathetic and neuronal pathways through the brain, all of them related to neuropeptide Y (Tough *et al.*, 2011; Camilleri, Nullens and Nelsen, 2012). The results show a 4–5-fold higher potency of DTX1 over OA on cytotoxicity (IC_{50} of 38.4 and 9.3 nM at 24 h, and 46.1 and 9.3 nM at 12 h for OA and DTX1, respectively), although the results show a potency of DTX1 15 fold higher than OA on transepithelial electrical resistance. Similar IC_{50} results were reported in Caco–2 cells in an earlier study, with IC_{50} of 49.7, 22.5 and 106 nM for OA, DTX1 and DTX2, respectively

(Ferron *et al.*, 2014). In both cases the toxin concentrations were certified. There is agreement on the higher oral toxicity of DTX1, its higher PP2A potency, and its higher alteration of paracellular permeability (Fernandez *et al.*, 2014). Although the study does not show a dose-response, it can be concluded that DTX1 is at least twice as potent as OA or DTX2 in modifying occluding integrity or intestinal mucosa integrity.

3.2.5 Conclusion

In conclusion, there seems to be a certain correlation between PP2A inhibition, diarrhoea induction, and effect on the intestinal mucosa caused by paracellular permeability, neuron effect or some other factor not necessarily related to a PP2A effect. This diarrhetic effect is not linked to animal survival: the diarrhoea effect is very fast (Vieira *et al.*, 2013) in animals that do not die or even do not suffer damage to the intestinal mucosa.

The potency to consider for the main toxins OA, DTX1 and DTX2, depends on the parameter to use as a reference, therefore a TEF approach would be:

- The TEFs recommended by the EFSA were 1.0, 1.0 and 0.6 for OA, DTX1 and DTX2 respectively, based on toxicity by i.p. injection and relative inhibitory effect on PP2A (EFSA, 2008c). Recent results based on oral LD_{50} suggest a TEF for DTX2 of 0.3.
- Based on cytotoxicity in a variety of cell lines, the relative potencies are 1.0, 3.1 and 0.52 for OA, DTX1 and DTX2, respectively.
- The relative potencies based on PP2A inhibition are 1.0, 1.6 and 0.5 for OA, DTX1 and DTX2 respectively
- Based on changes in membrane paracellular permeability, the relative potencies are 1.0, 2 to 15, and 0.6 for OA, DTX1 and DTX2, respectively.
- The data for DTX2 is reasonably consistent among the different assays and a TEF of 0.5 appears reasonable.
- There is, however, marked variation among the assays with regard to DTX1, with factors of 1.0, 1.6, 3.1 and between 2 and 15.

In the case of the OA group of toxins, several case reports from human intoxication are available, and these reports are analogue-specific. As outlined in the recent risk assessment by EFSA, a human poisoning event with DTX1 as main contaminant in Japan suggested a LOAEL of 48 µg DTX1 per person. This dose is similar to that reported to produce toxic effects in poisoning events in Sweden, Norway, UK and Portugal. Thus, it is not surprising that the currently used TEF of 1.0 in some countries appears protective for public health. Still, it should be noted that multiple *in vitro* studies suggest that the intrinsic potency of DTX1 could be higher than

that of OA. However, large uncertainty is associated with these studies (a factor of 10 difference between results depending on the cell line used).

The other toxin in the OA group is referred to as DTX3. DTX3 actually represents a mixture of 7-O-acyl ester derivatives of OA, DTX1 and DTX2. DTX3-group toxin is formed as metabolites of free OA, DTX1 and DTX2 in bivalves that have consumed toxic dinoflagellates. During analysis by LC-MS/MS techniques, an alkaline hydrolysis step during extraction yields the parental toxins and therefore no TEF specific to DTX3 was considered necessary by the Expert Group.[2]

2 Toxicity was seen with DTX3 after oral administration to mice. Relatively little hydrolysis may be expected in the mouse stomach due to the high pH in this organ. Under the highly acidic conditions pertaining in the human stomach, extensive hydrolysis may be anticipated.

TABLE 11. Expert Group recommended TEFs for OA and analogues

	TEF based on cytotoxicity	TEF based on PP2A inhibition	TEF based on membrane paracellular permeability	EFSA proposed TEF	Recommended TEF	Rationale
OA	1.0	1.0	1.0	1.0	1.0	
DTX1	3.1	1.6	2–15	1.0	1.0	In the case of the OA group of toxins, several case reports from human intoxication are available, and these reports are analogue specific. As outlined in the recent risk assessment by EFSA, a human poisoning event with DTX1 as main contaminant in Japan suggested a LOAEL of 48 µg DTX1 per person. This dose is similar to that reported in poisoning events in Sweden, Norway, UK and Portugal. Thus, it is not surprising that the currently used TEF of 1.0 in some countries appears protective for public health. Still, it should be noted that multiple *in vitro* studies suggest that the intrinsic potency of DTX1 could be higher than that of OA. However, large uncertainty is associated with these studies (a factor of approximately 5-fold difference between results depending on the cell line used). Therefore, the recommended TEF of 1.0 for DTX1 should be verified in future studies to corroborate the observations in humans, and also through more controlled studies *in vivo* (animals).
DTX2	0.52	0.5	0.6	0.6	0.5	Consistent among the different assays; based on acute oral and i.p. toxicity in mice, DTX2 is on average 0.5 times as toxic as DTX1). This value is also supported by the various *in vitro* data
DTX3						Explanation provided in main text as to why no TEF is recommended.

3.3 Domoic acid and analogues

DA is an excitotoxin produced by the red macro-alga *Chondria armata* (Takemoto and Daigo, 1958; Takemoto *et al.*, 1966), or by diatoms of the genera *Nitzschia, Pseudo-nitzschia* (Bates *et al.*, 1989; Wright *et al.*, 1989, 1990a, b; Lundholm *et al.*, 1994) and *Amphora* (Dhar *et al.*, 2015), and is distributed worldwide.

DA is an acidic crystalline water-soluble amino acid with three carboxyl groups (Takemoto and Daigo, 1958), very similar to the glutamatergic agonist kainate and the amino acid glutamate (Ramsdell, 2007; Vale, 2014).

3.3.1 Toxicity

The mechanism of toxicity is well understood, as DA activates alphaamino-5-methyl-3-hydroxyisoxazolone-4-propionate (AMPA)/kainate receptors, thereby increasing intracellular calcium levels, causing glutamate release and subsequent activation of N-methyl-D-aspartate (NMDA) receptors. The neurotoxicity is due to the activation of both AMPA/kainate and NMDA receptors (Vale, 2014). At high doses (10 µM), DA releases glutamate and causes neurotoxicity through NMDA receptor activation (Hogberg and Bal-Price, 2011). However, at low doses there is an apoptotic effect in neurons through AMPA/kainate that triggers oxidative stress and caspase 3 activation (Giordano *et al.*, 2007).

DA is a partial agonist of kainate receptors, with an affinity in the low nM range (Crawford *et al.*, 1999). It binds with high affinity to GluK1 and GluK2 kainate receptors, and with lower affinity to GluK4 and GluK5 (Vale, 2014). The symptoms of toxicity (nausea, vomiting, abdominal cramps, diarrhoea, headache, memory loss) include serious neurological damage, which is well known as a consequence of the deadly human intoxications that occurred in Quebec in 1987 (Perl *et al.*, 1990; Teitelbaum *et al.*, 1990; Doucette and Tasker, 2008). The damage affected in particular the hippocampus, amygdaloid nucleus and subfrontal cortex. The LOAEL in humans is estimated as 0.9 mg/kg, and the EFSA Contam Panel established an ARfD of 30 µg/kg b.w. (EFSA, 2009a). The Joint FAO/IOC/WHO *ad hoc* Expert Consultation proposed a provisional ARfD of 100 µg/kg b.w. and

concluded that this dose may also be considered as a provisional TDI (CODEX, 2006).[3]

DA is especially toxic in young neurons in newborn mice (Xi, Peng and Ramsdell, 1997; Mayer, 2000; Vale, 2014). Recent studies also show that DA may also have endocrine effects that relate to water balance, as it has been reported to accumulate in hypophysis (adeno- and neurohypophysis), and not in the *pars intermedia* (Crespo *et al.*, 2015). A recent article reports a new toxic effect not described before, a carditotoxic effect of 2.5 mg/kg DA in rats 30 days after an i.p. dose (Vieira *et al.*, 2016).

Toxicologic DA studies are conditioned by the quality of the test chemical used, as there are potentially very large differences in the toxicity unless it is certified (Crespo *et al.*, 2015). Therefore, the doses reported in the literature must be treated with caution unless thoroughly checked for the real concentration. Rats and mice are rather different in sensitivity to DA. with i.p. lethal doses of 2.5 mg/kg for rats (Crespo *et al.*, 2015) and 6 mg/kg for mice (Munday *et al.*, 2008b; Crespo *et al.*, 2015). There are differences in the oral bioavailability of DA in rats and mice, as mice were reported to have a higher sensitivity than rats (Iverson *et al.*, 1989).

Samples of domoic acid and isodomoic acids A, B and C, of 98–99% purity, were compared with regard to behavioural changes in mice after i.p. injection. At a dose of 5 mg/kg, domoic acid induced severe toxic changes, as indicated by hypoactivity, sedation, limb rigidity, stereotypic responses, loss of postural control, and forelimb tremors. In contrast, the isodomoic acid had little or no effect at this dose, and even at 20 mg/kg, isodomoic acid induced little change in the behaviour of the animals (Munday *et al.,* 2008b).

3 The reason for the discrepancy between EFSA and FAO/IOC/WHO lies in the use of different uncertainty factors by the two expert committees. The EFSA Panel used a Lowest Observed Adverse Effect Level (LOAEL) of 0.9 mg/kg for neurotoxicity in humans. They applied a factor of 3 for extrapolation from a LOAEL to a No Observed Adverse Effect Level (NOAEL) and then applied a further factor of 10 "to allow for human variability and also for the fact that sensitive methods for detection of neurotoxic effects had not been used in the investigation of affected individuals". They therefore proposed an ARfD of 30 micrograms/kg b.w. The FAO/IOC/WHO Panel used a LOAEL of 1.0 mg/kg, and then applied a safety factor of 10 to give a provisional ARfD of 100 μg/kg b.w. It was argued that this value was reasonable, since one individual who consumed 0.33 mg/kg b.w. did not become ill. It was also argued that cumulative effects of low doses of DA are unlikely, and on this basis it was concluded that the ARfD could be considered as a provisional TDI.

Of all the analogues of DA, only isodomoic acid A is relevant from a toxicity point of view, since all others have very low toxicity, with nM inhibition constants in the kainate receptor of 2.4, 4.4, 4990, 171, 600, 600 and 67 for DA and Isodomoic acids A, B, C, D, E, F, respectively (Vale, 2014). Only the analogues D, E and F are found in shellfish, and they are currently considered not toxic. DA transforms to epi-DA in storage or by ultraviolet light, and in general DA and epi-DA (the C5´-diastereomer) are considered as one toxin (Quilliam *et al.*, 1989c; Wright *et al.*, 1990b; EFSA, 2009a).

DA has a low bioavailability and it is rapidly eliminated in the kidney, with only 6 percent of an i.v. dose reaching the brain in rats (Maucher Fuquay *et al.*, 2012). The i.p. LD_{50} in mice was reported to be 6.2 mg/kg (Iverson *et al.*, 1989), 6 (Munday *et al.*, 2008b; Crespo *et al.*, 2015) and close to 4 (Grimmelt *et al.*, 1990; Peng and Ramsdell, 1996; Nagatomo *et al.*, 1999). The i.p. LD_{50} in rats is lower than in mice, as mentioned before, with reported values of 2.5 mg/kg (Crespo *et al.*, 2015), although higher values were also reported, e.g. 4 (Iverson *et al.*, 1989) and 7.5 (Tryphonas *et al.*, 1990); in young rats, toxicity is higher, with LD_{50} in 2-day-old animals of 0.25 mg/kg, and 0.7 mg/kg in 10-day-old animals (Xi, Peng and Ramsdell, 1997).

The administration of low doses (1.2 mg/kg) of DA to pregnant rats on day 13 causes persisting behavioural changes in newborn animals (Levin *et al.*, 2005).

The oral LD_{50} is much lower than the i.p. LD_{50}, with reported values of 71–83 mg/kg in mice and 80–82 mg/kg in rats (Iverson *et al.*, 1989).

3.3.2 Conclusion

No TEF is necessary as only DA is found in foods (sum of DA and epi-DA expressed as DA).

3.4 Azaspiracid and analogues

AZAs, produced by the genera *Azadinium and Amphidoma* (Tillmann *et al.*, 2009, 2014; Krock *et al.*, 2012), are a food safety problem first recognized in 1996 (McMahon and Silke, 1996) and later identified chemically (Satake *et al.*, 1998b). Their chemical structure is unusual, and the mechanism whereby they exert their toxic effects is at present unknown (Botana *et al.*, 2014b).

3.4.1 Toxicity

AZAs are easily absorbed orally (Aasen *et al.*, 2010, 2011; Aune *et al.*, 2012), with blood levels peaking within 30 minutes after oral administration and a half-life of 24 h in mice (Twiner, Hess and Doucette, 2014). The symptoms of intoxication in humans are vomiting, nausea, diarrhoea and stomach cramps, which appear

after a few hours and last for up to two days (Klontz *et al.*, 2009). No deaths from AZA ingestion have been reported. Human intoxications observed in the United Kingdom, United States of America, Italy and France permitted evaluation of the risk associated with the consumption of these compounds, and the EFSA Panel established an ARfD of 0.2 µg AZA equivalents/kg b.w. (EFSA, 2008a). The Joint FAO/IOC/WHO *ad hoc* Expert Consultation established a provisional ARfD of 0.04 µg/kg b.w. body weight, but they were unable to establish a TDI (FAO/IOC/WHO, 2004).

In mice, AZAs cause multiple organ damage after oral administration, with important injuries in intestinal epithelium, *lamina propria* and *villi,* and a lethal oral dose of 700 µg/kg b.w. (Ito *et al.*, 2000, 2002b). Prolonged administration (4 months) at doses of 1 to 50 µg/kg b.w. showed organ damage, with lung tumours and malignant lymphomas at the highest doses (Ito *et al.*, 2002b), although the relevance of these studies is far from conclusive due to extensive and severe toxicity noted at the higher doses (EFSA, 2008a).

According to the few data available so far, the simultaneous effect of AZAs with other lipophilic toxins is not functionally relevant (pectenotoxin, yessotoxin or OA), since it does not increase the toxicity of the other toxin groups. It has been shown, however, that the bioavailabilities of pectenotoxin 2 and OA are slightly increased (Aune, 2009) and slightly decreased (Aune *et al.*, 2012), respectively, by simultaneous administration of AZA.

AZAs were reported to be cytotoxic by targeting several receptors (Roman *et al.*, 2002) that participate in the apoptosis process (Twiner *et al.*, 2005; Botana *et al.*, 2014b), such as caspase activation and cytoskeletal disruption (Vilarino *et al.*, 2006, 2007), cytochrome release and DNA fragmentation (Twiner *et al.*, 2012b), increase in c-jun-N-terminal protein kinase (JNK) phosphorylation and inhibition of calcium oscillations (Vale *et al.*, 2007, 2008c; Cao *et al.*, 2010), and effects at the gene level in several fatty acid biosynthesis routes (Twiner *et al.*, 2008a). The effect of AZAs in ion channels may be linked to some of its toxic effects, as they decrease cell volume mediated by potassium and chloride efflux (Vale *et al.*, 2010), deplete ATP (Kellmann *et al.*, 2009), inhibit endocytosis (Bellocci *et al.*, 2010) and decrease procathepsin pools in endocytosis (Sala *et al.*, 2013).

An intriguing observation is that AZAs are only present in mussel samples with high levels of glutaric acid, and the combined effect of both AZA1 (50 nM) and glutaric acid (1 mM) increases the blockade of voltage dependent sodium channels (Chevallier *et al.*, 2015). This combined effect might explain the reported neurological symptoms described in AZA toxicity (Twiner, Hess and Doucette, 2014).

Data on the acute toxicity of AZAs to mice by i.p. injection are shown in Table 12.

TABLE 12. Acute toxicities of AZAs by i.p. injection

Compound	Parameter	Acute toxicity (μg/kg b.w.)	Source
AZA1	Lethality	200	Munday, 2014
AZA1	MLD	150	Satake *et al.*, 1998a
AZA1	LD_{50}	74	Marine-Institute, 2014
AZA1	LD_{50}	>10 and <55 in rats	Ferreiro *et al.*, 2016
AZA2	Lethality	Approximately 110	Munday, 2014
AZA2	LD_{50}	117	Marine-Institute, 2014
AZA2	LD_{50} (i.v.)	11 in rats	Ferreiro *et al.*, 2014
AZA3	Lethality	Approximately 140	Munday, 2014
AZA3	LD_{50}	164	Marine-Institute, 2014
AZA4	Lethality	Approximately 470	Munday, 2014
AZA5	Lethality	<1 000	Munday, 2014
AZA6	LD_{50}	100	Marine-Institute, 2014

Data on the acute oral toxicity of AZAs are shown in Table 3.8.

TABLE 13. Acute toxicities of AZAs by oral administration

Compound	Parameter	Acute toxicity (μg/kg b.w.)	Reference
AZA1	Lethality	> 700	Ito, 2008
AZA1	LD_{50}	775	Aune *et al.*, 2012
AZA1	LD_{50}	443	Marine-Institute, 2014
AZA2	LD_{50}	626	Marine-Institute, 2014
AZA3	LD_{50}	875	Marine-Institute, 2014

Repeated oral administration of AZA1 to mice at 50 μg/kg b.w. twice weekly over a period of up to 20 weeks caused death of all the animals, and at 20 μg/kg b.w. it killed 30 percent of the mice (Ito *et al.*, 2002b). There are conflicting reports as to what organs are affected, from general damage to liver, spleen or thymus with doses of 500 μg/kg b.w. (Ito *et al.*, 2000) to very little damage in these organs and mostly lesions in the stomach and intestine (Aasen *et al.*, 2010; Aune *et al.*, 2012). Probably a more important observation is that AZAs are direct blockers of the open state of hERG potassium channels (Twiner *et al.*, 2012c), and this translates into the *in vivo* cardiotoxicity of AZAs through hERG channels in rats (Ferreiro *et al.*, 2014), causing a prolongation of the PR electrocardiogram interval and arrhythmias with 11 μg/kg b.w. AZA2 i.v. This i.v. value is only 5 times higher than the LOAEL proposed by the EFSA working group in humans of 1.9 μg/kg b.w. (EFSA, 2008a). The i.v. bioavailability is 100 percent, and oral availability of AZAs is lower, with only 2 percent of an administered gavage dose in internal

mice organs after 24 hours (Aasen *et al.*, 2010). Considering oral bioavailability for AZA in humans as 20 percent and with cardiotoxicity potential in humans similar to that in rats, the derived LOAEL in humans could be potentially cardiotoxic (Ferreiro *et al.*, 2014, 2016).

The lethal dose of AZA2 in rats is below 55 μg/kg b.w. i.v. and close to 11 μg/kg b.w., since this dose caused the death of 50 percent of the animals; also, 11 μg/kg b.w. i.v. is enough to induce potentially lethal extrasystoles in 50 percent of the animals (Ferreiro *et al.*, 2014). A continuation of this study has shown that, in rats, 10 μg/kg b.w. i.p. after 4 doses in 15 days (one dose every 4 days) induces ascites, ultrastuctural damage in the heart. Hypotension is observed with only 1 μg/kg b.w. i.p. It is remarkable that this study, which was performed with AZA1, did not show any death with 10 μg/kg b.w., and all animals died with the first administrations of 55 μg/kg b.w. AZA1 (Ferreiro *et al.*, 2016). This suggests that AZA1 i.p. is about 5 times less toxic in rats than AZA2 (which was administered i.v.).

Based on this, the cardiotoxicity results suggest that the toxicity of AZAs might be higher than currently estimated.

3.4.2 In vitro relative potency

The recent *in vitro* studies of several recently identified analogues support the conclusion that relative potencies based on *in vitro* studies do not match the relative potencies based on *in vivo* effects, i.e. i.p. LD_{50} in mice (Table 14). Examples of this is the relative cytotoxicity in Jurkat T lymphocytes, being 1, 0.22 and 5.5 for AZA1, AZA33 and AZA34, respectively (Kilcoyne *et al.*, 2014a); 5.1 for 37-epi-AZA1 (Kilcoyne *et al.*, 2014a); 0.6, 0.4, 7, 4.5, 0.4 and 0.2 for AZA4, AZA5, AZA6, AZA8, AZA9, AZA10, respectively (Kilcoyne *et al.*, 2015); 1, 8.3 and 4.5 for AZA1, AZA2 and AZA3, respectively (Twiner *et al.*, 2012a); 1.3 and 1 for AZA2 and AZA3 using patch clamp-on voltage clamped hERK channels in HEK 293 cells (Twiner *et al.*, 2012c); 1.89 for AZA2 in 24 h treated 2–3 days *in vitro* cultured mice neurons (Vale *et al.*, 2008c), 1.36 and 3.22 for AZA2 and AZA3 in spontaneous calcium oscillations; and 0.89 and 4.32 for AZA2 and AZA3 in LDH release in mice neocortical neurons (Cao *et al.*, 2010). This variation in *in vitro r*elative potencies is probably linked to the potential different responses of the different analogues in each cell model and each transduction signal (Roman *et al.*, 2004; Vale *et al.*, 2007, 2008c, 2010; Alfonso *et al.*, 2008; Botana *et al.*, 2014b).

TABLE 14. Relative *in vitro* toxicity of AZAs

Compound	Cell type				
	Jurkat T (cytotoxicity)	HEK 293 (hERK current)	2–3 DIV mice neurons (cytotoxicity)	Neocortical neurons	
				(LDH release)	(calcium oscillations)
AZA1	1	1	1	1	1
AZA2	8.3	1.2	1.89	0.89	1.36
AZA3	4.5	1		4.32	3.22
AZA4	0.6				
AZA5	0.4				
AZA6	7				
AZA8	4.5				
AZA9	0.4				
AZA10	0.2				
AZA33	0.22				
AZA34	5.5				
37-epi-AZA1	5.1				

3.4.3 Conclusion

The identification of TEFs for each AZA analogue involves several variables that make it difficult to define criteria, especially taking into account that the toxicological target of AZAs is not yet defined. Based on the different toxicities, there is some consistency between cardiotoxicity in rats and other studies in mice, which along with the conclusions of the EFSA Panel, allowed the expert meeting to propose the TEFs as shown in Table 15.

TABLE 15. TEFs Recommended by the Expert Group

	TEF based on i.p. toxicity	TEF based on oral toxicity	EFSA proposed TEF	Recommended TEF	Rationale
AZA1	1.0	1.0	1	1.0	
AZA2	0.6	0.7	1.8	0.7	Based on recent oral data. (also consistent with recent i.p. data)
AZA3	0.45	0.51	1.4	0.5	Based on recent oral data. (also consistent with recent i.p. data)
AZA4			-		Cannot determine TEF due to lack of data.
AZA5			-		Cannot determine TEF due to lack of data.
AZA6	0.7		-	0.7	No oral data, based on recent i.p. data.

3.5 Brevetoxins

Brevetoxins target the neurotoxin receptor site 5 of domain IV of the alpha subunit of voltage-gated sodium channels, resulting in membrane depolarization, repetitive firing, and increases in sodium currents (Murrell and Gibson, 2009). BTXs have been found to activate voltage-gated sodium channels in the following manner: prolonging the mean open time, preventing inactivation of the channel, and making the channel activation more negative in value (Jeglitsch *et al. 1998;* Schreibmayer and Jeglitsch, 1992; Catterall, 1992). Rapid reductions in respiratory rate, cardiac conduction disturbances, and reduced body temperatures are *in vivo* effects produced by BTXs (Poli *et al.*, 1990; Templeton, Poli and Solow, 1989; van Apeldoorn, van Egmond and Speijers, 2001). Tremors, muscular contractions or fasciculations, Straub tail phenomenon, laboured breathing, and death have been observed in orally dosed mice. Mice injected with PBX3 presented salivation, lacrimation, urination and defecation, with hypersalivation being the most pronounced. At high doses, compulsive chewing and excessive mucous in the nasal cavity were reported. Morohashi *et al.* (1999) documented that BTX-B2 and BTX-B4 caused paralysis of hind limbs, diarrhoea, dyspnea and convulsion following i.p. injection. I.p. injection of BTX-B1 led to irritability, hind and/or hind-quarter paralysis, severe dyspnea and convulsions before death due to respiratory paralysis (Ishida *et al.* 1995; 1996).

Much of what is known about the acute toxicity of BTX analogues and metabolites has been determined in mice, mostly via i.p. administration (Table 16). Shimizu *et al. (*1986) reported PBX1 to be the most toxic of the BTX analogues. PBX2 was determined to be less toxic by oral ingestion compared with i.v. or i.p. administration. Of the metabolites identified in shellfish from New Zealand, BTX-B1 was found to be the most toxic (Ishida *et al.*, 1995). BTX-B4 was threefold more toxic than BTX-B2 and comparable to the toxicity of PBX3 (Baden *et al.,* 1982; Murata *et al.*, 1998; Morohashi *et al.*, 1999). *In vitro* studies using Jurkat calls demonstrated that PBX2 and PBX6, in comparison with PBX3, led to greater cellular effects, including apoptosis, as measured by caspase–3 activity (Walsh *et al.*, 2008). Recent studies have employed Caco–2 permeability assays to assess BTX metabolite toxicity (Henri *et al.*, 2014*).*

3.5.1 Conclusions

Given the limited data currently available for establishing TEFs, notably lacking human oral potency data, and since the Codex Standard method for BTXs is currently the MBA and the limit is expressed as mouse units, TEFs for BTXs are not proposed at this time. Rather the topic is covered in the section on data gaps.

TABLE 16. Acute toxicity of BTX analogues in mice

Brevetoxin	Observation/ Route	Toxicity µg/kg b.w.	Original source
PBX1	LD_{50}, 24-h, i.p.	>100	Dechraoui *et al.*, 1999
PBX2	LD_{50}, 24-h, i.v.	200	Baden *et al.*, 1982
	LD_{50}, 24-h, i.p.	200	Baden *et al.*, 1982
	LD_{50}, 24-h, oral	6600	Baden *et al.*, 1982
PBX3	LD_{50}, 24-h, i.v.	94	Baden *et al.*, 1982
	LD_{50}, 24-h, i.p.	170	Baden *et al.*, 1982
	LD_{50}, 24-h, oral	520	Baden *et al.*, 1982
	LD_{50}, 24-h, i.p.	250	Selwood *et al.*, 2008
BTX-B2	LD_{50}, 24-h, i.p.	400	Selwood *et al.*, 2008
S-desoxy-BTX-B2	LD_{50}, 24-h, i.p.	211	Selwood *et al.*, 2008
BTX-B1	MLD[1], <2 h, i.p.	50	Ishida *et al.*, 1995
BTX-B2	MLD, <1 h, i.p.	306	Murata *et al.*, 1998
BTX-B3	MLD, 24 h, i.p.	>300[2]	Morohashi *et al.*, 1995
BTX-B4	MLD, 6–24 h, i.p.	100	Morohashi *et al.*, 1999
BTX-B5	MLD[3], i.p.	300–500	Ishida *et al.*, 2004

Notes: [1] Minimum lethal dose, [2] No deaths recorded at 300 µg/kg. [3] Time to death not reported. Adapted from Plakas and Dickey, 2010, and Dickey *et al.*, 2011.

4

Data gaps and recommendations for research

4.1 DATA GAPS FOR EACH GROUP FOR WHICH TEFS WERE CONSIDERED

4.1.1 STX

- No information available about pharmacokinetics of each analogue, and limited information on the oral toxicity of saxitoxin analogues, especially those that are commonly found in seafood or that are found only rarely but at relatively high levels.
- Need for further information of specific TEFs of all analogues in pure and certified form.
- Need for toxicity studies with benzoate derivatives.
- Need for TEFs for TTX analogues commonly found in bivalves.
- Need for comparative studies on the oral toxicity of tetrodotoxin congeners.

4.1.2 OA

- There is no comparative data on analogues in a single study by oral administration with the same methodology. These data are required.
- There is little information clarifying the links between phosphatase inhibition and the physiological mechanisms that regulate intestinal physiology.
- As recommended by the Joint FAO/IOC/WHO *ad hoc Expert Consultation* (CODEX, 2006), information on the chronic toxicity of DSPs is needed.

- Consideration should be given to the determination of TEFs for DSPs by diarrhetic activity.
- The recommended TEF of 1.0 for DTX1 should be verified in future studies to corroborate the observations in humans, also through more controlled studies *in vivo* (in animals).

4.1.3 Domoic Acid

- Based on the information on chronic and low-dose neurotoxic effects, especially related to exposure of prenatal and very young individuals, more information is needed.
- The relevance of potential endocrine effects needs to be further investigated.
- Chronic and low-dose effects, especially related to pregnancy and in very young individuals, and potential endocrine effects, need serious attention with regard to DA toxicity.
- Studies needed on the toxicity of DA isomers present in food.

4.1.4 AZA

- Comparative study of all analogues with the same methodology is needed, e.g. for AZA4 and –5, for which oral toxicity data are not currently available.
- Detailed study is needed of oral toxicity, with special attention to cardiotoxicity; and possible interaction with YTXs should be investigated.
- Define the exact mechanisms of chronic and acute toxicity of AZAs (for cardiotoxicity, and GI tract symptoms). The relevance of lung toxicity observed for the AZAs for humans is currently unclear.
- In view of the deaths seen after repeated oral dosing of AZA1 to mice at levels well below the LD_{50} (Ito *et al.*, 2002b) and the possible induction of lung cancer by this substance (Ito, 2008), a long-term feeding study, conducted according to OECD guidelines, should be conducted either with AZA1 itself or a mixture of AZAs of known composition. In view of the high spontaneous incidence of lung cancer in mice, rats should be employed in such an experiment. Rats are much less susceptible to spontaneous pulmonary carcinogenesis than mice (Munday, 2014).

4.2 Considerations for other biotoxins

4.2.1 The special case of tetrodotoxin (TTX)

TTX is a marine toxin of bacterial origin and is produced, amongst others, by *Pseudomonas* spp. and *Vibrio* spp. (Bane *et al.*, 2014). It is becoming a concern in Europe given its presence in gastropods (Rodriguez *et al.*, 2008; Silva *et al.*, 2012) and in shellfish (Turner *et al.*, 2015a; Vlamis *et al.*, 2015). Because TTX in shellfish is a newly discovered phenomenon, there is no surveillance programme in place *per se.* Nevertheless, monitoring programmes that employ MBA for STX would also detect the presence of TTX. While the MBA does not distinguish between

TTX and STX, analytical methods for these two toxins differ. Thus, TTX can be detected as a different chemical entity and a TEF could be applied.

While the mode of action is similar to STX, the main difference between both toxin groups is the subtypes of Na_v with which they bind preferentially. In the case of TTX, the Na_v 1.7 is the main target (Walker *et al.*, 2012; Alonso *et al.*, 2016), although TTX can bind with less affinity to other Na_v subtypes. TTX binds to human Na_v 1.7 with 38-fold more potency than STX (Walker *et al.*, 2012). As for STX, TTX may target other receptors but they are not critical to explain its toxicity (Guatimosim *et al.*, 2001).

The toxicity of TTX is high, with 2 mg being a lethal dose to humans (Noguchi, Onuki and Arakawa, 2011). A mouse unit is 0.2 µg, which kills a 20 g mouse in 30 minutes. Based on the i.p. toxicity to mice, relative toxicities of TTX analogues described in the literature are 10 µg/kg b.w. for TTX (Noguchi, Onuki and Arakawa, 2011), 70 µg/kg b.w. for 11-deoxy-TTX (Bane *et al.*, 2014), 420 µg/kg b.w. for 6,11-dideoxy TTX (Jang and Yotsu-Yamashita, 2007), 16 µg/kg b.w. for 11-oxo-TTX (Nakamura and Yasumoto, 1985; Yotsu-Yamashita *et al.*, 2003), 64 µg/kg b.w. for 4-epi-TTX (Nakamura and Yasumoto, 1985; Munday, 2014), 60 µg/kg b.w. for 6-epi-TTX (Yasumoto *et al.*, 1988b), 490 µg/kg b.w. for 4,9-Anhydro-TTX (Nakamura and Yasumoto, 1985), 54 µg/kg b.w. for 11-nor-TTX–6(S)-ol (Yotsu *et al.*, 2014), and 70 µg/kg b.w. for 11-nor-TTX–6(R)-ol (Endo *et al.*, 1988).

Since a mouse unit for STX is 0.183 µg (9.15 µg/kg b.w.) (Schantz *et al.*, 1958; AOAC, 2005), then the potency of STX is 10 percent higher than TTX. With these figures, it is possible to construct a basic TEF list for TTX compared with STX: 1 for STX; 1.1 for TTX; 7.7 for 11-deoxy-TTX; 46 for 6,11-dideoxy TTX; 1.7 for 11-oxo-TTX; 7 for 4-epi-TTX; 6.6 for 6-epi-TTX (Yasumoto *et al.*, 1988b); 53.6 for 4,9-Anhydro-TTX; 5.9 for 11-nor-TTX–6(S)-ol; and 7.6 for 11-nor-TTX–6(R)-ol. Further information is needed for each TTX derivative.

The relative potency of TTX analogues, as reported as IC50 in *in vitro systems, are 4.1 nM (frog muscle* (Yang, Kao and Oshima, 1992a)) or 5.2 nM (squid axon; Kao and Yasumoto, 1985a) for TTX; 13.2 nM for 4-epi TTX (Kao and Yasumoto, 1985a); 96 nM for 6-epi TTX (Yang, Kao and Oshima, 1992a); 445 nM for 11-deoxy TTX (Yang *et al.*, 1992a); and 298 nM for 4,9-anhydro TTX (Kao and Yasumoto, 1985a).

The lethality of TTX decreases with the route of administration, from 10 µg/kg b.w. i.p., to 16 µg/kg b.w. subcutaneous, and 332 µg/kg b.w. oral (Kao, 1966; Moczydlowski, 2013).

While, traditionally, TTX contamination has been associated with puffer fish, the occurrence in bivalves is emerging, and as such the Expert Group recommended that this should be monitored. The Expert Group also noted the:

- Need to establish TEFs for TTX analogues commonly found in bivalves.
- Need for comparative studies on the oral toxicity of tetrodotoxin congeners.

4.2.2 Yessotoxin and analogues

Yessotoxins (YTX) are a group of ladder shaped compounds produced by the dinoflagellate *Protoceratium reticulatum* (Satake, MacKenzie and Yasumoto, 1997a; Miles *et al.*, 2005), which were first isolated from digestive glands of scallops *Patinopecten yessoensis* in Japan (Murata *et al.*, 1987; Satake *et al.*, 1997b). Their chemical identification was first reported by Murata *et al.* (1987) in Japan, and several analogues were first identified from shellfish harvested in Italy (Ciminiello *et al.*, 1997; Satake *et al.*, 1997b), Norway (Lee *et al.*, 1988), New Zealand and the Adriatic Sea (for this reason one of the analogues was named adriatoxin (Ciminiello *et al.*, 1998)). They can also be produced by *Lingulodinium polyedrum* (Draisci *et al.*, 1999) or *Gonyaulax spinifera* (Pistocchi *et al.*, 2012), and in total there are about 90 chemical derivatives, very few of them being abundant in nature (yessotoxin, 1a-homoYTX, 45-hydroxyYTX, 45-hydroxy–1ahomoYTX, carboxy-YTX) (Miles *et al.*, 2004a, b; Finch *et al.*, 2005). They have been reported in coastal areas worlwide (Vlamis and Katikou, 2014). Although YTX is rather toxic to mice after i.p. injection, these compounds do not show clear signs of toxicity after oral administration, even at high doses (Munday *et al.*, 2008a; EFSA, 2008b) – they were identified as toxins mostly because of their effect on the mouse bioassay. Their toxicology is now better known (Aune *et al.*, 2002; Aasen *et al.*, 2011), they do not produce diarrhoea, and the scientific debate today is on whether or not they are capable of causing harm in humans (Tubaro *et al.*, 2003; Tubaro *et al.*, 2004; Munday, 2014).

The mechanism of action of YTX remains to be fully elucidated. However, like AZAs that may have several targets, YTX and analogues are compounds that target several apoptotic pathways (Korsnes *et al.*, 2011, 2013, 2014; Tobio *et al.*, 2012) and induce autophagy triggered by stress (Rubiolo *et al.*, 2014). Even though many effects are derived from this effect, the final effect of YTX is an apoptotic effect especially intense in tumour cell lines (which makes YTX a potential anticancer drug lead) (Botana *et al.*, 2014b), and a rather mild effect in non-tumour cell lines (Fernandez-Araujo *et al.*, 2015b). Although not the only target, phosphodiesterase 4A seems to play a key role in the mode of action of YTX (Alfonso *et al.*, 2003; Fernandez-Araujo *et al.*, 2015a).

The EFSA Panel proposed an acute reference dose (ARfD) of 25 µg YTX equivalents/kg b.w. (EFSA, 2008b), and the European Union has defined a toxin limit of 3.75 mg yessotoxin equivalent/kg shellfish meat (EU, 2013). This was imposed as a precautionary measure, because there has never been a report of YTX intoxication in humans. Yessotoxin is an exotoxin, released by the producing cells (Hess and Aasen, 2007a). Its ecological role is not known, and its inclusion in the list of marine toxins is due to the fact that it co-exists with phosphatase inhibitors (OA and dinophysistoxins) and causes mice death in the biological mouse bioassay (Aune *et al.*, 2002).

Since there are no reports of effects in humans, all actual toxicology comes from rodent experiments. The acute i.p. toxicity of YTX has been reported with a large variation, from 80 (Ogino, Kumagai and Yasumoto, 1997) to 750 µg/kg b.w. (Aasen *et al.*, 2011). Several LD_{50} studies in different mice strains and in both sexes (Aune *et al.*, 2008) show no discrepancies with regard to both parameters, for YTX and homoYTX (Tubaro *et al.*, 2003, 2004; Aune *et al.*, 2008; Munday, Aune and Rossini, 2008a). The reported i.p. LD_{50} ranges were: 80–100 µg/kg b.w. body weight (Ogino *et al.*, 1997; Munday, 2014); 112–136 µg/kg b.w. (Munday, Aune and Rossini, 2008a); 269–462 µg/kg b.w. (Aune *et al.*, 2008); 286 and 301 µg/kg b.w. for YTX and didesulfated YTX, respectively (Ogino, Kumagai and Yasumoto, 1997); 220 µg/kg b.w. for 45–46–47-Tri-nor-YTX (Satake *et al.*, 1996); 500–750 µg/kg b.w. (Aune *et al.*, 2002; Munday, 2014); and 444 and 530 µg/kg b.w. for homoYTX and YTX, respectively (Tubaro *et al.*, 2003). Some compounds were reported toxic by the mouse bioassay, with lethal doses of 500 µg/kg b.w. for 45-hydroxy-YTX (Satake *et al.*, 1996); carboxy-YTX (Ciminiello *et al.*, 2000a); carboxy-homo-YTX (Ciminiello *et al.*, 2000b); and 1-desulfo-YTX (Daiguji *et al.*, 1998a). There is no report of deaths after i.p. administration of other YTX analogues, unless at high concentrations (Munday, 2014).

Acute dosing experiments suggest that YTX is the most toxic of all analogues, and that it is a slow-acting compound, with death after injection showing a longer delay with lower doses, from 30 minutes at high doses to 10 hours at doses close to the LD_{50} (Munday, Aune and Rossini, 2008a). No macroscopic changes were observed in animals after YTX injection in some studies, but a few authors have reported some cases of cardiac oedema and vacuolization of cardiac muscle, although some controls reported similar changes (Aune *et al.*, 2002). At the same time, there are studies that have shown no such effect after lethal doses (Tubaro *et al.*, 2004). Electron microscopy studies revealed some alterations at a cardiac level (Terao *et al.*, 1990), with swollen endothelial cells in the left ventricle, rounded mitochondria, and separated bundles of myofibrils and sarcoplasmic reticulum (Aune *et al.*, 2002). Terao *et al.* (1990) reported fatty degeneration and pancreatic and hepatic alterations after administration of di-desulfo-YTX.

The administration of acute oral doses have provided consistent results in several studies (Ogino, Kumagai and Yasumoto, 1997; Aune *et al.*, 2002; Tubaro *et al.*, 2004). They all describe a complete lack of effects even at high doses, and most of the toxin is recovered from the lower intestine and faeces (Munday, Aune and Rossini, 2008a). Nevertheless, some structural changes were observed after high oral dosing (up to 10 mg/kg), such as heart intracellular oedema or swelling, or pericapillary myocytes, causing the separation of organelles (Aune *et al.*, 2002; Tubaro *et al.*, 2004), while no effect was reported at low doses (Terao *et al.*, 1990). The significance of these ultrastructural changes are dubious, as some of the changes are reported in control animals, and they are not dose-dependent (Tubaro *et al.*, 2010; Munday, 2014). A recent acute study in rats, where potential electrocardiogram alterations were studied after i.v. administration, showed that 10 µg/kg b.w. YTX (and also DTX1 or OA) did not modify the electrical function of the heart (Ferreiro *et al.*, 2015), and the acute effects do not modify cardiac biomarkers.

A recent subacute study performed in rats treated for 15 days with four doses of YTX i.p., one every 4 days (days 1, 5, 9 and 13), was designed to study the effect of YTX (doses of 50–100 µg/kg b.w.) on electrocardiogram, arterial blood pressure and cardiac biomarkers. The results show a clear bradycardia, hypotension, autophagia in the heart tissue, and changes in kidney function (Botana *et al.,* manuscript submitted); there was an ultrastructural cardiomyocyte alteration that was dose-dependent (observed in 1/1 rat treated with 100, in 6/7 rats treated with 70 and in 5/8 rats treated with 50 µg/kg). Therefore, it is clear that YTX may be cardiotoxic, but the relevance of this to oral administration remains to be confirmed.

Repeated oral studies in rodents show that yessotoxin does not induce damage in organs or any death for up to 7 days with 2 mg/kg/day, or even 5 mg/kg/day administered seven times in 21 days (Munday, 2014). Ultrastructural analysis of tissues 90 days after the treatment show no sign of damage, though Tubaro *et al.* (2008b) reported some clusters of rounded mitochondria and disorganization of myofibrils in the heart tissue after seven days of oral exposure to 1 mg/kg, which may last for 30 days but disappears after 90 days. Although very large doses of YTX (5 mg/kg) administered orally give rise to levels of 0.02 percent and 0.01 percent of the total dose in the liver and the kidney, respectively (Aasen *et al.*, 2011), there are no data on the kinetics of YTX excretion in urine, hence a pharmacokinetic model remains to be defined. It is therefore safe to conclude, based on the significant evidence, that YTX is not a toxic compound in mice when given orally, since even doses of 50 mg/kg are not lethal (Munday, 2014).

According to the few studies available, YTX showed no interaction with other co-existing toxins when YTX combines with AZAs (Aasen *et al.*, 2011) or phosphatase inhibitors (Sosa *et al.*, 2013).

4.2.3 Pectenotoxin

Pectenotoxins (PTXs) are macrocyclic polyethers produced by the genus *Dinophysis* (Miles, 2007; Munday, 2008; Vlamis and Katikou, 2014) and were first isolated from *Patinopecten yessoensis, a Japanese scallop* (Yasumoto *et al.*, 1985; Yasumoto, 2000). There are several known compounds, but only a few are common in seafood, mostly PTX2. However, PTX2 may be converted to PTX1, PTX3 or PTX6. The toxins are not very stable, and they are converted to seco acids or epi-metabolites under alkaline conditions (Sasaki *et al.,* 1997; Sasaki, Satake and Yasimoto, 1998a; Daiguji *et al.*, 1998b; James *et al.*, 1999). Although PTX2 is metabolized by most shellfish to a mixture of pectenotoxin–2 seco acid and 7-epi-pectenotoxin–2 seco acid (Miles *et al.*, 2006), PTX11 is not converted to its seco acid derivative (Suzuki *et al.*, 2006).

The mechanism of action of PTX is well known, as it binds to actin (Allingham, Miles and Rayment, 2007) and causes cytoskeleton disorganization due to F-actin depolymerization (Leira *et al.*, 2002; Ares *et al.*, 2005, 2007; Espina *et al.*, 2008b). Therefore, PTXs are apoptotic *in vitro* (Leira *et al.*, 2002; Espina and Rubiolo, 2008a).

Since PTXs are produced by the same organism that produces OA and analogues, there were initial studies that reported a diarrhetic effect of PTXs, but this was later proved to be wrong (Miles *et al.*, 2004c). PTX2 is not toxic in rodent when given orally even at high doses (5 mg/kg) (Miles *et al.*, 2004c). There are no reports of human intoxication by PTXs. Other PTXs were proven to lack diarrhetic effect (Suzuki *et al.*, 2006; Ito *et al.*, 2008).

In a similar way to YTX, when given parenterally they cause death in mice. The administration of PTXs, even i.p., shows a large excretion in the urine and in the faeces, which suggest a large biliary excretion (Munday, 2014). At the same time, oral absorption is low (Ito *et al.*, 2008; Munday, 2008).

In general, where the presence of contamination with OA has been ruled out, indications are that PTXs are not toxic orally. The i.p. lethal doses reported show a lethal dose of 250 µg/kg b.w. for PTX1 (Yasumoto *et al.*, 1985); 219–411 µg/kg b.w. for PTX2 (Yasumoto *et al.*, 1985; Miles *et al.*, 2004c); 350 µg/kg b.w. for PTX3 (Murata *et al.*, 1986); 770 µg/kg b.w. for PTX4 (Yasumoto *et al.*, 1988a); 500 µg/kg b.w. for PTX6 (Ito *et al.*, 2008); and 244 µg/kg b.w. for PTX11 (Suzuki *et al.*, 2006); with all other analogues having no lethality, even at high doses.

Treatment of 7*R-PTXs (PTX1, –2, –3, –6 a*nd –11) under acidic conditions (e.g. Trifuoroacetic acid (TFA), pH 2) leads to an equilibrium mixture of spiroketal stereoisomers, 7R-, 7S-, and 6-membered-B-ring-isomers (Sasaki *et al.*, 1998b; Suzuki *et al.*, 2003). PTX2 is completely degraded under alkaline conditions such as those used in hydrolysis of OA, e.g. pH 11, NaOH, 40 minutes at 76°C (EURL, 2015).

PTX2 in bivalves is absorbed from algae and metabolized by two processes. In Japanese scallops, *P. yessoensis, PTX2 undergoes step-wise oxidation of the methyl group attached to C–18* (Suzuki *et al.*, 1998). Thus, the 18-methyl group in PTX2 is oxidised to an alcohol (PTX1), aldehyde (PTX3), and finally a carboxylic acid (PTX6) group. In many bivalve species, a different process has been demonstrated whereby the lactone moiety of PTX2 undergoes rapid enzymatic hydrolysis to PTX2sa (Suzuki *et al.*, 2001). PTX11 is not enzymatically converted to its seco acid (Suzuki *et al.*, 2006).

PTX2sa has also been reported to form fatty acid esters in shellfish (Wilkins *et al.*, 2006).

4.2.4 Palytoxin and analogues

Palytoxin was isolated from *Palythoa species* (Moore and Scheuer, 1971). It is a unique compound due to its large molecular weight and extreme acute i.p. toxicity (Katikou and Vlamis, 2014), although by voluntary feeding it is not as toxic (below 2500 µg/kg) (Munday, 2014). This compound group is receiving attention lately due to its reported presence in bivalves harvested in Europe (Aligizaki *et al.*, 2008), although an intoxication has never been reported in humans that was linked to consumption of bivalves. A fatal human case has been reported with consumption of contaminated crab in Philippines (Alcala *et al., 1988)* and with consumption of sardines in Madagascar (Onuma *et al.*, 1999). Several analogues of palytoxins produced by *Ostreopsis* (ovatoxins, ostreocins) were reported to cause respiratory problems on Mediterranean beaches due to aerosols (Ciminiello *et al.*, 2006, 2014), but this has not been linked to seafood. All the analogues share the same mode of action: the inhibition of the Na^+-K^+ pump (Botana, Vale and Vilariño, 2007). The toxicological relevance of this group in bivalves remains to be determined.

4.2.5 Cyclic imines

This is a diverse group of compounds, which include spirolides, gymnodimines, pinnatoxins and pteriatoxins (Molgó *et al.*, 2014), which are produced by the genera *Alexandrium, Prorocentrum, Karenia* and *Vulcanodinium.* They target the nicotinic receptors (Bourne *et al.*, 2010; Araoz *et al.*, 2011), and to date there is no clear evidence of their potential oral toxicity in either animals or humans (Munday, 2014), although they are orally absorbed and are distributed to the central nervous system (Alonso *et al.*, 2011; Otero *et al.*, 2012).

5

Recommendations for risk managers

1. In providing recommendations to risk managers, the Expert Group felt that it would be useful to offer some guidance on how to apply TEFs to calculate total potency in a given sample. The following hypothetical example of levels of the STX group in a bivalve are given. The toxin analogues have been converted to STX equivalents using TEFs. The last column indicates the total toxin level in STX equivalent. The Codex standard is 80 µg/100 g (0.8 mg/kg). Based on such a result, risk managers can decide whether the sample in question meets the Codex Standard.

Toxin analogue	Level (µg/100 g)	TEF	Level in saxitoxin equivalents (µg/100 g)
Saxitoxin	10	1.0	10
Neosaxitoxin	5	2.0	10
GTX1	15	1.0	15
GTX4	15	0.7	10.5
C1	5	0.01	0.05
C2	5	0.1	0.5
Total			**46.05**

2. The Expert Group recommended that the proposed TEF values be reviewed and re-evaluated in a 5–10-year time-frame, reflecting new data that might be available. This process could be facilitated through the establishment of a database on TEF-related data at FAO/WHO.

3. In view of the evolving geographical distribution of marine biotoxins, the Expert Group recommended that national toxin monitoring programmes be updated and expanded to include new and emerging toxins. Also, monitoring should occur during shellfish production.

4. The Expert Group recommended that the analytical methods and monitoring strategies be strengthened and expanded to include the different range of biotoxin analogues.

5. The Expert Group recommended that if methods cannot separate some of the analogues, highest TEF should be used to provide maximum consumer protection.

5.1 TEFS RECOMMENDED FOR EACH BIOTOXIN GROUP BY THE EXPERT GROUP

Saxitoxins

Compound	Oshima Relative Toxicity values (MU/ µmole)	Mouse LD_{50} (i.p.)	TEF based on LD_{50} by gavage	TEF based on LD_{50} by voluntary consumption	EFSA proposed TEF	Recommended TEF	Rationale
Saxitoxin	1	1.00	1.00	1.00	1.0	1.0	
NeoSTX	0.92	3.12	1.70	2.54	1.0	2.0	Both oral studies support higher toxicity than STX. A value of 2.0 is recommended and supported by some Na channel *in vitro* results (range of REP 0.7–3.7).
GTX1	0.99				1.0	1.0	No new data [1]
GTX2	0.36				0.4	0.4	No new data
GTX3	0.64				0.6	0.6	No new data
GTX4	0.73				0.7	0.7	No new data
GTX5	0.064	0.222	0.063	0.050	0.1	0.1	Relative potency values from oral LD_{50} studies suggest a lower TEF than from LD_{50} i.p. 0.1 TEF also in agreement with original Oshima TEF. As with NeoSTX, a number of *in vitro* Na channel assays also support a TEF of 0.1.
GTX6		0.122	0.038		0.1	0.05	New oral data show less than 0.1.
C1	0.006					0.01	No new data (rounded up) [2]
C2	0.096				0.1	0.1	No new data
C3	0.013					0.01	No new data
C4	0.058				0.1	0.1	No new data
dcSTX	0.51	0.785	0.457	0.368	1.0	0.5	From recent oral data (more weight on oral data, also supported by i.p. toxicity data)
dcNeoSTX		0.058	0.216	0.224	0.4	0.2	From recent oral data (more weight on oral data, also supported by *in vitro* data)
dcGTX2	0.15				0.2	0.2	No new data
dcGTX3	0.38				0.4	0.4	No new data

Notes: [1] In the case of saxitoxin analogues, for which no oral toxicity data were available, TEFs recommended are based on intraperitoneal toxicity data of Oshima (Appendix I). [2] When TEFs are less than 0.1, increments of 0.05 are used.

OA

	TEF based on cytotoxicity	TEF based on PP2A inhibition	TEF based on membrane Paracellular permeability	EFSA proposed TEF	Recommended TEF	Rationale
OA	1.0	1.0	1.0	1.0	1.0	
DTX1	3.1	1.6	2–15	1.0	1.0	In the case of the OA group of toxins, several case reports from human intoxication are available, and these reports are analogue specific. As outlined in the recent risk assessment by EFSA, a human poisoning event with DTX1 as main contaminant in Japan suggested a LOAEL of 48 µg DTX1 per person. This level is equivalent to an average of 50 µg OA per person in poisoning events in Sweden, Norway, UK and Portugal. Thus, it is not surprising that the currently used TEF of 1.0 in some countries appears protective for public health. Still, it should be noted that multiple *in vitro* studies suggest that the intrinsic potency of DTX1 could be higher than that of OA. However, large uncertainty is associated with these studies (a factor of approximately 5-fold difference between results depending on the cell line used). Therefore, the recommended TEF of 1.0 for DTX1 should be verified in future studies to corroborate the observations in humans also through more controlled studies *in vivo* (in animals).
DTX2	0.52	0.5	0.6	0.6	0.5	Consistent among the different assays; based on acute i.p. toxicity in mice, DTX2 is on average 0.5 times as toxic as DTX1). This value is also supported by the various *in vitro* data
DTX3						Explanation provided in main text as to why no TEF is recommended.

Domoic Acid

	EFSA proposed TEF	Recommended TEF
Domoic Acid (two epimers)	–	1.0

Notes: Domoic acid consists of two epimers, The same TEF of 1 should be applied. The sum should be reported.

AZAs

	TEF based on i.p. toxicity	TEF based on oral toxicity	EFSA proposed TEF	Recommended TEF	Rationale
AZA 1	1.0	1.0	1	1.0	
AZA 2	0.6	0.7	1.8	0.7	Based on recent oral data. (also consistent with recent i.p. data)
AZA 3	0.45	0.51	1.4	0.5	Based on recent oral data. (also consistent with recent i.p. data)
AZA 4			-		Cannot determine TEF due to lack of data.
AZA 5			-		Cannot determine TEF due to lack of data.
AZA 6	0.7		-	0.7	No oral data; based on recent i.p. data.

6

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ANNEX 1

OSHIMA RELATIVE TOXICITY[4]

TABLE 1. Specific i.p. toxicities of saxitoxin (STX) analogues

Toxin	Specific toxicity (MU/μmole)	Relative toxicity
STX	2 483	1
neoSTX	2 295	0.92
GTX1	2 468	0.99
GTX2	892	0.36
GTX3	1 584	0.64
GTX4	1 803	0.73
dcSTX	1 274	0.51
dcGTX2	382*	0.15
dcGTX3	935*	0.38
B1 (GTX5)	160	0.064
C1	15	0.006
C2	239	0.096
C3**	33	0.013
C4**	143	0.058

* After re-examination.

** Estimated by the measurement of GTX1, GTX4 formed by acid hydrolysis.

Source: Oshima, 1995b.

[4] Assessment and management of biotoxin risks in bivalve molluscs. 2011. FAO Fisheries and Aquaculture Technical Paper 551.

Shellfish can be contaminated with toxic substances derived from marine microalgae. In order to protect consumers from poisoning by these toxins, regulatory authorities have defined limits as to the maximum amount of such toxins in shellfish intended for human consumption, and analyses must be conducted in order to ensure that such limits are not exceeded.

Several groups of shellfish toxins have been described, such as the paralytic, diarrhetic and amnesic toxins. Within each group, several structurally-related toxins (cogeners) may be present that contribute to the overall toxic potential. Based on their chemical structure, the toxins included in the Codex Standard for Live and Raw Bivalve Molluscs (Codex Standard 292–2008) are classified into five groups, namely, the saxitoxin (STX), okadaic acid (OA), domoicacid (DA), brevetoxin (BTX) and azaspiracid (AZA) groups.

For risk assessment and management, knowledge of the amount of toxin congeners in the shellfish is not sufficient. There is also the need to know the relative toxicity of each of the congeners, so that the total toxicity of the material in the extract can be estimated. This requires the determination of Toxicity Equivalency Factors (TEFs).

The 34th Session of the Codex Committee on Fish and Fishery Products (CCFFP)requested FAO/WHO to provide scientific advice on TEFs for biotoxins included in the Codex Standard. FAO/WHO set up an Expert Group to provide scientific advice and recommendations on TEFs for biotoxins. The Expert Group developed an approach to be used for the development of TEFs for each group of biotoxins included in the Codex Standard, and in addition considered tetrodotoxin (TTX), due to its emergence in shellfish.

The Joint FAO/WHO Expert Meeting on Toxicity Equivalency Factors for Marine Biotoxins was convened in Rome on 22–24 February 2016. This report summarizes the outcomes of the meeting and identifies the TEFs for each of the six groups of toxins.

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