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Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods

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Abstract

Tetrodotoxin (TTX) and its analogues are produced by marine bacteria and have been detected in marine bivalves and gastropods from European waters. The European Commission asked EFSA for a scientific opinion on the risks to public health related to the presence of TTX and TTX analogues in marine bivalves and gastropods. The Panel on Contaminants in the Food Chain reviewed the available literature but did not find support for the minimum lethal dose for humans of 2 mg, mentioned in various reviews. Some human case reports describe serious effects at a dose of 0.2 mg, corresponding to 4 μg/kg body weight (bw). However, the uncertainties on the actual exposure in the studies preclude their use for derivation of an acute reference dose (ARfD). Instead, a group ARfD of 0.25 μg/kg bw, applying to TTX and its analogues, was derived based on a TTX dose of 25 μg/kg bw at which no apathy was observed in an acute oral study with mice, applying a standard uncertainty factor of 100. Estimated relative potencies for analogues are lower than that of TTX but are associated with a high degree of uncertainty. Based on the occurrence data submitted to EFSA and reported consumption days only, average and P95 exposures of 0.00–0.09 and 0.00–0.03 μg/kg bw, respectively, were calculated. Using a large portion size of 400 g bivalves and P95 occurrence levels of TTX, with exception of oysters, the exposure was below the group ARfD in all consumer groups. A concentration below 44 μg TTX equivalents/kg shellfish meat, based on a large portion size of 400 g, was considered not to result in adverse effects in humans. Liquid chromatography with tandem mass spectroscopy (LC–MS/MS) methods are the most suitable for identification and quantification of TTX and its analogues, with LOQs between 0.1 and 25 μg/kg.

Keywords: tetrodotoxin, marine bivalves, marine gastropods, human health risk

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Summary

Following a request from the European Commission, the Panel on Contaminants in the Food Chain (CONTAM Panel) was asked to deliver a scientific opinion on the risk related to the presence of TTX and TTX analogues (TTXs) in marine bivalves and gastropods.

TTX is a hydrophilic toxin, produced by bacteria that can be found in certain fish species but also marine gastropods and bivalves. There are no health-based guidance values for TTX worldwide and also no maximum levels of TTX in seafood in the European Union (EU).

Altogether, 25 naturally occurring analogues of TTX have been detected and many of these have also been shown to have toxicity potential. TTX is positively charged in neutral solutions. Under acidic conditions, TTX exists as a mixture in equilibrium with the ortho ester, the lactone form, 4-epiTTX and 4,9-anhydroTTX.

Recently, TTX and some analogues have been detected in gastropods and bivalves from European waters using liquid chromatography with tandem mass spectroscopy (LC–MS/MS) methods. The CONTAM Panel concluded that these methods are the most suitable methods for identification and quantification of TTXs. Besides, saxitoxins (STXs) may be included in the same analysis. Limit of quantification (LOQs) for TTX and analogues in marine bivalves, as reported by three Member States, vary between 0.1 and 25 μg/kg.

The mouse bioassay is the most widely applied detection method for TTXs outside the EU. However, this test cannot discriminate between TTX and its analogues and it can also not discriminate TTXs from STXs. Cell-based methods and immunoassays can be used for screening but cannot identify or quantify the different TTXs or discriminate between TTXs and STXs.

There is limited information about absorption and excretion of TTX and its analogues in humans and no information has been identified on its distribution and metabolism. It is assumed that TTX is rapidly absorbed in the human digestive tract, based on the short time span between ingestion and onset of the symptoms seen in human poisoning cases. In human poisoning cases, TTX has been detected in blood and urine of patients for several days after exposure.

TTX is acutely toxic in humans and in experimental animals. In humans, TTX exhibits its toxicity by extracellular blockade of the channel pore of voltage-gated sodium channels (Na_v). Thus, TTX affects both action potential generation and impulse conduction, resulting in a blockade of the neuron action potential and in muscle paralysis. This leads to a sequence of acute symptoms from perioral numbness and paraesthesia, lingual numbness, early motor paralysis, incoordination, slurred speech to generalised flaccid paralysis, aphonia and fixed/dilated pupils to hypoxia, hypotension, bradycardia, cardiac dysrhythmias and unconsciousness and in the end death. Death is caused by respiratory failure and cardiac collapse. There is no antidote against TTX poisoning.

In experimental animals, the effects that are observed upon acute exposure include skeletal muscle fasciculations, apathy, lethargy, ataxia, ascending progressive paralysis and death.

Acute intraperitoneal (i.p.) and subcutaneous (s.c.) LD_{50} values in mice range from 9 to 12.5 mg/kg body weight (bw). Upon intragastric application, much higher LD_{50} values of 232 and 532 mg/kg bw have been identified.

A no observed adverse effect level (NOAEL) of 75 μg/kg bw was established by the authors in a study with mice that received a single intragastric treatment. In this experiment, apathy was observed in all animals treated with doses of 125 μg/kg bw and above, but not at levels of 75 and 25 μg/kg bw. At higher doses, lethality was observed, i.e. at 250 μg/kg bw for four out of seven animals, at 500 μg/kg bw for four out of five animals and at 1,000 μg/kg bw for three out of three animals. This shows a relatively steep dose–response curve over a small dose range.

In the literature, a minimum lethal dose (MLD) of 2 mg is often mentioned for humans (corresponding to 40 μg/kg bw in a 50-kg Japanese adult). However, the original source of this MLD and the underlying data could not be retrieved. The doses at which TTX causes acute toxicity and lethality in humans are unclear, but a series of human case reports are available that indicate that acute poisoning can result from doses of 4–42 μg/kg bw or higher.

No subchronic or chronic studies on TTX in animals have been identified. TTX did not show any genotoxic activity in a battery of good laboratory practice (GLP)-compliant *in vitro* and *in vivo* assays conducted according to OECD guidelines.

Based on the pronounced toxicity of TTX upon acute exposure, the CONTAM Panel decided to derive an acute reference dose (ARfD). Both the use of human data, extrapolation from data on STX and the use of animal data were considered.

Due to the limitations of the available human data related to the estimation of the ingested dose of TTX, these data were not used to derive the ARfD, but they were used as supportive information. Similarly, the ARfD for STX was used as supportive data. STX shows in humans similar effects and in mice similar toxic potency both after i.p. and intragastric treatment. The mode of action of TTX and STX is similar but with some differences in the affinity for the different subtypes of Na_v channels.

Apathy was the most sensitive endpoint in the oral acute study in mice, observed in none of the animals at doses of 25 and 75 μg TTX/kg bw, but in all animals at 125 μg TTX/kg bw and above. At a dose of 250 μg/kg bw and higher, lethality occurred. Whereas a benchmark dose (BMD) could not be derived for apathy, a $BMDL_{10}$ of 112 μg/kg bw for lethality was calculated, which is only slightly above the NOAEL for apathy. Also, considering that with a group size of nine mice, applied at the dose of 75 μg/kg bw, it cannot be excluded that effects can occur, the next lower dose tested (25 μg/kg bw) was used as the reference point. This resulted in an ARfD of 0.25 μg/kg bw after applying a standard uncertainty factor of 100.

The dose of 25 μg/kg bw is 4.5-fold lower than the $BMDL_{10}$ calculated for lethality. The CONTAM Panel noted that the ARfD is 16-fold lower than the lowest dose at which severe effects have been observed in humans (4 μg/kg bw) and is twofold lower than the ARfD for STX (0.5 μg/kg bw).

When i.p. injected in mice, many TTX analogues exert similar effects as TTX but no data are available that allow derivation of NOAELs or lowest observed adverse effect levels (LOAELs). However, values such as LD_{50}, and LD_{99} upon i.p. application have been reported. These values have been used to estimate relative potencies of a number of analogues, including the ones thus far detected in bivalves and gastropods. The relative potencies calculated for 11-oxoTTX and 11-norTTX-6(R)-ol based on i.p. LD_{99} results compared to LD_{100} for TTX are 0.75 and 0.17, respectively, while relative potencies based on i.p. LD_{50} values in mice are 0.14 for 11-deoxyTTX and 0.19 for 11-norTTX-6(S)-ol. Relative potencies calculated from lethal potencies in mice are 0.16 for 4-epiTTX and 0.02 for 4,9-anhydroTTX, while the relative potency for 5,6,11-deoxyTTX is 0.01 based on comparison of the MLD with that for TTX. For some of the analogues, these potencies are to some extent supported by *in vitro* tests with the Neuro-2a bioassay. The Panel noted that the derivation of the relative potencies for TTX analogues is associated with high uncertainties since the underlying methods and data are poorly described.

The ARfD of 0.25 μg/kg bw is therefore a group ARfD that should apply to TTX and its analogues taking into account their relative potencies derived from a comparison with TTX using the most appropriate parameters. For analogues detected thus far in marine bivalves and gastropods, these are 0.75 for 11-oxoTTX, 0.14 for 11-deoxyTTX, 0.19/0.17 for S/R 11-norTTX-(6)-ol, 0.16 for 4-epiTTX, 0.02 for 4,9-anhydroTTX and 0.01 for 5,6,11-deoxyTTX.

A total of 8,268 analytical results of 1,677 samples of bivalves were submitted to the European Food Safety Authority (EFSA) by UK, Greece and the Netherlands. For all samples, analytical results were provided for TTX, whereas 1,136 analytical results were provided for 4-epiTTX, 1,080 for 5,6, 11-trideoxyTTX, 1,080 for 11-norTTX-6-ol, 1,080 for mono-deoxyTTX, 1,135 for 4,9-anhydroTTX and 1,080 for 11-oxoTTX. Samples were taken between 2006 and 2016, including mussels, oysters, cockles, clams, scallops and razor clams. No occurrence data were received for marine gastropods. In 1,544 (about 95%) of the samples, TTX was not detected or quantified, with LOQs ranging from 1 to 25 μg/kg. The upper bound median and 95th percentile levels reported to EFSA for TTX were 5.9 and 28 μg/kg. In most cases, this was based on raw meat, in some cases the digestive gland. Taking into account the types of water molluscs one by one, the highest upper bound mean level was reported for clams, 10.8 μg/kg; the highest 95th percentile level was reported for oysters, 79 μg/kg.

Due to the few quantified levels available, the relatively low levels of TTX analogues as compared to TTX and low estimated relative potencies (RPs) for the detected analogues, they were not included in the exposure assessment. Marine gastropods could not be included either, because no occurrence data were provided.

Acute exposure assessments for TTX were carried out with two different approaches, namely i) by using the consumption data in the EFSA Comprehensive database applying a probabilistic approach, and ii) by using a large portion size of 400 g for bivalves. This value has been used as large portion size in previous opinions on marine toxins. In the first approach, exposure was calculated using the probabilistic approach based on consumption days only.

Overall, the lower bound average acute exposure was zero while the upper bound was 0.01–0.09 μg/kg bw with the highest exposure calculated for the 'elderly' population. The highest estimated 95th percentile exposure of 0.03 μg/kg bw occurred in the elderly, adults and adolescents. However, only five out of 29 surveys allowed an estimate of the P95 exposure.

Regarding the different types of marine bivalves, the highest mean exposure levels for clams, mussels and oysters were 0.02, 0.03 and 0.09 μg/kg bw, respectively, whereas for the other bivalves, average acute exposure was zero. The highest 95th percentile exposure was estimated for oysters, being 0.08 μg/kg bw.

Using the large portion size of 400 g and P95 concentrations, the exposure ranged from 0.03 to 0.45 μg/kg bw, depending on the bivalve species.

The exposure estimates show that the average and 95th percentile acute exposure levels, either based on reported consumption or on a large portion size of 400 g, did not exceed the group ARfD of 0.25 μg TTX/kg bw in any of the consumer groups except in case of consumption of a large portion of oysters. This indicates no general concern for human health due to the consumption of marine bivalves except for consumption of a large portion of oysters.

Based on a large portion size (400 g), an adult body weight of 70 kg and a group ARfD of 0.25 μg/kg bw, the CONTAM Panel concluded that a concentration lower than 44 μg of TTX and/or the equivalent toxic amount of its analogues per kg shellfish meat is not expected to result in adverse effects in humans. The Panel noted that the P95 of the occurrence data for all shellfish is below this value. However, levels above 44 μg/kg have been reported for both mussels and oysters indicating an occasional concern for consumers of a large portion size of 400 g or larger.

It was not possible to perform a risk characterisation for marine gastropods because of limited consumption data and a lack of occurrence data.

It is recommended that further data on the sources and critical factors leading to the accumulation of TTX in marine bivalves and gastropods are collected.

There is also a need for further information on toxicokinetic and oral toxicity of TTX and its analogues. Even though TTXs are acute toxic and there is no warrant of chronic effects, this should be further investigated. Given the high uncertainties associated with derivation of the relative potencies of TTX analogues, better description of the methods and data, or adequate data are needed to estimate their relative potencies, preferentially after oral exposure. It should also be explored if STXs and TTXs should be combined in one health-based guidance value regarding their similar toxic effects and mode of action.

To provide more refined exposure assessments, more data on both occurrence data of TTXs and more consumption data of gastropods and bivalves are needed. More data are needed on the fate of TTX and its analogues during cooking. Data on concentrations of TTX and its analogues should preferably be obtained using EU approved and validated chemical-analytical methods. In addition, certified standards and reference materials for TTX and its analogues are needed to improve the quality of the occurrence data.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

Tetrodotoxins is the generic term for a group of toxins consisting of tetrodotoxins (=TTX) and analogues such as 4-epi-TTX, 6-epi-TTX, 11-oxo-TTX and tetrodonic acid. Tetrodotoxin (TTX) is the causative agent responsible for pufferfish/fugu poisoning, a fatal marine poisoning found predominantly in subtropical and temperate regions. It is found mainly in the organs of fish from the Tetraodontidae family, as well as other marine species such as the blue-ringed octopus and gastropods. The toxin and its structural analogues are thought to originate from a variety of marine bacteria, including *Vibrio* spp.

Clinical effects include a range of neuromuscular symptoms (such as paraesthesia of lips and tongue, dizziness and headache and gastrointestinal symptoms). Higher degree symptoms include ataxia, incoordination, cardiac arrhythmias, seizures and respiratory failure, leading to death.

In the EU, TTXs were detected in the course of a non-fatal human intoxication following consumption of a contaminated sea snail[1] harvested in Portugal and consumed in Spain containing 315 mg TTX/kg and 1,004 mg 5,6,11-trideoxyTTX/kg (Rodriguez et al., 2008; Fernandez-Ortega et al., 2010).

There has been no evidence of accumulation of TTX in bivalve molluscs grown in European waters until recently, and the threat from this toxin group was deemed negligible within the European Union (EU). However, detection of TTXs in European bivalve molluscs has been first reported by the United Kingdom for shellfish in England harvested in 2013 and 2014 at a maximum level of 137 μg TTX/kg (Turner et al., 2015), then by Greece in 2015 for samples obtained in 2012 at a maximum level of 223 μg TTX/kg (Vlamis et al., 2015). Moreover, a survey carried out in the Netherlands in 2015 revealed that TTXs can be found in mussels and oysters from Dutch production areas.

The current EU rules (Chapter V point 2 of Section VII of Annex III to Regulation 853/2004) establish that live bivalve molluscs: 'must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits:

a) for paralytic shellfish poison (PSP), 800 μg/kg;
b) for amnesic shellfish poison (ASP), 20 mg of domoic acid per kilogram;
c) for okadaic acid, dinophysistoxins and pectenotoxins together, 160 μg of okadaic acid equivalents per kilogram;
d) for yessotoxins, 3.75 mg of yessotoxin equivalent per kilogram; and
e) for azaspiracids, 160 μg of azaspiracid equivalents per kilogram.'

TTXs are not included in the list of marine biotoxins to be tested neither at EU level nor at international level (CODEX STAN 292-2008).

The European Food Safety Authority (EFSA), in its Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on marine biotoxins in shellfish (Question EFSA-Q-2006-065A), did not include this toxin in its evaluation.

1.1.2. Terms of Reference

Based in particular on the existing scientific literature, EFSA is requested to provide a scientific opinion on:

1) the occurrence, including concentration data of TTX and TTX analogues in bivalve molluscs and marine gastropods grown in EU and non-EU waters;
2) the toxicity for humans of TTX in bivalve molluscs and marine gastropods including: the toxic doses (e.g. minimum lethal dose (MLD), lowest observed adverse effect level (LOAEL)) and if possible the establishment of health-based guidance values (HBGVs) (e.g. acute reference dose (ARfD), tolerable daily intake (TDI));
3) the concentration of TTX and TTX analogues in bivalve molluscs and marine gastropods that is not expected to lead to health risks;

[1] *Charonia lampas lampas*.

4) the most efficient methods that could be used for the detection and quantification of this toxin group, including relevant parameters as limit of detection (LOD) and limit of quantification (LOQ) in relation to the concentration identified in Terms of Reference (ToR) 3.

In case insufficient data are available for the EFSA's assessment, EFSA should indicate which data should be collected by the Member States in order to respond to this request.

1.2. Additional information

1.2.1. Sources and origin of TTX

Any concentrations of TTX or its analogues reported in the present opinion refer to fresh weight material if not otherwise specified.

TTX and analogues were reported in gastropods (Rodriguez et al., 2008; Silva et al., 2012), oysters and mussels (Turner et al., 2015; Vlamis et al., 2015). Many non-shellfish species were reported to contain TTX (Bane et al., 2014). Although the origin of TTX is known to be associated to bacteria of the phylum Proteobacteria, containing *Pseudomonas*, *Pseudoalteromonas* and *Vibrio*, several phyla of bacteria (Actinobacteria, Bacterioides, Firmicutes, Proteobacteria) were reported as potential TTX sources (Jal and Khora, 2015). These bacteria were suggested to be linked to specific dinoflagellate blooms, such as *Prorocentrum minimum* (Vlamis et al., 2015; Rodríguez et al., 2017). The bacteria that produce TTX (i.e. genus *Bacillus*, *Shewanella*, *Roseobacter*, *Vibrio*, *Pseudomonas*, *Alteromonas*, *Aeromonas*, *Nocardiopsis*) are present in several species in the subcutaneous mucus, ovaries or in the intestine (Wu et al., 2005; Noguchi et al., 2006; Bane et al., 2014).

1.2.2. Previous assessments

There are three previous assessments on TTX available from EU Member States. TTX has not previously been considered by EFSA, the Scientific Committee on Food (SCF) or the Joint FAO/WHO Evaluation Committee (JECFA).

Following the paper by Turner et al. (2015), RIKILT investigated shellfish samples from Dutch harvest areas for TTX in the end of 2015 (RIVM-RIKILT, 2015). A number of oyster and mussel samples, collected during the summer period, showed detectable levels, with a maximum of 124.1 μg/kg in oysters. The highest level in mussels was 33.3 μg/kg. All positive samples were derived from the Oosterschelde in the south-west of the Netherlands, with levels above the LOQ of 10 μg/kg in 15 out of 55 samples from that area. Subsequently, the National Institute for Public Health and the Environment (RIVM)-RIKILT Front Office on Food and Consumer Product Safety was asked by the Netherlands Food and Consumer Product Safety Authority- Department for Risk Assessment and Research (NVWA-BuRO) for a risk assessment. In the absence of a HBGV, a rapid scan of the literature revealed that a dose of 2 mg was considered to be the MLD in several review papers, the initial ones derived from Japan. The Hazardous Substances Data Bank[2] mentions a range of 1–4 mg as the lethal dose. It was noted that a dose of 0.2 mg was mentioned in some papers as a dose leading to adverse effects but none of the papers mentioned the source of these figures, creating a high level of uncertainty. In a human poisoning case in Spain, following consumption of a sea snail, an intake of 1.5 mg was estimated based on a suggested intake of 5 g of digestive gland of sea snail containing 300 mg TTX/kg (Rodriguez et al., 2008; Fernandez-Ortega et al., 2010). In addition to TTX, the gland contained 1,000 mg 5,6,11-trideoxyTTX/kg. The patient recovered after artificial respiration. Overall, it was concluded that 4 μg/kg body weight (bw) seemed to be at the lower end of the dose range causing effects, with 10 μg/kg bw causing serious effects and 20–80 μg/kg bw being lethal without proper treatment. However, because of the large uncertainty on the reported toxic doses, no ARfD could be derived. Regarding differences in susceptibility between people, there are indications that this applies to individuals with heart problems or diseases of the nervous system. The RIVM-RIKILT FrontOffice was also asked to suggest a safe level in shellfish. Assuming that 4 μg/kg bw is a LOAEL, a factor of 3 would be needed to derive a no observed adverse effect level (NOAEL) and a factor of 10 to account for differences between individuals. This would result in a safe intake level of 0.13 μg/kg bw. Then, using the portion size of 400 g for a person of 60 kg bw, as applied by EFSA for other marine biotoxins (EFSA CONTAM Panel, 2010a–d), a safe level in shellfish would be 19.5 μg/kg. A similar level was derived for a child of 20 kg bw consuming 130 g shellfish. However, it was stressed

[2] https://toxnet.nlm.nih.gov/newtoxnet/hsdb.htm

that, regarding the uncertainties if the doses leading to adverse effects, it could not be excluded that lower levels in shellfish could also lead to effects. Based on this advice, NVWA-BuRO proposed to apply a zero tolerance for TTX in shellfish, which in practice meant that levels should be non-detectable with a required LOQ of 20 μg/kg or lower.

The UK FSA risk assessment (FSA, 2017) concluded that, because of the acute toxicity of TTXs, it would be appropriate to establish an ARfD, i.e. a dose level that would not be expected to result in adverse effects following a single eating occasion or over the course of a day. However the available data from human observations and from studies in experimental animals were not considered to provide an adequate basis for deriving an ARfD. Therefore, while noting a need for a more comprehensive literature search, a provisional approach was proposed based on the threshold of toxicological concern (TTC), which is used as a priority setting tool for chemical substances in food with no or few toxicological data. The TTC approach provides a framework for determining whether exposure to a substance is so low that the probability of adverse effects is low, based on classification according to chemical structure (EFSA Scientific Committee, 2012). The FSA noted that Toxtree[3] indicates that TTX (and its analogues) are not genotoxic and would fall into Cramer class III, which means that an intake of less than 1.5 μg/kg bw per day for the sum of TTXs would not be expected to be a safety concern. This value is half of the lowest dose at which symptoms have been reported, and one-tenth of the minimum lethal dose. Hence when the mode of action for TTX toxicity is also taken into consideration, it was concluded that this TTC value is a reasonable basis on which to undertake a preliminary risk assessment. Based on the large portion size of 400 g shellfish, identified in EFSA CONTAM Panel (2010c), the concentration of TTX in shellfish meat at which the TTC would not be exceeded for EU adults weighing 70 kg is 0.262 mg/kg shellfish meat. The age group with the highest exposure to contaminant in food is commonly toddlers (1–3 years). However, taking into account that toddlers would not be given bivalves in shell, it was considered unlikely that they would be given more than a small number of mussels on any single occasion.

The Scientific Committee of the Belgian Federal Agency for the Safety of the Food Chain was asked to set an action limit for TTX in bivalves (FAVV, 2016). The Committee reviewed the risk assessments in the Netherlands and the UK resulting in safe levels in bivalves of 16.67 and 262 μg/kg meat, respectively, based on a large portion size of 400 g. In Japan, a limit of 2,000 μg/kg is used for fish. The Committee noted the large variation between the different levels and concluded that it is better to wait for the risk assessment from EFSA. As a precautionary measure, it was advised to apply the LOQ of the analytical method as action limit.

1.2.3. Chemistry

TTX is a heterocyclic compound that consists of a guanidinium moiety connected to an oxygenated backbone that possesses a 2,4-dioxaadamantane structure with six hydroxyl groups (Chau et al., 2011). Altogether 25 naturally occurring analogues of TTX have been detected (Bane et al., 2014). TTX analogues found in pufferfish as well as in gastropods and bivalves can be classified into four groups (Yotsu-Yamashita et al., 2013):

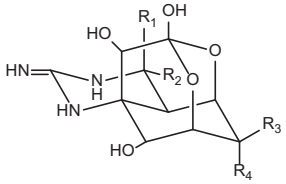
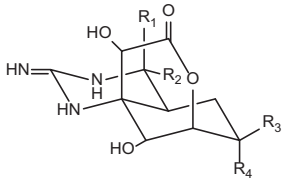
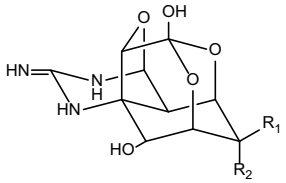
1) analogues chemically equivalent to TTX (e.g. 4-epiTTX and 4,9-anhydroTTX),
2) deoxy analogues (e.g. 5-deoxyTTX, 11-deoxyTTX, 6,11-dideoxyTTX, and 5,6,11-trideoxyTTX),
3) (3) 11-CH_2OH oxidised analogue (e.g. 11-oxoTTX),
4) C11 lacking analogues (e.g. 11-norTTX-6(*S*)-ol and 11-norTTX-6(*R*)-ol).

Table 1 shows nomenclature, molecular structures and weights and CAS numbers for the natural occurring TTXs that have been reported to occur in bivalves and gastropods. (Bane et al., 2014; EFSA database, see Section 3.3.2 Current occurrence).

TTX has a molecular weight of 319.1 g/M and the formula $C_{11}H_{17}N_3O_8$. TTX is hydrophilic and under acidic conditions, TTX exists as a mixture in equilibrium of the ortho ester, the lactone form, 4-epi-TTX and 4,9-anhydroTTX (Nishikawa and Isobe, 2013). It is stated in several papers (e.g. Bane et al., 2014; Turner et al., 2015; FAO/WHO, 2016) that TTX is heat stable but without reference to the original data.

[3] http://toxtree.sourceforge.net

Table 1: Tetrodotoxin (TTX) and its analogues detected in edible marine gastropods and bivalves

Name	Formula	Molecular weight	CAS number	Marine organisms[(a)]	Molecular structure
TTX	$C_{11}H_{17}N_3O_8$	319.1	4368-28-9	Gastropods Bivalves Also in pufferfish and crabs	 R_1 = H; R_2 = OH; R_3 = CH_2OH; R_4 = OH
11-oxoTTX	$C_{11}H_{17}N_3O_9$	335.1	123665-88-3	Gastropods Also in pufferfish and crabs	As TTX but R_3 = $CH(OH)_2$
4-epiTTX	$C_{11}H_{17}N_3O_8$	319.1	98242-82-1	Gastropods Bivalves Also in pufferfish and crabs	As TTX but R_1 = OH; R_2 = H
5,6,11-trideoxyTTX	$C_{11}H_{17}N_3O_5$	271.1	n.a.	Gastropods Bivalves Also in pufferfish	 R_1 = H; R_2 = OH; R_3 = CH_3; R_4 = H
4,9-anhydroTTX	$C_{11}H_{15}N_3O_7$	301.1	13072-89-4	Gastropods Bivalves Also in pufferfish and crabs	 R_1 = CH_2OH; R_2 = OH
11-norTTX-6-ol (*R*- or *S*-form)	$C_{10}H_{15}N_3O_7$	289.1	n.a.	Bivalves Also in pufferfish and crabs	As TTX but R_2 = OH; R_3 = OH/H; R_4 = H/OH (*R*/*S* form)

TTX: tetrodotoxin.
(a): Other edible species in which the substances are found are also mentioned in the table.

1.2.4. Legislation

Regulation (EC) No 853/2004[4] lays down specific rules for the hygiene in foodstuffs in the EU. Chapter V of Section VII of Annex III to the regulation establishes that live bivalve molluscs placed on the market for human consumption must not exceed limits for marine biotoxins. No such limits are currently listed for tetrodotoxins. However, according to the regulation (section VIII, chapter V), fishery products derived from poisonous fish of the families Tetraodontidae, Molidae, Diodontidae and Canthigas-teridae should not be marketed.

2. Data and methodologies

2.1. Data

2.1.1. Occurrence data

EFSA gathers data on the occurrence of chemical substances in food and feed at the EU level through continuous collections and/or calls for particular assessments. In addition, publicly available literature on occurrence of TTX in marine bivalves and gastropods was considered. Collection and appraisal of these occurrence data was carried out as described in Section 2.2.1 below.

2.1.2. Food consumption data

The EFSA Comprehensive European Food Consumption Database (Comprehensive Database) provides a compilation of existing national information on food consumption at individual level. It was first built in 2010 (EFSA, 2011; Huybrechts et al., 2011; Merten et al., 2011). Details on how the Comprehensive Database is used are published in the Guidance of EFSA (EFSA, 2011). The latest version of the Comprehensive Database[5] contains results from a total of 51 different dietary surveys carried out from 1997 to 2012 in 23 different European Countries covering 94,532 individuals. A summary table of the surveys included in the Comprehensive Database is presented in Annex A.

Within the dietary studies, subjects are classified in different age classes as described in Table 2; two additional surveys provided information on specific population groups: 'Pregnant women' (Latvia) and 'Lactating women' (Greece).

Table 2: Age classes considered in the EFSA Comprehensive European Food Consumption Database

Age class	Age range	Number of surveys	Number of countries
Infants	< 12 months old	6	6
Toddlers	≥ 12 months to < 36 months old	13	10
Other children	≥ 36 months to < 10 years old	24	17
Adolescents	≥ 10 years to < 18 years old	21	17
Adults	≥ 18 years to < 65 years old	29	21
Elderly	≥ 65 years to < 75 years old	18	15
Very elderly	≥ 75 years old	15	14

For acute exposure assessment, and considering the most recent dietary surveys, food consumption data were available from 41 different dietary surveys carried out in 23 different European countries. Overall, the food consumption data gathered by EFSA in the Comprehensive Database are the most complete and detailed data currently available in the EU. Consumption data were collected using single or repeated 24- or 48-h dietary recalls, as well as dietary records covering three to 7 days per subject. Owing to the differences in the methods used for data collection, direct country-to-country comparisons can be misleading.

2.1.3. Data on toxicokinetics and toxicity

The data were obtained as described in Section 2.2.2.

[4] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for the hygiene of foodstuffs. OJ L 139, 30.4.2004, p. 55–205.
[5] http://www.efsa.europa.eu/en/datexfoodcdb/datexfooddb

2.2. Methodologies

2.2.1. Collection and appraisal of EFSA occurrence data

The EFSA Evidence Management Unit (DATA Unit) initiated an ad-hoc collection of data to compile occurrence data on TTX and TTX analogues in marine bivalves and gastropods. The European national food authorities and similar bodies, research institutions and food business operators were invited to submit data.

The data (n = 1,677 samples in total) for the present assessment were provided by national authorities from Greece (n = 55), the Netherlands (n = 530) and the UK (n = 1,092). The data submission to EFSA followed the requirements of the EFSA Guidance on Standard Sample Description for Food and Feed (EFSA, 2010a). The occurrence data were managed following EFSA standard operational procedures (SOPs) on 'Data collection and validation' and on 'Data analysis of food consumption and occurrence data'.

A total of 1,677 samples of cockles, mussels, oysters, scallops, razor clams and other water mollusc data on TTX and its analogues were available in the EFSA database. For all of them, analytical results were provided to EFSA for tetrodotoxin (TTX), whereas only 1,135 analytical results were provided for 4-epiTTX, 1,080 for 5,6,11-trideoxyTTX, 1,080 for 11-norTTX-6-ol, 1,080 for mono-deoxyTTX, 1,135 for 4,9-anhydroTTX and 1,080 for 11-oxoTTX.

The UK reported analytical results for TTX, 4-epiTTX, 5,6,11-trideoxyTTX, 11-norTTX-6-ol, mono deoxy TTX, 4,9-anhydroTTX and 11-oxoTTX. The LOD and LOQ were never reported for the analogues. With the agreement of the data provider, the LOQ was determined as the minimum of the reported positive values for 4-epi TTX, 5,6,11-trideoxyTTX, 11-norTTX-6-ol and 4,9-anhydroTTX. In the case of 11-oxoTTX and mono-deoxyTTX, the highest LOQ identified among the other analogues has been used. In all cases, the LOD was calculated by dividing the LOQ by 3.

The Netherlands submitted analytical results only for TTX. In addition information was reported on the results of analyses of 4-epiTTX and 4,9-anhydroTTX. Solely TTX was found, except in one sample, where 4-epi TTX was also detected.

Greece reported analytical results for TTX, 4-epi TTX and 4,9 anhydroTTX. In addition, the TTX curve and assumption of equal molar response factor was used to estimate the levels of 5,6, 11-trideoxyTTX, 5-deoxyTTX, 11-deoxyTTX, 11-norTTX-6(R)-ol and 11-norTTX-6(S)-ol, but they were either non-detects or non-quantified samples. Information on LOQ/LOD of these five analogues was not reported and the data were not submitted to EFSA.

The reported LODs and LOQs are shown in Table 3 for TTX and all reported analogues. The Panel noticed the difference in LODs/LOQs reported by different Member States, possibly related to differences in the applied methods. This could have an influence on the exposure assessment, which is for the average exposure mitigated by the fact that most of the concentration data came from the UK.

Table 3: Reported LODs and LOQs (in μg/kg fresh weight) for TTX and its analogues

TTX/TTX analogue	Country					
	United Kingdom[(a)]		Netherlands		Greece	
	LOQ	LOD	LOQ	LOD	LOQ	LOD
TTX	1	0.5	10–25	3–5	7.2	2.2
11-norTTX-6-ol	6.4	2.13	–	–	n.r.	n.r.
11-oxoTTX	6.4	2.13	–	–	–	–
4,9-anhydroTTX	1.2	0.4	n.r.	n.r.	21.1	6.2
4-epiTTX	0.12	0.04	10–25	3–5	7.6	2.3
5,6,11-trideoxyTTX	1.03	0.34	–	–	n.r.	n.r.
mono-deoxyTTX	6.4	2.13	–	–	–	–
5-deoxyTTX	–	–	–	–	n.r.	n.r.
11-deoxyTTX	–	–	–	–	n.r.	n.r.

LOD: limit of detection; LOQ: limit of quantification; n.r.: not reported; – : not analysed; TTX: tetrodotoxin.

(a): In the case of analogues in samples from the UK, with the agreement of the Data Provider, LOQ = minimum of reported values, LOD = LOQ/3. For 11-oxoTTX and mono-deoxyTTX, the highest LOQ/LOD identified among the other analogues has been used as an assumption.

In addition, the French Research Institute for Exploitation of the Sea (IFREMER) analysed in 2015, 90 samples of digestive glands from bivalves (67 mussels + 23 oysters) collected from several sampling points along the French coast for TTX by liquid chromatography with tandem mass spectroscopy (LC–MS/MS). After confirmatory analysis of suspicious samples by liquid chromatography–high-resolution mass spectrometry (LC–HRMS), all samples were below the LOD (33 μg/kg of digestive glands, fresh weight). However, these data were not transmitted to EFSA and consequently could not be included in the exposure assessment.

At a later stage in developing the opinion, an additional 57 samples were received for TTX in mussels (n = 37) and oysters (n = 20) from the UK (Northern Ireland). The samples were taken between May and September 2016 and all of them were non-detects, the LOD being 0.5 μg/kg. These data were not included in the exposure assessment because they have been received too late.

Following the EFSA SOP on 'Data analysis of food consumption and occurrence data' to guarantee an appropriate quality of the data used in the exposure assessment, the initial data set was carefully evaluated applying several data cleaning and validation steps. Special attention was paid to different data elements such as 'Analytical method', 'Reporting unit', 'Expression of results' and the codification of the different food samples under the FoodEx classification.

The left-censored data (analytical data below the LOD/LOQ) were treated by the substitution method as recommended in the 'Principles and Methods for the Risk Assessment of Chemicals in Food' (WHO/IPCS, 2009). The same method is indicated in the EFSA scientific report 'Management of left-censored data in dietary exposure assessment of chemical substances' (EFSA, 2010b), as an option in the treatment of left-censored data. The guidance suggests that the lower bound (LB) and upper bound (UB) approach should be used for chemicals likely to be present in the food (e.g. naturally occurring contaminants, nutrients and mycotoxins). At the LB, results below the LOQ or LOD were replaced by zero; at the UB, the results below the LOD were replaced by the LOD and those below the LOQ were replaced by the value reported as LOQ. Additionally, a middle bound (MB) approach was used by assigning a value of LOD/2 or LOQ/2 to the left-censored data.

2.2.2. Collection and appraisal of publicly available toxicity, toxicokinetics and occurrence data

No previous comprehensive risk assessments or reviews have been identified that could be used as a starting point for the present opinion. Based on the short timeline given for the present mandate, it was not possible to retrieve and scrutinise all potentially relevant literature without setting a time limit. Based on a pilot search, it was decided to carry out an extensive literature search for publications from the year 2000 onwards (because that would cover the most recent and up to date studies and also reviews) and to combine it with the application of a 'forward snowballing' approach[6] for retrieving studies published before 2000 (see Jalil and Wohlin, 2012).

The extensive and comprehensive search for literature was conducted for peer-reviewed original research, reviews and grey literature from the years 2000 until 11 August 2016. The search strategy was designed to identify literature dealing with analytical determination, chemistry, formation, metabolism and toxicity of TTX and also with its occurrence in bivalves and gastropods, effect of processing and exposure to TTX. All search terms and Boolean operators used are presented in detail in Appendix A. The literature search was not restricted to publications in the English language, however, literature in other languages was only considered if an English abstract was available which provided enough details to check the data. The literature search was performed on 11 August 2016 in Web of Science[7] which was identified as an appropriate and exhaustive database for retrieving literature for the present evaluation.

The references resulting from the literature search were imported and saved using a software package (EndNote[8]), which allows effective management of references and citations. Of the 6,766 retrieved citations duplicate references were removed using the EndNote software. The remaining 2,704 references were screened using title and abstract to identify the relevant literature.

Search categories 'neurosciences or physiology' were excluded upfront in the individual subsearches (see Appendix A), as in the pilot search it was seen that papers in this category deal with the use of TTX for investigating the mode and mechanism of action of the blocking of sodium channels and it

[6] Identifying articles that have been cited in articles found in a search.
[7] Web of Science (WoS), formally ISI Web of Knowledge, Thomson Reuters. http://thomsonreuters.com/thomson-reuters-web-of-science/
[8] EndNote X5, Thomson Reuters. http://endnote.com/

was decided that such investigations are not of relevance for the present assessment. Upon screening of the abstracts, all papers dealing with chemical features, methods of identification and quantification of TTX and its analogues were considered as potentially relevant. Publications on the occurrence of TTX and its analogues in species covered by the ToR (i.e. marine bivalves and gastropods) were included, while information on occurrence in other species was excluded from further consideration. Publications on determination of TTX and its analogues in species not covered by the ToR were not considered as potentially relevant. Any papers on toxicokinetics and toxicodynamics, biomarkers and detoxification were included as possibly relevant. Publications on toxicity of TTX and its analogues (such as acute, subacute, subchronic and chronic toxicity, genotoxicity, reproductive toxicity) in any species were included as potentially relevant. Epidemiological studies, human exposure assessments and reports on human poisoning cases were also included. Applying expert judgement, 411 publications were identified as potentially relevant for the present assessment.

During the development of the opinion, additional potentially relevant publications, not retrieved in the above mentioned literature search (i.e. those published before the year 2000), have been identified.

2.2.3. Methodology for exposure assessment

Based on food consumption data from the Comprehensive Database and the TTX occurrence data described in Section 2.2.1, TTX acute exposure levels were estimated using a probabilistic approach. Due to the relatively low levels of TTX analogues as compared to TTX and low estimated relative potencies for the detected analogues, the CONTAM Panel decided not to carry out a specific exposure assessment for each of the TTX analogues.

Acute exposure was assessed for each reporting day by multiplying the total consumption amount for each food (each type of bivalve, e.g. oysters) by one occurrence level randomly drawn among the individual results available for that type. Respective intakes of the foods consumed that day were then summed and finally divided by the individual's body weight. This process was iterated 1,000 times for each reporting day. For the calculations, the UB approach was applied. The exposure was modelled using SAS software.

In addition, acute exposure was also estimated by using the former 400 g portion and the 95th percentile of actually observed contamination levels on TTX for the different species of water molluscs.

Marine gastropods could not be included in the exposure assessment as no occurrence and very few consumption data were available on these species.

2.2.4. Methodology for risk assessment

The CONTAM Panel applied the general principles of the risk assessment process for chemicals in food as described by WHO (2009), which include hazard identification and characterisation, exposure assessment and risk characterisation. In addition to the principles described by WHO (2009), EFSA guidance documents were applied whenever appropriate.

3. Assessment

3.1. Methods of detection and quantification

TTX acts via the blockage of voltage-gated sodium channels (Na_V) leading to acute effects in animals and humans (see Section 3.2.3 Mode of action). The initial test for the detection of TTX was the mouse bioassay based on these acute effects after intraperitoneal (i.p.) injection. More recent developments focussed on *in vitro* bioassays, immunochemical methods and chemical-analytical methods.

3.1.1. Bioassays

3.1.1.1. Mouse bioassay

The mouse bioassay was the first method developed and applied for the detection of TTX in pufferfish samples. The first papers appeared in the 1950s (Hashimoto and Migita, 1951) and a standardised method was proposed by McFarran (1966). This was adapted into an official method in Japan (Sato and Kodama, 2015). The method is based on the injection of a sample extract i.p. in two 4-week-old male mice of a specific strain (ddY) with a bodyweight of 19–21 g as a first step. If one of

the mice dies, a third mouse is injected. The extract is prepared by mixing 10 g of sample with 25 mL 0.1% acetic acid followed by heating for 10 min. Subsequently, the extract is cooled, filtered and the filtrate adjusted to 50 mL using 0.1% acetic acid. When the mice die within 30 min, the injected extract contains TTX at or above 1 mouse unit (MU). It was shown that 1 MU of TTX corresponds to 0.22 μg TTX (Kawabata, 1978, referred to by Chen and Chou, 1998), corresponding to a dose level of 11 μg/kg bw (after *i.p.* injection).

It was established that relationship between the injected dose and the time to death very reproducible and that hence the time of death gives a good indication of the injected dose. In practice, a table is used describing the relationship between the dose and the mean time to death. In case of a positive test response, dilution of the extracts may be needed to obtain a better estimation of the concentration in the extract.

Several papers present curves expressing the relationship between the dose and the average time to death of the mice for both TTX and saxitoxin (STX) (Konosu et al., 1968; Hashimoto and Noguchi, 1971). The curve for TTX is similar to that of STX, although a bit less steep. However, for STX, 1 MU corresponds to a dose of 0.18 μg (EFSA, 2009d). Although male mice of the ddY strain are prescribed within the Japanese protocol, Suzuki (2016) showed that ICR mice are equally sensitive, whereas e.g. male mice of the BALB/c, C3H/He, C57BL/6 and DBA/2 strains are less sensitive. In practice, this could result in a slight (25%) underestimation of the dose (shown to be 25% around an injected dose of 2 MU). This study shows the high reproducibility of the assay, at least with a pure standard. The mouse bioassay in practice also detects TTX analogues, although most of these show a relatively low response except for 11-oxoTTX (Yotsu-Yamashita and Mebs, 2003). The i.p. LD_{99} for mice for purified 11-oxoTTX in that study was estimated to be 16 μg/kg, so quite similar to the LD_{100} of 12 μg/kg bw for TTX (see Section 3.2.4 on relative potencies).

In practice, the test conditions for TTX and STX are very similar, with the exception that acetic acid rather than hydrochloric acid is applied for extraction of TTX. Furthermore, according to the specific protocols, an equivalent of 0.2 g fish is injected for TTX and 0.5 g shellfish for STX according to version 1 of the SOP (EURL-MB, 2014). The latter implies that shellfish samples with TTX levels higher than 440 μg/kg (1 MU or 0.22 μg per 0.5 g), might have caused a positive response in the mouse bioassay for STXs. The follow-up of false positive results in the MBA actually led to the discovery of TTX in shellfish in Greece (Vlamis et al., 2015), with levels up to 203 μg/kg digestive gland. These levels are lower than the 440 μg/kg described above. However, it was shown that a concentration of 100 μg/kg, added to the digestive gland of non-contaminated mussels, caused the death of four out of six mice with a median time to death of 310 min (range 240 min–24 h). So, it can be concluded that the use of the MBA for routine testing of STXs, could have resulted in positive test results for shellfish samples containing TTX at levels above 440 μg/kg, but potentially even at levels as low as 100 μg/kg, although effects occur after a longer period than the standard 60 min that should be applied for STXs according to the protocols in the EU.

3.1.1.2. Cell-based assays

Since the mode of action of TTX is similar to STX, all cell-based assays for STX (EFSA CONTAM Panel, 2009) will also detect TTX. Kogure et al. (1988) showed that neuroblastoma Neuro-2a cells can be used for detection of TTX. Cells are treated with ouabain/veratridine (O/V) which will decrease cell viability by increasing the intracellular sodium levels, but TTX (sodium channel blocker) will revert the response, i.e. increase the viability. Initially, this was scored by counting the number of cells. However, the use of a water soluble tetrazolium salt to check the viability, allows a much easier application of the assay (Hamasaki et al., 1996). The test could detect 0.1 ng/well, which should be sufficient to be applied in practice. Although the assay was used for potential TTX-production by bacteria and sediments, it is unclear whether it has been used for screening of fish or shellfish samples.

3.1.1.3. Immunoassays/SPR techniques

Methods based on antibodies in either competitive inhibition enzymatic immunoassay (ELISA, Raybould et al., 1992; Neagu et al., 2006; Stokes et al., 2012) or surface plasmon resonance biosensor (SPR) (Yakes et al., 2011; Campbell et al., 2013) are potentially useful for qualitative identification. Especially useful are those using antibodies, since they also provide high throughput screening. Nevertheless, at this time, these methods are not suitable for routine screening due to the lack of cross reactivity for all TTX analogues, and as such may not identify all the analogues of TTX. Although originally intended for STX, a radioreceptor assay that uses tritiated TTX allows the detection and quantification of TTX using synaptosomal membrane fractions (Doucette et al., 2000).

3.1.2. Chemical-analytical methods

TTX can be identified and quantified by several analytical methods, such as gas chromatography-mass spectroscopy (GC–MS), nuclear magnetic resonance (NMR), liquid chromatography with fluorescence detection (LC-FL) and LC-MS. The first analysis of TTX was done by GC–MS (Naritia et al., 1981) by conversion of TTX to the C9 base structure and later conversion to a trimethylsilane derivative. GC–MS is not suitable for quantitation since TTX is non-volatile (Alcaraz et al., 1999; Bane et al., 2014). NMR requires close to pure TTX samples to avoid the interference of matrix components. LC-FL (Yasumoto and Michishita, 1985) is useful to simply detect TTX and major TTX analogues (11-oxoTTX, 4-epiTTX and 4,9-anhydroTTX). However, it has the complication of the different fluorescence of other analogues (6-epi TTX is 20-fold more fluorescent than TTX, and 11-deoxy TTX is 100-fold less fluorescent than TTX (Yotsu et al., 1989; Pires et al., 2005). Although the simultaneous analysis of STX and analogues (paralytic shellfish poisoning (PSP) group) and TTX has been reported by one single LC-FL method (Rey et al., 2016), the differences in fluorescence between PSP and TTX is still a problem to solve.

This leaves LC–MS as the method of choice for the detection and quantification of TTX and analogues (see Table 4). LC–MS methods in general apply atmospheric pressured ionisation (API) with an electrospray-ionisation (ESI) interface that operates in positive ionisation mode (Shoji et al., 2001; Nakagawa et al., 2006; Tsai et al., 2006; Jen et al., 2008; Rodríguez et al., 2012). The separation is usually done with reverse-phase columns and solvents that contain an ion pair reagent, such as ammonium heptafluorobutyrate. Because TTX is a rather polar molecule, separation with reverse phase is poor and a lower noise and better elution and sensitivity is obtained with hydrophilic interaction liquid chromatography (HILIC, Nakagawa et al., 2006; Diener et al., 2007; Gerssen et al., 2011; Kudo et al., 2012; Yotsu-Yamashita et al., 2013). In HILIC, a gradient with initially low aqueous/high polar organic solvents is used, and not ion pair reagents, and a final higher water proportion to elute polar analytes. The typical positive ionisation provides a TTX ion of 320 (*m/z*) (McNabb et al., 2010). Although the main product ion for TTX is 162 (*m/z*), other TTX analogues can generate this same ion, and hence the MRM mode is commonly used to identify different analogues (Shoji et al., 2001; Jang et al., 2010; McNabb et al., 2010, 2014; Yotsu-Yamashita et al., 2011, 2013).

To avoid the problem of the lack of calibrants for the various TTX analogues, the alkaline hydrolysis (1N NaOH, 100°C, 45 min) of the compounds has been proposed, generating the C9 base 2-amino-6-(hydroxymethyl)quinazolin-8-ol, that is common to all, and then use this structure for indirect quantification (McNabb et al., 2014). Although this allows the estimation of total TTX and analogues, it does not account for toxicity and quantitation of each analogue. A confirmatory analysis is required to compare the profiles before and after hydrolysis (Turner et al., 2015).

Since 2012, there are certified standards available for TTX and 4,9-anhydroTTX but in general most of the published studies have not used them. In Table 4, the different LC–MS methods for the analysis of TTX and analogues are presented.

Table 4: Different LC–MS methods for the analysis of TTX and its analogues

Eluent	Column[(a),(b)]	*m/z*[(c)]	LOD	Matrix	Reference
A: 10 mM ammonium formate and 10 mM formic acid in water B: 80% acetonitrile and 20% water with 5 mM ammonium formate and 2 mM formic acid	ZIC-HILIC (5 μm, 150 x 2.1 mm)	272/162, 302/162, 304/162, 320/162	0.3 pmol	Pufferfish	Diener et al. (2007)
1 vol% acetonitrile, 20 mM ammonium heptafluorobutyrate, 10 mM ammonium formate (pH 4.0)	Reversed phase (250 x 4.6 mm)	320/162, 304/176	0.7 pmol	Pufferfish	Shoji et al. (2001)
10 mmol/L ammonium formate with formic acid (95:5, v/v), with 5 mM heptafluorobutyric acid and 2% acetonitrile	Reverse phase (5 μm, 2.1 x 15 mm)	320.1/162, 320.1/302.3, 320.1/256.2	0.13 ng/mL	Human urine and plasma	Fong et al. (2011)
90% (v/v) 20 mM ammonium acetate and 10% acetonitrile	Reverse phase (150 x 4.6 mm)	320.1	0.5 ng/mL	Mouse serum	Hayashida et al. (2004)

Eluent	Column[(a),(b)]	*m/z*[(c)]	LOD	Matrix	Reference
20 mM ammonium acetate–methanol (75:25)	Reverse phase (150 x 4.6 mm)	320.1/302, 320/284	0.1 µg/g	Pufferfish	Horie et al. (2002)
16 mM ammonium formate buffer (pH 5.5) and acetonitrile (3:7, v/v)	HILIC (5 µm, 150 x 2 mm)	320/162, 302/162, 304/162, 288/224, 272/162	0.5 nmol/g	Pufferfish	Jang et al. (2010)
A: 0.1% formic acid in water B: methanol	HILIC (150 x 4.6 mm)	320/302, 320/256, 320/162	1 ng/mL (LOQ)	Blood serum	Jen et al. (2008)
A: 5% acetonitrile B: 95% ACN with 1% acetic acid (pH 3.5)	HILIC (1.7 µm, 100 x 2.1 mm)	319.92/161.80 319.92/302.00	7.3 mg/kg	Gastropod	Nzoughet et al. (2013)
A: 10 mM ammonium formate and 10 mM formic acid in water B: 95% acetonitrile and 5% water with 5 mM ammonium formate and 2 mM formic acid	HILIC (3.5 µm, 150 x 2.1 mm)	320/302/162, 302/256/162, 304 /286/176, 290/272/162, 272/254/162	16 ng/mL	Pufferfish	Rodríguez et al. (2012)
16 mM ammonium formate buffer (pH 5.5)/acetonitrile (3:7, v/v)	HILIC (5 µm, 150 x 2 mm)	320/162, 302/162, 290/162, 272/162 or high resolution molecular weights 320.1088, 302.0983, 336.1038	0.6 pmol	Pufferfish	Yotsu-Yamashita et al. (2011), Nakagawa et al. (2006), Yotsu-Yamashita et al. (2013), Puilingi et al. (2015)

(a): ZIC: Zwitterionic hydrophilic interaction chromatography.
(b): HILIC: Hydrophilic interaction liquid chromatography.
(c): *m/z*: 320 (TTX, 4-epiTTX, 6-epiTTX), 306 (11-norTTX-6,6-diol), 304 (11-deoxyTTX), 302 (4,9-anhydroTTX, 6-epi-4,9-anhydroTTX), 290 (11-norTTX-6 (S)-ol, 11-norTTX-6(R)-ol), 272 (5,6,11-trideoxyTTX). See Shoji et al. (2001).

Table 5. provides an overview of advantages and disadvantages of available methods.

Table 5: Overview of the advantages and disadvantages of available methods

Method	Advantages	Disadvantages
Mouse bioassay	1) Estimates combined effect 2) Allows (semi) quantification, similar to STX	1) Requires the use of animals 2) Does not discriminate between TTX and STX 3) Does not provide information on the toxin profile 4) Not interlaboratory validated
Cell-based assays methods	1) Estimate combined effect as they use the mechanism of action of the toxin group 2) Allow high-throughput screening	1) Do not provide information of the toxin profile. 2) Do not discriminate STXs from TTXs 3) Not interlaboratory validated
Antibody-based methods (SPR, ELISA)	1) Allow estimation of the concentration within antibody cross reactivity 2) Allow high-throughput screening	1) Do not provide information of the toxin profile 2) Not interlaboratory validated 3) Do not estimate overall toxicity 4) Due to point 3, may overestimate largely the amount of equivalent TTX if high amounts of low active compounds are present and detected 5) Only detect the presence of the toxins that the antibody cross-reactivity allows

Method	Advantages	Disadvantages
Chemical-analytical methods	1) Provide information on toxin profile 2) Allow quantification of TTX and each analogue	1) Do not provide information about toxicity 2) Highly dependent on available TEF 3) Highly dependent on available standards 4) Not interlaboratory validated

ELISA: enzyme-linked immunoabsorbent assay; SPR: surface plasmon resonance; STX: saxitoxin; TTX: tetrodotoxin; TEF: toxic equivalency factor.

3.1.2.1. Concluding remarks on methods of detection and quantification

The mouse bioassay (MBA) has been the most widely applied test for TTXs, although not within the EU. It detects combined toxicity and cannot clearly discriminate between TTXs and STXs. Biochemical methods based on cell lines or antibodies offer a potential alternative for the MBA. In particular, the cell-based assays are able to detect the various analogues of TTX, based on their effects but depending on their toxic potency. Similar to the MBA, they cannot discriminate between the different TTX analogues and will also detect STX and its analogues. As such they can be used for screening to show the absence of toxins (both TTX and STX in one assay), but LC–MS is required for identification of the specific toxins and analogues. However, bioassays may detect novel analogues and in combination with LC–MS can be used for their identification, using a bioassay directed identification approach. Immunoassays will be more specific towards TTX and may be less suitable for broad detection of different analogues, depending on their cross-reactivity with such compounds. Prior to their use in routine control, suitable clean-up methods should be developed for the relevant matrices and these methods should subsequently be validated in interlaboratory trials.

For information on toxin profile and quantification the chemical-analytical methods, in particular LC–MS/MS methods are the most suitable. Some of these methods allow the simultaneous detection of TTXs and STXs. Although validated within laboratories, they require further validation in interlaboratory trials.

3.2. Hazard identification and characterisation

3.2.1. Toxicokinetics

The toxicokinetics of TTX in humans are not fully understood. TTX seems to be rapidly absorbed in the human digestive tract, based on the short delay between ingestion and onset of the symptoms (see Section 3.2.2).

Data from human poisoning cases showed that TTX can be detected in the urine after a few hours and up to 7 days after ingestion of contaminated fish (Oda et al., 1989; Fukushima, 1991; Kawatsu et al., 1999; O'Leary et al., 2004), with concentrations ranging from 0.4 to 650 ng/mL (Table 6). In plasma/serum, concentrations of TTX fall rapidly and may be undetectable after 6–24 h (Oda et al., 1989; Yamazaki and Shibuya, 1995; O'Leary et al., 2004; Fong et al., 2011). Reported concentrations in human cases ranged from < 1 to 320 ng/mL plasma/serum, as shown in Table 6.

Table 6: TTX concentrations in urine and serum/plasma reported in human cases of food poisoning[(a)]

Location	Patients	Detection method	LOD (ng/mL)	TTX in urine (ng/mL)	TTX in plasma or serum (ng/mL)	Reference
Japan	1	GC–MS	n.a.	281 on day 2 43 on day 3 16 on day 4 <LOD on day 5	36.3 on day 1 (3 h after ingestion) < LOD on days 2, 3, 4, 5	Oda et al. (1989)
Japan	11	GC–MS	0.5	27.2–650	2.5–320	Fukushima (1991)
Japan	1	n.a.	n.a.	n.a.	52.3 on day 1 < LOD on days 3, 6, 9, 42	Yamazaki and Shibuya (1995)

Location	Patients	Detection method	LOD (ng/mL)	TTX in urine (ng/mL)	TTX in plasma or serum (ng/mL)	Reference
Japan	6 (a–f)	n.a.	2	a) 86 (day n.a.) b) 96 on 1st day n.a., 6 on day 6 after 1st day c) 82 on 1st day n.a., 17 on day 5 after 1st day d) 8 on 1st day n.a., 6 on day 3 after 1st day e) 102 on day n.a. f) 74 on 1st day n.a., 13 on day 5 after 1st day, 6 on day 6 after 1st day, 7 on day 7 after 1st day	n.a.	Kawatsu et al. (1999)
Australia	7	LC-FL	5	28–258 (24 h after ingestion)	< LOD to 5 on day 1	O'Leary et al. (2004)
Taiwan	4	LC–MS	1.0	169–325 (15 h after ingestion)	< LOD to 8 (15 h after ingestion)	Hwang et al. (2005)
Japan	7	LC–MS/MS	0.1	15–150	0.9–1.8	Akaki and Hatano (2006)
Taiwan	4	LC–MS	4.9 (15.6 nM)	15–109.7 (47–344 nM, 10 h after ingestion)	< LOD to 13 (4.5–40.6 nM, 10 h after ingestion)	Tsai et al. (2006)
Taiwan	1	LC–MS/MS	0.1	n.a.	3.3 (12 h after ingestion)	Jen et al. (2008)
Spain	1	LC–MS	n.a.	285.38 (32 h after ingestion)	24.54 (32 h after ingestion)	Fernandez-Ortega et al. (2010)
		LC–MS/MS	n.a	211.1 for TTX 692.1 for 5,6,11-trideoxyTTX (32 h after ingestion)	26.4 for TTX 86.5 for 5,6,11-trideoxyTTX (32 h after ingestion)	Rodriguez et al. (2008)
Hong-Kong	8	LC–MS/MS	0.13	59.3 (8 h after ingestion)	< 2.5 (8 h after ingestion)	Fong et al. (2011)
				76.8 (8 h after ingestion)	< 2.5 (8.25 h after ingestion)	
				109.6 (8 h after ingestion)	< 2.5 (7.75 h after ingestion)	
				460.5 (6.5 h after ingestion) 351.9 (12.5 h after ingestion)	< 2.5 (6.5 h and 12.5 h after ingestion)	
				44.8 (6.45 h after ingestion) 17.6 (12.5 h after ingestion)	< 2.5 (6.45 h and 12.5 h after ingestion)	
				30.7 (8 h after ingestion)	< 2.5 (8 h after ingestion)	
				48.2 (9 h after ingestion)	< 2.5 (9 h after ingestion)	
				88.2 (5.5 h after ingestion)	na	
Korea	3	LC–MS/MS	0.32		3.9, 12.8, 29.7 (within 24 h after ingestion)	Cho et al. (2012)
Bangladesh	38	ELISA	1.6	0.4–75.4	< 1.6 to 13.7	Islam et al. (2011)

ELISA: enzyme-linked immunosorbent assay; GC–MS: gas chromatography–mass spectrometry; LC–MS: liquid chromatography–mass spectrometry; LC–MS/MS: liquid chromatography–tandem mass spectrometry; LC-FL: liquid chromatography-fluorescence; LOD: limit of detection; LOQ: n.a.: time to intake TTX not available.
(a): Modified from Leung et al. (2011).

No data are available regarding the distribution and the metabolism of TTX or TTX analogues in humans.

No studies were identified on the toxicokinetics of TTX or analogues in animals, using for instance radiolabelled compounds. However, from acute toxicity studies in mice (see Section 3.2.2, Toxicity), it can be seen that LD_{50} values after i.p. application are between 25 to 50 times higher than after oral administration.

TTX blood levels in treated female mice 2 h after a single administration by gavage were 6.46 + 1.91 ng/mL at 500 μg/kg bw, 1.29 + 0.18 ng/mL at 250 μg/kg bw and < LOQ at 125 μg/kg bw. Data were obtained by ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). TTX was quantified against a certified reference standard and the LOD was 0.5 ng/mL

and the LOQ was 1 ng/mL (Abal et al., 2017). More details on the study are presented in Section 3.2.2, Toxicity.

In conclusion, little is known on the absorption and excretion of TTX and its TTX and no information has been found on its distribution and metabolism.

3.2.2. Toxicity

No subchronic or chronic studies on TTX and its analogues have been identified.

3.2.2.1. Acute toxicity

Animal toxicity

Acute toxicity of TTX

According to Zimmer (2010) the overt clinical effects in experimental animals due to sublethal doses of TTX result in severe impairment of neurological/neuromuscular functions under the control of Na^+ channels and results in urination, hypersalivation, retching, diarrhoea, diminution or the absence of reflex, skeletal muscle fasciculations, lethargy, ataxia, ascending progressive paralysis, respiratory pattern changes and dyspnoea. Death occurs upon blockade of the phrenic nerve, the diaphragm and neurons in the central respiratory network. A direct effect of the toxin on the cardiovascular system has been excluded, following extensive review of results obtained in several animal models (rat, rabbit, dog and cat) considering TTX doses ranging from 2.5 to 80 μg/kg bw intravenously (i.v.) and 1 to 15 μg/kg bw i.p. (Zimmer, 2010).

Acute LD_{50} values for TTX have been established in different animal species exposed to the toxin through different routes of exposure (see Table 7). In mice, an LD_{50} of about 10 μg/kg bw has been observed after i.p. administration of TTX (see also Section 3.1.1 on bioassays).

An oral LD_{50} was determined in Swiss female mice (18–21 g) with an optimised 4-Level Up and Down Procedure (according to OECD, 2008), using a certified standard of the toxin (Abal et al., 2017). The methodology employed consisted of administration of a saline solution of TTX (98% pure, personal communication Paula Abal) by gavage as a single oral dose (10 mL/kg) to Swiss female mice at doses of 25, 75, 125, 250, 500 and 1,000 μg/kg bw. Based on the outcome, the number of mice treated was decreased/increased with each level (animal number being 3, 5, 7 and 9 at the first, second, third and fourth level, respectively). Also, the dose varied depending on the results obtained in the previous level (starting dose 1,000 μg/kg bw, dose decreased at the next level if more than 50% of the mice died, increased if less than 50% of the mice died within the study duration of 2 h). In practice, every next dose had to be decreased, until a dose of 125 μg/kg bw, with no dead animals within 2 h among the nine treated ones. No dead animals were observed within 2 h with two additional dose levels of 25 and 75 μg/kg bw. Symptoms after exposure to the toxin and time to death were registered (e.g. numbness, apathy, piloerection, paralysis of extremities, seizures). The number of dead animals increased from 0% (0/9) at 125 μg/kg bw to 57% (4/9), 80% (4/5) and 100% (3/3) at the dose levels of 250, 500 and 1,000 μg/kg bw, respectively. Based on this, the estimated LD_{50} for TTX was 232 μg/kg bw, the LD_{100} was 1,000 μg/kg bw. This confirms the steep dose–response curved observed after i.p. treatment of mice (Kao and Fuhrman, 1963).

First signs of poisoning (apathy in all treated animals and piloerection in two out of nine treated mice) occurred at a lowest dose of 125 μg/kg bw, while no behavioural signs were observed at the dose of 75 μg/kg bw (n = 9). At doses of 250 μg/kg bw and higher paralysis of extremities, seizures and death were observed. Macroscopic organ examination showed dilated stomachs with liquid and gas accumulation for doses of 125 μg/kg bw and higher and cardiac stiffness at 1,000 μg/kg bw.

Another study reported the acute toxicity of TTX in mice after various routes of application (Xu et al., 2003). For mice, this included intragastric application, showing a 50-fold lower toxicity than after i.p. or subcutaneous application. Also rabbits were tested, however, the results reported were unclear and are therefore not reported in Table 7 below.

Table 7: Summary of acute toxicity studies with laboratory animals

Species	Route of administration/ Purity of compound	LD_{50} (μg/kg bw)	References
Mouse	Intraperitoneal TTX source unknown	10.7	Xu et al. (2003)
Mouse	Intraperitoneal[(a)] Purity: 99.8%	9 (8.2–9.8)	Marcil et al. (2006)
Mouse	Intraperitoneal Purity not reported	10	Kao and Fuhrman (1963)
Mouse	Subcutaneous Purity not reported	12.5	Xu et al. (2003)
Mouse	Subcutaneous Purity not reported	10	Kao (1966)
Mouse	Intragastric Purity not reported	532	Xu et al. (2003)
Mouse	Oral (gavage) Purity: 98%	232	Abal et al. (2017)
Rat	Intramuscular[(a)] Purity: 99.8%	11.1 (10.5–11.7)	Marcil et al. (2006)

bw: body weight; TTX: tetrodotoxin.
(a): No experimental data provided.

Acute studies with pharmacological TTX preparations

To study the effects of TTX on pain reduction, Marcil et al. (2006) injected rats and mice with sublethal doses of TTX (a sterile, non-pyrogenic aqueous solution for parental administration containing TTX 15 μg/mL, 99.8% pure). Male Wistar rats (170–220 g) and male Swiss Weber mice (20–30 g) were treated subcutaneously (s.c.) with TTX at doses of 0.3, 1, 3 or 6 μg/kg bw (dilution in saline 0.9%) 30 min before pain induction. TTX adverse effects were monitored and recorded up to 90 min, the time needed to complete the longest battery of pain tests. No respiratory distress, motor impairment or paralysis was evident but treated animals displayed antinociceptive[9] effects. The ED_{50} was between 0.62 and 3 μg/kg bw depending on the pain model, showing no respiratory distress, motor impairment or paralysis.

Guzman et al. (2007) injected mice with a solution of TTX (15 μg/mL, 99.8% pure). Male (26–34 g) and female (21–25 g) CD-1 mice were administered s.c. with TTX at doses of 2, 4 and 8 μg/kg bw and sacrificed 24 h (all doses) and 48 h (TTX 8 μg/kg bw) later for bone marrow dissection. No mortality and no clinical signs were evident in mice receiving 2 μg TTX per kg bw. Piloerection, ataxia, hunched back, dyspnoea, decreased motor activity, muscular weakness and palpebral ptosis were observed at 8 μg/kg bw in mice. Less severe dyspnoea and decreased motor activity was evident at 4 μg/kg bw. Authors did not specify the time of occurrence of behavioural effects. It is thus inferred that TTX adverse effects occur along the timeframe before sacrifice. Also, male Sprague–Dawley rats (169–195 g) were injected s.c. twice (second dose given 14 h after the first and 2 h prior to sacrifice) with TTX at doses of 2.4 and 8 μg/kg bw. Rats were underactive and displayed nervous behaviour, irregular and fast respiration, flattened posture, unsteady gait and partially closed eyelids before sacrifice occurring 16 h after TTX administration. Reduced body tone was also evident in high dose animals (8 μg/kg bw).

In Marcil et al. (2006), sublethal doses of TTX (single administration, 0.3–6 μg/kg of a 99.8% pure solution) administered s.c. to rats and mice were not associated with the occurrence of clinical signs within 30–90 min. On the contrary, clinical signs were evident in a dose-dependent manner in mice exposed to TTX (single administration, 2–8 μg/kg bw of a 99.8% pure solution), but observed in a 2–48 h time frame (Guzman et al., 2007). In this study, no effects were evident at 2 μg/kg (Guzman et al., 2007).

Acute toxicity studies with TTX from extracts and pufferfish meat

Some studies describe the toxicity induced by i.p. injection of extract, homogenate (Zaki et al., 2001; Saoudi et al., 2011) or oral administration (Saoudi et al., 2009) of meat obtained from different

[9] Reducing sensitivity to painful stimuli.

types of pufferfish (*Lagocephalus sceleratus and Lagocephalus lagocephalus*) as a source of TTX. Reversible increase in different neurotransmitters (serotonin, acetylcholine, histamine and noradrenaline) (Zaki et al., 2001), decrease of serum enzyme activity (i.e. aldolase (ALS), alanine aminotransferase (ALT), alkaline phosphatase (ALP), y-glutamyl transpeptidase (GTP) and lactate dehydrogenase (LDH)), hepatic damage (increased lipid peroxidation, focal necrosis, hepatic chord, infiltration of lymphocytes, congestion in central veins and sinusoids; Saoudi et al., 2009, 2011) were observed. No information on the amount of TTX or the presence of analogues was provided.

3.2.2.2. Concluding remarks on acute toxicity

The acute LD_{50} values for TTX in mice range from 8.2 to 10.7 μg/kg bw following i.p. exposure. Similar values, 10–12.5 μg/kg bw were reported upon s.c. administration. LD_{50} levels in mice increased to 232 and 532 μg/kg bw after oral administration.

A NOAEL of 75 μg/kg bw (respective LOAEL was 125 μg/kg at which apathy was observed in 9/9 animals) was established by the authors in an acute oral study in mice with a single administration (Abal et al., 2017). In this study, at higher doses lethality was observed, i.e. at doses of 250 μg/kg bw (four out of seven animals or 57%), 500 μg/kg bw (four out of five animals or 80%) and 1,000 μg/kg bw (three out of three animals or 100%). The CONTAM Panel noticed the relatively short duration of the observation period (2 h) after which all surviving animals were killed. This precluded the observation of potential deaths and other effects occurring at later time-points, as observed by Vlamis et al. (2015) with shellfish extracts spiked with TTX at relatively low levels.

3.2.2.3. Genotoxicity studies

TTX did not show any genotoxic activity in a battery of good laboratory practice (GLP)-compliant *in vitro* and *in vivo* assays conducted by Guzman et al. (2007) according to OECD guidelines, and including an Ames test (bacterial reverse-mutation assay), an *in vitro* human lymphocyte chromosome-aberration assay, an *in vivo* mouse bone-marrow micronucleus assay and an *in vivo* rat-liver unscheduled DNA synthesis (UDS) assay. The TTX used in this study was commercially obtained. For *in vitro* assays, TTX powder (purity of 97.1%) was dissolved in a 0.02% acetic acid solution. For *in vivo* assays the pharmaceutical dosage form 'Tetrodotoxin Injectable' was used (purity of 99.8%).

Maximum test concentrations in *in vitro* assays were determined by the TTX limit of solubility in the formulation vehicle. In the Ames test, TTX was tested at concentrations of up to 200 μg/plate in four *Salmonella* Typhimurium histidine auxotrophic strains (TA98, TA100, TA1535 and TA1537) and an *Escherichia coli* tryptophan-requiring strain (WP2 uvrA pKM101).

In the chromosome-aberration assay, human lymphocytes were exposed to TTX at concentrations of up to 50 μg/mL for 3 and 20 h in the absence of S9, and for 3 h in the presence of S9.

For the *in vivo* assays, maximum tested dose levels were determined by the acute lethal toxicity of TTX after s.c. injection. Positive control animals were dosed orally (gavage). In the mouse micronucleus assay, TTX dose levels of 2, 4 and 8 μg/kg bw (in 0.02% acetic acid) were administered by s.c. injection to groups of five male and female CD-1 mice, and bone-marrow samples from femurs were taken 24 and 48 h (high-dose animals only) after administration.

In the UDS assay, male Sprague–Dawley rats were given TTX by s.c. injection (in 0.004% acetic acid) on two occasions with a 14-h interval at dose levels of 2.4 and 8 μg/kg bw, the last dose being administered 2 h before liver perfusion and hepatocyte culturing. Relevant vehicle and positive control cultures and animals were included in all assays.

Crude extracts from skin and liver of the porcupinefish (*Diodon hystrix*) containing TTX (0.5 mg/mL), caused spindle fibre aberration in the human lymphocyte chromosome aberration assay, but this was not the case with extracts from intestine, eyes and gonads. All the five extracts showed lack of DNA damage in the comet assay (Lokesh et al., 2016).

A quantitative structure–activity relationship (QSAR) analysis was conducted using DEREK, VEGA and the Danish (Q)SAR data base, to obtain information on the potential properties and activities of 4,9-anhydroTTX and 11-oxoTTX. No structural alert for genotoxicity was identified for either analogue (data available from EFSA on request).

3.2.2.4. Human observations

TTX is a potent neurotoxin, inhibiting nerve and muscle conduction by blocking sodium channels. Symptoms of toxicity can occur within 10–45 min of ingestion, although a delayed response of 3–6 h has also been reported (Hwang and Noguchi, 2007). Bane et al. (2014) and Lago et al. (2015) described four grades of TTX poisoning (both citing Fukuda and Tani, 1941):

Grade 1: perioral numbness and paraesthesia, with or without gastrointestinal symptoms; (nausea, vomiting, diarrhoea, and abdominal pain);

Grade 2: lingual numbness (of face and other areas), early motor paralysis and incoordination, slurred speech with normal reflexes;

Grade 3: generalised flaccid paralysis, respiratory failure, aphonia and fixed/dilated pupils;

Grade 4: severe respiratory failure and hypoxia, hypotension, bradycardia, cardiac dysrhythmias and unconsciousness may occur.

In fatal cases, progressive paralysis leads to death by respiratory failure and cardiac collapse (Lago et al., 2015). Liu et al. (2011) proposed that electrophysiological changes, such as nerve conduction velocity and somatosensory-evoked nerve potentials could be used to determine the extent, course and range of nerve system damage in patients with acute TTX poisoning, but did not associate the severity of the changes to specific doses of TTX.

TTX poisoning has most frequently been reported in Japan, associated with consumption of pufferfish (fugu), which is considered a delicacy. Regulations were introduced in Japan in 1983 to define species of pufferfish that are allowed to be caught and marketed, and for careful preparation of them, but there continue to be cases of pufferfish poisoning (Noguchi and Ebesu, 2001; Noguchi et al., 2011a,b). These authors reviewed data for pufferfish poisoning incidents in Japan over the period 1965–2010, including annual data for the period 1995-2010 with no clear trend over this latter period.

Cases of TTX poisoning have also been reported in a number of other countries including Australia, Bangladesh, Brazil, China, Israel, Morocco, Singapore, Spain, Taiwan and the USA, resulting from consumption mainly of pufferfish, but also from goby fish and marine gastropods.

The doses of TTX that cause symptoms of acute toxicity, or lethality, in humans are unclear. Values for lethal doses are reported in the literature, either without attribution or by citing secondary literature and reviews, and the CONTAM Panel was not able to trace the origins of these values, and hence their inherent uncertainties and assumptions. Hwang et al. (2005), citing Tani (1945), noted that the MLD[10] of TTX for a human by oral administration is estimated to be 10,000 MU, corresponding to 2 mg based on the observation that the minimum dose that kills a mouse (1 MU) is equal to 0.2 μg of TTX. Noguchi and Ebesu (2001) and Noguchi et al. (2011a,b) mention the same MLD for humans but without citing a source. Since 50 kg is commonly used as a default bodyweight in the TTX scientific literature, presumably relating to Asian individuals, the amount of 2 mg is sometimes extrapolated to 40 μg/kg bw for these values. The Hazardous Substance Databank (HSDB)[11] notes that 'a fatal dose may be as little as 1–4 mg per person', citing a text book (Klaasen, 2001) as the source, which in turn does not provide any information on the basis of this assertion. Some papers also refer to a lowest dose resulting in symptoms of 0.2 mg (e.g. Tsang and Tang, 2007; Katikou et al., 2009), which corresponds to 4 μg/kg bw for a 50-kg individual. Again the basis for this observation was initially unclear as no references are provided in these papers. However, the CONTAM Panel was able to identify two reviews describing intoxications at this level (Noguchi and Ebesu, 2001; Hwang and Noguchi, 2007). If this value were robust, it could be regarded as a LOAEL for sensitive individuals. However, there is a lack of information on the accuracy of the exposure estimate. This is particularly problematic for case reports related to marine biotoxins, since there is uncertainty with respect to the concentration of toxin in the food that was actually consumed. There is potential for the reported exposures to be either under- or overestimated, and without information on the ways that these estimates were derived, and the number of individual case reports reviewed, it is not possible to characterise the degree of uncertainty.

Table C.1 in Appendix C summarises the reports of TTX poisoning retrieved in the literature searches or identified from other sources for the same time period, that do not provide information on possible consumption and therefore exposure cannot be estimated. The following paragraphs provide detail on poisoning cases reported since 2000, for which TTX exposure has been estimated.

The review of Noguchi and Ebesu (2001) described a number of case reports, with limited detail, including two that provided estimates of exposure. The primary reference cited by the authors is an

[10] There is unclarity on the terminology, e.g. the review of Bane et al. (2014) transposed the MLD 'minimum lethal dose' reported by Noguchi and Ebesu (2001) and Noguchi et al. (2011a,b) into a 'median lethal dose'. Similar inconsistencies are seen throughout the literature. Given that a median lethal dose is a statistical outcome of an acute toxicity study, it seems likely that where a human MLD is referred to, it actually means a minimum lethal dose, however there is also uncertainty in this assumption.

[11] https://toxnet.nlm.nih.gov/newtoxnet/hsdb.htm, accessed 5 October 2016

earlier publication in Japanese (Noguchi and Akaeda, 1998). A 48-year old male in Japan became ill within 1 h of eating 'more than 4 livers' and some flesh of wild puffer. He subsequently died despite hospitalisation. The leftover liver contained 715–4,260 MU/g and it is stated that he ingested more than 10,000 MU. Since, 10,000 MU would correspond to only 2–14 g liver, it is likely that he must have consumed very much more than 10,000 MU and the mention of 10,000 is likely to be based on the assumption that this is the MLD. The CONTAM Panel noted that this report does not provide good information on the lethal dose. A second case describes a 59-year old female who cooked and ingested 3.5 livers of wild pufferfish (estimated weight 300 g). After 50 min, she developed numbness in the lips and tip of the tongue, followed by numbness of the fingers and vomiting, and then more severe symptoms (dyspnoea, mydriasis and paralysis of the extremities) 5 h later in the hospital. She recovered following artificial respiration. After 9 h, she developed arrhythmia for which she was treated with drugs. The report states that the leftover liver contained 3.5 MU/g, and estimated that the total amount of TTX ingested was 900–1,000 MU (equivalent to 0.18–0.2 mg TTX). The CONTAM Panel noted that it is not clear what the 'leftover liver' was or if the concentration was actually comparable to the livers she consumed.

A total of 36 individuals (18 men and 18 women) were admitted to hospital in Khulna, Bangladesh, after consuming pufferfish (Ahmed, 2006). Seven died as a result of respiratory failure, and the others recovered following treatment. Six days after the incident, pufferfish were collected from a fish-landing centre adjacent to the implicated area, and stored frozen for transfer to a laboratory. The partially thawed fish were then dissected into skin, muscle, liver gonad and viscera and TTX concentration was estimated. Concentrations in the poisoning-related samples were 13.2–18.9 MU/g in the skin, 2.7–4.4 MU/g in the muscle, < 2.0–4.9 MU/g in the liver, < 2.0–132 MU/g in the gonads and 14.8–37 MU/g in the viscera (excluding liver). The victims ate the whole fish including the gonads, but excluding the viscera. The authors reported the average toxicity in the whole body without the viscera as about 17 MU/g, and estimated that the lowest amount of fish consumed by one of the fatalities was about 150 g, which would be equivalent to 2,550 MU. Since 10,000 MU corresponds to approximately 2.2 mg TTX, the ingested amount would be about 0.56 mg, which corresponds to 11 μg/kg bw for a 50-kg person. However, the CONTAM Panel noted that this estimate is subject to considerable uncertainty resulting from the time between the outbreak and sampling of fish (6 days after the incident), the use of the average concentration and assumption on lowest amount consumed by the fatal cases.

Hwang and Noguchi (2007) reported a case of TTX poisoning that occurred in Taiwan in 1998. A 52-year old woman with uraemia and her healthy 55-year-old husband ate fish soup. About 4 h later, the woman developed a headache, lingual and oral tingling and numbness in the distal parts of all four limbs. Her symptoms became increasingly severe leading to respiratory distress, cyanosis and comatose-like state, from which she eventually recovered following hospital treatment. The husband remained asymptomatic. The remains of the cooked fish were found to contain 25 MU/g and it was estimated that both the man and the woman consumed about 0.2 mg TTX. The authors suggested that there was a synergistic effect of uraemia and TTX (Hwang and Noguchi, 2007).

Frozen fish purchased from an Asian market in Chicago was consumed in soup by a family of three. (Cohen et al., 2009). The woman (47-year-old) suffered symptoms of TTX poisoning within 30 min and was admitted to hospital, where she recovered following treatment. The husband (55-year-old) consumed less of the soup and experienced less severe symptoms, not requiring hospitalisation. The 10-year-old daughter tasted only one spoonful of the soup and did not experience any symptoms. Three samples of left-over soup were analysed by LC–MS, and found to contain TTX at 4.63 ± 1.67 μg/g TTX. STX was not detected. Based on the amount of soup consumed (700 and 230 mL, respectively) and the mean TTX concentration, it was estimated that the female patient consumed 'as much as 3 mg TTX', and the husband consumed 'as much as 1 mg TTX'. Based on a 70-kg adult, these correspond to 42 and 14 μg/kg bw, respectively. Samples of similar fish, labelled as 'frozen fish' were purchased from the same market and identified as pufferfish of the genus Lagocephalus (Cohen et al., 2009). The CONTAM Panel noted that in this case report, TTX was measured by chemical analysis of samples of the fish soup that caused the TTX poisoning, and therefore, the exposure estimates are likely to be more reliable than in outbreaks where the analysis is conducted on fish obtained from the same region subsequent to that causing the poisoning.

The first report of poisoning due to TTX in Europe is from Malaga, Spain. A 49-year-old man consumed trumpet clam (*Charonia lampas sualiae*) that had been caught off the southern coast of Portugal and sold in a local fish market (Rodriguez et al., 2008; Fernandez-Ortega et al., 2010; Fernández-Fígares et al., 2013). He boiled it for 45 min, then ate the ventral portion and a few minutes later started to feel perioral numbness, which extended to both arms, followed by abdominal

pain, nausea and vomiting. His symptoms worsened and he was admitted to hospital for treatment. He recovered and was discharged 72 h after admission. By MBA, the non-ingested shellfish was found to contain 1.51 and 255 mg/kg in the meat and digestive gland, respectively. The presence of TTX in the digestive gland was confirmed by LC–MS, and quantified at a level of 248.5 mg/kg. In the flesh, TTX could not be detected (LOD not presented). Analysis of TTX analogues was not reported in this paper. The authors noted that 'consumption of the digestive glands of the mollusc, about 5 g, was enough to produce the symptoms described' (Fernandez-Ortega et al., 2010). Consumption of about 5 g at the TTX level of 248.5 mg/kg would lead to an exposure of 1.24 mg TTX or 18 μg/kg bw in a 70-kg adult but from the publications it is unclear if the patient consumed part of the digestive gland. Rodriguez et al. (2008) also reported on this case and stated that analysis of the digestive gland by LC–MS showed that it contained 315 mg/kg of TTX and 1,004 mg/kg of 5,6,11-trideoxyTTX. Both were not detected in the meat. This TTX analogue seems much less toxic than TTX (see Section 3.2.4 on relative potencies of TTX analogues) although Rodriguez et al. (2008) assumed a higher relative potency (about 0.13) in order to explain the discrepancy between the level of TTX detected with LC–MS and the overall toxicity in the mouse bioassay. Taking into account that potency, the amount of 5 g digestive gland would lead to an exposure of about 2 mg, being the often reported MLD for humans. The CONTAM Panel noted that based on the three publications, there is considerable uncertainty on the actual amount consumed and whether this was only the meat or also the digestive gland. Based on the mouse bioassay, the amount of TTX that could have been ingested with the meat appears to be much lower than if he would have consumed part of the digestive gland. However, based on personal communication (Luis Botana), the patient reported to have ingested an undefined part of the digestive gland.

You et al. (2015) conducted a case series analysis of an outbreak of TTX poisoning associated with consumption of goby fish in three towns of the Guangdong province of China in 2012. The number of cases that were hospitalised was 22 (9 males, 13 females, aged 31–78 years), showing mild to severe symptoms but no deaths. TTX was measured by LC–MS/MS in one sample of remaining cooked goby from one of the cases, and in two samples of uncooked gobies from the same batch responsible for the poisoning purchased from the same markets. The concentration was 2.09 mg/kg in the cooked sample. In the two uncooked samples, the concentrations were 1.86 and 2.78 mg/kg in the muscle, and 2.0 and 3.0 mg/kg in the viscera. Based on the amounts the patients reported they had consumed (100–300 g), the authors estimated that they had ingested more than 0.63 mg of TTX, which corresponds to more than 12.6 μg/kg bw for a 50-kg adult.

3.2.2.5. Concluding remarks on human observations

Overall, the human case reports indicate that poisoning can result from TTX exposures in the region of 4–42 μg/kg bw and higher, but these values are subject to multiple limitations (e.g. not defined amounts of actual consumption, material analysed might not represent material that has actually been consumed, concentration estimates of TTX might not be accurate due to a lack of certified standards). Moreover, the CONTAM Panel was unable to identify the original data that underlay the MLD of 2 mg that is reported by several authors in the literature. The Panel noted inconsistencies in the use of the term MLD in human cases.

3.2.3. Mode of action

The targets of TTX are the voltage-gated sodium channels (Na_v), which belong to a superfamily of channels that also include potassium (K_v), calcium (Ca_v) and cyclic nucleotide-gated channels (Jan and Jan, 1992). Na_v channels are multimeric complexes containing a pore forming α-subunit and one or more β-subunits, and show a high similarity with Ca_v (Strong et al., 1993). The crystal structure of the channel has been resolved to understand the interaction of β- and α-subunits (Payandeh et al., 2011). There are four known β-isoform subunits (β1– β4); β1 are associated to the nervous system, and β2 are associated to the skeletal muscle. β-subunits belong to the cell adhesion molecules (CAMs) immunoglobulin superfamily (Patino and Isom, 2010). The coexpression with β-subunit is required for the α-subunit to show native Na_v functions. The pore of the channel is the transmembrane α-subunit that contains four domains, each one with six transmembrane hydrophobic domains. Based on the α-subunit, Na_v are classified in nine isoform types (Na_v 1.1.–1.9) that show different tissue presence and pharmacological features. TTX is active on Na_v 1.1, 1.2, 1.3, 1.4, 1.6 and 1.7 subtypes in the nanomolar range ($IC_{50} < 10$ nM). Na_v 1.6 is sensitive to 1 nM TTX (Wingerd et al., 2012). All other channels (Na_v 1.5, 1.8 and 1.9) show a low sensitivity to TTX being in the μM range. Na_v 1.1, 1.2, 1.3,

1.6 and 1.7 are abundant in the nervous system, and Na_v 1.4 is common in the skeletal muscle (Zimmer, 2010; Teramoto and Yotsu-Yamashita, 2015). The tissues involved in TTX symptoms include respiratory system, involving blockade of the phrenic nerve, the diaphragm and neurons in the central respiratory network, skeletal muscle and tissues from the digestive track (Zimmer, 2010).

The sensitivity of the various Na_v channels to Na+ and to voltage resides on the α-subunit, and it is this subunit being responsible for the initiation and propagation of the action potential in excitable cells (Catterall, 1984; Hartshorne and Catterall, 1984; Alexander et al., 2013). Na^+ currents are either TTX-sensitive (TTX-s) or TTX-resistant (TTX-r) (Roy and Narahashi, 1992; Kiernan et al., 2003). TTX-s currents contribute to action potential generation and to the control of peripheral nerve excitability (Baker and Bostock, 1997). TTX-r currents characterise small neurons (Brock et al., 1998), many of which are involved in nociception.

The mechanism of action of TTX consists of the extracellular blockade of the channel pore by binding to site 1, hence inhibiting Na conductance (Narahashi, 1974; Hille, 1975). TTX, thus, has effects both on action potential generation and on impulse conduction (Isbister and Kiernan, 2005). The result is the blockade of the neuron action potential and muscle paralysis. This mechanism is shared by similar water-soluble heterocyclic guanidinium alkaloids, including STX and analogues (Hartshorne et al., 1980; Huang et al., 2012; Walker et al., 2012; FAO/WHO, 2016; Pratheepa and Vasconcelos, 2017). The chemical structures of TTX and STX have certain similarities and dissimilarities. The molecular volumes for both STX and TTX are similar (251 and 252 $Å^3$, respectively), despite their disparate molecular shapes, TTX is a monovalent and STX is a divalent cation, which explains some differences in the affinities of TTX and STX for different Na_v isoforms (Zhang et al., 2010; Walker et al., 2012; Pratheepa and Vasconcelos, 2017). Walker et al. (2012) studied the affinity of TTX and STX for Na_V 1.7 and Na_V 1.4 in CHO cells transfected with cDNA for human Na_v1.7 or rat Na_v1.4. STX blocked rNav1.4 with an IC_{50} value of 2.8 ± 0.1 nM compared to 17.1 ± 1.2 nM for TTX. The IC_{50} values for hNav1.7 were 702 ± 53 nM for STX and 18.6 ± 1.0 nM for TTX. The human Na_v1.7 possesses a 2-aa sequence variation compared with all other Na_v isoforms, which results in differences in the binding affinity for TTX and STX. Structurally related GTX-3 also displayed a significant reduction in potency against hNa_v1.7 compared with rNa_v1.4 (1,513 ± 55 vs. 14.9 ± 2.1 nM, respectively).

The interaction of the TTX molecule is based on the combined guanidinium group with the presence of six hydroxyl groups, especially the C-11 OH (Choudhary et al., 2003). The different number of hydroxyl groups is also responsible for the different potencies of TTX analogues (see Section 3.2.4 Relative potency of TTX analogues). The blockade of the sodium channel pore is explained by means of a network of hydrogen-bonds between TTX and three acidic residues on the outer side of the selectivity filter, with the guanidine group adopting a lateral orientation (Chen and Chung, 2014). TTX blockade of Na^+ channels is consistent with the significant changes evident in Na^+ channel dependent nerve excitability parameters in humans upon intoxication (Kiernan et al., 2005).

Oda et al. (1989) carried out serial nerve conduction studies in a single patient and documented reduced amplitudes of compound motor and sensory potentials, slowing of conduction velocities, and lengthening of distal motor latencies and F-wave latencies. Neurophysiological investigations incorporating nerve excitability studies were done in four patients with TTX poisoning within 24 h of ingestion, and the results compared with previously established normative data (Isbister et al., 2002; Kiernan et al., 2005). Nerves in these four patients were of high threshold, had slow conduction, and had reduced amplitude compound potentials, suggesting some axons were unable to conduct. The effect on amplitude was greater in sensory neurons than motor axons, consistent with the prominence of sensory symptoms (dysaesthesia and numbness) over motor involvement (weakness) in the patients (Isbister et al., 2002; Kiernan et al., 2005).

According to a review conducted by Zimmer (2010), TTX has no direct effect on the heart. The transient or permanent reduction of the heart rate is most likely the result of a complex systemic reaction to TTX intoxication. Pratheepa and Vasconcelos (2017) also reported that the sodium channels in the cardiac muscles have less binding affinity to the toxin, due to the presence of cysteine residue in cardiac sodium channel at position 385 (Tyr385).

Based on the high selectivity of TTX for certain sodium channels, it is tested in pharmacological research for example as a possible therapeutic agent for treatment of human pain conditions and to dissect different aspects of membrane excitability and synaptic translation (Narahashi, 2001; Guzman et al., 2007).

The *in vitro* methods described below use as endpoints some physical parameters resulting from this blockage, such as cell or organ voltage. Reported inhibitory concentrations (IC_{50}) for blocking of the response by TTX and analogues in *in vitro* systems are presented in Table 8. No information is available on the origin and the purity of the extracts.

Table 8: *In vitro* IC_{50} values for blocking 50% of the response for TTX and analogues

Compound	IC_{50}	Test system	Reference
TTX	5.2 nM at pH 7.80 14.2 nM at pH 8.80	Squid axon	Hu and Kao (1986)
	3.8 nM at pH 6.50 and 7.25 4.3 nM at pH 8.25	Frog muscle	Yang and Kao (1992)
	4.1 nM at pH 7.25	Frog muscle	Yang et al. (1992)
	25 nM ± 2.4 ($Na_v1.4$)	Oocytes of *Xenopus* sp. injected with RNA to express $hNa_v1.4$	Chahine et al. (1994)
	4.3 ± 0.92 nM	Oocytes of *Xenopus* sp. injected with RNA to express rat peripheral sodium channel	Sangameswaran et al. (1997)
	0.4 and 1.6 nM (Nav1.6) In two oocytes	Oocytes of *Xenopus* sp. injected with rRNA to express $rNa_v1.6$	Dietrich et al. (1998)
	7.8 ± 1.3 nM ($Na_v1.2$) 2.0 ± 0.4 nM ($Na_v1.3$) 4.5 ± 1.0 nM ($Na_v1.4$) 1,970 ± 565 nM ($Na_v1.5$) 3.8 ± 1.5 nM ($Na_v1.6$) 5.5 ± 1.4 nM ($Na_v1.7$) 1,330 ± 459 nM ($Na_v1.8$)	Oocytes of *X. laevis* injected with RNA to express $rNa_v1.2$, rNav1.3, $rNa_v1.4$, hNav1.5, $mNa_v1.6$, hNaV1.7 or $rNa_v1.8$	Rosker et al. (2007)
	17.1 ± 1.2 nM ($Na_v1.4$) 18.6 ± 1.0 nM ($Na_v1.7$)	CHO cells transfected with cDNA encoding $rNa_v1.4$ or $hNa_V1.7$	Walker et al. (2012)
4-epiTTX	13.2 nM at pH 7.80	Squid axon	Kao and Yasumoto (1985)
6-epiTTX	96 nM at pH 7.25	Frog muscle	Yang et al. (1992)
11-deoxyTTX	445 nM at pH 7.25	Frog muscle	Yang et al. (1992)
4,9-anhydroTTX	298 nM at pH 7.80	Squid axon	Kao and Yasumoto (1985)
	1,260 ± 121 nM ($Na_v1.2$) 341 ± 36 nM ($Na_v1.3$) 988 ± 62 nM ($Na_v1.4$) 78,500 ± 11,600 nM ($Na_v1.5$) 7.8 ± 2.3 nM ($Na_v1.6$) 1,270 ± 251 nM ($Na_v1.7$) > 30,000 nM ($Na_v1.8$)	Oocytes of *X. laevis* injected with RNA to express $rNa_v1.2$, $rNa_v1.3$, $rNa_v1.4$, $hNa_v1.5$, $mNa_v1.6$, $hNa_V1.7$ or $rNa_v1.8$	Rosker et al. (2007)

IC_{50}: inhibitory concentration blocking 50% of the response; TTX: tetrodotoxin.

3.2.4. Relative potency of TTX analogues

Relative potencies (RPs) for TTX analogues, potentially standardised into toxic equivalency factors (TEFs), are required to estimate the total toxicity of a sample based on all analogues identified and quantified. The information available on the relative potencies of TTX analogues is rather scarce but includes the analogues thus far detected in bivalves and gastropods in EU waters (11-norTTX-(6)-ol (*S*/*R*), 4-epiTTX, 4,9-anhydroTTX and 5,6,11-deoxyTTX, see Section 1.2.3). A partial list can be established based on literature and was recently also presented in a Joint FAO/WHO technical paper (FAO/WHO, 2016) and a corresponding publication (Botana et al., 2016) Data on additional analogues were found in the literature, but some of these were on synthetic analogues, not occurring naturally and are therefore not included. The data and estimated RPs are presented in Table 9.

In most cases, details on the study and interpretation of the results were very limited. It is also evident that the way lethality in mice was presented is rather diverse using parameters like MLD, LD_{50}, LD_{99}, IC_{50} (all in μg/kg bw) or toxic potency (MU/mg compound). In this regard, the CONTAM Panel also noted that the use of the term MLD seems highly variable and confusing. Kao and Fuhrman (1963) reported MLD, LD_{50} and LD_{100} values for TTX after i.p. treatment of CF#1 mice of 8, 10 and 12 μg/kg bw, using seven doses and 6–16 animals per dose. This is in agreement with the fact that the MLD is the minimum lethal dose, i.e. the lowest dose that can kill a mouse (see also Section 3.2.2 on toxicity). However, Yotsu-Yamashita and colleagues refer to the MLD as being the LD_{99}

(Yotsu-Yamashita M., personal communication, Tohoku University, Sendai), which is in line with Noguchi and Arakawa (2008) stating that it is the dose killing 100% of the mice. The dose that killed only half of the mice was indicated as the LD_{50} and should thus be lower than the LD_{99}. The study by Kao and Fuhrman (1963) shows that the actual values for these parameters, at least for TTX, are within a very small dose range (8–12 μg/kg bw) after i.p. treatment. It should also be pointed out that in many studies the number of mice used is not indicated, and that in others only few mice were injected with the analogue, probably due to the small amount available. As an example, the study on 6,11-dideoxyTTX reports an IC_{50} of 7.5 μg/kg bw for the dose that killed one of the two mice treated (Jang and Yotsu-Yamashita, 2007). Doses of 4 and 15 μg/kg bw killed none or both of the two mice, respectively. For 5,6,11-deoxyTTX, death was reported to occur after a period much longer than the 30 min, on which the MLD of 0.2 μg (1 MU) for TTX in the MBA is normally based. This supports the observations of Vlamis et al. (2015) with contaminated and spiked blank mussels, showing the death of mice after several hours and at TTX levels corresponding to exposures below the MLD. Nakamura and Yasumoto (1985) reported lethal potencies for two analogues, 4,9-anhydroTTX and 4-epiTTX as being 92 and 710 MU/mg, and compared them with the lethal potency of TTX being 4,500 MU/mg. These values can be converted into the amount of compound corresponding to 1 MU, being respectively 10.9, 1.4 and 0.22 μg/MU, the MU being the minimum amount that would kill a 20 g mouse within 30 min. Corresponding RPs for these two analogues would thus be 0.02 and 0.16.

The RPs shown in Table 9, were estimated by comparing the lethality parameter obtained for the analogue with the appropriate parameter for TTX (set at 1), i.e. lethality TTX/lethality analogue. A lower value thus indicates a lower potency. The CONTAM Panel noted that correction for the molecular weight would result in a small difference (up to 15%) for some values but for many analogues not be visible in the rounded values.

Table 9: Lethal doses and relative potencies (RPs) for TTX and some of its analogues in mice, in most cases after i.p. treatment

Compound	Parameter	RP	Molecular weight	Reference
	i.p. LD_{50} (μg/kg bw)			
TTX	**10**	**1**	319.1	see Table 7, Kao and Fuhrman (1963)
***11-deoxyTTX*[c]**	**70**	**0.14**	303.1	Yasumoto et al. (1988)
6-epiTTX[c]	60	0.17	319.1	Yasumoto et al. (1988)
11-norTTX-6(S)-ol[e]	**54**	**0.19**	289.1	Yotsu et al. (1992)
5-deoxyTTX[c]	970	0.01	303.1	Satake et al. (2014)
	i.p. $LD_{100/99}$[a] (μg/kg bw)			
TTX	**12**	**1**	319.1	Kao and Fuhrman (1963)
***11-oxoTTX*[c]**	**16**	**0.75**	335.1	Yotsu-Yamashita and Mebs (2003)
11-norTTX-6(R)-ol[c]	**70**	**0.17**	289.1	Endo et al. (1988)
	i.p. Lethal potency (MU/mg)			
TTX	**4,500**	**1**	319.1	Nakamura and Yasumoto (1985)
4,9-anhydroTTX[d]	**92**	**0.02**	301.1	Nakamura and Yasumoto (1985)
4-epiTTX[d]	**710**	**0.16**	319.1	Nakamura and Yasumoto (1985)
	i.p. MLD[b] (μg/kg bw)			
TTX	**8**	**1**	319.1	Kao and Fuhrman (1963)
5,6,11-deoxyTTX[f]	**750**	**0.01**	271.1	Yotsu-Yamashita et al. (1995)
	i.v. MLD[b] (μg/kg bw)			
TTX	**8**	**1**	319.1	Tsuda et al. (1964)
Tetrodonic acid[g]	> 300,000	0.00	319.1	Tsuda et al. (1964)
	i.p. IC_{50} (μg/kg bw)			

Compound	Parameter	RP	Molecular weight	Reference
6,11-dideoxyTTX	420	0.02 *vs*. LD_{50} TTX	287.1	Jang and Yotsu-Yamashita (2007)

i.p.: intraperitoneal; i.v.: intravenous; MLD: minimum lethal dose; TTX: tetrodotoxin.
Analogues given in bold were detected in marine bivalves or gastropods (those not detected in the EU are in addition also in italics).
(a): LD_{100} (lethal dose 100%) for TTX, LD_{99} (lethal dose 99%) for the two analogues.
(b): The use of the term MLD is unclear; see Section 3.2.2 on toxicity.
(c): No details provided.
(d): Lethal potencies, standardised for TTX according to Kawabata (1978) (5 mice/dose). No MLD (minimum lethal dose) or LD_{50} provided.
(e): Acetate form, no details provided.
(f): Mentioned in a footnote, deaths occurred between 5 and 24 h.
(g): MLD, based on Mongrel mice. In Tsuda (1966), values are reported as LD_{50} (lethal dose 50%) values.

Table 10 describes relative potencies of TTX analogues as determined in the Neuro2a-assay.

Table 10: Relative potencies (RPs) for TTX analogues, as determined in the Neuro2a-assay

Compound	EC_{50} (nM)	RP	Reference
TTX	2.1 4.6 4.7	1	Kudo et al. (2014) Yotsu-Yamashita et al. (2003) Saruhashi et al. (2016)
11-deoxyTTX	130 270	0.016 0.017	Kudo et al. (2014) Yotsu-Yamashita et al. (2003)
6,11-dideoxyTTX	400	0.056	Kudo et al. (2014)
11-oxoTTX	2.9	1.6	Saruhashi et al. (2016)
6-deoxyTTX	6.5	0.32	Kudo et al. (2014)

EC_{50}: Effective concentration 50%.

As can be seen in Table 10, some analogues were also tested in the Neuro-2a assay in the presence of ouabain/veratridine, using the conversion of WST (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt), as the end-point for viability. RPs are based on the EC_{50} (in nM). This revealed for 11-deoxyTTX a 10-fold lower RP than in the MBA, while for 11-oxoTTX, the relatively high potency was confirmed, although a factor of 3 higher in the Neuro-2a assay, for 6,11-dideoxyTTX, the RP was rather similar.

3.2.4.1. Concluding remarks on relative potencies

It can be concluded that various TTX analogues have been tested in mice, most of them by i.p. injection. The Panel noted that in general the description of the studies is rather limited and that there are various ways in which the toxicity was expressed (LD_{50}, LD_{99}, IC_{50}, MLD and lethal potency). There were some additional studies with the Neuro-2a cell assay, to some extent supporting the data from the mouse bioassay. Overall, the data should be considered as indicative and may at best allow an order of magnitude estimation of the relative potencies. Nevertheless, these studies show that most analogues are much less toxic than TTX.

As can be seen in Table 9 estimated relative potencies, based on comparison with the most appropriate parameter for TTX, were 0.75 for 11-oxoTTX, 0.14 for 11-deoxyTTX, 0.19/0.17 for S/R 11-norTTX-(6)-ol, 0.16 for 4-epiTTX, 0.02 for 4,9-anhydroTTX and 0.01 for 5,6,11-deoxyTTX. The CONTAM Panel noted that the uncertainties associated with estimation of these relative potencies are very high.

3.2.5. Consideration of setting a health-based guidance value (HBGV)

TTX is a potent neurotoxin, causing inhibition of nerve and muscle conduction by blocking sodium channels, with symptoms occurring within minutes to hours of consumption of contaminated foods. Because of the acute toxicity of TTX, the CONTAM Panel concluded that an ARfD should be established, for the amount of TTX that can be ingested in a period of 24 h or less without appreciable health risk to consumers (as defined by OECD, 2010). Because of the overall limitations in the data available, different possible approaches to derive an ARfD were considered which were i) the use of the human intoxication data ii) extrapolation from STX and iii) the use of newly available data from an acute oral toxicity study in mice.

It is often reported that the minimum lethal dose of TTX for humans is in the region of 40 μg/kg bw and that the lowest dose resulting in symptoms is 4 μg/kg bw. However, the CONTAM Panel was not able to identify the origins of these reports, and therefore, it is unknown how many cases were assessed in reaching these conclusions, nor whether the ascertainment of dose in the cases was robust. The reports of TTX toxicity generally do not allow estimation of the dose of TTX that resulted in the symptoms in humans. Five recent reports refer to serious symptoms, and in some instances fatalities, at doses in the range of 4–42 μg/kg bw. There is considerable uncertainty in these estimates, relating to whether the food in which TTX was measured was representative of that consumed, how much of the food was consumed and the bodyweight of the affected subjects. Perhaps the most reliable data are from a case, in which pufferfish was cooked in soup, and it was possible to measure the TTX concentration in unconsumed soup. In this incident, a dose of 42 μg/kg bw resulted in severe symptoms, whereas 14 μg/kg bw led to less severe symptoms not requiring hospitalisation. However, even with this case there is uncertainty regarding the estimation of the amount of soup consumed and the bodyweights to the affected individuals (the pufferfish was purchased from an Asian market in the USA). Therefore, the CONTAM Panel concluded that the human data did not provide a standalone basis for derivation of an ARfD because of the considerable uncertainties associated with the reported effect doses.

The mode of action of TTX is similar to that of STX, with some differences in the affinity for the different subtypes of voltage-gated sodium channels (Na_v), but not identical to that of STX. The CONTAM Panel previously established an ARfD of 0.5 μg STX equivalents/kg bw (EFSA CONTAM Panel, 2009). This was based on the LOAEL in the region of 1.5 μg STX equivalents/kg bw, derived from the available reports of human poisoning, affecting more than 500 individuals. The Panel noted that many individuals did not suffer adverse reactions at much higher intakes, and therefore, it was expected that this LOAEL was very close to the threshold for effects in the most sensitive individuals. The Panel therefore applied a factor of 3 to the LOAEL in order to estimate a NOAEL of 0.5 μg STX equivalents/kg bw but no additional factor for variation among humans. The database for STX is much more extensive than that for TTX. The Panel also noted that the difference in toxic potency observed after i.p. vs oral treatment in mice was also rather similar between STX (i.p. and oral LD_{50} values of 9.0–11.6 and 260–263 μg/kg bw respectively; EFSA CONTAM Panel, 2009) and TTX (i.p. and oral LD_{50} values of 8.2–10.7 and 232–532 μg/kg bw, respectively; see Section 3.2.4 on relative potencies), suggesting no large differences in toxicokinetics, at least in mice. Extrapolating from STX to TTX would imply an ARfD of 0.5 μg/kg bw.

The newly available oral toxicity data on TTX in mice provide an acute LOAEL of 125 μg/kg bw (incidence 9/9 animals), and an acute NOAEL of 75 μg/kg bw based on apathy. However, there is a narrow interval between the dose where lethality occurred (250 μg/kg bw) and the dose where there was no apathy observed. The observations precluded the application of a benchmark dose (BMD) modelling for apathy. However, lethality could be modelled and resulted in a $BMDL_{10}$ of 112 μg/kg bw which is only slightly above the NOAEL for apathy (75 μg/kg bw, for details on the BMD calculations see Appendix D). Furthermore, with a group size of nine individuals, it cannot be excluded that at that a dose of 75 μg/kg bw no effects can occur. Therefore, the CONTAM Panel decided to use the next lower dose tested (25 μg/kg bw) as the reference point to derive an ARfD. This dose is 4.5-fold lower than the $BMDL_{10}$ for lethality. Applying a standard uncertainty factor (UF) of 100, an ARfD of 0.25 μg/kg bw was derived. The CONTAM Panel noted that the ARfD is 16-fold lower than the dose at which severe effects have been observed in humans (4 μg/kg bw) and is 2-fold lower than the ARfD for STX.

The CONTAM Panel noted that other TTX analogues may have a similar mode of action. Such analogues have been detected in marine bivalves and gastropods, like 11-oxoTTX (not in European waters), 4-epiTTX, 5,6,11-trideoxyTTX, 4,9-anhydroTTX and 11-norTTX-6-ol (*R*- and/or *S*-form). Some studies isolated these analogues and tested them by i.p. or i.v. injection in mice for lethality. With the exception of 11-oxoTTX, the analogues seem at least 5- to 100-fold less toxic than TTX. Relative potencies have been estimated for TTX analogues based on these studies (see Section 3.2.4). The Panel noted that the description of the methods, the results and derivation of a lethal dose is rather poor in these studies. Nevertheless they should allow an order of magnitude estimation of the toxic potency. Based on the detected levels, these analogues (with the possible exception of 11-oxoTTX) appear to contribute very little to the toxicity, when assuming similar kinetics. The Panel points out that the derivation of relative potencies as described in Section 3.2.4 is associated with very high uncertainties.

The ARfD of 0.25 μg/kg bw is therefore a group ARfD that should apply to TTX and its analogues taking into account their estimated relative potencies as compared to TTX, being 0.75 for 11-oxoTTX, 0.14 for 11-deoxyTTX, 0.19/0.17 for S/R 11-norTTX-(6)-ol, 0.16 for 4-epiTTX, 0.02 for 4,9-anhydroTTX and 0.01 for 5,6,11-deoxyTTX.

No data were available to consider possible longer term effects of TTX, or to establish a tolerable daily intake (TDI).

3.3. Occurrence data

3.3.1. Previously reported occurrence data

In this section, the reports of occurrence of TTX and its analogues (TTXs) found in marine bivalves and gastropods in European waters are summarised and the results are shown in Table 11. Summaries of studies from non-European waters are shown in Table C.1 in Appendix C and a brief overview is given below. It should be noted that 4,9-anhydroTTX (see Table 11) and anhydroTTX (see Table C.1 in Appendix C) are considered to be the same analogue.

Reports of TTX from human poisoning cases are already mentioned in Section 3.2.2 on toxicity and Table B.1 of Appendix B and are therefore not included in Table 11 and Table C.1. For the European studies, the results are primarily given as concentrations of TTXs while for the non-European studies results are primarily given in MU. Only in few of the latter cases, TTXs are quantified, and in these cases, the results are often not given exactly. Results given in mg/kg and MU/g can be converted to each other as 1 MU was shown to correspond to 0.22 μg TTX except when otherwise specified such as in Hwang et al. (2005) where 1 MU is assumed to correspond to 0.178 μg TTX. It should be noted that in some studies the presence of other marine toxins is indicated and therefore not all the toxicity can be assigned to TTX and its analogues.

The first finding of TTX and its analogues in marine gastropods from European waters was the poisoning case reported by Rodriguez et al. (2008; see Section 3.2.2). Here, TTX and 5,6,11-trideoxyTTX were found in the digestive gland (DG), while neither of the substances were found in the flesh.

The first report on findings of TTX and its analogues in bivalves in European waters was by Turner et al. (2015). TTX and four of its analogues were found in mussels (*Mytilus edulis*) and Pacific oysters (*Crassostrea gigas*), harvested from the south coast of England in 2013 and 2014. The results are shown in Table 11. In general, TTX was always found when its analogues were found except in two samples, where 5,6,11-trideoxyTTX was the only finding. TTX and six TTX analogues (4-epiTTX; 5,6,11-trideoxyTTX; 4,9-anhydroTTX; 11-norTTX-6-ol; mono-deoxyTTX; and 11-oxoTTX) were included in the method and quantitation of TTX analogues was conducted assuming the same analytical response factor as TTX. In the absence of standards, an extract from a grey side-gilled sea slug studied by McNabb et al. (2010) was used for identification of analogues. Data on the UK samples are included in the EFSA database.

Vlamis et al. (2015) have investigated the presence of TTXs in mussels (*Mytilus galloprovincialis*) from marine waters around Greece. Also, a few samples of Venus clams (*Venus verrucosa*) have been analysed TTXs were found in mussels from 2012 that were sampled as part of the official Greek control program for shellfish. Four out of six samples were tested positive in the Yasumoto 1978 protocol for lipophilic toxins (Yasumoto et al., 1978) which is based on an initial extraction with acetone, potentially followed by a partitioning step to separate lipophilic toxins from the more hydrophilic toxins. According to Vlamis et al. (2015), the test with the acetone extract presented an atypical nervous symptomology prior to mice death. The four samples were tested negative in the MBA for PSP and < LOD of 0.1 mg/kg for ASP toxins. All six samples were then analysed for the presence of TTXs by LC–MS. Mainly digestive glands (DG) were analysed but also a single sample of whole flesh (WF = whole body or any part edible, see definitions in Regulation 2074/2005[12]). This revealed the presence of TTXs at levels between 0.0462 and 0.203 mg/kg in DG and 0.179 mg/kg in WF (see Table 11). Besides, TTX also 4-epiTTX and 4,9-anhydroTTX were identified. Subsequently, control data from 2006 to 2012 were reviewed and 17 samples (16 mussels and 1 Venus clam) with atypical positive results to asymptomatic negative results in the MBA protocol (Yasumoto et al., 1978) were selected and DG or WF from these samples were analysed. The concentrations ranged from 0.047 to 0.092 mg/kg in mussels DG only for TTX, and was 0.080 mg/kg in the single WF sample analysed. In clam DG, the sum of TTXs was 0.177 mg/kg. In all 17 samples, 4-epiTTX and 4,9-anhydroTTX were

[12] Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC)No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. OJ L 338. 22.1.2005. p. 27–59.

below the LOD. In 2014, samples of mussels were taken during the period April–July from the same water as the previously positive samples and WF were analysed. The concentrations ranged from 0.025 to 0.043 mg/kg only for TTX (4-epiTTX and 4,9-anhydroTTX were below the LOD). Also, randomly collected routine samples of shellfish (15 mussels, 1 Venus clam) in June–July 2014 were analysed but no TTXs were found. All the Greek results are shown in Table 11 and the results are also included in the EFSA database. In all the studies, concentrations between LOD and LOQ are quantified and included in the sum of TTXs. Certified TTX together with naturally contaminated samples provided retention times, and these were used for identification of 5,6,11-trideoxyTTX, 5-deoxyTTX, 11-deoxyTTX, 11-norTTX-6(R)-ol and 11-norTTX-6(S)-ol, but none of those analogues were found.

In the Netherlands, the possible presence of TTX and its analogues was investigated in mussels and oysters sampled in 2015 (RIVM-RIKILT, 2015). A total of 152 shellfish samples were analysed, originating from different Dutch production areas. Only in samples collected from certain parts in the Eastern Scheldt estuary, TTX levels exceeded the LOD of 3 μg TTX/kg. Altogether, 55 samples were collected from these areas, namely 12 samples of oysters and 43 samples of mussels. Of these 55 samples, 15 samples had levels of TTX above the LOD, and 11 had concentrations above the LOQ, in this first report being 10 μg/kg. The maximum concentration found was in oysters at a level of 0.124 mg TTX/kg. These samples are included in the EFSA database which also includes new samples from 2016 which showed concentrations up to a maximum of 0.253 mg TTX/kg (see Section 3.3.2 on current occurrence).

A total of 134 samples of marine organisms, including gastropods and bivalves, were collected along the Portuguese coast from July 2009 to November 2010 and analysed for the content of TTXs (Silva et al., 2012). In samples of the gastropods *Gibbula umbilicalis*, *Monodonta lineata* and *Charonia lampas* (trumpet shell), TTX, 4-epiTTX, mono-deoxyTTX and 5,6,11-trideoxyTTX were found (see Table 11). The two analogues 4-anhydroTTX and 11-norTTX-6-ol were looked for but not found. The term mono-deoxyTTX is used because it could not be identified at which carbon the oxygen was lost. Quantification of TTX and its analogues were calculated against a TTX standard assuming that all the toxins had the same molar response. The gastropod sample from Rodriguez et al. (2008) and a positive sample of *Lagocephalus sceleratus* were used as a reference.

All other publications about findings of TTXs in marine gastropods and bivalves are from samples from New Zealand and Asia (i.e. China, Japan, Vietnam and Taiwan). McNabb et al. (2014) detected TTXs in a bivalve (*Paphies australis*) at levels up to 0.8 mg/kg. In all the publications about Asian samples, TTXs are reported in gastropods not only in *Nassarius* spp. but also in species of the genera *Oliva*, *Babylona*, *Choronia* and *Zeuxis*, TTX was found. In species of genera *Nassarius*, *Baylonia* and *Charonia*, also TTX analogues were found (see Appendix C Table C.1). In the studies from Asia, the levels were measured with the MBA for either TTX or PSP and expressed as MU/g. Quantified mean concentrations in gastropods ranged from 4 MU/g to 370 MU/g in DG.

As it can be seen from Table 11 and Table C.1 in Appendix C, the concentrations of TTXs in bivalves and gastropods from European waters are much lower than in samples from non-European waters, with the exception of the snail causing the intoxication case in Spain. The concentration of TTX observed in positive samples from European waters is between 0.003 and 0.253 mg/kg, while it is between 0.88 mg/kg and 81.4 mg/kg in samples from non-European waters. Neither from the European nor from the non-European samples is it possible to compare the contents in different tissues. In the European samples, only Vlamis et al. (2015) reported about analysis of DG and WF in a limited number of samples and concluded that there were no differences in the concentrations. In a few of the non-European samples edible portion and DG are analysed in gastropods but concentrations in DG are both higher or lower than in other tissues (see Table C.1). It is therefore not possible to conclude, across the studies, which tissues or parts have the highest concentrations of TTXs in bivalves.

Table 11: Content of TTXs in marine gastropods and bivalves from European waters

Reference	Species (number of samples)	TTX and analogues found (number of positive samples)	Concentration in positive samples (mg/kg)	Tissue[(a),(b)]	Additional information[(c)]
Rodriguez et al. (2008)	*Charonia lampas* (1)	TTX 5,6,11-trideoxyTTX	315 1,004	DG	No TTXs found in other tissues
Turner et al. (2015)	*Mytilus edulis* (6)	TTX (2) 5,6,11-trideoxyTTX (1)	0.003–0.039 0.0038	WF WF	Highest sum was 0.137 mg/kg (not corrected for differences in molecular weight)
	Crassostrea gigas (23)	TTX (12) 4-epiTTX (5) 5,6,11-trideoxyTTX (12) 4,9-anhydroTTX (1)	0.0027–0.12 0.0004–0.0039 0.0013–0.011 0.0018	WF WF WF WF	
Vlamis et al. (2015)	2012, Mussels (6)	TTX (6) 4-epiTTX (3) 4,9-anhydroTTX (1) TTX (1)	0.0462–0.203 0.0076–0.0156 0.0229 0.179	DG DG DG WF	Sum of TTXs: 0.046–0.2223 WF from sample with 0.203 mg/kg TTX and 0.076 mg/kg 4-epiTTX in DG
	2006–2012, Mussels (16) Venus clam (1)	TTX (15) TTX (1) TTX (1)	0.047–0.092 0.080 0.177	DG WF WF	Sum of TTXs: 0.061–0.195
	2014, Mussel (15)	TTX (15) 4-epiTTX (1)	0.0251–0.0434 0.0091	WF WF	Sum of TTXs: 0.0251–0.047 Samples collected in area with positive findings in 2012
	2014, Mussels (15) Venus clam (1)	None None		DG (all) WF (9 samples) DG and WF	Four samples of TTX below LOQ but above LOD
					LOD and LOQ in tissues TTX = 0.0022 and 0.0072 mg/kg; 4-epiTTX = 0.0023 and 0.0076 mg/kg and 4,9-anhydroTTX = 0.0062 and 0.0211 mg/kg
RIVM-RIKILT (2015)	Mussels Oysters	TTX (15/10)	Highest 0.137	WF	All positive samples from same area: 55 samples out of a total of 152 are from this area; 15 above LOD; 10 above LOQ LOQ: 0.01 mg/kg

Reference	Species (number of samples)	TTX and analogues found (number of positive samples)	Concentration in positive samples (mg/kg)	Tissue[(a),(b)]	Additional information[(c)]
Silva et al. (2012)	*G. umbilicalis* *M. lineata* *Charonia lampas*	MonodeoxyTTX TTX 4-epiTTX 5,6,11-trideoxyTTX	0.063 0.090 0.021 0.006	WF	LOD of 1.7 ng/mL and LOQ of 5 ng/mL Whole animal (edible part) analysed

LC–MS/MS: liquid chromatography–tandem mass spectrometry; LOD: limit of detection; LOQ: limit of quantification; TTX: tetrodotoxin.
(a): WF: whole flesh = whole body according to Commission Regulation No 2074/2005.
(b): DG: digestive gland = hepatopancreas according to Commission Regulation No 2074/2005.
(c): LC–MS/MS was used in all studies.

3.3.2. Current occurrence data

A total of 1,677 samples of marine bivalves analysed for the presence of TTX were available in the final dataset. Most of the available data (n = 1,544 or 92%) were either non-detected or non-quantified values. In case of analogues, altogether only 62 of the total 6,590 reported values (0.94%) were numerical values. Taking into account that few data were available on analogues combined with relatively low levels and low potencies of the detected analogues, the CONTAM Panel decided not to include them in the exposure assessment.

The number of analysed and quantified values for TTX and its analogues are presented in Table 12. In most of the reported samples, the whole flesh of bivalves was analysed. This includes the meat of the animals and their digestive glands. Only Greece reported some results where digestive glands were analysed separately.

The Greek data differentiated between the following cases:

1) Mussel digestive gland – only digestive gland was analysed (n = 28).
2) Mussel digestive gland/whole flesh – both tissues were analysed separately, deriving from the same sample (same batch of mussels). The result was considered the same for both (n = 8) by the data provider, thus these results were reported only once.
3) Mussel whole flesh – digestive gland and meat was analysed together (n = 17).

Similarly, besides the above described samples, two samples for Venus clam (one for digestive gland/whole flesh and one for digestive gland only) were reported from Greece.

Considering the low number of quantified levels in the digestive gland among the total number of samples and due to very limited data on the distribution of TTX between the digestive gland and flesh of the animal (Table C.1, Appendix C), the CONTAM Panel assumed that there was no difference in TTX content of the different tissues and therefore decided to include all data. Samples were analysed by LC–MS/MS, LC–MS (quadrupole) and LC–MS/MS (triple quadrupole).

Table 12 presents an overview about the samples received from EU MS.

Table 12: Number of analysed, non-detected, non-quantified and quantified values for TTX and its analogues

Country	*Parameter*	Total analysed	Non detected or non quantified value (< LOD or < LOQ)	Quantified value
		N	N	N
United Kingdom	TTX	1,092	1,057	35
	11-norTTX-6-ol	1,080	1,079	1
	11-oxoTTX	1,080	1,080	0
	4,9-anhydroTTX	1,080	1,073	7
	4-epiTTX	1,080	1,058	22
	5,6,11-trideoxyTTX	1,080	1,053	27
	MonodeoxyTTX	1,080	1,080	0
Greece[(a)]	TTX	55	16	39
	4,9-anhydroTTX	55	54	1
	4-epiTTX	55	51	4
Netherlands[(b)]	TTX	530	471	59

LOD: limit of detection; LOQ: limit of quantification; TTX: tetrodotoxin.

(a): According to the data provider, the 55 samples were analysed for 5,6,11-trideoxyTTX; 5-deoxyTTX; 11-deoxyTTX; 11-norTTX-6(R)-ol; and 11-norTTX-6(S)-ol analogues as well. The results were either non-detects or non-quantified. These data were not submitted to EFSA.

(b): According to the data provider, the 530 samples were analysed for 4,9-anhydroTTX and 4-epiTTX as well, where all the results were either non-detects or non-quantified except for one level for 4-epiTTX in a sample of oysters (14.5 μg/kg). These data were not submitted to EFSA.

Table 13 provides an overview of the descriptive statistics of the data by the type of bivalves. The UB median and 95th percentile levels reported to EFSA, taking into consideration all received data on TTX in bivalves, were 5.9 and 28 μg/kg, respectively. Concerning the different types of water molluscs, the highest UB mean of 10.8 μg/kg was reported for clams, and the highest 95th percentile level of

79 μg/kg was reported for oysters. Highest reported level among all data was 253 μg/kg in oysters, highest reported level in mussels were 179 and 203 μg/kg for WF and DG, respectively.

Table 13: Statistics of occurrence data of TTX in marine bivalves sampled between 2006 and 2016 as provided by three European countries: the United Kingdom, the Netherlands and Greece

Type of water mollusc	No. of samples	% Left-censored data	Mean concentration (μg/kg)			95th percentile concentration (μg/kg)[e]		
			LB	MB	UB	LB	MB	UB
All water molluscs	**1,677**	**92**	**4.1**	**5.0**	**5.9**	**28**	**28**	**28**
Water molluscs (unspecified)[a]	12	100	0.0	0.3	0.5	–	–	–
Clam[b]	28	75	10.3	10.6	10.8	–	–	–
Cockle	95	100	0.0	0.8	1.6	0.0	2.5	5.0
Mussel[c]	1,084	93	3.8	4.8	5.7	30.1	30.1	30.1
Oyster	299	85	8.4	9.3	10.2	79.0	79.0	79.0
Scallop	61	100	0.0	0.3	0.5	0.0	0.3	0.5
Razor clam[d]	98	100	0.0	1.0	1.9	0.0	2.5	5.0

LB: lower bound; MB: middle bound; UB: upper bound.
(a): Non-specified bivalves.
(b): Includes hard clams, surf clams and Venus clams.
(c): Includes *Mytilus edulis*, *Mytilus galloprovincialis*.
(d): Includes ensis razor clams.
(e): 95th percentile concentration cannot be given when number of samples is less than 60.

Samples were taken with different sampling strategies as given by the data providers according to the classification of Standard Sample Description for Food and Feed (EFSA, 2010a). From the total 1,677 samples, 83 samples from the Netherlands were reported as suspect samples. In order to follow a conservative approach, these samples were included as well in the exposure assessment.

The total of 1,677 samples taken between years 2006 and 2016 by the UK, Greece and the Netherlands are presented in Table 14 according to sampling year. After discovery of the presence, Greece performed some retrospective studies, looking at stored samples with false-positive results (Vlamis et al., 2015).

Table 14: Total number of samples available for each sampling year by the respective country

Country	Year of sampling							
	2006	2008	2009	2012	2013	2014	2015	2016
United Kingdom	0	0	0	0	15	643	434	0
Greece	3	6	3	12	0	31	0	0
Netherlands	0	0	0	0	0	0	257	273
Total	3	6	3	12	15	674	691	273

For the 1,636 samples where the exact sampling date was reported, the seasonal variability of occurrence of TTX was investigated. Most of the samples were taken in the period from June to September. Number of samples by month, country and percentage of quantified values per month are presented in Table 15.

Table 15: Number of samples taken by the reporting countries by months and proportion of quantified values

Sampling month	Country			Total samples	No of quantified values	% of quantified values[a]
	UK	NL	GR			
January	0	18	0	18	0	0
February	0	37	0	37	0	0

Sampling month	Country			Total samples	No of quantified values	% of quantified values[(a)]
	UK	NL	GR			
March	4	35	1	40	1	3
April	25	19	5	49	5	10
May	13	16	11	40	11	28
June	88	70	12	170	32	19
July	317	185	15	517	54	10
August	262	65	0	327	2	1
September	328	68	1	397	4	1
October	14	17	4	35	4	11
November	0	0	3	3	3	100
December	0	0	3	3	3	100
Sum	1,051	530	55	1,636	119	

GR: Greece; NL: Netherlands; UK: United Kingdom.
(a): Percentage of quantified values out of the total samples taken in the given month.

The distribution of the analytical results per month in each country and all countries together are shown in Figure 1. It is evident that in the UK and the Netherlands, contaminated samples were only observed in the summer season. This was not the case in Greece, with contaminations also at the end of the year.

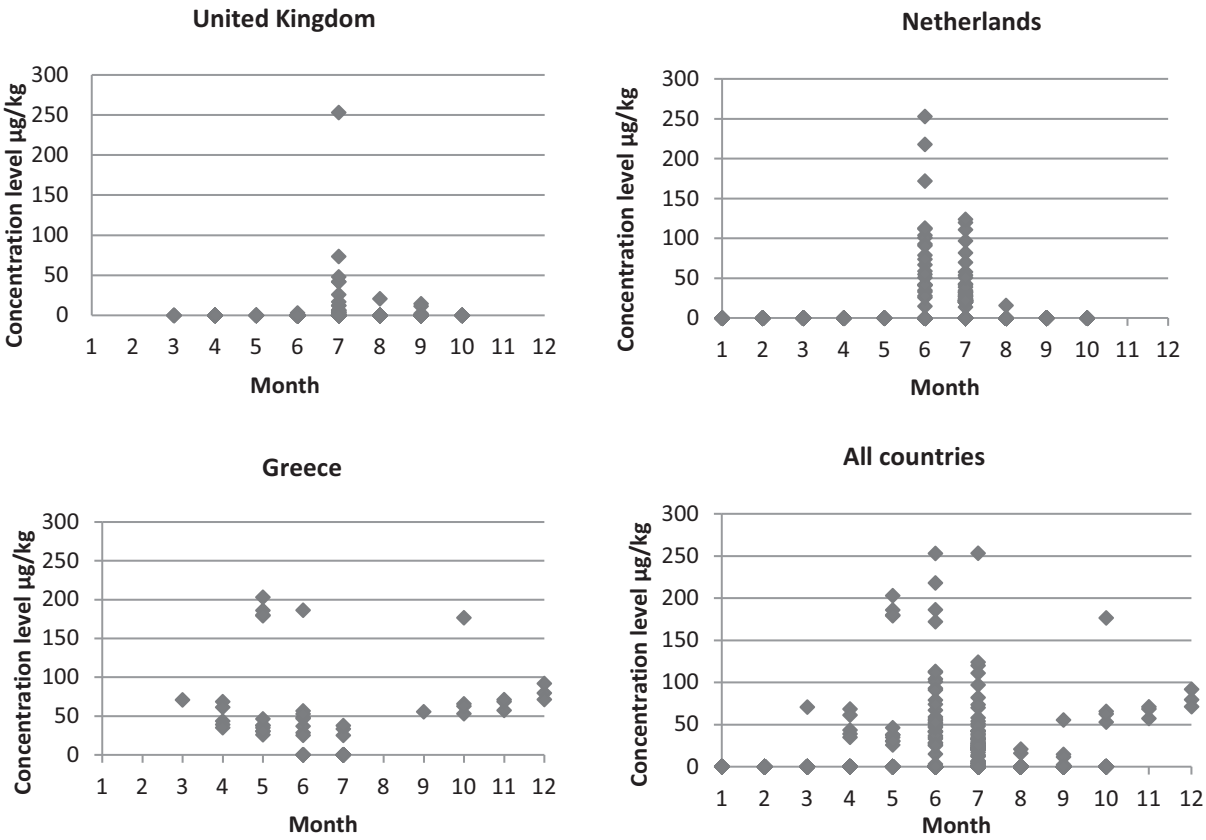


Figure 1: Distribution of TTX concentrations in European marine bivalves by months in the different reporting countries

3.4. Impact of food processing and storage

Very little information is available about the influence of processing on the concentration of TTX. Although TTX is mentioned to be heat stable in various reviews, only one processing study has been identified.

TTX is heat-stable and will not decompose during the traditional cooking process (Islam et al., 2011). In other reviews (e.g. in Bane et al., 2014; Turner et al., 2015; FAO/WHO, 2016) it is also stated that TTX is heat stable.

In the project ECsafeSEAFOOD[13], the level of TTX in commercially available pufferfish was assessed after steaming of pufferfish (*Lagocephalus sceleratus*). The gonads from a pufferfish were steamed at 100°C for 10 min and then extracted and analysed. The concentrations measured were 14.34 mg TTX equivalents/kg (w/w) in raw gonads as compared to 18.35 mg TTX equivalents/kg in cooked gonads. The toxin was not decomposed by the treatment.

EFSA (2009a,d) has published opinions on the saxitoxin group and domoic acid and both kind of toxins are hydrophilic similar to TTXs. According to these EFSA opinion, both for the saxitoxin group and domoic acid, studies have shown that home cooking processes, such as boiling or steaming, can reduce the level of toxins in shellfish meat, due to partial leaching of the toxins into the cooking liquid.

3.4.1. Concluding remarks on food processing

It can be assumed that TTX will not be degraded during normal home-made cooking processes. No data were identified on TTX analogues but since TTX and its analogues are chemically similar it can be assumed that they will behave in the same way to processing.

3.5. Current exposure assessment

3.5.1. Consumption of marine bivalves

Since 2008, the CONTAM Panel adopted a series of opinions on the safety of marine biotoxins (EFSA, 2008a–c, 2009a–e; EFSA CONTAM Panel 2009, 2010a–d). In all these opinions, in order to assess the exposure, the CONTAM Panel used 400 g as a standard for a large portion of shellfish meat consumed in Europe. This standard amount was first used in 2008, in the scientific opinion on okadaic acid and analogues (EFSA, 2008b) and was based on consumption data from five Member States. These food consumption data were derived from dietary surveys from 1985 carried out with different methodologies, including food records (from 2 to 7 days) and food frequency questionnaires. In these studies, the 95th percentiles consumption of shellfish meat ranged from 70 to 465 g, whereas the maximum reported portion sizes ranged from 239 to 1,500 g. However, the Panel noted that the portion sizes of 1,000 and 1,500 g of shellfish did not likely include only the edible part and could include the shells as well.

The estimated 400 g large portion size was then reviewed in 2010 in a statement of the CONTAM Panel, as in the meantime new food consumption data on shellfish were made available to EFSA by different EU countries, mainly within the EFSA Comprehensive Database. In particular, the 95th percentile consumption of shellfish meat ranged from 63 to 251 g in the EFSA Comprehensive Database. This information was not considered sufficient to warrant a revision of the CONTAM Panel's earlier established estimate on the consumption figure of 400 g shellfish meat. The CONTAM Panel based this decision on the uncertainty due to limited information on shellfish consumption in Europe and due to the limited representative data on shellfish types other than mussels. In addition, an appreciable uncertainty was highlighted in the 95th percentile consumption of shellfish meat from the Comprehensive Database, presenting a wide range of confidence intervals.

Since then, the Comprehensive Database has been updated and the current state is that consumption of bivalves was reported in 29 surveys from 17 countries out of the 51 surveys from 23 countries for which food consumption data are available. Number of consumption days, the mean and 95th percentile for consumed quantities are reported in Annex A for water molluscs and for each individual type of bivalves, i.e. mussels, clams, razor clams, oysters and cockles.

As suggested in the guidance on the Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment (EFSA, 2011a), the 95th percentile has been considered as reliable only when the number of consumption days is at least 60. According to the above-mentioned

[13] http://www.ecsafeseafood.eu/

document, percentiles calculated over a number of subjects/days lower than 60 (for the 95th percentile) and lower than 300 (for the 99th percentile) have considerable uncertainty and may not be statistically robust.

When considering all water molluscs together (so without differentiating between individual species), and all population groups, mean consumption of shellfish meat (in a single day) ranged from 1.2 to 450 g/day (0.02–6.7 g/kg bw per day), taking into account consumption days 1–404 for the 68 population groups in the 29 surveys. The 95th percentile ranged from 5.6 to 180 g/day (0.07 to 3.27 per kg bw) when taking into account the seven population groups in five surveys with at least 60 consumption days per population group.

It needs to be pointed out that the highest means are associated with a high uncertainty because they are based on results from population groups with only 1 or 2 consumption days.

Only in the adult group from the surveys of Spain and France, the number of consumption days for bivalves exceeded 300, allowing calculation of the 99th percentile, being 95 and 210 g/day (1.4 and 3.8 g/day per kg bw), respectively. Only in 9 out of the 2,102 days the consumption of bivalves reported in the Comprehensive Database exceeded 400 g/day.

The CONTAM Panel noted that the consumption data on marine gastropods are limited (32 consumption days in two surveys on whelks and winkles).

3.5.2. Results from current exposure assessment

The results of the probabilistic exposure assessment for TTX are presented in detail in Annex B. According to the guidance on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' the 95th percentile can be considered statistically robust if it is calculated with surveys where the number of consuming days is at least 60 per age group per survey. Similarly, the 99th percentile can be taken into account if the number of consuming days is at least 300. Thus, in the results presented below the calculated mean values are applicable for all the surveys, but the 95th percentiles are calculated for only those where consuming days were at least 60 per age group per survey.

Overall, considering consuming days only the average acute exposure level ranged from 0.00 to 0.09 μg/kg bw, and 95th percentile acute exposure level ranged from 0.00 to 0.03 μg/kg bw depending on the age group and survey. It should be noted that the studies with the highest average intakes did not allow the calculation of a 95th percentile as they are based on few consumption days.

In the 'adult' group, the average acute exposure level ranged from 0.00 to 0.01 μg/kg bw and the 95th percentile acute exposure levels ranged from 0.00 to 0.03 μg/kg bw.

In the 'adolescent' group, the average acute exposure level ranged from 0.00 to 0.01 μg/kg bw. The 95th percentile acute exposure level was available at one survey where it was 0.03 μg/kg bw.

Average acute exposure level in the 'other children' group ranged from 0.00 to 0.03 μg/kg bw. The 95th percentile acute exposure level was not calculated as the surveys were below 60 subjects/consuming days.

Average acute exposure level in the 'toddlers' group ranged from 0.00 to 0.02 μg/kg bw. The 95th percentile acute exposure level was not calculated as the surveys were below 60 subjects/consuming days.

The highest estimated average exposure level of 0.09 μg/kg bw occurred in the 'elderly' group.

Regarding the different types of water molluscs (see Annex B, worksheet 'Group'), the highest mean exposure level for clams was 0.02 μg/kg bw, corresponding values for mussels and oysters were 0.03 and for 0.09 μg/kg bw, respectively.

For razor clams, scallops, cockles and non-specified water molluscs, average acute exposure was 0.

The highest 95th percentile exposure, considering only the consumption days, was estimated for oysters (0.08 μg/kg bw).

Acute exposure to TTX was also estimated by multiplying the above mentioned large portion size of 400 g by the 95th percentile of TTX occurrence data and divided by a standard body weight of 70 kg. In this case, the acute exposure ranged from 0.03 to 0.45 μg/kg bw depending on the types of water molluscs. Results are presented in Table 16.

Table 16: Acute exposure to TTX by type of bivalve calculated with deterministic approach, based on a 400 g large portion size and P95 occurrence levels

Type of water mollusc	N	95th percentile concentration (μg/kg)[e]			UB Exposure (μg/kg bw per day)
		LB	MB	UB	
Water molluscs (all)	1,677	28	28	28	0.16
Water molluscs (unspecified)[a]	12	–	–	–	–
Clam[b]	28	–	–	–	–
Cockle	95	0.0	2.5	5.0	0.03
Mussel[c]	1,084	30.1	30.1	30.1	0.17
Oyster	299	79.0	79.0	79.0	0.45
Scallop	61	0.0	0.3	0.5	0.00
Razor clam[d]	98	0.0	2.5	5.0	0.03

bw: body weight; LB: lower bound; MB: middle bound; N: number of samples; UB: upper bound.
(a): Non specified bivalves.
(b): Includes hard clams, surf clams and Venus clams.
(c): Includes *Mytilus edulis, Mytilus galloprovincialis*.
(d): Includes ensis razor clams.
(e): 95th percentile cannot be given when number of samples is less than 60.

3.6. Risk characterisation

When considering consumption days only, the average acute exposure to TTX ranged from 0.00 to 0.09 μg/kg bw, the P95 from 0.00 to 0.03 μg/kg bw. It should be noted that only five surveys allowed the estimation of a P95 for one or more age classes or bivalve type, and this did not include the surveys with the highest average intakes where the estimation is based on only few observations.

In previous opinions on marine biotoxins, the CONTAM Panel used a large portion size of 400 g. This portion size is higher than the highest P95 observed in food consumption surveys, roughly by a factor of two. Nevertheless, the Panel decided to use this 400 g as a conservative approach. Combining this intake with the P95 of the occurrence data for the whole group of water molluscs, being 28 μg/kg, and assuming a body weight of 70 kg, would result in an intake of 0.16 μg/kg bw. The highest exposure was estimated for oysters (P95 level of 79 μg/kg), showing an estimated intake of 0.45 μg/kg bw. For mussels, this value was 0.17 μg/kg bw.

The average and 95th percentile exposure levels did not exceed the group ARfD of 0.25 μg/kg bw regarding the consumption days or subjects except that for consumption of a large portion of oysters (exposure was 0.45 μg/kg bw). Consequently, the CONTAM Panel did not identify a concern for the general population upon consumption of marine bivalves from European waters, with the possible exception of a large portion of oysters with high TTX level.

When combining the large portion size of 400 g, consumed by a person of 70 kg bw, with the ARfD of 0.25 μg/kg bw, it can be estimated that the highest level in shellfish not expected to result in adverse effects in consumers would be 70 x 0.25/0.4 = 44 μg/kg raw shellfish meat.

The Panel noted that some samples reported to EFSA exceeded this level, potentially implying a health risk for some of the observed levels in case of a large portion size.

3.7. Uncertainty analysis

In human case studies, estimated TTX doses are associated with a high level of uncertainty, due to e.g. undefined amounts of actual consumption, the fact that material analysed might not represent what has actually been consumed, and inaccurate concentration estimates of TTX due to the method used or the lack of certified standards. Using the mouse bioassay to determine the TTX content in food consumed or human samples does not enable to discriminate whether indeed TTX or STX caused the effects. There is uncertainty on the MLD of 2 mg per person that is reported in the literature but for which no underlying data could be identified. The use of the term MLD in human, but also in animal studies, is inconsistent throughout the literature. Due to these overall uncertainties, the CONTAM Panel decided not to base the ARfD on results from human case studies.

Toxic effects of TTX, observed after a single intragastric dose in mice were used to derive the group ARfD. The applied method excludes absorption of the compound in the very upper part of the digestive system (i.e. mouth and oesophagus), which based on the rapid onset of effects, seem potential sites of TTX absorption in humans. The CONTAM Panel also noted that the observation period in the study (2 h) is relatively short, even for an acute toxicity study, and that deaths have been described in mice after 2 h following i.p. treatment with shellfish extracts spiked with TTX.

Effects in mice occurred at oral doses more than 30-fold higher than the lowest dose reported to cause serious toxicity in humans (4 μg/kg bw). This suggests considerable interspecies differences between mice and humans, and between humans. As such, the applied uncertainty factors amounting to 100, do not necessarily lead to an overestimation of the risk.

The CONTAM Panel noted that the derivation of relative potencies for TTX analogues is associated with a high level of uncertainty since the underlying methods and data are poorly described.

Due to the fact that there are no occurrence data on TTX and its analogues in European marine gastropods and no consumption data, these could not be included in the exposure assessment and therefore constitutes an uncertainty.

Because of the low number of consumption surveys, 5 out of 29, that allowed an estimation of the P95, the 95th exposures might be underestimated. Application of a 400 g portion size could both over- or underestimate the high exposure.

There is insufficient information about the distribution of TTX and its analogues in the different tissues of bivalves (i.e. digestive gland versus whole flesh). Therefore, the CONTAM Panel could not take potential differences into account. There is also insufficient information about the variation in TTX levels between individual animals but it can be assumed that these differ significantly. However, a large portion size contains a large number of individual shellfish and as such may average out individual differences. This may not be the case for smaller portions.

In the exposure assessment, occurrence data from samples collected following different sampling strategies are included. This could have led to the inclusion of non-representative samples meaning that the average and P95 levels are higher than if only samples from objective sampling would have been used leading to an overestimation of the actual exposures.

Due to a low number of quantified levels of TTX analogues, the relatively low levels of TTX analogues as compared to TTX when detected and the low relative potencies of the analogues detected, these were not included in the exposure assessment. This may lead to a small underestimation of the exposure.

The occurrence data used in the exposure assessment came predominantly from the UK, and to a minor extent from the Netherlands and Greece and are therefore not representative for the EU which could have led to both an over or underestimation of the exposure. Additional occurrence data from the UK (Northern Ireland) and France could not be considered in the present exposure assessment because they were submitted late. As all the samples were non-detects, their inclusion would have resulted in lower TTX exposure.

Not for all edible bivalves and gastropods, consumption data are included in the EFSA Comprehensive Consumption database leading to an underestimation of the overall exposure.

3.7.1. Summary of the main uncertainties

In Table 17 a summary of the uncertainty evaluation is presented.

Table 17: Evaluation of the main uncertainties impacting the assessment

Sources of uncertainty	Direction[(a)]
Derivation of an ARfD based on mouse data	+/−
Non-inclusion of marine gastropods in the exposure assessment	−
Non-inclusion of TTX analogues in the exposure assessment	−
Relative potencies for TTX and some of its analogues	+/−
Occurrence data not representative for the EU	+/−
Non representative sampling strategy	+
Assumption of a 400 g portion size	+/−
P95 exposure estimation based on only few consumption surveys	−

(a): + = uncertainty with potential to cause overestimation of exposure/risk; − = uncertainty with potential to cause underestimation of exposure/risk, +/− = extent of potential over/underestimation might differ in direction.

The overall uncertainty associated with the present assessment is considered as high and it would rather overestimate than underestimate the risk.

4. Conclusions

4.1. Introduction

TTX is a hydrophilic heat-stable toxin, produced by bacteria that can be found in certain fish species but also marine gastropods and bivalves. Recently, TTX and some of its analogues have been detected in gastropods and bivalves from European waters. There are no HBGVs for TTX worldwide and also no maximum levels in seafood in the EU. TTX is a sodium channel blocker and can cause serious poisoning and even death after ingestion. Altogether, 25 naturally occurring analogues of TTX have been detected and many of these have also been shown to have toxicity potential. TTX is positively charged in neutral solutions. Under acidic conditions, TTX exists as a mixture in equilibrium with the ortho ester, the lactone form, 4-epiTTX and 4,9-anhydroTTX.

4.2. Analytical methods

- The MBA is the most widely applied detection method for TTXs outside the EU. However, the test cannot discriminate between TTXs and STXs.
- Cell-based assays are an alternative to the mouse bioassay but likewise cannot discriminate between TTXs and STXs.
- Immunoassays are more specific towards TTX but less suitable for screening for analogues, since their detection depends on cross-reactivity. When detected, this may not be according to the toxic potencies.
- The MBA and cell-based assays can be used to show the absence of TTXs and STXs but in case of positive test results, only chemical-analytical methods can identify the toxins and their analogues.
- Chemical-analytical methods, in particular LC–MS/MS, are the most suitable detection methods because they allow identification and quantification of TTX and its analogues. STXs may be included in the same analysis. LOQs for TTX and analogues in marine bivalves, as reported by three Member States, vary between 0.1 and 25 μg/kg.

4.3. Toxicokinetics

- There is very little information on toxicokinetics of TTXs in laboratory animals. In the case of mice, LD_{50} values for i.p. treatment are 25–50-fold lower than for oral treatment. This was also observed for STX.
- There is limited information about absorption and excretion of TTX and its analogues in humans and no information has been identified on its distribution and metabolism. TTX has been detected in blood and urine of patients for several days after exposure. TTX is assumed to be rapidly absorbed in the human digestive tract, based on the short time span between ingestion and onset of the symptoms seen in human poisoning cases.

4.4. Toxicity

- No subchronic or chronic studies on TTX have been identified.
- TTX is acutely toxic in humans and in experimental animals.
- In experimental animals, effects observed upon acute exposure include skeletal muscle fasciculations, apathy, lethargy, ataxia, ascending progressive paralysis and death.
- Acute i.p. and s.c. LD_{50} values in mice range from 9 to 12.5 μg/kg bw. Upon intragastric application, much higher LD_{50} values of 232 and 532 μg/kg bw have been identified.
- In an acute study, apathy was seen in all mice at a doses of 125 μg/kg bw TTX and higher but in none of the mice at 25 and 75 μg/kg bw upon single dose intragastric treatment. Doses of 250 μg/kg bw and higher resulted in the death of animals within the 2-h observation period.
- TTX is not genotoxic.
- In humans, TTX is a potent neurotoxin, leading to a sequence of acute symptoms from perioral numbness and paraesthesia, lingual numbness, early motor paralysis, incoordination, slurred speech to generalised flaccid paralysis, aphonia and fixed/dilated pupils to hypoxia,

hypotension, bradycardia, cardiac dysrhythmias and unconsciousness. Death is caused by respiratory failure and cardiac collapse. There is no antidote against TTX poisoning.

- The doses at which TTX causes acute toxicity and lethality in humans are unclear, but there are a series of human case reports available that indicate that acute poisoning can result from doses of 4–42 μg/kg bw and higher. In the literature, a MLD of 2 mg per person is often mentioned (corresponding to 40 μg/kg bw in a 50-kg adult). The source of the MLD and the underlying data could not be retrieved.
- For TTX analogues, no data are available allowing derivation of NOAELs or LOAELs; however, LD_{50} values upon i.p. application have been reported.

4.5. Mode of action

- TTX exhibits its toxicity by extracellular blockade of the channel pore of voltage-gated sodium channels (Na_v). Thus, TTX affects both action potential generation and impulse conduction resulting in a blockade of the neuron action potential and in muscle paralysis. Affected are the respiratory system, the diaphragm, skeletal muscles and tissues in the digestive tract.

4.6. Relative potency of TTX and its analogues

- When i.p. injected in mice, many TTX analogues exert similar effects as TTX. Relative potencies compared to TTX have been estimated for a series of TTX analogues based on these *in vivo* tests with mice where lethality was the endpoint and are supported by *in vitro* tests with the Neuro-2a bioassay. TTX is more potent than most of its tested analogues.
- The relative potencies for 11-oxoTTX and 11-norTTX-6(R)-ol derived from i.p. LD_{99} results compared to LD_{100} for TTX are 0.75 and 0.17, respectively. Relative potencies derived from i.p. LD_{50} values in mice in comparison to TTX are 0.14 for 11-deoxyTTX and 0.19 for 11-norTTX-6 (S)-ol. Relative potencies calculated from lethal potencies in mice are 0.16 for 4-epiTTX and 0.02 for 4,9-anhydroTTX. Relative potency for 5,6,11-deoxyTTX is 0.01 based on comparison of the MLD with that for TTX.
- The Panel noted that the derivation of relative potencies for TTX analogues is associated with significant uncertainties since underlying methods and data are poorly described.

4.7. Consideration of setting a HBGV

- Based on the pronounced toxicity of TTX upon acute exposure, the CONTAM Panel decided to derive an ARfD. The use of human data, extrapolation from data on STX and the use of animal data were considered. Because of the overall limitations of the available human data, these were not used to derive the ARfD, but only as supportive information. Similar was true for STX which in humans shows similar effects and in mice similar toxic potency (both after i.p. and oral treatment) and mode of action as TTX.
- Acute toxicity data on TTX in mice provide an acute LOAEL of 125 μg/kg bw (incidence 9/9 animals), and an acute NOAEL of 75 μg/kg bw based on apathy. However, there is a narrow interval between the dose where lethality occurred (250 μg/kg bw) and the dose where no apathy was observed.
- It was not possible to derive a BMD for apathy while lethality could be modelled, resulting in a $BMDL_{10}$ of 112 μg/kg bw. This is only slightly above the NOAEL for apathy (75 μg/kg bw). Furthermore, with a group size of nine individuals at the dose of 75 μg/kg bw, it cannot be excluded that effects can occur. The next lower dose tested (25 μg/kg bw) was therefore selected as the reference point to derive an ARfD. This dose is 4.5-fold lower than the $BMDL_{10}$ for lethality.
- Applying a standard UF of 100, an ARfD of 0.25 μg/kg bw was derived. This ARfD is 16-fold lower than the dose at which severe effects have been observed in humans (4 μg/kg bw) and twofold lower than the ARfD for STX (0.5 μg/kg bw).
- The ARfD is a group ARfD that should apply to TTX and its analogues, taking into account their relative potencies as compared to TTX, calculated to be 0.75 for 11-oxoTTX, 0.14 for 11-deoxyTTX, 0.19/0.17 for S/R 11-norTTX-(6)-ol, 0.16 for 4-epiTTX, 0.02 for 4,9-anhydroTTX and 0.01 for 5,6,11-deoxyTTX.
- No data on long-term effects of TTX have been identified, and therefore, a TDI has not been set for TTX and its analogues.

4.8. Occurrence data

- A total of 8,268 analytical results for 1,677 samples were submitted to EFSA by the UK, Greece and the Netherlands. For all samples, analytical results were provided for TTX, whereas only 1,136 analytical results were provided for 4-epiTTX, 1,080 for 5,6,11-trideoxyTTX, 1,080 for 11-norTTX-6-ol, 1,080 for mono-deoxyTTX, 1,135 for 4,9-anhydroTTX and 1,080 for 11-oxoTTX.
- Samples of bivalves were taken between 2006 and 2016, including mussels, oysters, cockles, clams, scallops and razor clams. No occurrence data were received for marine gastropods.
- In 92% of the samples, TTX was not detected or quantified. LOQs on TTX ranged from 1 to 25 μg/kg, where LODs ranged from 0.5 to 5 μg/kg. The UB median and 95th percentile levels reported to EFSA, taking into consideration all received data on TTX in bivalves were 5.9 and 28 μg/kg, respectively. In most cases, this was based on raw meat, in some cases the digestive gland.
- For different species of water molluscs, the highest upper bound mean was reported for clams, 10.8 μg/kg and the highest 95th percentile level was reported for oysters, 79 μg/kg.
- TTX analogues were detected in about 1% of the samples.

4.9. Exposure assessment

- Due to the relatively low number of quantified levels and relatively low levels of TTX analogues as compared to TTX and the low relative potencies of the analogues, the CONTAM Panel decided not to include them in the exposure assessment and only TTX data were used.
- Because no occurrence and limited consumption data were available for marine gastropods, these could not be included in the exposure assessment.
- Acute exposure assessments for TTX were carried out with two different approaches, namely i) by using the consumption data in the EFSA Comprehensive database applying a probabilistic approach, and ii) by using a large portion size of 400 g for bivalves.
- In the probabilistic assessment and based on consumption days only, the highest estimated average acute exposure level of 0.09 μg/kg bw occurred in the 'elderly' (based on only two consumption days) and the highest estimated 95th percentile exposure of 0.03 μg/kg bw in the elderly, adults and adolescents. However, only five out of 29 surveys allowed an estimate of the P95 exposure.
- Regarding the different types of marine bivalves, the highest mean exposure levels from clams, mussels and oysters were 0.02, 0.03 and 0.09 μg/kg bw, respectively, whereas for razor clams, scallops, cockles and non-specified water molluscs, average acute exposure was zero.
- The highest 95th percentile exposure, considering only the consumption days, was estimated from oysters, being 0.08 μg/kg bw.
- Acute exposures to TTX estimated using a large portion size of 400 g and P95 concentrations ranged from 0.03 to 0.45 μg/kg bw, depending on the bivalve species.

4.10. Risk characterisation

- The average and 95th percentile acute exposure levels, based on reported consumption did not exceed the group ARfD of 0.25 μg TTX/kg bw in any of the consumer groups indicating no general concern for human health due to the consumption of marine bivalves. This also applied for a 400 g portion size using the 95th percentile occurrence data with exception of oysters for which the group ARfD was exceeded about twofold.
- It was not possible to include marine gastropods in the risk characterisation because of lack of data.
- Based on a large portion size (400 g), an adult body weight of 70 kg and a group ARfD of 0.25 μg/kg bw, the CONTAM Panel concluded that a concentration lower than 44 μg of TTX and/or its equivalent toxic amount of its analogues per kg shellfish meat is not expected to lead to adverse effects in humans. The Panel noted that the P95 of the occurrence data for all shellfish is below this value. However, levels above 44 μg/kg have been reported for both mussels and oysters indicating an occasional concern for consumers of a large portion size of 400 g or larger.

5. Recommendations

- More occurrence data on TTX and its analogues in edible parts of marine bivalves and gastropods from different EU waters are needed to provide a more reliable exposure assessment.
- Occurrence data on marine gastropods are needed from different EU Member States.
- Data on concentrations of TTX and its analogues should be obtained using EU approved and validated chemical-analytical methods. In addition, certified standards and reference materials for TTX and analogues are needed.
- Information on the fate of TTX and its analogues during cooking is needed to refine exposure assessments.
- Studies on the sources and critical factors leading to the accumulation of TTX in marine bivalves and gastropods are needed.
- Further information on toxicokinetics of TTX and its analogues is needed.
- Further information on the acute oral toxicity of TTX and its analogues is needed. Chronic effects should also be investigated.
- Given the high uncertainties associated with derivation of the relative potencies of TTX analogues, adequate and well described evidence is needed to estimate their relative potencies, preferentially after oral exposure.
- As STX and TTX exert similar toxic effects via a similar mode of action, the possibility to combine STX and its analogues together with TTX and its analogues in one HBGV should be explored.

References

Abal P, Louzao MC, Antelo A, Alvarez M, Cagide E, Vilariño N, Vieytes MR and Botana LM, 2017. Acute oral toxicity of tetrodotoxin in mice: determination of lethal dose 50 (LD_{50}) and No Observed Effect Level (NOAEL). Toxins, 9, 75. doi: 10.3390/toxins9030075

Ahmed S, 2006. Puffer fish tragedy in Bangladesh: an incident of *Takifugu oblongus* poisoning in Degholia, Khulna. African Journal of Marine Sciences, 28, 457–458.

Akaki K and Hatano K, 2006. Determination of tetrodotoxin in puffer-fish tissues, and in serum and urine of intoxicated humans by liquid chromatography with tandem mass spectrometry. Shokuhin Eiseigaku Zasshi, 47, 46–50.

Alcaraz A, Whipple RE, Gregg HR, Andresen BD and Grant PM, 1999. Analysis of tetrodotoxin. Forensic Science International, 99, 35–45.

Alexander SP, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA, Spedding M, Peters JA, Harmar AJ and Collaborators C, 2013. The Concise Guide to Pharmacology 2013/14: ion channels. Britisch Journal of Pharmacology, 170, 1607–1651.

Baker MD and Bostock H, 1997. Low-threshold, persistent sodium current in rat large dorsal root ganglion neurons in culture. Journal of Neurophysiology, 77, 1503–1513.

Bane V, Lehane M, Dikshit M, O'Riordan A and Furey A, 2014. Tetrodotoxin: chemistry, toxicity, source, distribution and detection. Toxins, 6, 693–755.

Behera A, Dash BK and Barik BK, 2008. Rare puffer fish poisoning—A case report. Medico-Legal Update, 8, 5–6.

Bentur Y, Gurevych B and Lurie Y, 2007. Lessepsian immigration and tetrodotoxin poisoning in the Eastern Mediterranean. Abstracts of the 2007 North American Congress of Clinical Toxicology Annual Meeting, October 19–24, 2007, New Orleans, Louisiana. Clinical Toxicology, 45, 605–648. doi: 10.1080/15563650701610056

Botana LM, Hess P, Munday R, Arnich N, DeGrasse SL, Feeley M, Suzuki T, van den Bergh M, Fattori V, Garrido Gamarro E, Tritscher A, Nakagawa R and Karunasagar I, 2016. Derivation of toxicity equivalency factors for marine biotoxins associated with bivalve molluscs. Trends in Food Science and Technology, 59, 15–24.

Brock JA, McLachlan EM and Belmonte C, 1998. Tetrodotoxin-resistant impulses in single nociceptor nerve terminals in guinea-pig cornea. Journal of Physiology, 512, 211–217.

Campbell K, Barnes P, Haughey SA, Higgins C, Kawatsu K, Vasconcelos V and Elliott CT, 2013. Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood. Analytical and Bioanalytical Chemistry, 405, 7753–7763.

Catterall WA, 1984. The molecular basis of neuronal excitability. Science, 223, 653–661.

Chahine M, Bennett PB, George AL and Horn R, 1994. Functional expression and properties of the human skeletal muscle sodium channel. Pflügers Archiv, 427, 136–142.

Chau R, Kalaitzis JA and Neilan BA, 2011. On the origins and biosynthesis of tetrodotoxin. Aquatic Toxicology, 104, 61–72.

Chen R and Chung SH, 2014. Mechanism of tetrodotoxin block and resistance in sodium channels. Biochemical Biophysical Research Communications, 446, 370–374.

Chen CY, Chou HN, Chen YM and Lee TH, 2002. Detection of tetrodotoxin by HPLC in shellfishes and goby from south Taiwan. Journal of Natural Toxins, 11, 63–68.

Cho HE, Ahn SY, Son IS, In S, Hong RS, Kim DW, Woo SH, Moon DC and Kim S, 2012. Determination and validation of tetrodotoxin in human whole blood using hydrophilic interaction liquid chromatography-tandem mass spectroscopy and its application. Forensic Science International, 217, 76–80.

Choudhary G, Yotsu-Yamashita M, Shang L, Yasumoto T and Dudley SC Jr, 2003. Interactions of the C-11 hydroxyl of tetrodotoxin with the sodium channel outer vestibule. Biophysical Journal, 84, 287–294.

Chowdhury FR, Ahasan HA, Al Mamun A, Rashid AK and Al Mahboob A, 2007a. Puffer fish (Tetrodotoxin) poisoning: an analysis and outcome of six cases. Tropical Doctor, 2007, 263–264.

Chowdhury FR, Ahasan HA, Rashid AK, Al Mamun A and Khaliduzzaman SM, 2007b. Tetrodotoxin poisoning: A clinical analysis, role of neostigmine and short-term outcome of 53 cases. Singapore Medical Journal, 48, 830–833.

Cohen NJ, Deeds JR, Wong ES, Hanner RH, Yancy HF, White KD, Thompson TM, Wahl M, Pham TD, Guichard FM, Huh I, Austin C, Dizikes G and Gerber SI, 2009. Public health response to puffer fish (tetrodotoxin) poisoning from mislabeled product. Journal of Food Protection, 72, 810–817.

Cole JB, Heegaard WG, Deeds JR, McGrath SC and Handy SM, 2015. Tetrodotoxin Poisoning Outbreak from Imported Dried Puffer Fish — Minneapolis, Minnesota, 2014. Morbidity and Mortality Weekly Report. 63, 1222–1225.

Diener M, Christian B, Ahmed MS and Luckas B, 2007. Determination of tetrodotoxin and its analogues in the puffer fish *Takifugu oblongus* from Bangladesh by hydrophilic interaction chromatography and mass-spectrometric detection. Analytical and Bioanalytical Chemistry, 389, 1997–2002.

Dietrich PS, McGivern JG, Delgado SG, Koch BD, Eglen RM, Hunter JC and Sangameswaran L, 1998. Functional analysis of a voltage-gated sodium channel and its splice variant from rat dorsal root ganglia. Journal of Neurochemistry, 70, 2262–2272.

Doucette GJ, Powell CL, Do EU, Byon CY, Cleves F and McClain SG, 2000. Evaluation of 11-[3H]-tetrodotoxin use in a heterologous receptor binding assay for PSP toxins. Toxicon, 38, 1465–1474.

EFSA (European Food Safety Authority), 2008a. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – azaspiracids. EFSA Journal 2008;6(10):723, 52 pp. doi: 10.2903/j.efsa.2008.723

EFSA (European Food Safety Authority), 2008b. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – okadaic acid and analogues. EFSA Journal 2008;6(1):589, 62 pp. doi: 10.2903/j.efsa.2008.589

EFSA (European Food Safety Authority), 2008c. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – yessotoxin group. EFSA Journal 2009;7(2):907, 62 pp. doi: 10.2903/j.efsa.2009.907

EFSA (European Food Safety Authority), 2009a. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on marine biotoxins in shellfish – domoic acid. EFSA Journal 2009;7(7):1181, 61 pp. doi: 10.2903/j.efsa.2009.1181

EFSA (European Food Safety Authority), 2009b. Statement of the Panel on Contaminants in the Food Chain on a request from the European Commission on the Influence of processing on the levels of lipophilic marine biotoxins in bivalve molluscs. EFSA Journal 2009;7(3):1016, 10 pp. doi: 10.2903/j.efsa.2009.1016

EFSA (European Food Safety Authority), 2009c. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on marine biotoxins in shellfish – pectenotoxin group. EFSA Journal 2009;7(6):1109, 47 pp. doi: 10.2903/j.efsa.2009.1109

EFSA (European Food Safety Authority), 2009d. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – Saxitoxin group. EFSA Journal 2009;7(4):1019, 76 pp. doi: 10.2903/j.efsa.2009.1019

EFSA (European Food Safety Authority), 2009e. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Summary on regulated marine biotoxins. EFSA Journal 2009;7(8):1306, 23 pp. doi: 10.2903/j.efsa.2009.1306

EFSA (European Food Safety Authority), 2010a. Standard sample description for food and feed. EFSA Journal 2010;8(1):1457, 54 pp. doi: 10.2903/j.efsa.2010.1457

EFSA (European Food Safety Authority), 2010b. Management of left-censored data in dietary exposure assessment of chemical substances. EFSA Journal 2010;8(3):1557, 96 pp. doi: 10.2903/j.efsa.2010.1557

EFSA (European Food Safety Authority), 2011. Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment. EFSA Journal 2011;9(3):2097, 34 pp. doi: 10.2903/j.efsa.2011.2097

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2009. Scientific Opinion on marine biotoxins in shellfish – Palytoxin group. EFSA Journal 2009;7(12):1393, 38 pp. doi: 10.2903/j.efsa.2009.1393

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2010a. Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Brevetoxin group. EFSA Journal 2010;8(7):1677, 29 pp. doi: 10.2903/j.efsa.2010.1677

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2010b. Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group. EFSA Journal 2010;8(6):1627, 38 pp. doi: 10.2903/j.efsa.2010.1627

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2010c. Statement on further elaboration of the consumption figure of 400 g shellfish meat on the basis of new consumption data. EFSA Journal 2010;8(8):1706, 20 pp. doi: 10.2903/j.efsa.2010.1706

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2010d. Scientific Opinion on marine biotoxins in shellfish – Cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins). EFSA Journal 2010;8(6):1628. 39 pp. doi: 10.2903/j.efsa.2010.1628

EFSA Scientific Committee, 2012. Scientific Opinion on Exploring options for providing advice about possible human health risks based on the concept of Threshold of Toxicological Concern (TTC). EFSA Journal 2012;10(7):2750, 103 pp. doi: 10.2903/j.efsa.2012.2750

EFSA Scientific Committee, 2017. Update: Guidance on the use of the benchmark dose approach in risk assessment. EFSA Journal 2017;15(1):4658, 41 pp. doi: 10.2903/j.efsa.2017.4658

Endo A, Khora SS, Murata M, Naoki H and Yasumoto T, 1988. Isolation of 11-nortetrodotoxin-6(R)-ol and other tetrodotoxin derivatives from the puffer fugu niphobles. Tetrahedron Letters, 29, 4127–4128.

EURL-MB (European Union Reference Laboratory for Marine Biotoxins), 2014. EURLMB Standard Operating Procedure for PSP toxins by Mouse Bioassay. Version 1, March 2014. Available online: http://www.aesan.msssi.gob.es/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/EURLMB_SOP_PSP_by_MBA_Version_1.pdf

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization), 2016. Technical paper on Toxicity Equivalency Factors for Marine Biotoxins Associated with Bivalve Molluscs. Rome. pp. 1–133. Available online: http://www.fao.org/3/a-i5970e.pdf

FAVV (Federal Agency for the Safety of the Food Chain), 2016. Advise for action limits for tetrodotoxin (TTX) in bivalve molluscs. Available online: http://www.favv.be/wetenschappelijkcomite/adviezen/2016/_documents/Advies20-2016_SciCom2016-19_TTXbivalvemolluscs_000.pdf

Fernández-Fígares M, Fernández V, Postigo MJ and Feron P, 2013. Acute paralysis after seafood ingestion. Clinical Neurophysiology, 43, 299–302.

Fernandez-Ortega JF, Santos JM, Herrera-Gutierrez ME, Fernandez-Sanchez V, Loureo PR, Alfonso-Rancaño A and Tellez-Andrade A, 2010. Seafood Intoxication by Tetrodotoxin: First Case in Europe. Journal of Emergency Medicine, 39, 612–617.

Fong BMW, Tam S, Tsui SH and Leung KSY, 2011. Development and validation of a high-throughput double solid phase extraction-liquid chromatography-tandem mass spectrometry method for the determination of tetrodotoxin in human urine and plasma. Talanta, 83, 1030–1036.

FSA (Food Standards Agency), 2017. Provisional risk assessment to support risk management options for tetrodotoxin. Available from: https://www.food.gov.uk/sites/default/files/uk-provisional-risk-assessment-july-2016.pdf#overlay-context=user

Fukuda A and Tani A, 1941. Records of puffer poisonings report 3. Nippon Igaku Oyobi Kenko Hoken, 3528, 7–13.

Fukushima S, 1991. Examination of the poisoning level of tetrodotoxin in body fluids. Japanese Journal of Forensic Toxicology, 9, 126–127.

Gerssen A, Mulder PP and de Boer J, 2011. Screening of lipophilic marine toxins in shellfish and algae: development of a library using liquid chromatography coupled to orbitrap mass spectrometry. Analytica Chimica Acta, 685, 176–185.

Guzman A, Fernandez de Henestrosa AR, Marın AP, Ho A, Gonzalez Borroto JI, Carasa I and Pritchard L, 2007. Evaluation of the genotoxic potential of the natural neurotoxin Tetrodotoxin (TTX) in a battery of *in vitro* and *in vivo* genotoxicity assays. Mutation Research, 634, 14–24.

Hamasaki K, Kogure K and Ohwada K, 1996. A biological method for the quantitative measurement of tetrodotoxin (TTX): tissue culture bioassay in combination with a water-soluble tetrazolium salt. Toxicon, 34, 490–495.

Hartshorne RP and Catterall WA, 1984. The sodium channel from rat brain. Purification and subunit composition. Journal of Biological Chemistry, 259, 1667–1675.

Hartshorne RP, Coppersmith J and Catterall WA, 1980. Size characteristics of the solubilized saxitoxin receptor of the voltage-sensitive sodium channel from rat brain. Journal of Biological Chemistry, 255, 10572–10575.

Hashimoto Y and Migita M, 1951. On the assay method of puffer poison. Nippon Suisan Gakkaishi, 16, 341–345 (in Japanese).

Hashimoto Y and Noguchi T, 1971. Occurrence of tetrodotoxin-like substance in goby *Gobius criniger*. Toxicon, 9, 79–84.

Hayashida M, Hayakawa H, Wada K, Nihira M and Ohno Y, 2004. Sensitive determination of tetrodotoxin using column switching liquid chromatography-mass spectrometry with electrospray ionization in mouse serum. Journal of Analytical Toxicology, 28, 46–49.

Hille B, 1975. The receptor for tetrodotoxin and saxitoxin. A structural hypothesis. Biophysical Journal, 15, 615–619.

Homaira N, Rahman M, Luby SP, Rahman M, Sabbir Haider M, Faruque LI, Khan D, Parveen S and Gurley S, 2010. Multiple Outbreaks of Puffer Fish Intoxication in Bangladesh, 2008. American Journal of Tropical Medicine and Hygiene, 83, 440–444 doi: 10.4269/ajtmh.2010.10-0168

Horie M, Kobayashi S, Shimizu N and Nakazawa H, 2002. Determination of tetrodotoxin in puffer-fish by liquid chromatography-electrospray ionization mass spectrometry. The Analyst, 127, 755–759.

How CK, Chern CH, Huang YC, Wang LM and Lee CH, 2003. Tetrodotoxin poisoning. American Journal of Emergency Medicine, 21, 51–54.

Hu SL and Kao CY, 1986. The pH dependence of the tetrodotoxin-blockade of the sodium channel and implications for toxin binding. Toxicon, 24, 25–31.

Huang H-N, Lin J and Lin H-L, 2008. Identification and quantification of tetrodotoxin in the marine gastropod Nassarius by LC–MS. Toxicon, 51, 774–779.

Huang CG, Schild L and Moczydlowski EG, 2012. Use-dependent block of the voltage-gated Na+ channel by tetrodotoxin and saxitoxin: Effect of pore mutations that change ionic selectivity. Journal of General Physiology, 140, 435–454.

Huybrechts ISI, Boon PE, Ruprich J, Lafay L, Turrini A, Amiano P, Hirvonen T, De Neve M, Arcella DMJ, Westerlund A, Ribas-Barba L, Hilbig A, Papoutsou S, Christensen T, Oltarzewski MVS, Rehurkova I, Azpiri M, Sette S, Kersting M, Serra-Walkiewicz A, Majem VJ, Trolle E, Tornaritis M, Busk L, Kafatos A, Fabiansson S, De Henauw S and Van Klaveren, JD, 2011. Dietary exposure assessments for children in Europe (the EXPOCHI project): rationale, methods and design. Archives of Public Health, 69, 1169–1184.

Hwang D-F and Noguchi T, 2007. Tetrodotoxin Poisoning. Advances in Food and Nutrition Research, 52, 141–236. doi: 10.1016/S1043-4526(06)52004-2. ISSN: 1043-4526.

Hwang D-F, Shiu Y-C, Hwang P-A and Lu Y-H, 2002. Tetrodotoxin in Gastropods (Snails) Implicated in Food Poisoning in Northern Taiwan. Journal of Food Protection, 65, 1341–1344.

Hwang PA, Tsai YH, Deng JF, Cheng CA, Ho PH and Hwang DF, 2005. Identification of tetrodotoxin in a marine gastropod (*Nassarius glans*) responsible for human morbidity and mortality in Taiwan. Journal of Food Protection, 68, 1696–1701.

Isbister GK and Kiernan MC, 2005. Neurotoxic marine poisoning. Lancet Neurology, 4, 219–228.

Isbister GK, Son J, Wang F, Maclean CJ, Lin CSY, Ujma J, Balit CR, Smith B, Milder DG and Kiernan MC, 2002. Puffer fish poisoning: a potentially life-threatening condition. Medical Journal of Australia, 177, 650–653.

Islam QT, Razzak MA, Islam MA, Bari MI, Basher A, Chowdhury FR, Sayeduzzaman AB, Ahasan HA, Faiz MA, Arakawa O, Yotsu-Yamashita M, Kuch U and Mebs D, 2011. Puffer fish poisoning in Bangladesh: clinical and toxicological results from large outbreaks in 2008. Transactions of the Royal Society of Tropical Medicine and Hygiene, 105, 74–80.

Jal S and Khora SS, 2015. An overview on the origin and production of tetrodotoxin, a potent neurotoxin. Journal of Applied Microbiology, 119, 907–916.

Jalil S and Wohlin C, 2012. Systematic literature studies: Database searches vs. backward snowballing. 6th ACM-IEEE International Symposium on Empirical Software Engineering and Measurement. Lund. Available online: http://www.diva-portal.org/smash/get/diva2:834640/FULLTEXT01.pdf

Jan LY and Jan YN, 1992. Tracing the roots of ion channels. Cell, 69, 715–718.

Jang JH and Yotsu-Yamashita M, 2007. 6,11-dideoxytetrodotoxin from the puffer fish, *Fugu pardalis*. Toxicon, 50, 947–951.

Jang JH, Lee JS and Yotsu-Yamashita M, 2010. LC/MS analysis of tetrodotoxin and its deoxy analogues in the marine puffer fish Fugu niphobles from the southern coast of Korea, and in the brackishwater puffer fishes *Tetraodon nigroviridis* and *Tetraodon biocellatus* from Southeast Asia. Marine Drugs, 8, 1049–1058.

Jen H-C, Lin S-J, Lin S-Y, Huang Y-W, Liao I-C, Arakawa O and Hwang D-F, 2007. Occurrence of tetrodotoxin and paralytic shellfish poisons in a gastropod implicated in food poisoning in southern Taiwan. Food Additives and Contaminants, 24, 902–909.

Jen HC, Lin SJ, Tsai YH, Chen CH, Lin ZC and Hwang DF, 2008. Tetrodotoxin poisoning evidenced by solid-phase extraction combining with liquid chromatography-tandem mass spectrometry. Journal of Chromatography, B, 871, 95–100.

Jen H-C, Nguyen TA-T, Wu Y-J, Hoang T, Arakawa O, Lin W-F and Hwang D-F, 2014. Tetrodotoxin and paralytic shellfish poisons in gastropod species from Vietnam analyzed by high performance liquid chromatography and liquid chromatography-tandem mass spectrometry. Journal of Food and Drug Analysis, 22, 178–188.

Kao CY, 1966. Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. Pharmacological Reviews, 18, 997–1049.

Kao CY and Fuhrman FA, 1963. Pharmacological studies on tarichatoxin, a potent neurotoxin. Journal of Pharmacology, 140, 31–40.

Kao CY and Yasumoto T, 1985. Actions of 4-epitetrodotoxin and anhydrotetrodotoxin on the squid axon. Toxicon, 23, 725–729.

Katikou P, Georgantelis D, Sinouris N, Petsi A and Fotaras T, 2009. First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece). Toxicon, 54, 50–55.

Kawabata T, 1978. Puffer toxin. Environmental Health Bureau, ministry of Health and Welfare. In: *The Manual for Methods of Food Sanitation Tests II*, vol. 2. Environmental Health Bureau, Food Hygiene Association, Tokyo, Japan. pp. 21–22.

Kawatsu K, Shibata T and Hamano Y, 1999. Application of immunoaffinity chromatography for detection of tetrodotoxin from urine samples of poisoned patients. Toxicon, 37, 325–333.
Kiernan MC, Baker MD and Bostock H, 2003. Characteristics of late Na currents in adult rat sensory neurones. Neuroscience, 119, 653–660.
Kiernan MC, Isbister GK, Lin CS, Burke D and Bostock H, 2005. Acute tetrodotoxin-induced neurotoxicity after ingestion of puffer fish. Annals of Neurology, 57, 339–348.
Klaasen CD (ed)., 2001. *Casarett and Doull's Toxicology – The Basic Science of Poisons*. McGraw-Hill, New York, 1236 pp.
Kogure K, Tampline M, Simidu U and Colwell RR, 1988. A tissue culture assay for tetrodotoxin, saxitoxin and related toxins. Toxicon, 26, 191–197.
Konosu S, Inoue A, Noguchi T and Hshimoto Y, 1968. Comparison of crab toxin with saxitoxin and tetrodotoxin. Toxicon, 6, 113–117.
Kudo Y, Yasumoto T, Konoki K, Cho Y and Yotsu-Yamashita M, 2012. Isolation and structural determination of the first 8-epi-type tetrodotoxin analogues from the newt, *Cynops ensicauda popei*, and comparison of tetrodotoxin analogues profiles of this newt and the puffer fish, *Fugu poecilonotus*. Marine Drugs, 10, 655–667.
Kudo Y, Finn J, Fukushima K, Sakugawa S, Cho Y, Konoki K and Yotsu-Yamashita M, 2014. Isolation of 6-deoxytetrodotoxin from the pufferfish, *Takifugu pardalis*, and a comparison of the effects of the C-6 and C-11 hydroxy groups of tetrodotoxin on its activity. Journal of Natural Products, 77, 1000–1004.
Lago J, Rodríguez LP, Blanco L, Vieites JM and Cabado AG, 2015. Tetrodotoxin, an Extremely Potent Marine Neurotoxin: Distribution, Toxicity, Origin and Therapeutical Uses. Marine Drugs, 13, 6384–6406. doi:10.3390/md13106384
Leung KS-Y, Fong BM-W and Tsoi Y-K, 2011. Analytical Challenges: Determination of Tetrodotoxin in Human Urine and Plasma by LC-MS/MS. Marine Drugs, 9, 2291–2303.
Lin W-F and Hwang D-F, 2012. Analysis of Poisoning Cases, Monitoring and Risk Warning for marine Toxins (TTX, PSP and CTXs) in Taiwan. Journal of Food and Drug Analysis, 20, 764–771.
Lin S-J, Liao H-J and Hwang DF, 1996. Toxicity of gastropods *Babylonia formosae* and *Charonia sauliae* in Taiwan. Journal of Natural Toxins, 5, 307–315.
Liu D, Zhang J, Han B, Pen L and Liu D, 2011. An Electrophysiological Study of Acute Tetrodotoxin Poisoning. Cell Biochemistry Biophysics, 59, 13–18.
Liu FM, Fu YM and Shih DYC, 2004. Occurrence of tetrodoxin poisoning in *Nasarius Papillosus Alectrion and Nasarius Gruneri Niloha*, 2004. Journal of Food Drug Analysis, 12, 189–192.
Lokesh R, Adwaid M, Riven C, Vignesh M, Vishakha K and Gopiesh K, 2016. Genotoxicity of Tetrodotoxin Extracted from Different Organs of *Diodon hystrix* Puffer Fish from South East Indian Coast. Research Journal of Toxins, 8, 8–14.
Luo X, Yu R-C, Wang X-J and Zhou M-J, 2012. Toxin composition and toxicity dynamics of marine gastropod *Nassarius* spp. collected from Lianyungang, China. Food Additives and Contaminants, 29, 117–127.
Mahmud Y, Tanu MB and Noguchi T, 1999. First Occurrence of a Food Poisoning Incident Due to Ingestion of *Takifugu oblongus*, Along with a Toxicological Report on three marine puffer species in Bangladesh. Journal of the Food Hygienic Society of Japan, 40, 473–480.
Mahmud Y, Arakawa O and Noguchi T, 2000. An epidemic survey on freshwater puffer poisoning in Bangladesh. Journal of Natural Toxins, 9, 2000.
Marcil J, Walczak J-S, Guindon J, Ngoc AH, Lu S and Beaulieu P, 2006. Antinociceptive effects of tetrodotoxin (TTX) in rodents. British Journal of Anaesthesia, 96, 761–768.
McFarran EF, 1966. Differentiation of the poisons of fish, shellfish and plankton. In: Russell FE and Saunders PR (eds.). *Animal Toxins*. Pergamon Press, Oxford, 85 pp.
McNabb P, Selwood AI, Munday R, Wood SA, Taylor DI, Mackenzie LA, van Ginkel R, Rhodes LL, Cornelisen C, Heasman K, Holland PT and King C, 2010. Detection of tetrodotoxin from the grey side-gilled sea slug - *Pleurobranchaea maculata*, and associated dog neurotoxicosis on beaches adjacent to the Hauraki Gulf, Auckland, New Zealand. Toxicon, 56, 466–473.
McNabb PS, Taylor DI, Ogilvie SC, Wilkinson L, Anderson A, Hamon D, Wood SA and Peake BM, 2014. First detection of tetrodotoxin in the bivalve *Paphies australis* by liquid chromatography coupled to triple quadrupole mass spectrometry with and without precolumn reaction. Journal of AOAC International, 97, 325–333.
Merten C, Ferrari P, Bakker M, Boss A, Hearty A, Leclercq C, Lindtner O, Tlustos C, Verger P, Volatier JL and Arcella D, 2011. Methodological characteristics of the national dietary surveys carried out in the European Union as included in the European Food Safety Authority (EFSA) Comprehensive European Food Consumption Database. Food Additives and Contaminants Part A, 28, 975–995.
Nakagawa T, Jang J and Yotsu-Yamashita M, 2006. Hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry of tetrodotoxin and its analogues. Analytical Biochemistry, 352, 142–144.
Nakamura M and Yasumoto T, 1985. Tetrodotoxin derivatives in puffer fish. Toxicon, 23, 271–276.
Narahashi T, 1974. Chemicals as tools in the study of excitable membranes. Physiological Reviews, 54, 813–889.
Narahashi T, 2001. Pharmacology of tetrodotoxin. Journal of Toxicology: Toxin Reviews, 20, 67–84.
Naritia H, Noguchi T, Maruyama J, Ueda Y, Hashimoto K, Watanabe Y and Hida K, 1981. Occurrence of tetrodotoxin in a trumpet shellfish "boshubora" *Charonia sauliae*. Nippon Suisan Gakkai Shi, 47, 934–941.

Neagu D, Micheli L and Palleschi G, 2006. Study of a toxin-alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination. Analytical and Bioanalytical Chemistry, 385, 1068–1074.
Nishikawa T and Isobe M, 2013. Synthesis of Tetrodotoxin, a Classic but Still Fascinating Natural Product. The Chemical Record, 13, 286–302.
Noguchi T and Akaeda H, 1998. Pufferfish poisoning. Japanese Journal of Toxicology, 11, 339–345.
Noguchi T and Arakawa O, 2008. Tetrodotoxin – Distribution and Accumulation in Aquatic Organisms, and Cases of Human Intoxication. Marine Drugs, 6, 220–242. doi:10.3390/md20080011
Noguchi T and Ebesu JSM, 2001. Puffer poisoning: Epidemiology and treatment. Journal of Toxicology: Toxin Reviews, 20, 1–10.
Noguchi T, Arakawa O and Takatani T, 2006. TTX accumulation in pufferfish. Comparative Biochemistry and Physiology Part D, Genomics & Proteomics, 1, 145–152.
Noguchi T, Onuki K and Arakawa O, 2011a. Tetrodotoxin poisoning due to pufferfish and gastropods, and their intoxication mechanism. ISRN Toxicology, 2011, 1–10.
Noguchi T, Miyazawa K, Daigo K and Arakawa O, 2011b. Paralytic shellfish poisoning (PSP) toxin- and/or tetrodotoxin-contaminated crabs and food poisoning by them. Toxin Reviews, 30, 91–102.
Nzoughet JK, Campbell K, Barnes P, Cooper KM, Chevallier OP and Elliott CT, 2013. Comparison of sample preparation methods, validation of an UPLC–MS/MS procedure for the quantification of tetrodotoxin present in marine gastropods and analysis of pufferfish. Food Chemistry, 136, 1584–1589.
Oda K, Araki K, Totoki T and Shibasaki H, 1989. Nerve conduction study of human tetrodotoxication. Neurology, 39, 743–745.
OECD (Organisation for Economic Co-operation and Development), 2008. OECD Guidelines for the testing of chemicals 425. Acute Oral Toxicity-Up and Down Procedure. Organisation for Economic Co-operation and Development, Paris. pp. 1–69. Available online: https://www.oecd.org/chemicalsafety/testing/45799595.pdf
OECD (Organisation for Economic Co-operation and Development), 2010. Guidance for the derivation of an acute reference dose. Series on Testing and Assessment No. 124 ENV/JM/MONO (2010) 15 Organisation for Economic Co-operation and Development, Paris, 45 pp. Available online: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2010)15&doclanguage=en
O'Leary MA, Schneider JJ and Isbister GK, 2004. Use of high performance liquid chromatography to measure tetrodotoxin in serum and urine of poisoned patients. Toxicon, 44, 549–553.
Patino GA and Isom LL, 2010. Electrophysiology and beyond: multiple roles of Na+ channel beta subunits in development and disease. Neurosciences Letters, 486, 53–59.
Payandeh J, Scheuer T, Zheng N and Catterall WA, 2011. The crystal structure of a voltage-gated sodium channel. Nature, 475, 353–358.
Phua D-H, 2013. Dying for delicacy – tetrodotoxin poisoning from dried preserved puffer fish. XXXIII International Congress of the European Association of Poisons Centres and Clinical Toxicologists (EAPCCT) 28–31 May 2013, Copenhagen, Denmark. Clinical Toxicology, 51, 252–378.
Pires OR, Sebben A, Schawartz EF, Morales RAV, Bloch C and Schwartz CA, 2005. Further report of the occurrence of tetrodotoxin and new analogues in the Anuran family Brachycephalidae. Toxicon, 45, 73–79.
Pratheepa V and Vasconcelos V, 2017. Binding and Pharmacokinetics of the Sodium Channel Blocking Toxins (Saxitoxin and the Tetrodotoxins). Mini-Reviews in Medicinal Chemistry, 17, 320–327.
Puilingi CG, Kudo Y, Cho Y, Konoki K and Yotsu-Yamashita M, 2015. Tetrodotoxin and its analogues in the pufferfish *Arothron hispidus* and *A. nigropunctatus* from the Solomon Islands: A comparison of their toxin profiles with the same species from Okinawa, Japan. Toxins, 7, 3436–3454.
Raybould TJ, Bignami GS, Inouye LK, Simpson SB, Byrnes JB, Grothaus PG and Vann DC, 1992. A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples. Journal of Clinical Laboratory Analysis, 6, 65–72.
Rey V, Botana AM, Álvarez M, Antelo A and Botana LM, 2016. Liquid chromatography with a fluorimetric detection method for analysis of paralytic shellfish toxins and tetrodotoxin based on a porous graphitic carbon column. Toxins, 8, 1–16.
RIVM-RIKILT, 2015. Risk assessment of tetrodotoxins in shellfish. Available online: https://www.google.nl/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&ved=0ahUKEwj1pPeRzd_SAhUDCBoKHdhzAioQFggtMAI&url=https%3A%2F%2Fwww.nvwa.nl%2Fbinaries%2Fnvwa%2Fdocumenten%2Frisicobeoordeling%2Fvoedselveiligheid%2Farchief%2F2016m%2Fbeoordeling-tetrodotoxines-in-schelpdieren%2FBeoordeling%2Btetrodotoxines%2Bin%2Bschelpdieren-1.pdf&usg=AFQjCNGABUZP6eZcyhb588I0AnMW6HLN-Q
Rodriguez P, Alfonso A, Vale C, Alfonso C, Vale P, Tellez A and Botana LM, 2008. First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* in Europe. Analytical Chemistry, 80, 5622–5629.
Rodríguez P, Alfonso A, Otero P, Katikou P, Georgantelis D and Botana LM, 2012. Liquid chromatography-mass spectrometry method to detect Tetrodotoxin and its analogues in the puffer fish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters. Food Chemistry, 132, 1103–1111.
Rodríguez I, Alfonso A, Alonso E, Rubiolo JA, Roel M, Vlamis A, Katikou P, Jackson SA, Menon ML, Dobson A and Botana LM, 2017. The association of bacterial C9-based TTX-like compounds with *Prorocentrum minimum* opens new uncertainties about shellfish seafood safety. Scientific Reports, 7, 40880. doi:10.1038/srep40880

Rosker C, Lohberger B, Hofer D, Steinecker B, Quasthoff S and Schreibmayer W, 2007. The TTX metabolite 4,9-anhydro-TTX is a highly specific blocker of the Na(v1.6) voltage-dependent sodium channel. American Journal of Physiology. Cell Physiology, 293, C783–C789.

Roy ML and Narahashi T, 1992. Differential properties of tetrodotoxin-sensitive and tetrodotoxin resistant Na channels in rat dorsal root ganglion neurons. Journal of Neuroscience, 12, 2104–2111.

Sangameswaran L, Fish LM, Koch BD, Rabert DK, Delgado SG, Ilnicka M, Jakeman LB, Novakovic S, Wong K, Sze P, Tzoumaka E, Stewart GR, Herman RC, Chan H, Eglen RM and Hunter JC, 1997. A novel tetrodotoxin-sensitive, voltage-gated sodium channel expressed in rat and human dorsal root ganglia. Journal of Biological Chemistry, 272, 14805–14809.

Saoudi M, Abdelmouleh A, Ellouze F, Jamoussi K and El Feki A, 2009. Oxidative stress and hepatotoxicity in rats induced by poisonous pufferfish (*Lagocephalus lagocephalus*) meat. Journal of Venomous Animals and Toxins including Tropical Diseases, 15, 424–443.

Saoudi M, Messarah M, Boumendjel A, Abdelmouleh A, Kammoun W, Jamoussi K and El Fek A, 2011. Extracted tetrodotoxin from puffer fish *Lagocephalus lagocephalus* induced hepatotoxicity and nephrotoxicity to Wistar rats. African Journal of Biotechnology, 10, 8140–8145.

Saruhashi S, Konoki K and Yotsu-Yamashita M, 2016. The voltage-gated sodium ion channel inhibitory activities of a new tetrodotoxin analogue, 4,4a-anhydrotetrodotoxin, and three other analogues evaluated by colorimetric cell-based assay. Toxicon, 119, 72–76.

Satake Y, Adachi M, Tokoro S, Yotsu-Yamashita M, Isobe M and Nishikawa T, 2014. Synthesis of 5- and 8-deoxytetrodotoxin. Chemistry an Asian Journal, 9, 1922–1932.

Sato S and Kodama M, 2015. Puffer toxin. Food Hygiene Inspection Guidelines In: *Shokuhin Eisei Kensa Shishin, Rikagaku-hen [Standard Methods of Analysis in Food Safety Regulation]*. Japan Food Hygiene Association, Tokyo. pp. 813–820. In Japanese. ISBN 978-4-88925-071-8.

Shiu Y-C, Lu Y-A, Tsai Y-H, Chen S-K and Hwang DF, 2003. Occurrence of Tetrodotoxin in the Causative Gastropod *Polinices didyma* and another Gastropod *Natica lineata* Collected from Western Taiwan. Journal of Food and Drug Analysis, 11, 159–163.

Shoji Y, Yotsu-Yamashita M, Miyazawa T and Yasumoto T, 2001. Electrospray ionization mass spectrometry of tetrodotoxin and its analogues: liquid chromatography/mass spectrometry, tandem mass spectrometry, and liquid chromatography/tandem mass spectrometry. Analytical Biochemistry, 290, 10–17.

Shui LM, Chen K, Wang JY, Mei HZ, Wang AZ, Lu J-Y and Hwang DF, 2003. Tetrodotoxin-Associated Snail Poisoning in Zhoushan: A 25-Year Retrospective Analysis. Journal of Food Protection, 66, 110–114.

Silva M, Azevedo J, Rodriguez P, Alfonso A, Botana LM and Vasconcelos V, 2012. New gastropod vectors and tetrodotoxin potential expansion in temperate waters of the Atlantic Ocean. Marine Drugs, 10, 712–726.

Simões E, Abreu Mendes TM, Adão A and Haddad V Jr, 2014. Poisoning after ingestion of pufferfish in Brazil: report of 11 cases. Journal of Venomous Animals and Toxins including Tropical Diseases, 20, 54.

Stokes AN, Williams BL and French SS, 2012. An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. Biological Procedures Online, 14, 1–5.

Strong M, Chandy KG and Gutman GA, 1993. Molecular evolution of voltage-sensitive ion channel genes: on the origins of electrical excitability. Molecular Biology and Evolution, 10, 221–242.

Sui LM, Chen K, Hwang PA and Hwang DF, 2002. Identification of tetrodotoxin in marine gastropods implicated in food poisoning. Journal of Natural Toxins, 11, 213–220.

Suzuki H, 2016. Differences in susceptibility of mouse strains to tetrodotoxin. Toxicon, 119, 168–170.

Tani I, 1945. *Study of the Toxicity of Japanese Fugu*. Teikoku Toshoun Co., Tokyo. pp. 1–103.

Taniyama S, Isami Y, Matsumoto T, Nagashima Y, Takatani T and Arakawa O, 2009. Toxicity and Toxin Profile of Tetrodotoxin Detected in the Scavenging Gastropod *Nassarius (Alectrion) glans* "Kinshibai". Shokuhin Eiseigaku Zasshi, 50, 22–28.

Teramoto N and Yotsu-Yamashita M, 2015. Selective Blocking Effects of 4,9-Anhydrotetrodotoxin, Purified from a Crude Mixture of Tetrodotoxin Analogues, on NaV1.6 Channels and Its Chemical Aspects. Marine Drugs, 13, 984–995.

Thompson TM, Cohen N, Brinker N, Pearlman M and Wahl M, 2008. Puffer Fish (Tetrodotoxin) Poisoning in Chicago, IL 2008 Abstracts of the 2008 North American Congress of Clinical Toxicology Annual Meeting, September 11–16, 2008, Toronto, Canada. Clinical Toxicology, 46, 591–645. doi: 10.1080/15563650802255033

Tsai YH, Hwang DF, Cheng CA, Hwang CC and Deng JF, 2006. Determination of tetrodotoxin in human urine and blood using C18 cartridge column, ultrafiltration and LC-MS. Journal of Chromatography B, 832, 75–80.

Tsang YY and Tang ASP, 2007. Tetrodotoxin Poisoning. Food Safety Focus (9th Issue, April 2007). Centre for Food Safety. The Government of the Hong Kong Special Administrative Region. Available online: http://www.cfs.gov.hk/english/multimedia/multimedia_pub/multimedia_pub_fsf_09_01.html

Tsuda K, 1966. Über Tetrodotoxin, Giftstoff der Bowlfische. Naturwissenschaften, 53, 171–176.

Tsuda K, Ikuma S, Kawamura M, Tachikawa R, Sakai K, Tamura C and Amakasu O, 1964. Tetrodotoxin. VII. On the structures of tetrodotoxin and its derivatives. Chemical Pharmacological Bulletin, 12, 1357–1374.

Turner A, Powell A, Schofield A, Lees D and Baker-Austin C, 2015. Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Eurosurveillance, 20, 1–7.

Villa AF, Chataigner D, Arakawa O, Guegueniat P, Hommel D, De Haro L and Garnier R, 2010. Familial Tetrodotoxin Poisoning in French Guiana. Abstracts of the 2010 International Congress of the European Association of Poisons Centres and Clinical Toxicologists, 11-14 May 2010, Bordeaux, France. Clinical Toxicology, 48, 240–318. doi: 10.3109/15563651003740240

Vlamis A, Katikou P, Rodriguez I, Rey V, Alfonso A, Papazachariou A, Zacharaki T, Botana AM and Botana LM, 2015. First Detection of Tetrodotoxin in Greek Shellfish by UPLC-MS/MS Potentially Linked to the Presence of the Dinoflagellate *Prorocentrum minimum*. Toxins, 7, 1779–1807.

Walker JR, Novick PA, Parsons WH, McGregor M, Zablocki J, Pande VS and Du Bois J, 2012. Marked difference in saxitoxin and tetrodotoxin affinity for the human nociceptive voltage-gated sodium channel (Nav1.7). Proceedings of the United States National Academy of Science, 109, 18103–18107.

Wang XJ, Yu RC, Luo X, Zhou MJ and Lin XT, 2008. Toxin-screening and identification of bacteria isolated from highly toxic marine gastropod *Nassarius semiplicatus*. Toxicon, 52, 55–61.

Wheeler MW and Bailer AJ, 2008. Model averaging software for dichotomous dose response risk estimation. Journal of Statistical Software, 26, 1–15.

WHO (World Health Organization), 2009. Environmental Health Criteria 240. Principles and methods for the assessment of chemicals in food. Available online: http://www.who.int/foodsafety/chem/principles/en/index1.html

Wingerd JS, Vetter I and Lewis RJ, 2012. Voltage-gated sodium channels as therapeutic targets. In: Botana LM and Loza MI (eds.). *Therapeutic Targets. Modulation, Inhibition and Activation*. John Wiley & Sons, Hoboken, NJ. pp. 63–122.

Wu Z, Xie LH, Xia G, Zhang J, Nie Y, Hu J, Wang S and Zhang R, 2005. A new tetrodotoxin-producing actinomycete, *Nocardiopsis dassonvillei*, isolated from the ovaries of puffer fish *Fugu rubripes*. Toxicon, 45, 851–859.

Wu Y-J, Lin C-L, Chen C-H, Hsieh CH, Jen H-C, Jian S-J and Hwang DF, 2014. Toxin and species identification of toxic octopus implicated into food poisoning in Taiwan. Toxicon, 91, 96–102.

Xu Q, Kai H, Lisha G and Zhang H, 2003. Toxicity of tetrodotoxin towards mice and rabbits. Journal of Hygiene Research (China), 32, 371–374.

Yakes BJ, Deeds J, White K and DeGrasse SL, 2011. Evaluation of surface plasmon resonance biosensors for detection of tetrodotoxin in food matrices and comparison to analytical methods. Journal of Agricultural Food Chemistry, 59, 839–846.

Yamazaki M and Shibuya N, 1995. Motor nerve conduction velocity is useful for patients with tetrodotoxin. Anesthesia and Analgesia, 80, 857.

Yang L and Kao CY, 1992. Actions of chiriquitoxin on frog skeletal muscle fibers and implications for the tetrodotoxin/saxitoxin receptor. Journal of General Physiology, 100, 609–622.

Yang L, Kao CY and Yasumoto T, 1992. Actions of 6-epi tetrodotoxin and 11-deoxytetrodotoxin on the frog skeletal muscle fiber. Toxicon, 30, 635–643.

Yang C-C, Liao S-C and Deng J-F, 1996. Tetrodotoxin poisoning in Taiwan: An analysis of poison center data. Veterinary and Human Toxicology, 4, 282–286.

Yasumoto T and Michishita T, 1985. Fluorometric determination of tetrodotoxin by high performance liquid chromatography. Agricultural and Biological Chemistry, 49, 3077–3080.

Yasumoto T, Oshima Y and Yamaguchi M, 1978. Occurrence of a new type of shellfish poisoning in the Tohoku district. Bulletin of the Japanese Society of Scientific Fisheries, 44, 1249–1255.

Yasumoto T, Yotsu M and Murata M, 1988. New tetrodotoxin analogues from the newt *Cynops ensicauda*. Journal of the American Chemical Society, 110, 2344–2345.

Yotsu M, Endo A and Yasumoto T, 1989. An improved tetrodotoxin analyzer. Agricultural and Biological Chemistry, 53, 893–895.

Yotsu M, Hayashi Y, Khora SS, Sato S and Yasumoto T, 1992. Isolation and structural assignment of 11-nortetrodotoxin-6(S)-ol from the puffer *Arothron-nigropunctatus*. Bioscience Biotechnology and Biochemistry, 56, 370–371.

Yotsu-Yamashita M and Mebs D, 2003. Occurrence of 11-oxotetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*, and further studies on the levels of tetrodotoxin and its analogues in the newt's efts. Toxicon, 41, 893–897.

Yotsu-Yamashita M, Yamagishi Y and Yasumoto T, 1995. 5,6,11-Trideoxytetrodotoxin from the puffer fish, *Fugu poecilonotus*. Tetrahedron Letters, 36, 9329–9332.

Yotsu-Yamashita M, Urabe D, Asai M, Nishikawa T and Isobe M, 2003. Biological activity of 8,11-dideoxytetrodotoxin: lethality to mice and the inhibitory activity to cytotoxicity of ouabain and veratridine in mouse neuroblastoma cells, Neuro-2a. Toxicon, 42, 557–560.

Yotsu-Yamashita M, Jang J-H, Cho Y and Konoki K, 2011. Optimisation of simultaneous analysis of Tetrodotoxin, 4-epitetrodotoxin, 4,9-anhydrotetrodotoxin and 5,6,11-trideoxytetrodotoxin by hydrophilic interaction liquid chromatography-tandem mass spectrometry. Forensic Toxicology, 29, 61–64.

Yotsu-Yamashita M, Abe Y, Kudo Y, Ritson-Williams R, Paul VJ, Konoki K, Cho Y, Adachi M, Imazu T, Nishikawa T and Isobe M, 2013. First identification of 5,11-dideoxytetrodotoxin in marine animals, and characterization of major fragment ions of tetrodotoxin and its analogs by high resolution ESI-MS/MS. Marine Drugs, 11, 2799–2813.

You J, Yue Y, Xing F, Xia W, Lai S and Zhang F, 2015. Tetrodotoxin poisoning caused by Goby fish consumption in southeast China: a retrospective case series analysis. Clinics, 70, 24–29.
Zaki SZA, Mady A, Ahmed SM and Youssef NM, 2001. Effect of tetrodotoxin (TTX) on some brain neurotransmitters in rats. Journal of Natural Toxins, 10, 307–316.
Zaman L, Arakawa O, Shimosu A and Onoue Y, 1997. Occurrence of paralytic shellfish poison in Bangladeshi freshwater puffers. Toxicon, 35, 423–431.
Zhang MM, Gruszczynski P, Walewska A, Bulaj G, Olivera BM and Yoshikami D, 2010. Cooccupancy of the outer vestibule of voltage-gated sodium channels by micro-conotoxin KIIIA and saxitoxin or tetrodotoxin. Journal of Neurophysiology, 104, 88–97.
Zimmer T, 2010. Effects of tetrodotoxin on the mammalian cardiovascular system. Marine Drugs, 8, 741–762.

Abbreviations

γ-GTP	gamma-glutamyl transpeptidase
ALP	alkaline phosphatase
ALS	aldolase
ALT	alanine aminotransferase
API	atmospheric pressured ionisation
ARfD	acute reference dose
ASP	amnesic shellfish poisoning
BMD	benchmark dose
Ca_v	voltage-gated calcium channel
CAS	Chemical Abstracts Service
CAM	cell adhesion molecules
cDNA	complementary DNA
CHO	Chinese hamster ovary cells
CONTAM Panel	EFSA Panel on contaminants in the food chaiin
DG	digestive gland
ED_{50}	effective dose 50%
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
EURLMB	European reference laboratory for marine biotoxins
FAO	Food and Agriculture Organization of the United Nations
FAVV	Federal Agency for the Safety of the Food Chain
FSA	Food Standards Agency
GC	gas chromatography
GLP	good laboratory practice
GTX-3	grayanotoxin 3
HILIC	hydrophilic interaction chromatography
hNa_v	human voltage-gated sodium channel
HSDB	Hazardous Substances Data Bank
IC_{50}	inhibitory concentration 50%
ICR	mouse strain
IFREMER	French Research Institute for Exploitation of the Sea
i.p.	intraperitoneal
IPCS	International Programme on Chemical Safety
ISO	The International Organization for Standardization
i.v.	intravenous
JECFA	Joint FAO/WHO Expert Committee on Food Additives
K_v	voltage-gated potassium channel
LB	lower bound
LC	liquid chromatography
LC-FL	liquid chromatography with fluorescence detection
LC–ESI–MRM-MS	liquid chromatography–electrospray ionisation–multiple reaction monitoring-mass spectrometry
LC-HRMS	liquid chromatography-high resolution mass spectrometry
LC–MS	liquid chromatography with mass spectroscopy
LC–MS/MS	liquid chromatography with tandem mass spectroscopy

LD_{50}	lethal dose 50%
LD_{99}	lethal dose 99%
LD_{100}	lethal dose 100%
LDH	lactate dehydrogenase
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
MB	medium bound
MBA	mouse bioassay
MLD	minimum lethal dose
MRM	multiple reaction monitoring
MS	mass spectroscopy
MU	mouse unit
MW	molecular weight
m/z	mass-to-charge ratio
N2a-assay	bioassay with neuro-2a cells
Na_v	voltage-gated sodium channel
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
NVWA-BuRO	Netherlands Food and Consumer Product Safety Authority - Department for Risk Assessment & Research
OECD	Organisation for Economic Co-Operation and Development
O/V	ouabain/veratridine
PSP	paralytic shellfish poisoning
QSAR	quantitative structure–activity relationship
RIVM	National Institute for Public Health
rNa_v	rat voltage-gated sodium channel
RP	relative potency
s.c.	subcutaneous
SCF	Scientific Committee on Food
SOP	standard operational procedure
SPR	surface plasmon resonance
STX	saxitoxin
TDI	tolerable daily intake
TEF	toxic equivalency factor
TLC	thin-layer chromatography
ToR	Term of Reference
TTC	threshold of toxicological concern
TTX	tetrodotoxin
TTX-s	TTX-sensitive
TTX-r	TTX-resistant
UB	upper bound
UDS	unscheduled DNA synthesis
UPLC	ultra performance liquid chromatography
UPLC–MS/MS	ultra performance liquid chromatography - tandem mass spectrometer
WF	whole flesh
WHO	World Health Organization
w/w	weight/weight
ZIC	zwitterionic hydrophilic interaction chromatography- hydrophilic interaction chromatography

Appendix A – Identification and selection of relevant scientific literature and reports

Analytical methods	
Search terms	TOPIC: (Tetrodotoxin OR TTX) AND TOPIC: (chem* OR analy* OR identi* OR charact* OR detect* OR determin* OR method* OR GC OR HPLC OR LC-MS OR ICP-MS) AND TOPIC: (animal* OR bivalve OR mollusc* OR gastropo* OR Marine* OR Fish OR Organism*) NOT TOPIC: (frog OR newt OR worm OR crab OR terrestrial OR star fish OR star fish OR salamander OR sea slug OR zebrafish OR cats OR jellyfish OR rat OR mouse OR monkey OR dog OR horse)
Numbers of papers retrieved	336
Occurrence	
Search terms	TOPIC:(Tetrodotoxin OR TTX) *AND* TOPIC: (concentration* OR occurrence) *AND* TOPIC: (Bivalve* OR Mollusc* OR Mussel OR clams OR oysters OR cockles OR scallops OR Gastropo* OR nassariid* OR snail* OR Gibulla OR Charonia OR octopus* OR squid OR cuttlefish OR mantle)
Numbers of papers retrieved	51
Metabolism, Kinetics	
Search terms	TOPIC: (Tetrodotoxin OR TTX) AND TOPIC: (toxicokinetic* OR metabolism OR distribution OR excretion OR absorption OR distribution OR biomarker OR mode of action OR biotransformation OR elimination OR reduction OR detoxification OR extraction) Refined by: [excluding] WEB OF SCIENCE CATEGORIES: (NEUROSCIENCES OR PHYSIOLOGY)
Numbers of papers retrieved	458
Toxicity	
Search terms	TOPIC: (Tetrodotoxin OR TTX) AND TOPIC: (acute OR chronic OR tox* OR genotox* OR muta* OR DNA OR damage OR repair OR clastogen* OR aneugen* OR chromosom* OR cancer* OR carcino* OR tumor* OR tumour* OR organ* OR tissue* OR immun* OR neuro* OR developmental OR teratogen* OR repro* OR liver OR kidney* OR brain* OR lung* OR cardiovascular OR rat* OR mouse OR mice OR rabbit* OR hamster* OR primate* OR monkey*) Refined by: [excluding] WEB OF SCIENCE CATEGORIES: (NEUROSCIENCES OR PHYSIOLOGY) AND [excluding] DOCUMENT TYPES: (PROCEEDINGS PAPER OR MEETING ABSTRACT)
Numbers of papers retrieved	5,467
Epidemiology	
Search terms	TOPIC: (Tetrodotoxin OR TTX) AND TOPIC: (epidemio* OR exposure* OR case* OR poison* OR cross-sectional) Refined by: [excluding] WEB OF SCIENCE CATEGORIES: (NEUROSCIENCES OR PHYSIOLOGY)
Numbers of papers retrieved	454
Database used	**Web of Science**
Total Number Retrieved	**6,766**
Number after duplicates removed	**2,704**
Number considered relevant	**411**

Appendix B – Case reports on TTX poisoning

Table B.1: Case reports of TTX poisoning

Location (year)	Implicated food	No of cases	Fatalities	TTX concentration	Reference
Taiwan (1988–1995)	Pufferfish, gastropods, goby fish	20 incidents involving 52 cases (27 M, 21 F, 4 not specified)	7	NR	Yang et al. (1996)
China 1977–1988 and 1998–2001	Gastropods: *Zeuxis samiplicutus*	42 incidents involving 309 cases	16	50–300 (in snails inducing death; calculated in the paper using 1 MU = 0.18 μg TTX)	Shui et al. (2003)
Cox's Bazar district, Bangladesh (1998)	Pufferfish roe	8	5	11.8–21.3 MU/g in the skin, 2.8–4.9 MU/g in the muscle, < 2–5.9 MU/g in the liver, < 2–3.6 MU/g in the testis, 24.5–323.8 MU/g in the ovary and 12.8–46.3 MU/g in the viscera (except liver) 2	Mahmud et al. (1999)
Bangladesh (1988–1996)	Pufferfish	10 outbreaks involving *55* cases	17	< 4 MU/g assumed from data reported earlier by Zaman et al. (1997)	Mahmud et al. (2000)
New South Wales, Australia (2001–2002)	Pufferfish	11	–	NR	Isbister et al. (2002)
Taiwan (2001)	Gastropods: *Zeuxis sufflatus* and *Niotha clatharata*	4	–	*Z. sufflatus*: 586 MU (~ 104 μg) *N. clatharata*: 254 MU (~ 58 μg) (mean per specimen)	Hwang et al. (2002))
South Zheijiang, China	Gastropod: *Zeuxis samiplicutus*	31 (18 M, 13 F)	–	111 ± 45 MU (mean ± SD) 1	Sui et al. (2002)
Taiwan (2001)	Pufferfish	6	1	NR	How et al. (2003)
Kaohsiung City, Taiwan (2002)	*Nassarius papillosus* and *N gruneri* gastropods	2 (1 M, 1 F)	–	*N papillosus* – 320 MU/g *N gruneri* – 386 MU/g	Liu et al. (2004)
Australia (2004)	Toadfish	7 (5 M, 2 F)	–	NR	O'Leary et al. (2004)
Tungsa Island, Taiwan (2004)	Gastropod: *Nassarius glans*	6 (22–48 y)	2	5,188 ± 1,959 MU/specimen [1 MU = 0.178 μg]	Hwang et al. (2005)
Taiwan (2001)	Unknown fish	6	1	NR	Tsai et al. (2006)
Haifa, Israel (NR)	Puffer fish	2 (1 M, 1 F)	–	NR	Bentur et al. (2007) (abstract only)
Khulna, Bangladesh (2005)	Pufferfish liver	6 (3 M, 3 F, aged 4–35 years)	0	NR	Chowdhury et al. (2007a)
Bangladesh (2001–2006)	Pufferfish	53	8	NR	Chowdhury et al. (2007b)
Taiwan (2005)	Gastropod	1	0	42–60 mg/kg; STX: 3–6 mg/kg	Jen et al. (2007)

Location (year)	Implicated food	No of cases	Fatalities	TTX concentration	Reference
Burla, India (2007)	Pufferfish	8	2	NR	Behera et al. (2008)
Kaohsiung, Taiwan (2006)	Gastropod: *Niotha clathrata*	3	–	0.009–0.088 mg/specimen	Jen et al. (2008)
Chicago, USA (NR)	Pufferfish	2 (1 M, 1 F)	–	NR	Thompson et al. (2008)
Malaga, Spain (NR)	Trumpet shellfish (*Charonia lampas sauliae*)	1 (M, aged 49 years)	–	249 mg/kg	Fernandez-Ortega et al. (2010)
Narshingdi, Dhaka and Naore districts, Bangladesh (2008)	'Large marine pufferfish'	63 (32 M, 31 F, median age 25 years)	14	NR	Homaira et al. (2010)
French Guyana (NR)	Unknown fish	3 (2 M adults + 1 child age 2 years)	1 (adult)	NR	Villa et al. (2010) (abstract only)
Maiskhal, Bangladesh (2008)	Pufferfish eggs	6	2 (4–50 years)	NR	Islam et al. (2011)
Japan (1957–2008)	Gastropods	6 outbreaks involving 11 cases	3	4,290 MU/g reported for one of the incidents	Noguchi et al. (2011b)
Japan (1995–2010)	Pufferfish	477 outbreaks involving 698 cases	40	NR	Noguchi et al. (2011a)
Taiwan (2004)	Gastropod: *Nassarius glans*	5	2	NR	Noguchi et al. (2011a)
Korea (2010)	Unknown fish	3	1	NR	Cho et al. (2012)
Taiwan (1988–2011)	Pufferfish > gastropod > goby	192	22	Data reported for 3 outbreaks: 525 MU/g in fish; 1,100 MU/g in unidentified fish roe; 3,450 MU/g in adulterated mullet roe[(a)]	Lin and Hwang (2012)
Singapore (NR)	Dried pufferfish	1 M	–	NR	Phua (2013)
Duque de Caxias City, Brazil (NR)	Spotted pufferfish	11	–	NR	Simões et al. (2014)
Taipei, Taiwan (2010)	Octopus *Hapalochlaena fasciata*	2 M	–	118 ± 7.5 μg (mean ± SD) per specimen	Wu et al. (2014)
Minneapolis, USA (2014)	Dried pufferfish	4 (1 M, 2 F, 2 not specified)	–	Mean 19.8 μg/g (range 5.7–72.3 μg/g)	Cole et al. (2015)

NR: not reported; M: male; F: female; MU: mouse unit; SD: standard deviation.
(a): Toxins identified by chemical assays, but quantified data were not provided.

Appendix C – Concentrations of TTX in marine gastropods and bivalves of non-European waters

Table C.1: Concentrations of TTX and STX (based on use of PSP MBA) in gastropods and bivalves of non-European waters

Species (No of samples)	Results from MBA (MU/g)	MBA used	TTXs found	Concentration of TTX (mg/kg)	Additional information	Reference
P. australis (pipi)	–	–	TTX A peak assumed to be 4-epiTTX was observed	Up to 0.8	Sampled: New Zealand, 2011 Chemical method: LC–MS, two different methods LOQ: 0.5 mg/kg and 0.1 mg/kg TTX from positive sample used as standard	McNabb et al. (2014)
N. lineata (321) *N. livescens* (17)	13.3, 17.7, 52.8 17.2, 56.2	PSP	TTX (3) TTX (2) Peaks corresponding to anhydroTTX and 4-epiTTX were observed	2.37, 3.15, 9.40 3.07, 10	Sampled: Taiwan, 1997/1998 Chemical method: LC–FL LOQ: 0.2 mg/kg Authentic standards of TTX and anhydroTTX were used In total, 557 samples of gastropods analysed	Chen et al. (2002)
Nassarius	6.76 ± 2.63 (mean ± SD)	TTX	TTX	1.35	Sampled: China Chemical method: LC–MS Authentic standard for TTX was used LOD < 0.02 μg/l	Huang et al. (2008)
O. hirasei *O. lignaria* *O. annulata*	42 ± 28 51 ± 17 39 ± 18	PSP	TTX	5–6 mole% calculated on background of relative peak heights in chromatogram from LC-FL	Sampled: Vietnam, 2007 Chemical method: LC–FL; LC–MS Authentic standard of TTX used Detection limit: 1 μg/mL Also STX (73–82 mole%) and GTX2,3 (12–22 mole%) were identified	Jen et al. (2014)
B. formosa (242) *C. sauliae* (14)	DG: 4–29 (18) DG: 4.1–4.5 (4)	TTX	TTX AnhydroTTX TTX AnhydroTTX	Not quantified	Sampled: Taiwan, 1994/1995 Chemical method: Electrophorese, LC–FL, UV, GC–MS Authentic TTX and anhydroTTX standards obtained from liver from puffer fish were used as references	Lin et al. (1996)

Species (No of samples)	Results from MBA (MU/g)	MBA used	TTXs found	Concentration of TTX (mg/kg)	Additional information	Reference
N. semiplicatus	183 MU/g	PSP	TTX TrideoxoTTX 4-epiTTX AnhydroTTX OxoTTX	Total: 5–125 mg/kg	Sampled: China Chemical method: HILIC–MS LOD = 1.27 mg/kg Due to lack of standards, analogues were quantified using standard curve for TTX and assuming same molar response as TTX	Luo et al. (2012)
N. lineata (120) *P. didyma* (fresh; 120) *P. didyma* (cooked; 7)	DG: ND-11 (4 ± 4) Edible portion[a]: ND-26 (8 ± 6) DG: ND-10 (3 ± 3) Edible portion: ND-14 (7 ± 3) DG: ND-19 (10 ± 6) Edible portion: ND-54(28 ± 21)	TTX	TTX (35) TTX (15) TTX (4)		Sampled: Taiwan, 1999–2000 Chemical method: LC; GC–MS Authentic standards of TTX, 4-epiTTX and anhydroTTX was used as references LOD/LOQ not given Cooked: Samples from poisoning case	Shiu et al. (2003)
Z. samiplicutus (35)	DG: 370 ± 118 Edible portion[a]: 307±192	TTX	TTX AnhydroTTX TTX AnhydroTTX		Sampled: China, 2001 Chemical method: Electrophoresis, TLC and LC–FL	Sui et al. (2002)
N. semiplicatus (about 300)	Whole edible portion[b]: 200 DG: Not given Muscle: Not given Rest[c]: Not given	PSP	TTX trideoxyTTX anhydroTTX 4-epiTTX OxoTTX Total TTXs[d] Total TTXs[d] Total TTXs[d]	26.1 39 3.53 3.37 12.2 51.2 58.8 14.7	Sampled: China Chemical method: LC–MS Detection limit: 5 mg/mL Quantification using TTX standard and supposing the same molar response for all TTXs Results are given as mean; single residues not stated DeoxyTTX was also found in all tissues but not quantified	Wang et al. (2008)
N. glans	Positive	Not stated	TTX OxyTTX		Sampled: Japan, 2007 and 2008 Chemical method: LC–MS In Japanese; only abstract in English	Taniyama et al. (2009)

Species (No of samples)	Results from MBA (MU/g)	MBA used	TTXs found	Concentration of TTX (mg/kg)	Additional information	Reference
N. glans	Positive	Not stated	TTX 4,9-anhydroTTX 4-epiTTX 11-oxoTTX		Sampled: Japan, 2009 Chemical method: LC–MS In Japanese; only abstract in English	Taniyama et al. (2009)
N. coronatus	Positive		TTX			
O. annulata	Positive		TTX			
O. concavospira	Positive		TTX			
Zeuxis sp.	Positive		TTX			
Niotha albescens	Negative		TTX			

DG: digestive gland; TTX: tetrodotoxin; MBA: mouse bioassay; MU: mouse unit; LC–MS: liquid chromatography–mass spectroscopy; TLC: thin-layer chromatography; GC–MS: gas chromatography–mass spectroscopy; LOD: limit of detection; LOQ: limit of quantification; HILIC–MS: hydrophilic interaction chromatography–mass spectroscopy.
(a): Edible portion: muscle, salivary gland, brain and mouth organs.
(b): Whole edible portion: Is not defined in the paper.
(c): Rest includes salivary gland, brain and mouth organs.
(d): Sum of TTX, trideoxyTTX, anhydroTTX, 4-epiTTX and oxoTTX; the sums are given in the paper but single residues in each tissue are shown as stacked bar so contents of each of the TTXs can only be determined by read off.

Appendix D – Derivation of a BMD for acute effects of TTX applying a BMR_{10}

A. Data description

The dose-dependent lethality observed within 2 h upon intragastric administration of TTX to mice in the study from Abal et al. (2017) has been selected for derivation of a benchmark dose (BMD) for TTX following EFSA guidance on the use of the BMD (EFSA Scientific Committee, 2017). The findings selected for deriving a BMD are described in Section 3.2.2 of this opinion.

Substance	Dose (μg/kg bw)	Dead animals	N	Sex
TTX	25	0	9	F
	75	0	9	F
	125	0	9	F
	250	4	7	F
	500	4	5	F
	1,000	3	3	F

N: number of animals; bw: body weight.

B. Selection of the BMR:

The BMD is defined as the dose that corresponds with an extra risk of 10% compared with the background risk. The benchmark response (BMR) is the estimated risk corresponding with the BMD of interest. A 90% confidence interval around the BMD will be estimated, the lower bound is reported by BMDL and the upper bound by BMDU. Based on the number of animals, it was decided to apply a BMD_{10} and not a BMD_{01}, despite the severity of the endpoint.

C. Software used:

Results are obtained using the R-package 'bmdModeling'. Fitting BMD models is based on the R-package Proast 61.3. Averaging results from multiple fitted BMD models is based on the methodology in Wheeler and Bailer (2008).

D. Results

Model	Number of parameters	Log-likelihood	AIC	BMD	BMDL	BMDU	Converged	Accepted AIC
Null	1	−24.15	50.30	NA	NA	NA	Yes	
Full	6	−7.28	26.56	NA	NA	NA	Yes	
Logistic	2	−10.01	24.02	159.94	103.38	228.63	Yes	Yes
Log-logistic	3	−8.43	22.86	153.78	95.16	211.13	Yes	Yes
Log-probit	3	−8.28	22.56	154.43	97.46	212.34	Yes	Yes
Weibull	3	−9.16	24.32	142.30	77.47	214.42	Yes	Yes
Gamma	3	−8.64	23.28	152.71	89.35	214.54	Yes	Yes
Two-stage	3	−9.35	24.70	120.61	90.48	167.64	No	No

Alerts that were indicated when fitting the models: Errors in calculation: Null, Probit model: need at least two non-NA values to interpolate.

Estimated model weights

Logistic	Log-logistic	Log-probit	Weibull	Gamma
0.14	0.25	0.29	0.12	0.2

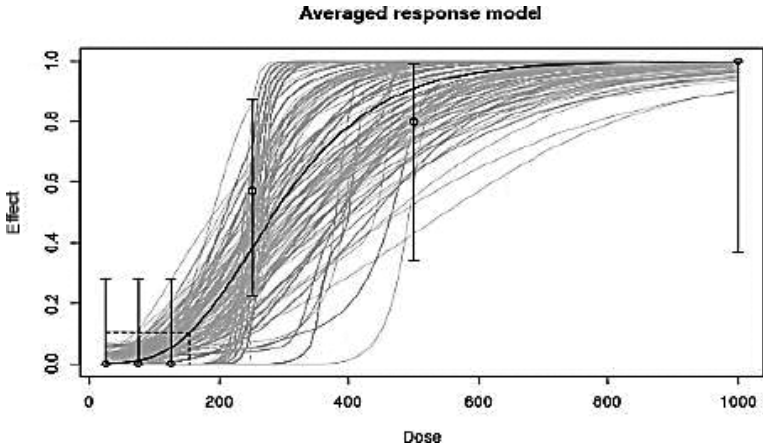


Figure D.1: Average response model

G. Conclusion

Given the 500 generated data sets, the BMDL is the 5th percentile of all parametric bootstrap BMD values and the BMDU is the 95th percentile.

Estimated the BMD based on the averaged response model which is a weighted average of the accepted models' response values.

BMD = 153.23 μg/kg bw; BMDL = 112.12 μg/kg bw; BMDU = 246.59 μg/kg bw.

Annex A – TTX Consumption data

Annex A can be found in the online version of this output ('Supporting information' section): http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2017.4752/abstract

Description: TTX Consumption data – excel file

Annex B – TTX Acute exposure individuals

Annex B can be found in the online version of this output ('Supporting information' section): http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2017.4752/abstract

Description: TTX Acute exposure individuals – excel file