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Derivation of toxicity equivalency factors for marine biotoxins associated with Bivalve Molluscs

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ABSTRACT

Background: Seafood toxins pose an important risk to human health, and maximum levels were imposed by regulatory authorities throughout the world. Several toxin groups are known, each one with many analogues of the major toxin. Regulatory limits are set to ensure that commercially available seafood is not contaminated with unsafe levels.

Scope and approach: The mouse bioassay was used to measure the toxicity in seafood extracts to determine if a sample exceeded regulatory limits. The advantage of this approach was to provide an estimation of the total toxicity in the sample. As instrumental methods of analysis advance and serve as replacements to the mouse bioassay, the challenge is translating individual toxin concentrations into toxicity to determine whether regulatory limits have been exceeded. Such analyses provide accurate quantitation of the toxin analogues, by they have widely dissimilar potencies. Thus, knowledge of the relative toxicities is required for risk assessment and determining overall toxicity. The ratios between the toxicity of the analogues and that of a reference compound within the same toxin group are termed "Toxicity Equivalency Factors" (TEFs).

Key findings and conclusions: In this document, the requirements for determining TEFs of toxin analogues are described, and recommendations for research to further refine TEFs are identified. The proposed TEFs herein, when applied to toxin analogue concentrations determined using analytical methods, will provide a base to determine overall toxicity, thereby protecting human health.

1. Introduction

Bivalve molluscs may be contaminated with marine biotoxins produced by microalgae and these toxins are an important cause of seafood intoxications, with symptoms that vary from mild diarrhoea to permanent neuropathy or death. Their presence is expanding worldwide, for reasons that are not fully understood, but appear to be linked to climate change, eutrophication and international trade (Hallegraef, 2015).

The limits for marine biotoxins for international trade are set by

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the CODEX Committee on Fish and Fishery Products (CCFFP), that has developed the Standard for Live and Raw Bivalve Molluscs (Codex, 2008). This Standard identifies maximum levels in mollusc flesh for 5 toxin groups, saxitoxin (STX), <0.8 mg/STX equivalents (eq.)/kg, okadaic acid (OA), <0.16 mg/OA eq./kg, domoic acid (DA), 20 mg/kg, brevetoxin (BTX), 200 mouse units/or eq./kg, and azaspiracid (AZA), 0.16 mg/kg. Each group of seafood toxins is comprised of many analogues of the major toxin, yet the regulatory levels are represented according to the total toxicity of the analogues. Traditionally regulatory limits were assessed using the mouse bioassay (MBA), which involves the intraperitoneal injection of seafood extracts (AOAC, 2005a; T. Yasumoto, Murata, Oshima, Matsumoto, & Glardy, 1984; T. Yasumoto, Oshima, & Yamaguchi, 1978b). The advantage of the MBA is that it provides an estimate of the total toxicity of the sample. Instrumental analytical approaches are becoming available as alternatives to the MBA; such methods include liquid chromatography with ultraviolet, fluorescence or mass spectrometric detection (AOAC, 2005b; EU, 2011; These, Klemm, Nausch, & Uhlig, 2011). These methods permit the quantitation of toxin analogues when compared to a certified standard of the toxin (Antelo, Alfonso, & Alvarez, 2014).

Quantitation of the toxin analogues is not, however, sufficient for monitoring and regulatory decision making, since the different analogues may have widely dissimilar toxic potencies. For such assessment, it is necessary to know the relative toxicities of the components of the toxin mixture. These are termed "Toxicity Equivalency Factors" (TEFs), which are 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 (Botana et al., 2010; Van den Berg et al., 2006).

Accurate TEFs are essential for the monitoring and control of regulatory limits set for groups of related compounds. The 34th Session of CODEX Committee on Methods of Analysis and Sampling (CCMAS) encouraged CCFFP to investigate TEFs for the marine biotoxins listed in the Standard. For this purpose, an Expert Group was created by Food and Agricultural Organization (FAO) and World Health Organization (WHO) to elaborate and propose a list of TEFs for each toxin group for which limits are recommended in the Codex standards for Live and Raw Bivalve Molluscs.

An additional toxin group, tetrodotoxin (TTX), was also considered given its reported presence in shellfish (A. D. Turner, McNabb, Harwood, Selwood, & Boundy, 2015; Vlamis et al., 2015). While TTXs are not specifically mentioned in the CODEX standard, they have the same mode of action as STXs and can be grouped along with the PSTs.

2. Deriving TEFs

The calculation of the amounts of different substances, sharing the same mechanism of action, into the equivalent value for a single compound is a complex process. It requires an understanding of both the mechanism of action of the toxins, and how this mechanism translates into toxicity. In many cases, such an understanding is not available, as with OA and its analogues, the dinophysistoxins (DTXs). This toxin group, referred to as DSTs (diarrhetic shellfish toxins) has been known for many years (T. Yasumoto et al., 1978b). Their toxicity has been suggested to result from inhibition of protein phosphatases, particularly PP2A (Bialojan & Takai, 1988), thereby disrupting duodenal paracellular permeability due to alterations of tight junction integrity (Tripuraneni, Koutsouris, Pestic, De Lanerolle, & Hecht, 1997). However, recent research results call into question both the target (Espina et al., 2010; Wang et al., 2012) and the mechanism of toxicity of this group (Munday, 2013).

The Expert Group agreed on an approach for establishing TEFs which is summarized in Fig. 1. With respect to the relevance of toxicity data in the derivation of TEFs the following order of priority was agreed:

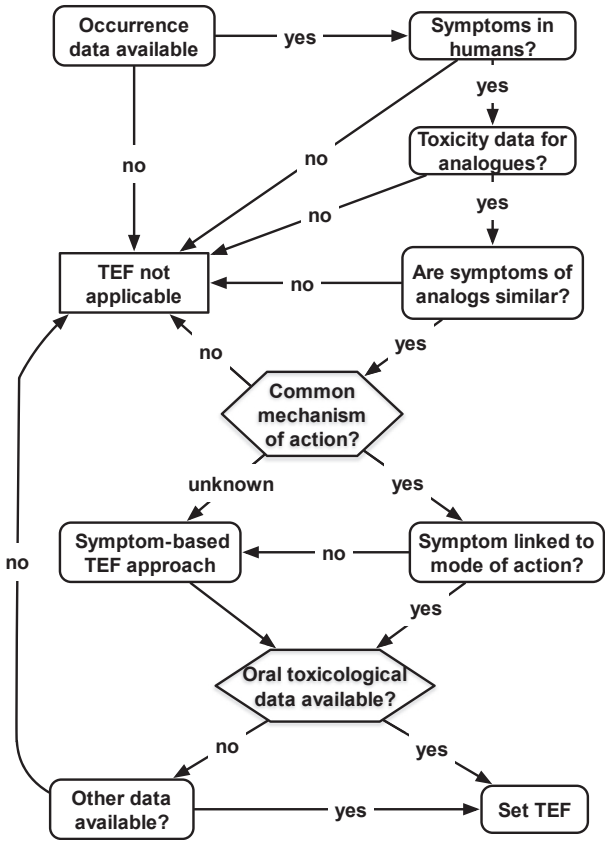

Fig. 1. Scheme of decisions to define and apply a TEF.

1. Data from human intoxications, the most relevant data for the human situation.
   2, Acute toxicity data through oral administration to animals, relevant to the route of human exposure.
3. Acute toxicity data through intraperitoneal (i.p.) administration to animals is less valuable, since this is less relevant for the route of human exposure. It should also be noted that there is no correlation between LD_{50} values obtained by i.p. injection and those by oral administration.
4. *In vitro* data. Such data are particularly useful when the mechanism of action of the toxin is known, and the *in vitro* test system is relevant to this mechanism.

For those toxins with no clearly defined mode of action, with several known targets, such as AZAs (Botana et al., 2014) or with no reported lethal effect in humans, such as DSTs (EFSA, 2008c), the data reported in the literature may be confusing. While values for an LD_{50}, a minimum lethal dose (MLD) or the non-specific term "lethality" have been reported (Munday, 2014). It is of little use to define a TEF for humans based on the dose of AZA that kills a mouse. Therefore, the toxic potency of AZAs in humans is somewhat biased by reference to effects in rodents, although there is presently no other way to quantify them. Another important bias is the lack of information on the chronic effect of toxins that cause death after repeated sub-lethal doses (Ferreiro et al., 2016b), and which may also be toxic through the long-term ingestion of non-lethal amounts, such as described for DA (Truelove, Mueller,

Pulido, & Iverson, 1996; Vieira et al., 2015).

The approach applied by the Expert Group to establish TEFs is summarized in Fig. 1. Table 1 lists the uncertainties associated to TEF definitions for each toxin group.

3. Saxitoxin group

This group of toxin analogues has saxitoxin (STX) as the reference compound, and they share a common structure of tetrahydropurine. These toxins are mainly produced by dinoflagellates of the genus *Alexandrium*, but *Pyrodinium* and *Gymnodinium* are also potential sources (Wiese, D'Agostino, Mihali, Moffitt, & Neilan, 2010). More than 50 compounds have been reported (Wiese et al., 2010) and at least 18 have been demonstrated to have toxicological relevance. They are soluble in water and thermostable at acidic pH; at alkaline pH they are quickly degraded (Kodama & Sato, 2008).

STX and analogues exert their toxic effects in animals by binding to the voltage-gated sodium channel (Na_v) (Payandeh, Scheuer, Zheng, & Catterall, 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, & 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 that are the targets for many toxins, including several phycotoxin groups. Site 1 is the receptor for TTX and STXs and site 5 is the receptor for ciguatoxins (CTXs) and BTXs (Hartshorne & Catterall, 1981). The major molecular mechanism of toxicity of both TTX and STX is to block the channel pore, thereby 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.

Sommer and Mayer reported a quantitative MBA for STX (Sommer & Meyer, 1937), which is based on the dose-death time relationship in mice dosed intraperitoneally with this toxin. This MBA, which is now an approved AOAC method (Hungerford, 1995), has been widely used for comparing the toxicity of STX analogues (Oshima, 1995). The assumption is that the dose-death time relationship is the same for all analogues, yet that is not case, (Munday, Thomas, Gibbs, Murphy, & Quilliam, 2013), which calls into question the validity of this assay for the calculation of TEFs.

Table 2 shows the relative potencies determined by MBA as presented in the scientific literature. There is a correlation between the relative specific activity and relative toxicity by ip. injection with some STX derivatives; however, with NeoSTX, GTX 1&4 and dcGTX 2&3, there is no such correlation. This is attributable to differences in the dose-death time relationship (Munday et al., 2013). The differences among the values shown in Table 2 in many cases most likely reflect the use of impure compounds, although the estimates for NeoSTX reported (Munday et al., 2013) and (Vale, Alfonso, et al., 2008), using certified toxins, are significantly different. As certified STX analogues became available, a list of relative potency was proposed by the European Food Safety Authority (EFSA) based on the effect of certified toxins on neuronal cultures and on MBA (EFSA, 2009). These values were reevaluated using oral administration (gavage or feeding) (Munday et al., 2013). In some cases, the results were similar to those obtained through i.p. administration, but differences were observed for other congeners. The TEF for dcSTX was 0.64 in the MBA and 0.785 by the i.p. route compared to 0.37 by feeding and 0.46 by gavage. The TEF for dcNeoSTX was 0.4 in the MBA and 0.058 by i.p. injection compared to 0.22 by both gavage and feeding. Importantly, the TEF for the oral toxicity of NeoSTX was higher (1.7 by gavage, 2.5 by feeding) compared to 1.16 by i.p. injection. The TEF for the oral toxicity of NeoSTX was (1.7 by gavage, 2.5 by feeding) compared to 1.16 in the MBA and 3.12 by i.p. injection.

There are *in vitro* methods that compare the effects of STX with its congeners, as shown in Table 3. The EFSA TEFs for GTX-1&4, GTX-2&3 and C1,2 are consistent with those determined by oral administration. In contrast, the TEF for NeoSTX was significantly higher than that proposed by EFSA, while the proposed TEFs for GTX5, GTX6, 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 TEF of 0.8 (Vale, Alfonso, et al., 2008), 0.64 (Munday et al., 2013), 0.478 (Suzuki & Machii, 2014) and 0.37 (Suzuki & Machii, 2014), and NeoSTX from 1 (Alonso, Alfonso, Vieytes, & Botana, 2016; EFSA, 2009) to 2.54 (Munday et al., 2013). It is interesting to note that there is a better match between the results obtained with Na_v subtype 1.2 channel blockage (Alonso et al., 2016) and with oral administration to mice (Munday et al., 2013).

4. Okadaic acid group

This group of toxins has OA as the reference compound. OA was originally isolated from the sponge *Halichondria okadaii* (Tachibana et al., 1981) and linked to diarrhetic shellfish poisoning (DSP) (T. Yasumoto, Oshima, & Yamaguchi, 1978a) through dinophysistoxin-1 (DTX1), produced by *Dinophysis fortii*. Dinophysistoxin-2 (DTX2)

Table 1
Uncertainties in the definition of TEFs, from high (+++) to low (−) or no relevance if complete information is not available.

Toxin group	Mode of action (to explain toxicity)	Animal data relevant to human effect	Known potency of each analogue	Chronic toxicity information available
STX[a]	−	−	++	−
OA[b]	++	++	−	++
DA[c]	−	+	−	+++
BTX[d]	+	+	+	+
AZA[e]	+++	++	+	++

[a] Most of the required information is available for common toxins, but new toxins, such as benzoate derivatives, and dcSTX or NeoSTX require further research about their potency. Benzoate derivatives toxicity and an enhanced understanding of the pharmacokinetics of the group are also needed.

[b] Analogues that lack phosphatase inhibition have potent cellular effects (Espina et al., 2010), therefore the mechanism for diarrhoea needs to be understood (Louzao et al., 2015; Munday, 2013). Other phosphatase inhibitors do not show a diarrhetic effect. This suggests other factors are involved in the mechanism of toxicity, i.e. neuropeptide Y inhibition (Louzao et al., 2015). No damage to the mucosa was observed while severe diarrhoea was induced (Vieira et al., 2013). Oral studies with the same methodology are also required.

[c] Target is well known (Hogberg & Bal-Price, 2011), but long term effects are unclear with regard to endocrine (Crespo et al., 2015), cardiotoxic (Vieira et al., 2016; Vranyac-Tramoundanas, Harrison, Sawant, Kerr, & Sammut, 2011), or prenatal toxicity (Levin, Pizarro, Pang, Harrison, & Ramsdell, 2005).

[d] Several aspects of toxicity needs further investigation, such as effects on smooth muscle mediated by the autonomic nervous system, cardiotoxicity, or body temperature (Abraham et al., 2005; Berman & Murray, 2000; Gordon, Kimm-Brinson, Padnos, & Ramsdell, 2001).

[e] Several target candidates, but no identified mode of action (Botana et al., 2014; Twiner, Doucette, et al., 2012; Vilarino et al., 2007). Many compounds without mechanistic studies (Marine-Institute, 2014). Unclear long term toxicity (EFSA, 2008a; Ferreiro et al., 2016b).

Table 2
Relative toxicity 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, 0.1[5]	0.222
GTX-6	0.10, <0.1[5]	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	
TTX[6]	10 μg/Kg (i.p. lethality)	1 (compared to TTX)
11-deoxy-TTX	70	0.142
6,11-dideoxy TTX	420	0.023
11-oxo-TTX	16	0.625
4-epi-TTX	64	0.156
6-epi-TTX	60	0.166
4,9-Anhydro-TTX	490	0.020
11-nor-TTX-6(S)-ol	54	0.185
11-nor-TTX-6(R)-ol	70	0.143

Data are taken from Table 13 of the 2009 ESFA report on saxitoxin group toxins (EFSA, 2009) and modified as indicated by superscript numerals.
1. (Munday et al., 2013). 2. (Vale, Alfonso, et al., 2008). 3. (Suzuki & Machii, 2014). 4. Munday, unpublished results. 5 (Watanabe, Suzuki, & Oshima, 2010). A mouse unit for STX is 0.183 μg (9.15 μg/kg b.w.) (AOAC, 2005a; Schantz, McFarren, Schafer, & Lewis, 1958), and the potency of STX is 10 percent higher than TTX. 5. A basic TEF list for TTX, compared to STX (T. Yasumoto, Yotsu-Yamashita, Murata, & Naoki, 1988). Further information is needed for each TTX derivative. 6. From FAO/WHO (2016). Technical paper on Toxicity Equivalency Factors for Marine Biotoxins Associated with Bivalve Molluscs. Rome.108 pp.

was discovered as a third main analogue (Hu et al., 1992) in Irish mussels associated with diarrhetic episodes. OA and DTXs are produced by *Dinophysis* and *Prorocentrum* species.

OA is a polyether characterised by a carboxylic acid group and three spiro-keto ring assemblies, one of which connects a five with a six-membered ring. OA, DTX1 and DTX2 withstand a wide pH and temperature range in methanolic NaOH solution. Strong mineral acids cause their rapid degradation in 20 min at 76 °C even with shellfish matrix in the extract (T. Yasumoto, Murata, Oshima, & Sano, 1985), but food itself can buffer the acid and the toxins may be stable in the stomach after a meal (Alfonso et al., 2008).

There are different types of esters of OA and DTXs. They are all fatty acid esters (palmitic being the most common) of OA, DTX1 and DTX2, of variable chain length and referred to as DTX3. 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 overall toxic potential of shellfish samples. All of the esters are quantitatively cleaved through treatment with strong base, e.g. 0.3 M methanolic NaOH at 76 °C for 10–40 min (Marr, Hu, Pleasance, Quilliam, & Wright, 1992).

The target of OA and analogues is suggested to be protein phosphatases (PP), especially PP2A (ID_{50} 1,2 nM) and, as secondary targets, PP1 (ID_{50} 315 nM) and PP2B (ID_{50} 4530 nM) (Bialojan & Takai, 1988; Takai, Bialojan, Troschka, & Ruegg, 1987). Table 4 shows the intraperitoneal (i.p.) and *in vitro* (i.v.) toxicities of this group of compounds. There is remarkable consistency among the cell lines tested, with DTX-1 showing a 2–4 fold higher activity than OA, and DTX-2 showing less toxicity than OA, by a factor of between 0.35 and 0.73.

DTX-1 shows similar toxicity in mice when administered either intraperitoneally or by oral administration, with fluid accumulation in the gastrointestinal tract of mice dosed with DTX1 at 0.4 and 0.32 μg/mouse for OA and DTX1, respectively (Tubaro, Sosa, Bornacin, & Jungerford, 2008). The lethal dose of DTX1 by oral administration has been reported as below 300 μg/kg b.w. (all animals dead) in fasted animals (Munday, 2014; Ogino, Kumagai, & Yasumoto, 1997), while other studies reported no deaths in mice or rats given DTX-1 orally at 750 mg/kg b.w. (Ito & Terao, 1994; Terao, Ito, Ohkusu, & Yasumoto, 1993). No published reports regarding the oral toxicity of DTX2 are available, although a work not yet published (Louzao, *pers comm.*) has concluded that the oral LD_{50} is 2150 μg/kg b.w. (death at 24 h, mice fasted for 12 h, administration by gavage) and that the LD_{100} is 3000 μg/kg b.w., all animals dying in less than 5 h. No damage to the GI tract mucosa was observed in this study. Although the toxicity of DTX-1 by gavage appears to be higher than that of OA, the variability among published values precludes an estimate of TEFs based on oral toxicity. A recent study on the cardiotoxic effects of OA (20 μg/kg b.w.) and DTX1 (16 μg/kg) in rats showed no cardiotoxic effects of these compounds in acute experiments as assessed either by the electrocardiogram or by biomarkers (Ferreiro et al., 2015). The TEFs recommended by the Expert group for OA group are indicated in Table 6.

5. Azaspiracid group

This group of toxins has AZA1 as the reference compound. The

Table 3
Relative toxicities of STX and derivatives in mice through oral administration (gavage/voluntary feeding) and relative activities toward sodium channels *in vitro*.

Compound	Relative toxicity by voluntary feeding/ gavage *	Relative activity toward sodium channels *in vitro* by type of assay method[1]								
		1 squid axon	2 rat cortex	3 frog sciatic nerve	4 frog muscle fibre	5 rat muscle	6 cultured neurons	7 cerebellar neurons	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				

Note 1 - Assay methods:
* Relative toxicity by voluntary feeding/gavage (Munday et al., 2013).
1: Relative blockade of sodium channels in the squid giant axon (Kao et al., 1985).
2: Relative binding to sodium receptors of the rat cerebral cortex (Llewellyn, 2006; Usup, Leaw, Cheah, Ahmad, & Ng, 2004).
3: Relative blockade of impulses in frog sciatic nerve (Strichartz, 1984).
4: Relative blockade of sodium current in frog skeletal muscle fibre (Kao & Walker, 1982; Yang, Kao, & Oshima, 1992).
5: Relative blockade of sodium channels from rat muscle plasma membrane (Guo et al., 1987; E.; Moczydlowski, Hall, Garber, Strichartz, & Miller, 1984).
6: Blockade of veratridine-induced changes in membrane potential in cultured neurons (Vale, Alfonso, et al., 2008).
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).

Table 4
Toxicities of OA and its analogues by i.p. injection (Munday, 2014). Large discrepancies are most likely due to the use of non-certified calibrants.

Compound	Parameter	Acute toxicity (μg/kg b.w.)
OA	LD_{50}	192 (Tachibana et al., 1981)
OA	LD_{50}	200 (pers. comm. T. Yasumoto, 1991)
OA	No death	200 (Ito & Terao, 1994)
OA	LD_{50}	204 (Aune et al., 2012)
OA	LD_{50}	210 (Dickey, Bobzin, Faulkner, Bencsath, & Andrzejewski, 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 & Terao, 1994)
DTX1	MLD	160 (Murata, Shimatani, Sugitani, Oshima, & Yasumoto, 1982; T; Yasumoto & Murata, 1990)
DTX1	LD_{50}	160 (pers. comm. T. Yasumoto, 1991) (Dominguez et al., 2010)
DTX1	LD_{100}	375 (Ito & Terao, 1994)
DTX2	LD_{50}	352 (Aune et al., 2007)
DTX3	LD_{100}	375 (Ito & Terao, 1994)
DTX3	MLD	500 (T. Yasumoto et al., 1985)
DTX4	LD_{50}	610 (Hu, Curtis, Walter, & Wright, 1995)

In vitro cell toxicities of OA, DTX-1 and DTX-2

Compound	Relative toxicity in the specified cell line					
	SH-SY5Y (Louzao et al., 2015)	Neuro-2a (Solino, Sureda, & Diogene, 2015)	NG108-15 (Solino et al., 2015)	MCF-7 (Solino et al., 2015)	Caco-2 (Ferron, Hogeveen, Fessard, & Le Hegarat, 2014)	HT29-MTX (Ferron et al., 2014)
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

first intoxication by AZAs was recognised in 1996 (McMahon, 1996). These compounds are produced by the genera *Azadinium* and *Amphidoma* (Krock et al., 2012; Tillmann, Salas, Jauffrais, Hess, & Silke, 2014). Their structure is unusual in that they have a unique spiro ring assembly and a cyclic amine instead of a cyclic imine group; a carbocyclic or lactone ring is absent (Satake, Ofuji, Naoki, et al., 1998); their mechanism of toxicity is presently unknown (Botana et al., 2014). Long term effects are inconclusive (EFSA, 2008b; Ito et al., 2002), although damage to multiple organs was reported following oral administration to mice, with injury to the intestinal epithelium, lamina propria and villi, and a lethal oral dose of 700 μg/kg b.w. (Ito et al., 2002).

AZAs are readily absorbed after oral administration to mice (Aune et al., 2012). They were first detected 30 min after administration to pigs, with peak levels achieved after 4 h (Twiner, Hess, & Doucette, 2014). In humans, they cause vomiting, nausea, diarrhoea and stomach cramps within a few hours after ingestion (Klontz, Abraham, Plakas, & Dickey, 2009). No deaths from AZA ingestion have been reported. The EFSA working group established an acute reference dose (ARfD) of 0.2 μg AZA equivalents/kg body weight (b.w.) (EFSA, 2008b). The Joint FAO/IOC/WHO *ad hoc* Expert Consultation established a provisional ARfD of 0.04 μg/kg b.w. body weight but were unable to establish a Tolerable Daily Intake (A. CODEX, 2006).

AZAs target several apoptotic modulators (Botana et al., 2014; Roman et al., 2002; Twiner et al., 2005), such as caspase, cytoskeleton (Vilarino, Nicolaou, Frederick, Vieytes, & Botana, 2007), cytochrome release (Twiner, Hanagriff, Butler, Madhkoor, & Doucette, 2012), c-jun-N-terminal protein kinase (JNK), calcium levels (Cao, LePage, Frederick, Nicolaou, & Murray, 2010; Vale, Wandscheer, et al., 2008), fatty acid biosynthesis (Twiner et al., 2008). AZAs decrease cell volume mediated by potassium and chloride efflux (Vale, Nicolaou, Frederick, Vieytes, & Botana, 2010), deplete ATP (Kellmann et al., 2009), inhibit endocytosis (Bellocci, Sala, Callegari, & Rossini, 2010) and decrease procathepsin pools in endocytosis (Sala, Bellocci, Callegari, & Rossini, 2013). The observation that AZAs are present only in mussel samples with high levels of glutaric acid is intriguing, and a combination of AZA and glutaric acid blocks voltage-dependent sodium channels (Chevallier et al., 2015), which could explain the neurotoxicity linked to AZA (Twiner et al., 2014).

AZAs block open hERG potassium channels (Twiner, Doucette, et al., 2012), and this translates into the *in vivo* acute (11 μg/kg) and subacute (four doses of 10 μg/kg in 15 days) cardiotoxicity of AZAs through hERG channels in rats (Ferreiro et al., 2016b; Ferreiro et al., 2014). Ultrastructural changes in the hearts of rats were observed at a dose of 1 μg/kg b.w. i.p. The possible cardiotoxic effect of this group requires further investigation.

Acute toxicity data on AZAs are shown in Table 5. The TEFs recommended by the Expert group for AZAs are indicated in Table 6.

Table 5
Toxicities of AZAs.

Intraperitoneal injection			
Compound	Parameter	Acute toxicity (μg/kg b.w. b.w) (reference)	
AZA1	Lethality	200 (Munday, 2014)	
AZA1	MLD	150 (Satake, Ofuji, James, Furey, & Yasumoto, 1998)	
AZA1	LD_{50}	74 (Marine-Institute, 2014)	
AZA1	LD_{50}	>10 and <55 in rats (Ferreiro et al., 2016a)	
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	<1000 (Munday, 2014)	
AZA6	LD_{50}	100 (Marine-Institute, 2014)	
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)

In vitro toxicity

Compound	Cell type				
	Jurkat T (cytotoxicity)	HEK 293 (hERK current)	2-3 Days *in vitro* mice neurons (cytotoxicity)	Neocortical neurons (LDH release)	Neocortical neurons (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				

6. Domoic acid

Domoic acid is a globally distributed excitotoxin produced by the red macroalga *Chondria armata* (Takemoto & Daigo, 1958), and by diatoms of the genera *Nitzschia*, *Pseudo-nitzschia* (Bates et al., 1989) and *Amphora* (Dhar et al., 2015). The worldwide distribution of the toxin producing organisms makes the presence of DA rather ubiquitous in the world oceans. A TEF of one is applicable, as only DA and its diastereoisomer, epi-DA, have been shown to be of toxicological relevance (sum of DA and epi-DA expressed as DA).

7. Brevetoxins

Brevetoxins target the neurotoxin receptor site five voltage gated sodium channels, resulting in membrane depolarization, prolongation of open time, prevention of channel inactivation, induction of the channel activation at more negative potentials, thereby causing repetitive firing and increases in sodium currents (Atchison, Luke, Narahashi, & Vogel, 1986; Trainer, Moreau, Guedin, Baden, & Catterall, 1993). These effects lead to rapid reductions in respiratory rate, cardiac rhythm alterations, and hypothermia (Templeton, Poli, & LeClaire, 1989).

Brevetoxins have a polyether backbone and can be grouped into two types. BTX1 (also referred to in the literature as PbTx1) represents the A-type toxins and has been reported to be the most toxic of the BTX analogues (Shimizu, Chou, Bando, Van Duyne, & Clardy, 1986). BTX2 (also referred to as PbTx2), representing the B-type toxins, is the most abundantly produced by the source dinoflagellate *Karenia brevis* (Shimizu et al., 1986). Following a neurotoxic shellfish poisoning outbreak in New Zealand in 1992–1993, it was found that BTXs are extensively metabolized in shellfish (Ishida et al., 1995). Of the metabolites identified in shellfish from New Zealand BTX-B1 was found to be most toxic (Ishida et al., 1995). BTX-B4 was threefold more toxic than BTX-B2 and comparable to the toxicity of BTX3 (also referred to as PbTx3) (Baden & Mende, 1982; Morohashi et al., 1999; Poli, Mende, & Baden, 1986). There is limited human oral data available for establishing TEFs; currently, the CODEX Standard method for BTXs is the MBA and the regulatory limit is expressed as mouse units. For this reason, TEFs for BTXs are not currently proposed.

8. The special case of tetrodotoxin and emerging toxins

TTX is a marine toxin of bacterial origin and is produced, amongst others, by *Pseudomonas* and *Vibrio* spp. (Bane, Lehane, Dikshit, O'Riordan, & Furey, 2014). It is becoming a concern in Europe given its presence in gastropods (Rodriguez et al., 2008; Silva et al., 2012) and in shellfish (A. Turner, Powell, Schofield,

Table 6
TEFs recommended by the Expert Group for each biotoxin group.

Saxitoxin 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**	Oral studies show more potency than STX. A value of 2.0 is recommended, and supported by Na channel *in vitro* results.
GTX1	0.99				1.0	**1.0**	No new data
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**	Oral LD_{50} data suggest a lower TEF than i.p. LD_{50}. As for NeoSTX, *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: increments of 0.05 for TEF<0.1)
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**	New oral data (and supported by i.p. toxicity data)
dcNeoSTX		0.058	0.216	0.224	0.4	**0.2**	New oral data (and 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

In case of saxitoxin analogues, for which no oral toxicity data were available, TEFs recommended are based on i.p.data

Okadaic acid group

	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**	There are several analogue specific reports from human intoxications. Human intoxications in Japan suggest a LOAEL of 48 μg DTX1 per person, equivalent to events of 50 μg OA per person in Sweden, Norway, UK and Portugal (EFSA, 2008c). Current used TEF of 1.0 is protective for public health. However *in vitro* studies suggest potency of DTX1 is higher than OA. The uncertainties of these studies (5-fold difference between cell lines) suggest a TEF of 1.0 for DTX1 should be assumed until further data is available.
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, DTX-2 is on average 0.5 times as toxic as DTX1. This value is also supported by the various *in vitro* data
DTX3						The TEF of the hydrolysis product of AO, DTX1 or DTX2 would apply.

Domoic Acid

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

Azaspiracids

	TEF based on i.p. toxicity	TEF based on oral toxicity	EFSA proposed TEF	Recommended TEF	Rational
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			–		No data
AZA5			–		No data
AZA6	0.7		–	**0.7**	No oral, only i.p. data.

Lees, & Baker-Austin, 2015; Vlamis et al., 2015). Because TTX in shellfish is a newly-discovered phenomenon, there is presently no surveillance programme for TTX in place. The mode of action is similar to that of STX, with main difference between the toxin groups being the subtypes of Na_v for which they preferentially bind. In the case of TTX, the Na_v 1.7 is the main target (Alonso et al., 2016; Walker et al., 2012), although TTX can bind with lower 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).

The human lethal dose of TTX is 2 mg (Noguchi, Onuki, & Arakawa, 2011). Based on the intraperitoneal toxicity to mice, relative toxicities of TTX analogues have been reported in the literature (Bane et al., 2014).

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; E. G.; Moczydlowski, 2013).

The Expert Group suggested an emerging need to establish TEFs for TTX analogues that may be found in bivalves, indicating a requirement for oral toxicity data on the analogues.

It was also suggested that other emerging toxins, such as palytoxin, ovatoxins, ostreocins and cyclic imines should be further investigated to determine the actual risk to consumers (Munday, 2014).

Disclaimer

The views expressed in this publication are those of the author(s) and do not necessarily reflect the views of FAO and WHO.

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