New Zealand Food Safety
Haumaru Kai Aotearoa

Tetrodotoxin in non-commercial New Zealand bivalve shellfish

New Zealand Food Safety Technical Paper No: 2020/17

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ISBN No: 978-1-99-002537-2 (online)
ISSN No: 2624-022X (online)

June 2020

New Zealand Government

Ministry for Primary Industries
Manatū Ahu Matua

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Scientific Interpretative Summary

This SIS is prepared by New Zealand Food Safety (NZFS) risk assessors to provide context to the following report for MPI risk managers and external readers.

Report No. 2986 Review of literature to help identify risks associated with tetrodotoxin in seafood, including bivalve molluscs.

Report No. 3173 Tetrodotoxin in non-commercial New Zealand bivalve shellfish.

New Zealand Food Safety (NZFS) contracted the Cawthron Institute to review available literature and to carry out a survey to help determine if the presence of tetrodotoxin (TTX) in non-commercial New Zealand bivalve shellfish presents a risk to consumers.

TTX is a potent neurotoxin found in a variety of organisms from both marine and terrestrial environments. TTX has been responsible for human intoxications and deaths around the world, mostly through consumption of puffer fish, where the toxin is commonly found in their ovaries and liver. TTX is heat-stable and in fact cooking may increase its toxicity.

In New Zealand, TTX was first reported in 2011 in pipi samples, and consequently was included in the non-commercial marine biotoxin monitoring programme over the period June 2015 to October 2016. 697 samples were analysed over this period, the majority being green-lipped mussels. All samples were below 2 mg/kg, the upper limit to classify puffer fish as non-toxic in Japan. An analysis for the presence of TTX in historical samples of non-commercial was also completed (samples taken 2001-2003 and 2007-2009), of which 46% of the samples contained TTX, consistent with the 2015-2016 sampling.

As a follow-on to this work, Cawthron analysed a further 766 samples from 8 different bivalve matrices over a period of 15 months (Dec 2016 – Mar 2018). TTX levels were found to be low and similar to those observed in other countries, except for pipi. All pipi samples analysed were found to contain detectable levels of TTX, and pipi from one sampling site contained TTX consistently more than the recommended European Food Safety Authority (EFSA) safe guidance level of 0.044 mg /kg. However, they were not more than the Japanese regulatory limit of 2 mg/kg. Furthermore, there have been no reports of human illness directly attributable to the consumption of shellfish containing TTX in New Zealand.

TTX was not detected in cockles taken from the same sampling site where pipi samples had TTX levels exceeding the EFSA safe guidance level. The reason why pipi contain TTX at locations where other filter feeding shellfish do not remains unclear and warrants further investigation.

To manage the risk for a particular food contaminant, it is important to understand its origin, the mechanism by which it enters the food chain and the levels observed. There are gaps in the biosynthetic pathway for TTX production, which make it difficult to determine the origin of this potent neurotoxin. Currently, New Zealand Food Safety and Cawthron Institute, through the Safe New Zealand Seafood research programme, are carrying out research to address these key data gaps, and consequently support future regulatory decisions.

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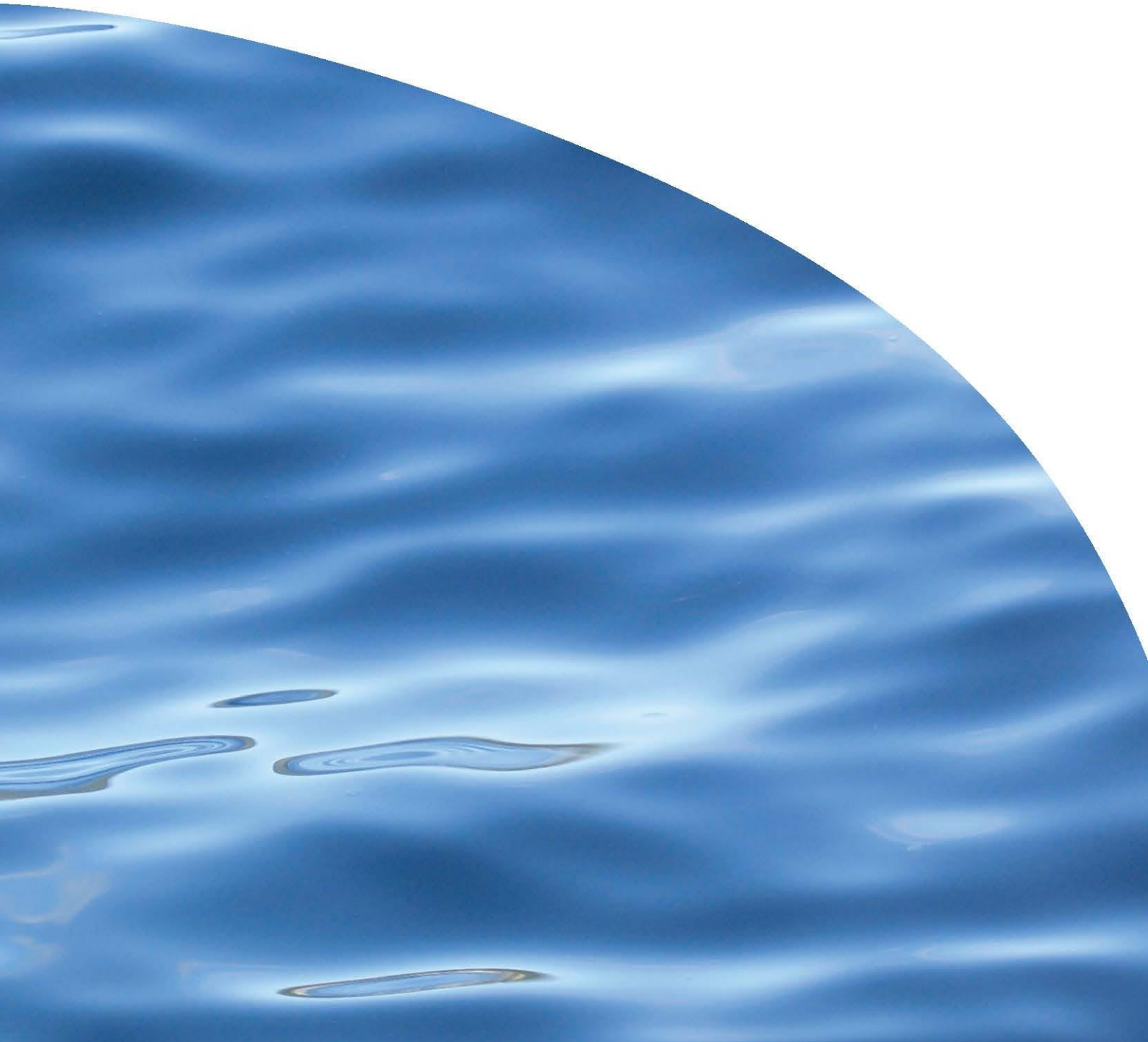
REPORT NO. 2986A

REVIEW OF LITERATURE TO HELP IDENTIFY RISKS ASSOCIATED WITH TETRODOTOXIN IN SEAFOOD, INCLUDING BIVALVE MOLLUSCS



REVIEW OF LITERATURE TO HELP IDENTIFY RISKS ASSOCIATED WITH TETRODOTOXIN IN SEAFOOD, INCLUDING BIVALVE MOLLUSCS

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Prepared for MPI

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ISSUE DATE: 30 March 2020

RECOMMENDED CITATION: Boundy MJ, Harwood DT 2020. Review of literature to help identify risks associated with tetrodotoxin in seafood, including bivalve molluscs. Prepared for MPI. Cawthron Report No. 2986A. 46 p.

EXECUTIVE SUMMARY

Tetrodotoxin (TTX) is a potent neurotoxin that has been responsible for countless human intoxications and deaths around the world, particularly in Japan from consumption of pufferfish. The distribution of TTX and its analogues, in the environment is remarkably diverse, being found in a variety of organisms from both marine and terrestrial environments. Increasing reports of detection of TTX in farmed species such as bivalve molluscs have drawn considerable attention to the toxin, reinvigorating scientific interest and questioning the need for regulation to be introduced.

There have been reports of TTX in bivalve molluscs from Japan, New Zealand, the United Kingdom, Greece, China, and the Netherlands. Although the detection rate was high in the New Zealand samples, the concentrations observed were low, with 98% of the samples below 0.020 mg/kg, 2% of the samples between 0.020 and 0.20 mg/kg. Only one sample with a concentration above 0.20 mg/kg was detected, and this was also above the regulatory limit applied to the paralytic shellfish toxins by mouse bioassay. All of the New Zealand samples analysed to date were below 2 mg/kg, which is the limit used to classify non-toxic species of pufferfish in Japan. Concentrations of TTX observed in bivalve shellfish to date are very low in comparison to other species, such as specimens of New Zealand grey side-gilled sea slugs (*Pleurobranchaea maculata*), which contain up to 1400 mg/kg. This is approximately one thousand times greater than the highest concentration observed in New Zealand bivalves to date.

While TTX is structurally dissimilar to saxitoxin, it has similar sodium channel blocking action and potency. The regulatory limit for paralytic shellfish toxins applied by New Zealand and many other countries to shellfish is 0.8 mg STX.2HCl eq/kg, based on a 100 g feed size. The presence of TTX in shellfish is currently not regulated by any country, although it is strictly controlled in pufferfish in Asian countries, and this fish species is forbidden from sale in many other countries. Due to the recent interest and concern surrounding TTX in bivalve molluscs, the European Food Safety Authority has established a panel to assess the risk and regulation of TTX in bivalves, and their opinion is imminent. Depending on the information available to the panel on the toxicology and abundance TTX in shellfish, this may or may not result in a recommendation to regulate.

New Zealand shellfish are exported to international markets. Access to these markets can be affected by their sanitation requirements, such as marine biotoxin regulation. It is important to be aware of any regulatory developments around the presence of TTX in bivalve shellfish, particularly in the EU, and fully understand the implications for shellfish grown in New Zealand waters should they be enforced.

逢はぬ恋 思切夜や ふぐと汁
I cannot see her tonight.
I have to give her up.
So I will eat fugu. -Yosano Buson. 18th Century

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GLOSSARY

Text	Description
Canthigasteridae	A genus in the pufferfish family (Tetraodontidae), often referred to as a "toby", or a sharpnose puffer
Cultured	Grown or propagated in an artificial medium
Diodontidae	A family of fish of order Tetraodontiformes, defined by their characteristic two teeth (upper and lower), known mostly as porcupine fish, although sometimes collectively called pufferfish
EC_{50}	Half maximal effective concentration refers to the concentration of a druf, antibody or toxicant which induces a response halfway between the baseline and maximum after a specified exposure time.
Equilibrium	The condition existing when a chemical reaction and its reverse reaction proceed at equal rates
Fugu	The Japanese word for pufferfish and the meal prepared from it. (See pufferfish)
GC-MS	Gas chromatography coupled with mass spectrometric detection
Guanidine	A function group comprised of a central carbon bound to three nitrogens, with a single double bond
Hemilactal	Ortho acid diester
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
Hygroscopicity	The ability of a material to absorb or release water as a function of humidity
Ichthyosarcotoxism	Poisoning caused by the ingestion of fish whose flesh contains a toxic substance
Isoform	Any of two or more functionally similar proteins that have similar but not identical amino acid sequences and are encoded by different genes or RNA transcripts from the same gene which have had different exons removed
LC-FL	Liquid chromatography coupled with fluorescence detection
LC-MS	Liquid chromatography coupled with mass spectrometric detection
LC-UV	Liquid chromatography coupled with ultraviolet absorbance detection
LD_{50}	The concentration of substance which resulted in the death of 50% of the test subjects
Maculotoxin	The original name given to tetrodotoxin when it was identified in blue-ringed octopus (Hapalochlaena maculosa) before it was shown to be identical to tetrodotoxin
Mouse bioassay	A functional method for analysis of biologically active toxins by

	intraperitoneal administration to mice
Minimum lethal dose	The lowest concentration that was observed to have a lethal effect in any of the test subjects
Molidae	A family of ocean sunfishes in the order Tetraodontiformes
MU	Mouse unit, the amount of toxin that is required to kill a 20-gram male mouse within 30 min after intraperitoneal administration.
Mutagenicity	The ability of a compound to alter genetic material, thus increasing mutations above the background level
Neurotoxin	A poison which acts on the nervous system
Organism	An individual animal, plant, or single-celled life form.
Pufferfish	Any of a family (Tetraodontidae) of chiefly tropical, scaleless, marine fishes that when threatened can distend themselves to a large, roundish form. Many of these fish are highly poisonous. Also known as blowfish, globefish, and toadfish
Relative response factor	An adjustment factor to compensate for the different sensitivity response of a target analyte and a calibration solution used where the target analyte is calibrated from a different analogue
SIR	Selective ion recording, a targeted acquisition method in mass spectrometry monitoring intact precursor ions
STX.2HCl	Saxitoxin dihydrochloride
Synthesis	Making a compound artificially in the laboratory through a chemical process
Tarichatoxin	The original name given to tetrodotoxin when it was identified in newts (Taricha sp.) before it was identified as chemically to tetrodotoxin
TEF	Toxin equivalency factor
Tetraodontidae	A family of fish of order Tetraodontiformes, defined by their characteristic four teeth (two upper, two lower)
Tetraodontiformes	An order of highly derived ray-finned fish, also called the Plectognathi. Most are marine and dwell in and around tropical coral reefs
Tetrodotoxication	Intoxication caused by ingestion of tetrodotoxin, typically from pufferfish
TTX	Tetrodotoxin
4-epi-TTX	4-epi-tetrodotoxin
6-epi-TTX	6-epi-tetrodotoxin
11-deoxy-TTX	11-deoxy-tetrodotoxin
6-epi-11-deoxy-TTX	6-epi-11-deoxy-tetrodotoxin
6,11-dideoxy-TTX	6,11-dideoxy-tetrodotoxin
8,11-dideoxy-TTX	8,11-dideoxy-tetrodotoxin
TTX-8-O-hemisuccinate	Tetrodotoxin-8-O-hemisuccinate

CqTX	Chiriquitoxin
11-nor-TTX-6(S)-ol	11-nor-tetrodotoxin-6(S)-ol
11-nor-TTX-6(R)-ol	11-nor-tetrodotoxin-6(R)-ol
11-nor-TTX-6,6-diol	11-nor-tetrodotoxin-6,6-diol
11-oxo-TTX	11-oxo-tetrodotoxin
TTX-11-COOH	Tetrodotoxin-11-carboxylic acid
5-deoxy-TTX	5-deoxy-tetrodotoxin
5,11-dideoxy-TTX	5,11-dideoxy-tetrodotoxin
4-epi-5,11-dideoxy-TTX	4-epi-5,11-dideoxy-tetrodotoxin
1-hydroxy-5,11-dideoxy-TTX	1-hydroxy-5,11-dideoxy-tetrodotoxin
5,6,11-trideoxy-TTX	5,6,11-trideoxy-tetrodotoxin
4-epi-5,6,11-trideoxy-TTX	4-epi-5,6,11-trideoxy-tetrodotoxin
4,9-anhydro-TTX	4,9-anhydro-tetrodotoxin
4,9-anhydro-6-epi-TTX	4,9-anhydro-6-epi-tetrodotoxin
4,9-anhydro-11-deoxy-TTX	4,9-anhydro-11-deoxy-tetrodotoxin
4,9-anhydro-6,11-dideoxy-TTX	4,9-anhydro-6,11-dideoxy-tetrodotoxin
4,9-anhydro-TTX-8-O-hemisuccinate	4,9-anhydro-tetrodotoxin-8-O-hemisuccinate
4,9-anhydro-TTX-11-O-hemisuccinate	4,9-anhydro-tetrodotoxin-11-O-hemisuccinate
4,9-anhydro-5,6,11-trideoxy-TTX	4,9-anhydro-5,6,11-trideoxy-tetrodotoxin
4,9-anhydro-5,11-dideoxy-TTX	4,9-anhydro-5,11-dideoxy-tetrodotoxin
4,9-anhydro-5-deoxy-TTX	4,9-anhydro-5-deoxy-tetrodotoxin

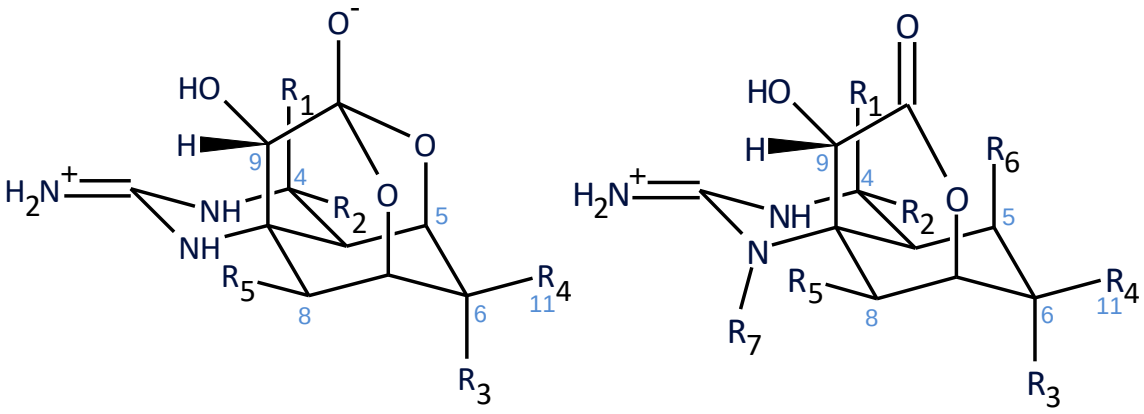
1. INTRODUCTION

1.1. What is tetrodotoxin?

Tetrodotoxin (TTX) is a low-molecular mass neurotoxin found in a variety of organisms, mostly in the ovaries and livers of pufferfish (*syn* fugu, blowfish, globefish and toadfish) [1]. It is named from its initial identification and isolation from tetraodontiformes, which have been related to intoxication events throughout history. The distribution of TTX is remarkably diverse occurring in both marine and terrestrial environments [2, 3]. It has been reported in fish [3], gastropods [4], crabs [5-7], marine flatworms [8], ribbon worms [9], arrow worms [10], annelid worms [3], starfish [11], grey side-gilled sea slug [12], blue-ringed octopus [13, 14], newts [15], frogs [16], terrestrial flatworms [17], and has become a hot topic regarding its presence in bivalve molluscs [18-22]. Poisoning due to the toxin has long been a serious issue in Japan where pufferfish are a highly desirable delicacy [1]. TTX has also been identified under various other names based on other species from which it has also been identified, such as tarichatoxin [23] (from *Taricha* newts), and maculotoxin [14] (from *Hapalochlaena maculosa*, blue-ringed octopus).

TTX has a highly unusual chemical structure [3], which was first elucidated in 1964 [1, 24-27]. It is a zwitterion, with a positively charged guanidine group, and a negatively charged hemilactal alcohol group [27]. A range of TTX analogues has been reported from natural sources, or synthesised in the laboratory (Figure 1). TTX is water soluble and heat-stable [20]. Cooking pufferfish flesh may increase toxicity, which has been attributed to decomposition of antioxidants allowing toxicity enhancement [28].

Figure 1. Structure of TTX analogues with hemilactal (left) and lactone (right) type backbones



Left: Hemilactal type analogues

Toxin	R1	R2	R3	R4	R5
TTX	H	OH	OH	CH_2OH	OH
4-epiTTX	OH	H	OH	CH_2OH	OH
6-epiTTX	H	OH	CH_2OH	OH	OH
11-deoxyTTX	H	OH	OH	CH_3	OH
6-epi-11-deoxyTTX	OH	H	OH	CH_3	OH
6,11-dideoxyTTX	H	OH	H	CH_3	OH
8,11-dideoxyTTX[a]	H	OH	OH	CH_3	H
TTX-8-O-hemisuccinate[a]	H	OH	OH	CH_2OH	$OCO(CH_2)_2COO^-$
Chriquitoxin (CqTX)[b]	H	OH	OH	$CH(OH)CH(NH_3^+)COO^-$	OH
11-nor-TTX-6(S)-ol	H	OH	OH	H	OH
11-nor-TTX-6(R)-ol	H	OH	H	OH	OH
11-nor-TTX-6,6-diol	H	OH	OH	OH	OH
11-oxo-TTX	H	OH	OH	$CH(OH)_2$	OH
TTX-11-COOH[b]	H	OH	OH	COO^-	OH

Right: Lactone type analogues

Toxin	R1	R2	R3	R4	R5	R6	R7
TTX (lactone)[c]	H	OH	OH	CH_2OH	OH	OH	H
6-epi-TTX (lactone)[c]	H	OH	CH_2OH	OH	OH	OH	H
11-deoxyTTX (lactone)[c]	H	OH	OH	CH_3	OH	OH	H
11-nor-TTX-6(S)-ol (lactone)[c]	H	OH	OH	H	OH	OH	H
11-nor-TTX-6(R)-ol (lactone)[c]	H	OH	H	OH	OH	OH	H
11-nor-TTX-6,6-diol (lactone)[c]	H	OH	OH	OH	OH	OH	H
5-deoxyTTX	H	OH	OH	CH_2OH	OH	H	H
5,11-dideoxyTTX	H	OH	OH	CH_3	OH	H	H
4-epi-5,11-dideoxyTTX	OH	H	OH	CH_3	OH	H	H
1-hydroxy-5,11-dideoxyTTX[b]	H	OH	OH	CH_3	OH	H	OH
5,6,11-trideoxyTTX	H	OH	H	CH_3	OH	H	H
4-epi-5,6,11-trideoxyTTX	OH	H	H	CH_3	OH	H	H

a – Analogue which was synthesised, and has not been naturally observed

b – Analogue which has been identified only in newts

c – Lactone tautomer of hemilactal form

TTX exists in an equilibrium with its 4-epi and 4,9-anhydro analogues under acidic conditions (Figure 2), favouring TTX in an 8:1:1 ratio [29, 30], consistent with the ratios observed in pufferfish [30]. Similarly, other analogues of TTX have been reported to have 4-epi- and 4,9-anhydro- forms. Transformations from TTX to its deoxy or oxo analogues have not been observed *in vivo* [29]. Purified 4,9-anhydroTTX can be converted back to the equilibrated ratio using strong hydrochloric acid [24]. TTX decomposes under mild alkaline conditions, whereas 4,9-anhydro-TTX is stable [1].

When cultured pufferfish were fed a diet contaminated with TTX, it was accumulated and was the dominant toxin in tissue [31, 32]. However, intramuscular administration of purified TTX, 4-epi-TTX or 4,9-anhydro-TTX to cultured pufferfish showed that 4,9-anhydro-TTX was the dominant analogue after 16 days [29]. These results suggest that the absorption of TTXs through the gastrointestinal tract is important in pufferfish for efficient accumulation of TTXs and to prevent transformation or degradation of TTX.

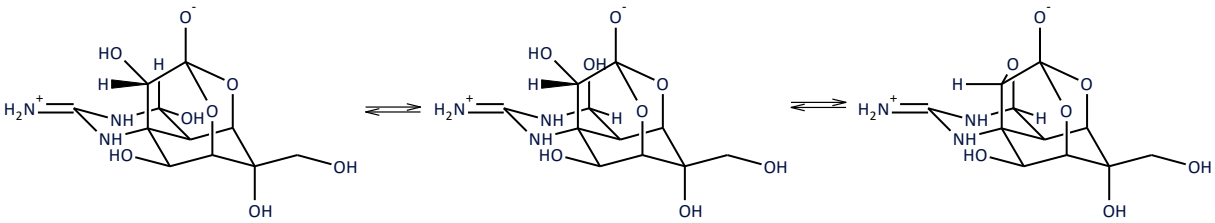


Figure 2. Interconversion of TTX (left), with 4-epi-TTX (middle), and 4,9-anhydro-TTX (right)

1.2. A brief history of TTX intoxications

The history of TTX poisoning is as difficult to trace as many other human ailments, because it merges into the realm of folklore [33]. Records of pufferfish poisoning have been described in ancient literature from various parts of the world, particularly from Japan and China [34] and from Egypt [35]. In Japan, the oldest record of puffer poisoning is found in the Nara and Heian eras (710-1185 AD). Pufferfish frequently appeared in senryu, haiku and poetry in the Edo era (1603-1868) [3]. In China, puffers caused human intoxication as long as 2000 years ago [34]. In Egypt, a pufferfish identified as *Tetraodon lineatus* was shown on an Egyptian tomb of the fifth dynasty (2500 BC), with evidence that the Egyptians of the time knew this fish to be poisonous [35].

Pufferfish were first brought to the attention of Europe by Kämpfer in 1727, a German physician at the Dutch embassy in Nagasaki [36]. At the time, different species of pufferfish were known. Some species were occasionally eaten as a delicacy, but the head, guts, bones and other remains were thrown away and the flesh carefully washed and cleaned before it was fit to be eaten, and yet many still died from eating it. Another species was known to be poisonous even after careful preparation and cleaning, and this was only consumed by those who sought to end their lives. Later, pufferfish poisoning was experienced first-hand by European explorers. In September

1774, Captain James Cook described in his journal during the voyage around the world in the HMS Resolution, an intoxication of himself and two shipmates after they tasted the liver and roe of a fish caught by a local in New Caledonia. They found themselves seized with an extraordinary weakness and numbness over their limbs. After they vomited and perspired they recovered. A pig which had eaten the entrails of the fish was subsequently found dead [37].The fish was sketched and later identified as *Lagocephalus sceleratus*, the silver cheeked toadfish. Only six-weeks before this incident, the crew had eaten an unidentified red fish, resulting in ichthyosarcotoxism with symptoms characteristic of ciguatera fish poisoning [37, 38].

In Italy in 1977, human poisoning was reported after consumption of imported pufferfish mislabelled as anglerfish [39]. Human intoxication from TTX from product caught within Europe was first reported from a trumpet shell, *Charonia lampas lampas*, purchased at a market in southern Spain and caught off the coast of Portugal in October 2007. The digestive gland was determined to be extremely toxic by mouse bioassay (255 mg STX.2HCl eq/kg), although no saxitoxins (STXs) were found when using a specific analytical technique (HPLC with fluorescence detection). The identity of the causative toxin was confirmed as TTX by LC-MS/MS [40, 41], and the digestive gland was found to contain 249 mg/kg of TTX. It was estimated that approximately 5 g of the digestive gland was consumed.

To date, incidents of human poisoning by TTX have been reported from fish, crabs, and/or gastropods in Australia [42], Bangladesh [43-46], Cambodia [47], China [48-50], Egypt [51], Fiji [52], Hawaii [53], Hong Kong [54, 55], Israel [56], Italy [39], Japan [57], Lebanon [58], Madagascar [59], Malaysia [60], Mexico [61], Singapore [62-64], Spain [41], Taiwan [65-68], Thailand [69, 70], the Philippines [71], the United States [72, 73], and Vietnam [74].

1.3. Relationship to saxitoxins

While saxitoxin (STX) has quite a different structure to TTX, both molecules possess very similar sodium channel blocking action [75]. As with TTX, there are many STX analogues with varying molecular structures and toxicities (Figure 3). The STX analogues are regulated in shellfish as the paralytic shellfish toxin (PST) group and are known to cause paralytic shellfish poisoning (PSP). Not all sodium channels are the same, with varying binding affinities for each analogue across the different sodium channel isoform binding sites [76, 77]. Notably, newts of the genus *Taricha* are resistant to TTX but not to STX, which suggests that it cannot be assumed that the toxicity characteristics of STX and TTX will be the same [78].

Most literature surrounding intoxications by pufferfish ingestion have not confirmed TTX as the causative toxin from the organism: it is assumed to be the cause through the link between the result from the mouse bio-assay, and the causative fish

(pufferfish). Similarly, it is common practice to assume that PST is the cause when toxicity is found in bivalve shellfish when using the PSP mouse bioassay [79]. The mouse bioassay protocol for TTX is nearly identical to the PSP mouse bioassay with samples prepared with a greater dilution, making it somewhat less sensitive (10 MU/g, 2 mg/kg compared to 0.3-0.4 mg/kg for the PSP mouse bioassay). Many of the organisms that contain TTX may also contain paralytic shellfish toxins [79-82]. Historically, toxicity measured in shellfish by the mouse bioassay was assumed to be due to PST, while toxicity measured in pufferfish was assumed to be due to TTX. As the historical analyses were non-specific, it is possible that TTX has been present in shellfish for quite some time.

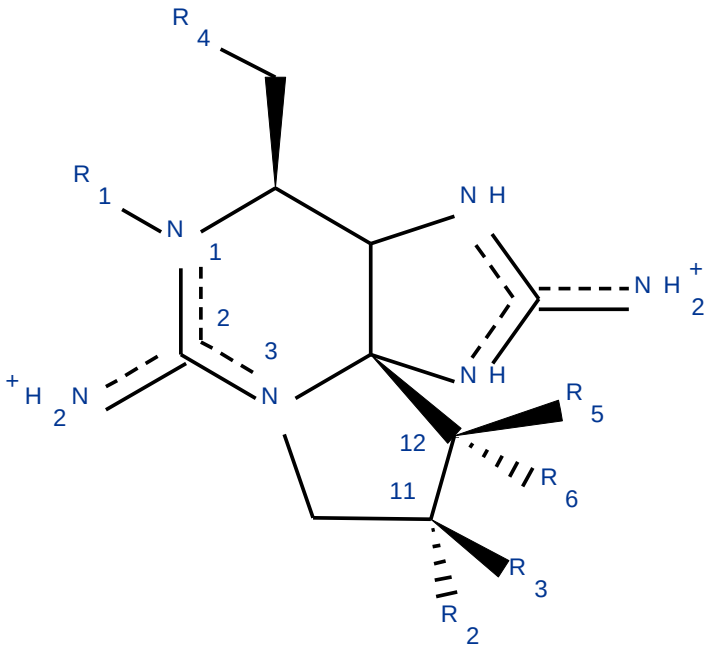


Figure 3. Structure of saxitoxin analogues

Toxin	R1	R2	R3	R4	R5	R6
C1	H	H	OSO_3^-	$OCONHSO_3^-$	OH	OH
C2	H	OSO_3^-	H	$OCONHSO_3^-$	OH	OH
C3	OH	H	OSO_3^-	$OCONHSO_3^-$	OH	OH
C4	OH	OSO_3^-	H	$OCONHSO_3^-$	OH	OH
GTX1	OH	H	OSO_3^-	$OCONH_2$	OH	OH
GTX2	H	H	OSO_3^-	$OCONH_2$	OH	OH
GTX3	H	OSO_3^-	H	$OCONH_2$	OH	OH
GTX4	OH	OSO_3^-	H	$OCONH_2$	OH	OH
GTX5	H	H	H	$OCONHSO_3^-$	OH	OH
GTX6	OH	H	H	$OCONHSO_3^-$	OH	OH
dcGTX1	OH	H	OSO_3^-	OH	OH	OH
dcGTX2	H	H	OSO_3^-	OH	OH	OH
dcGTX3	H	OSO_3^-	H	OH	OH	OH
dcGTX4	OH	OSO_3^-	H	OH	OH	OH
11αOH-GTX5 (M1α)	H	OH	H	$OCONHSO_3^-$	OH	OH
11βOH-GTX5 (M1β)	H	H	OH	$OCONHSO_3^-$	OH	OH
M3	H	OH	OH	$OCONHSO_3^-$	OH	OH
M5						
doSTX	H	H	H	H	OH	OH
dcSTX	H	H	H	OH	OH	OH
dcNEO	OH	H	H	OH	OH	OH
STX	H	H	H	$OCONH_2$	OH	OH
NEO	OH	H	H	$OCONH_2$	OH	OH
11βOH-dcSTX (dcM2β)	H	OH	H	OH	OH	OH
11βOH-dcSTX (dcM2β)	H	H	OH	OH	OH	OH
11βOH-STX (M2β)	H	OH	H	$OCONH_2$	OH	OH
11βOH-STX (M2β)	H	H	OH	$OCONH_2$	OH	OH
11,11diOH-STX (M4)	H	OH	OH	$OCONH_2$	OH	OH
12αdo-dcSTX	H	H	H	OH	H	OH
12βdo-dcSTX	H	H	H	OH	OH	H
12αdo-doSTX	H	H	H	H	H	OH
12βdo-doSTX	H	H	H	H	OH	H
12,12dido-dcSTX	H	H	H	OH	H	H

2. DISTRIBUTION AND PRODUCTION OF TETRODOTOXIN

2.1. Reports of TTX in Bivalve Molluscs

2.1.1. Japan

In 1993, TTX was reported at concentrations of up to 40 MU/g (~8 mg/kg) in scallop (*Patinopecten yessoensis*) digestive glands. This was observed during an *Alexandrium tamarense* bloom, and it was suggested that intracellular bacteria within the *Alexandrium* cells may have been responsible for the TTX production [18]. Despite the high levels of TTX present in the scallop digestive gland, this was a relatively minor contributor to the total toxicity (reported as 3427 MU/g of PST, which equates to ~610 mg STX.2HCl eq/kg) observed at the maximum concentration during the bloom. TTX was confirmed as being present using cellulose acetate membrane electrophoresis, thin layer chromatography, and high performance liquid chromatography with fluorescence detection and FABS-MS after the toxin was partially purified from the scallop tissue.

2.1.2. New Zealand

TTX was first reported in a clam species (pipi; *Paphies australis*) collected from Whangapoua during April 2011. Concentrations were reported up to 0.8 mg/kg [19], which is considerably lower than that seen in the Japanese scallop material. Assesments of TTX concentration were made using two separate analytical methods that employed liquid chromatography tandem mass spectrometry. The first analysed the intact toxin, and the second monitored a derivatisation product generated by dehydration under highly alkaline conditions.

Over the past year (June 2015 – October 2016), TTX has been monitored in New Zealand shellfish as part of the non-commercial marine biotoxin monitoring programme. Samples were analysed using a recently developed and implemented HILIC-MS/MS method that is now being used at Cawthron for routine regulatory monitoring of the paralytic shellfish toxin group [83, 84]. This method has the benefit of being able to simultaneously quantify TTX with the addition of quality control measures, and additional processing steps [85]. In total, 697 samples were analysed, of which 327 (47%) samples contained TTX at between 0.0001 and 0.020 mg/kg, 12 samples (2%) contained between 0.020 and 0.20 mg/kg, and a single Greenshell™ mussel sample (0.1%) contained TTX above 0.20 mg/kg. All samples were below 2 mg/kg, the upper limit used to classify pufferfish as non-toxic in Japan. Most of the samples analysed were Greenshell mussels (n=460, 66%).

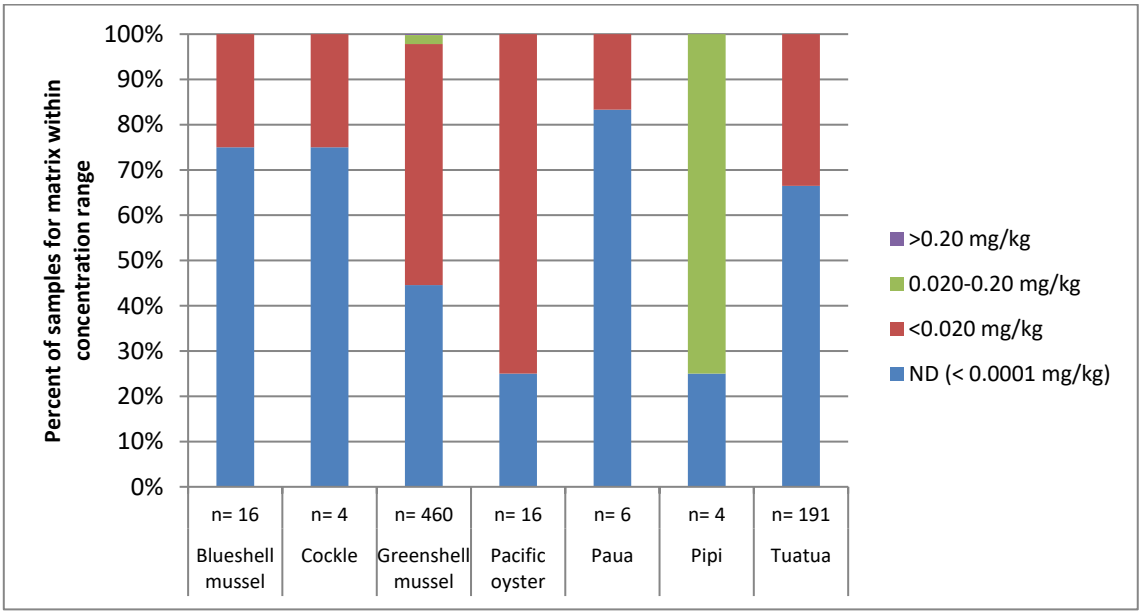


Figure 4. Non-commercial shellfish matrices analysed for tetrodotoxin, ND = not detected.

An observation from reviewing this data was that there appeared to be a time-related trend at two sites on the west-coast North Island (SF015, Cornwallis, Manukau Harbour; SF017, Kawhia Harbour). At both sites there was an increase in TTX concentrations in April-June 2016 (Figure 5). This suggests a potential seasonal variation which may warrant further study. All samples were Greenshell mussels. Investigation into areas such as these may provide further information on how shellfish become contaminated with TTX in order to better guide management plans around this issue.

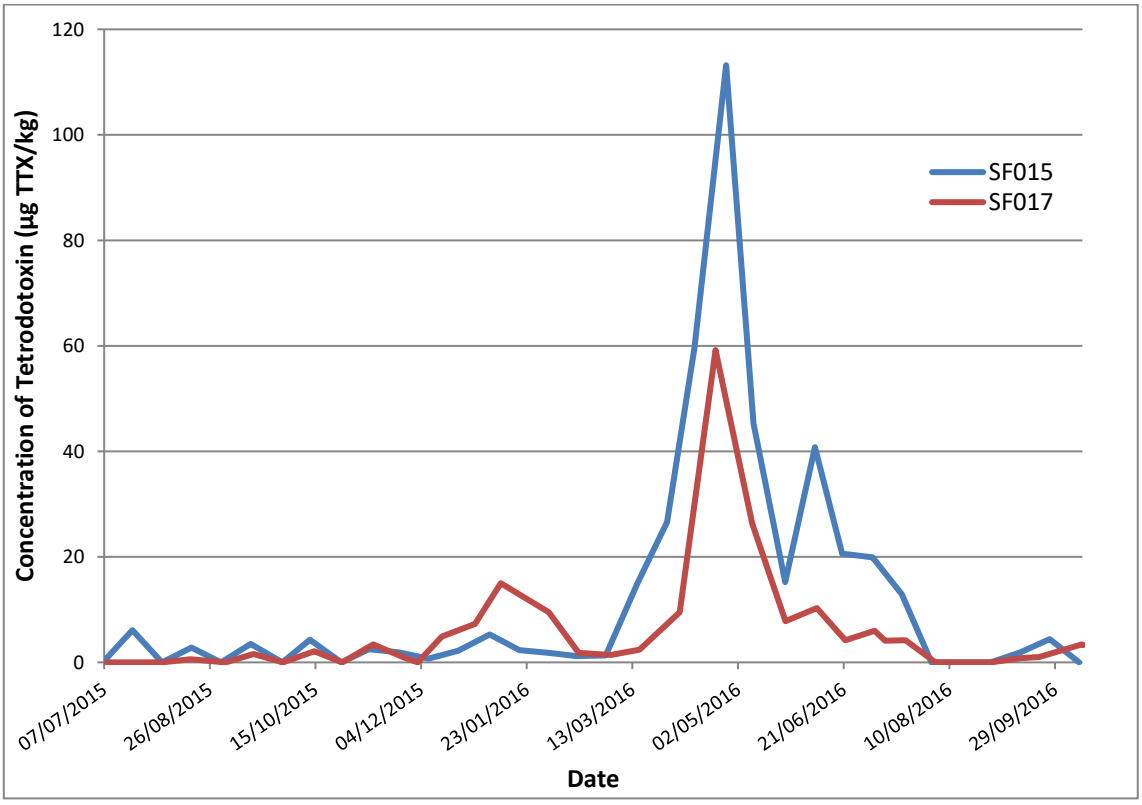


Figure 5. Plot showing time-related TTX concentrations in shellfish sourced from non-commercial sites SF015 and SF017 between July 2015 to October 2016.

Another site of interest was within a populated area of the Hauraki Gulf (SC032D). This site was sampled to probe the impact range of a reasonably large PST bloom event that occurred within the Mahurangi inlet north of Auckland city. Of the two shellfish samples taken for analysis (pool of n≥12 shellfish during each sampling event) during the study period, the first contained low concentrations of TTX (0.0041 mg/kg). However, the second sample, taken 2 weeks later, contained the highest level of TTX observed in New Zealand shellfish to date (1.6 mg/kg) (Table 1). This second sample also contained paralytic shellfish toxins at a level of 0.4 mg STX.2HCl eq/kg. To assess the contribution of TTX to the total toxicity of the sample, it was subjected to the PSP mouse bioassay [86]. The total toxicity from the mouse bioassay was 7278 MU/kg, or 1.3 mg STX.2HCl eq/kg. This is above the regulatory limit of 0.8 mg STX.2HCl eq/kg for this toxin class [87]. Samples taken from nearby sites also contained low concentrations of TTX (<0.020 mg/kg), with results summarised in Table 1. Due to the limited sampling around this event, it is not possible to determine if there was accumulation or depuration, and if the TTX contamination was spread through the region, or if it was localised to a small number of shellfish through contact with a highly toxic organism.

Table 1. TTX levels in samples taken from SC032D and nearby sites from May-July 2016

Date Sampled	Matrix	Site	Distance from SC032D (km)	mg/kg
11/05/2016	Pacific Oyster	SC032F	11	0.0019
01/06/2016	Greenshell Mussel	SC032F	11	0.020
06/06/2016	Greenshell Mussel	SC032F	11	0.010
06/06/2016	Greenshell Mussel	SC032	5	ND
19/06/2016	Greenshell Mussel	SC032D	-	0.0041
03/07/2016	Greenshell Mussel	SC032D	-	1.6
11/07/2016	Pacific Oyster	SC032F	11	0.0036
17/07/2016	Rock Oyster	SC032F	11	0.0026

ND = not detected

The location and number of all non-commercial samples analysed as part of the non-commercial marine biotoxin monitoring programme are shown in Figure 6. Most of the samples were from the North Island, since historical information indicates these sites represent the most at-risk areas from the impacts of harmful algal bloom events. The average concentration of TTX and PST, determined at each site are shown in Figure 7 and Figure 8 respectively. Trace levels of TTX were observed in both the North and South Island, with low level detections at most sites. The average concentrations of PST in these samples were much higher than that of TTX.

To determine if the presence of TTX in New Zealand shellfish is a recent phenomenon, archived samples (2001-2003 n=18, 2007-2009 n=9) were analysed. Of these samples 12 (46%) contained TTX. The highest concentration in the samples taken between 2001 and 2003 was 0.019 mg/kg, and the highest concentration in the samples from 2007-2009 was 0.021 mg/kg. The detection rate of TTX in the library samples were consistent with the detections observed in the fresh samples taken from June 2015- October 2016.

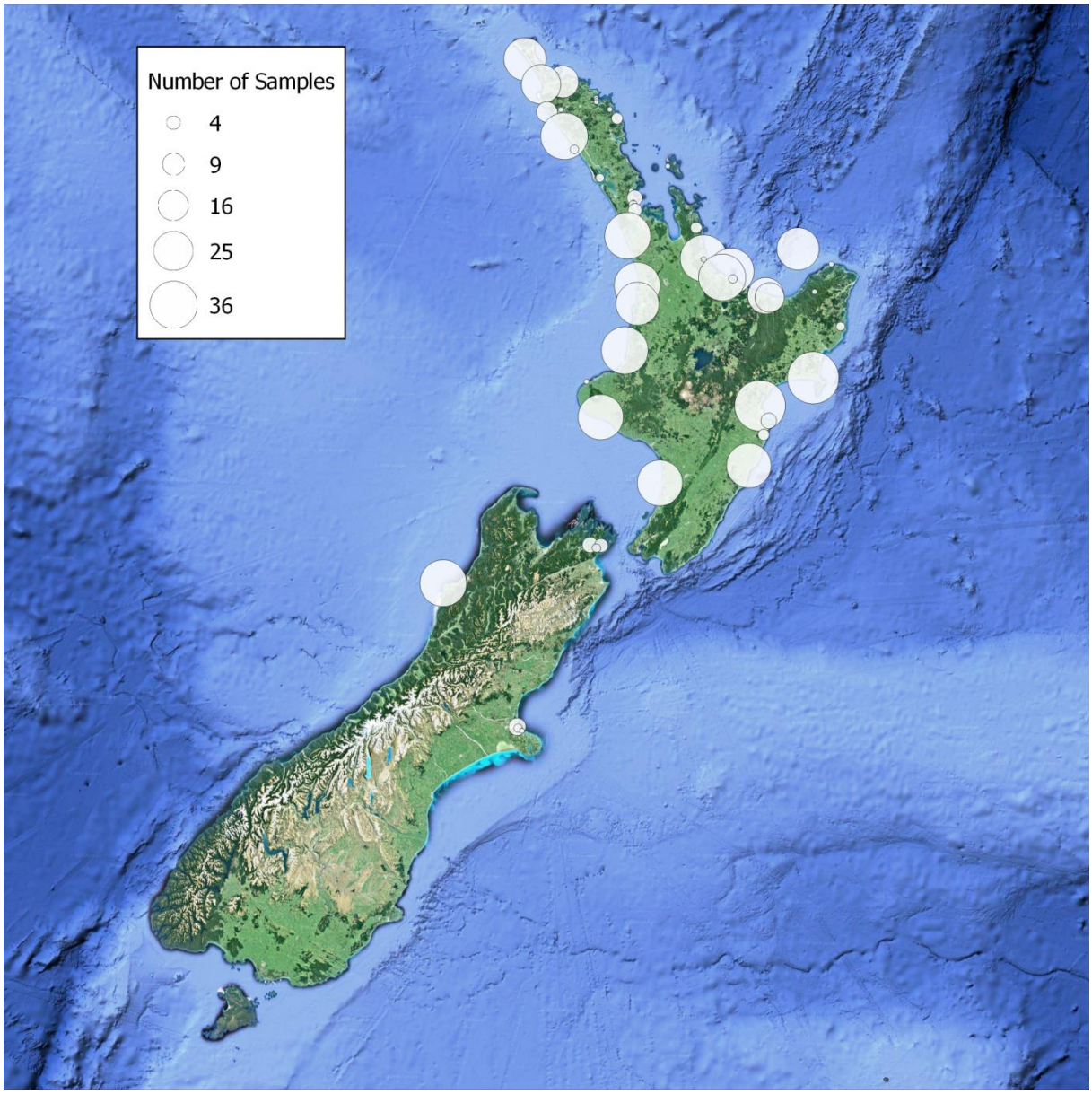


Figure 6. Location and number of non-commercial samples analysed for paralytic shellfish toxins between June 2015 and October 2016

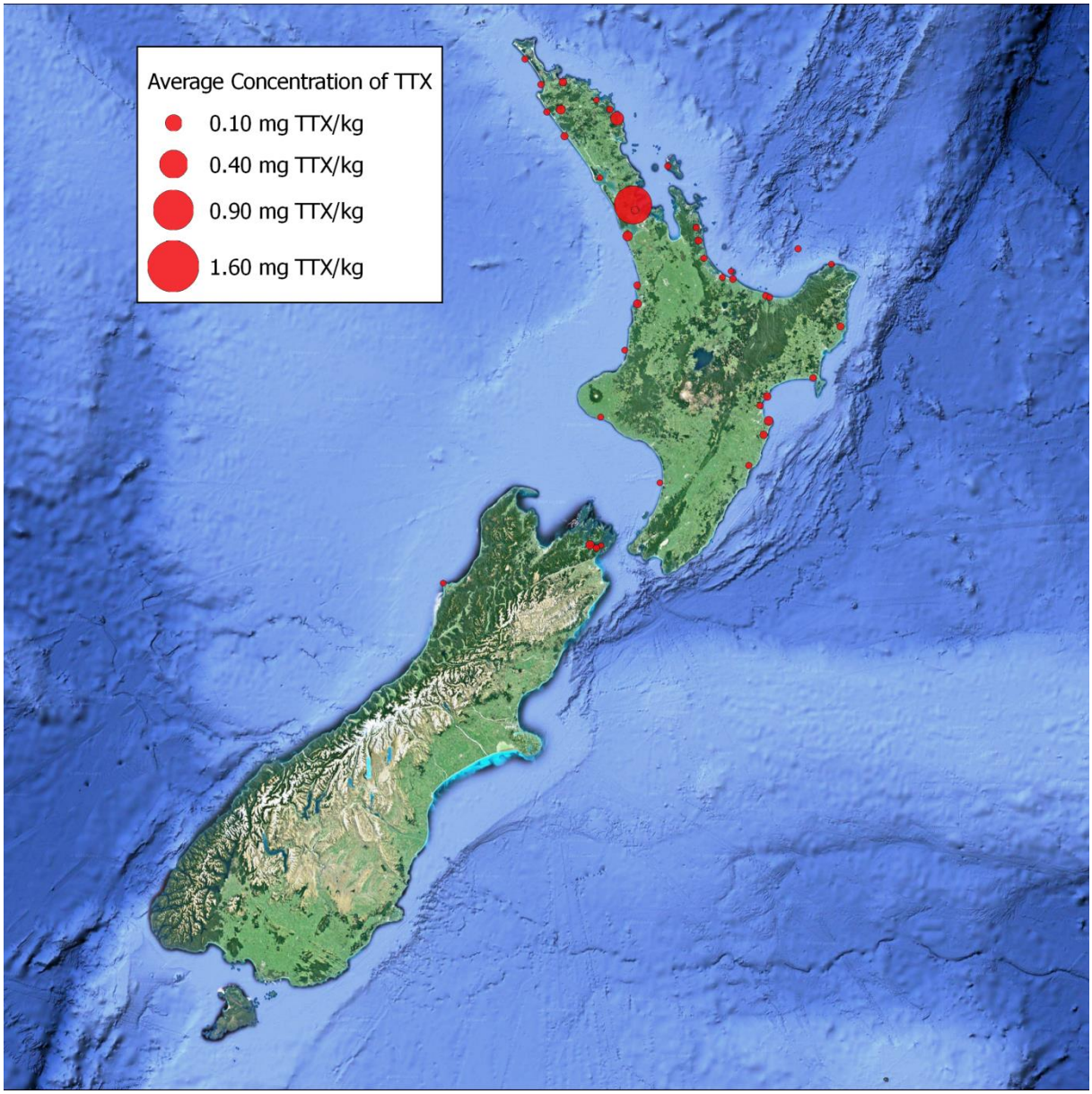


Figure 7. Location and average concentration of TTX observed in non-commercial samples taken between June 2015 and October 2016

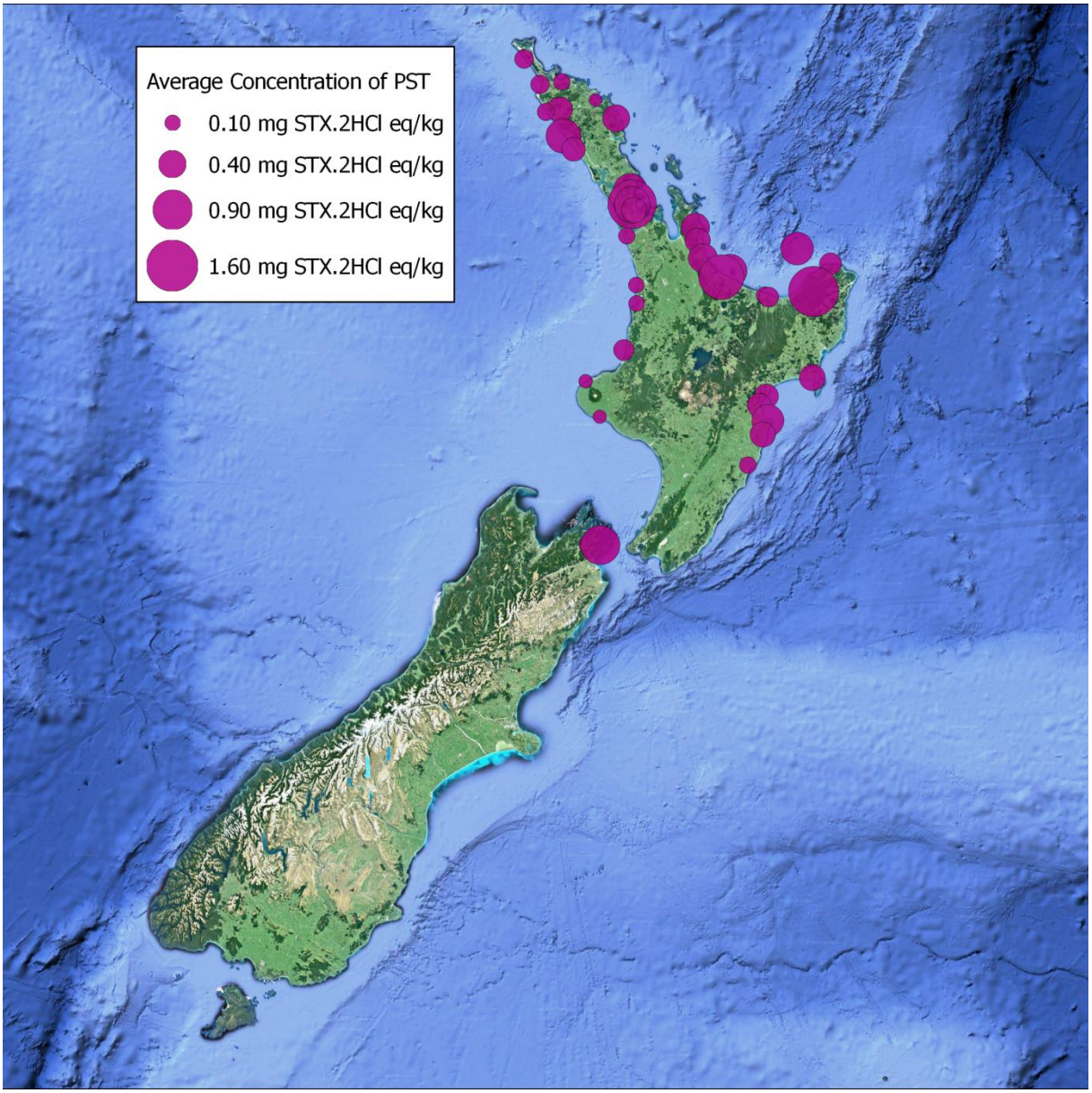


Figure 8. Location and average concentration of PST observed in non-commercial samples taken beween June 2015 and October 2016

2.1.3. United Kingdom

TTX was first reported in European bivalve molluscs in 2014. Twenty-nine shellfish samples of mussels (*Mytilus edulis*) and Pacific oysters (*Crassostrea gigas*), harvested from two sites on the south coast of England between February 2013 and October 2014 were screened for TTXs. The samples were stored frozen, then analysed using HILIC-MS/MS for tetrodotoxin analogues and the presence of TTX was confirmed using LC-MS/MS after derivatisation under alkaline conditions to the C_9 base. Of the 29 samples, 14 contained TTX at concentrations ranging from 0.003 – 0.12 mg/kg [20]. The C9-base was detected after derivatisation in the 5 samples that

had the highest concentration of TTX, ranging from 0.02 - 0.12 mg/kg, in agreement with the values found without derivatisation.

2.1.4. Greece

During official shellfish monitoring for the presence of marine biotoxins in Greece in 2012, a series of unexplained positive mouse bioassay screens was observed [21]. This was not attributed to any regulated toxin, but occurred during a *Prorocentrum minimum* algal bloom. Analysis by LC-MS showed the presence of TTX at up to 0.223 mg/kg. A review of historical reference samples (mussels *Mytilus galloprovincialis* and clams, *Venus verrucosa*) from 2006-2012 showed TTX at levels from 0.061-0.194 mg/kg. Recent analysis of cultures of three strains of *Prorocentrum minimum* grown under different temperature, salinity and light conditions did not show any production of TTX, indicating that *Prorocentrum minimum* is unlikely to be the origin of TTX in this case [88].

2.1.5. China

In 2015, as part of validation of an LC-MS method, 20 manila clams (*Ruditapes philippinarum*) purchased from markets in China were analysed, of which one was reported to contain detectable levels of TTX (0.00222 mg/kg) [22]. The concentration reported was very low. However, the report demonstrates the increasing awareness and monitoring of TTX in export markets.

2.1.6. The Netherlands

Dutch researchers have also been analysing shellfish for the presence of TTX (unpublished data). These results were presented during the Emerging Toxins Workshop, September 2016 in Baiona, Spain, by researchers from the Dutch National Reference Institute (RIKILT). They reported detection of TTX in bivalve molluscs, with samples from 2015 showing a maximum of 0.124 mg/kg, and samples analysed in 2016 showing a maximum of 0.253 mg/kg. The data suggested seasonal and regional variation. In an evaluation of the limited toxicological data of TTX, it was suggested from a risk assessment undertaken by a Dutch committee that an action limit of 0.020 mg/kg should be implemented until there is better toxicological understanding of the toxin group. This, along with the detection of TTX in European bivalve shellfish and gastropods has led to a review by the European Food Safety Authority (EFSA).

2.2. Reports of tetrodotoxin in marine species

2.2.1. Tetraodontiformes (pufferfish)

Organisms of the order Tetraodontiformes, particularly of the family Tetradontidae, are the most well-known sources of TTX. Most species of wild pufferfish are known to be potentially toxic, although cultured pufferfish are considered to be of low toxicity [89, 90]. Pufferfish show regional and seasonal variations in their toxicological profiles [91], and tissue distribution and toxicity are different for males and females [92]. Maximum toxicity is observed during spawning and this is associated with an increase in ovary mass containing high levels of TTX. This is followed by a rapid decrease in TTX levels immediately after spawning [93], when highly toxic eggs are laid [94]. It has been observed that some pufferfish species consume the eggs of other pufferfish and this represents a potential source of toxification [95]. Unlike ovaries, the testes have not been observed to accumulate high levels of TTX [93].

2.2.2. Gastropods

TTX has been reported in a wide range of marine gastropods, such as a variety of sea snails [4, 66, 96-105] and a sea slug [12]. It is generally found at the highest concentration in the digestive gland of contaminated gastropods, although it was found predominantly in the muscle on lined moon snails (*Natica lineata*) [98, 99]. Retention of TTX within the muscle of lined moon snails was due to binding to a 434 kDa protein [106]. Similar protein binding has been reported in other species of gastropods [106], and crabs [107]. Similar proteins have also been shown to specifically bind STX, but not TTX, in Xanthid crabs [108]. The occurrence of TTX primarily in the digestive gland of the gastropods suggests that accumulation is via ingestion. For the Trumpet shell this was reported to originate primarily from starfish, a food source of the mollusc that contains the toxin [11].

Grey side-gilled sea slugs (*Pleurobranchaea maculata*) are of particular concern in New Zealand. Large numbers occasionally wash-up on highly populated beaches around Auckland. Dogs that ate slugs from the inter-tidal zone died, and there was a real and imminent threat should a toddler put a toxic slug in their mouth [12]. The sea slugs contained high concentrations of TTX, with levels of up to 1414 mg/kg being recorded [109]. To give this level context, it is 1000 times higher than the highest level observed in molluscan shellfish to date. Similar to cultured pufferfish, no TTX was detected in a single side-gilled sea slug raised from toxic eggs laid in captivity [110]. This suggests that the TTX accumulates in this species through its food source.

2.2.3. Worms

In 1986, several species of marine flatworms were shown to contain a toxin that induced paralysis [111], and TTX was identified in *Planocera multitentaculata* [112].

As in pufferfish, flatworms contain the majority of toxin in the reproductive organs and can lay highly toxic eggs [8]. Marine flatworms are carnivorous, and because their body is very thin, they can slip between the valves of oysters, and consume the bivalve flesh. They are known to eat commercial bivalve species including oysters, scallops, mussels and giant clams [113]. It has been observed that most finfish do not eat flatworms, although pufferfish may consume them. Real-time PCR has shown that grey side-gilled sea slugs also consume flatworms as a possible source of TTX, although it is unlikely to be a sole source [114]. TTX has been reported in other species of marine worms, such as ribbon worms (*Cephalothrix linearis*) [9], (*Lineus fuscoviridis* and *Tubulanus punctatus*) [115], and is present in the venom of marine arrow worms, which facilitates the immobilization of prey such as copepods [10]. Ribbon worms were observed on and around oyster farms, with some worms containing sufficient TTX to be lethal to humans [116]. They represent a potential vector for TTX accumulation in shellfish.

2.3. Origin

To be able to manage the associated risk for a particular food contaminant it is important to understand its origin, the mechanism in which they enter the food supply and the levels observed. For TTX this is difficult as controversy remains as to the origin of this potent neurotoxin. The two most common hypotheses are that it is produced by symbiotic bacteria, or that it is accumulated through the diet.
According to the literature, various bacterial species are able to produce TTX, and its analogues [117]. Species of bacteria reported to produce TTX span several genera, including; *Acinetobacter* [118], *Aeromonas* [118-122], *Alteromonas* [118, 123-125], *Bacillus* [50, 118, 126-128], *Caulobacter* [129], *Enterobacter* [130], *Flavobacterium* [129], *Lysinibacillus* [131], *Marinomonas* [122, 132], *Microbacterium* [127, 133], *Mirococcus* [118], *Moraxella* [118], *Nocardiopsis* [134], *Photobacterium* [119, 132], *Planococcus* [132], *Plesiomonas* [121], Pseudoalteromonas [135], *Pseudomonas* [118, 120, 121, 126, 127], *Rahnella* [130], *Raoultella* [127], *Roseobacter* [136], *Serratia* [127, 133, 137], *Shewanella* [122, 125, 132, 138, 139], *Streptomyces* [140], *Tenacibaculum* [122], and *Vibrio* [120-122, 125, 126, 132, 141-145].
Thirteen genera of pufferfish contain TTX, of which only 3 have been reported to contain TTX-producing bacteria. Analysis of the associated bacterial cultures show that the amount of TTX is low when compared to the TTX-bearing animals. Gaps in the biosynthetic pathways for TTX production remain [146], which hinders the understanding of TTX production and accumulation in higher organisms.

Two methods of analysis have commonly been used to investigate the production of TTX in bacterial cultures. These are:
1) high performance liquid chromatography with post column derivatization under alkaline conditions followed by fluorescence detection (LC- FL) [147]. This approach has low specificity and gives false positives due to matrix interferences. This includes the polypeptone, yeast and starch culture medias used to grow bacteria [148].

2) gas chromatography-mass spectrometry (GC-MS) [149]. This requires a two-step derivatization, first to the C_9 base from hydrolysis under basic conditions, and then to a trimethylsilyl (TMS) derivative that is compatible with GC-MS. This approach has also been shown to generate false positives from extraction of culture media [148].

In a review of bacteria that were reported to produce TTX, no evidence of production of TTX was found when using modern highly-specific methods [150]. This indicates that there has been a large overestimation of the number of bacteria that produce TTX. Additionally, the majority of the bacterial production publications did not demonstrate production of TTX over time and did not provide controls to eliminate contamination from the TTX source material. It is possible that TTX may have contaminated the culture during the experimentation. One study demonstrated that adding extracts from toxic pufferfish increased the amount of TTX in the culture, which was then attributed to TTX production [127]. This is a flawed assumption.

As the TTX concentrations demonstrated in the bacterial cultures are very low, and thus do not explain the extremely high levels found in toxic species, bioaccumulation in the food chain and from the diet appears more likely. Accumulation of TTX from the food chain is supported by the observation that TTX accumulates in pufferfish when they are fed material contaminated with TTX [151], and detoxification occurs when toxic pufferfish are maintained in tanks containing purified water and fed TTX-free food [90, 152]. In a thorough survey of potential sources of TTX found in New Zealand grey side-gilled sea slugs, no TTX was detected in any of the expected prey species analysed [110], except for flatworms [114]. However, flatworms are rare and would not be expected to be the sole source of TTX, indicating insufficient evidence of accumulation via the food chain. No culturable bacteria isolated from the sea slugs or their flatworm prey produced TTX [153], and a single sea slug raised from eggs laid by a toxic sea slug in captivity was found to contain no TTX, indicating that environmental factors or diet are required for toxin accumulation [110].

Unable to be cultured, or yet-to-be-cultured, microbes have been largely ignored as a focus of biosynthetic studies [154]. These may be the ultimate source of TTX.

3. TOXICOLOGY

3.1. Human intoxication

TTX intoxication is characterized by a range of symptoms, and these have been categorized into four degrees of tetrodotoxication [34, 155]. Depending on the amount of toxin ingested, symptoms usually appear within 10-15 minutes, though some cases have been reported to be asymptomatic as long as 3-6 hours after exposure. Oral paresthesia is usually the initial symptom and gradually spreads to the extremities.

In severe cases, symptoms progress to respiratory failure, hypotension, seizures and loss of deep tendon and spinal reflexes. Although most patients remain fully conscious, some individuals may exhibit impaired mental faculties and even become comatose. If the patient survives past 24 hours, the prognosis for recovery is good. Otherwise, death is caused by progressive ascending paralysis involving the respiratory muscles [34].

Some unusual symptoms which have also been associated with TTX intoxications are hypertension in individuals predisposed to the condition [156], cranial diabetes insipidus [157], and temporary blindness [158].

As a result of multiple intoxications resulting from consumption of TTX-containing pufferfish it is widely reported that the lethal dose for humans (50kg bodyweight) is approximately 10,000 MU, which is equivalent to approximately 2 mg [155]. Individuals may have symptoms when ingesting 1,000 MU (approximately 0.2 mg/kg). This figure is supported with correlation with estimates of amount of toxin consumed during intoxication events [155]. However, the origin of this widely accepted information is unclear, with reports not accurately citing their sources or leading to references which are unobtainable due to their age.

3.2. Toxicity of tetrodotoxin and its analogues to experimental animals

3.2.1. Relative acute toxicities of tetrodotoxin analogues by intraperitoneal injection

The LD_{50} of TTX and several analogues have been published (summarized in Table 2, with several paralytic shellfish toxins shown for comparison). TTX can be regarded as highly toxic, with an LD_{50} by intraperitoneal injection of between 8 and 10.7 ug/kg [159-162]. This is similar to STX, which has an LD_{50} of 8.3 ug/kg (7.4 - 9.3, 95% confidence). STX was shown to be more potent to female mice than male [163], while TTX was shown to be more potent to male Kunming mice than female [162]. No significant difference was observed between ddY male and female mice, and no significant difference between ddY and ICR mice. However, there were differences between ddY and five inbred strains which were found to be significantly more resistant to TTX [164]. The potential differences in strains may result in added uncertainty when comparing toxicities between publications.

TTX is the most toxic of the known TTX analogues, with less oxidized analogues demonstrating significantly lower toxicity. The hydroxyl groups of TTX have been shown to be essential for binding within the voltage-gated sodium channel, the mechanism through which TTX imparts its toxic effects. This is similar to the C12 hydroxyl groups in STX [75, 165]. Several of the deoxy analogues have not been isolated in sufficient quantities for determination of acute toxicities.

Of the known TTX analogues, 11-oxo-TTX is regarded as the next most potent. There have however been contradictory reports of its toxicity in literature (16-120 µg/kg). This discrepancy has been attributed to the hygroscopicity of 11-oxo-TTX, resulting in incorrect concentration of the standard which may have underestimated its toxicity [166]. In a frog skeletal muscle assay, 11-oxo-TTX was demonstrated to have strong binding affinity to the sodium channel, approximately 3-5 fold higher than TTX suggesting potential for high toxicity [167]. It is a dominant analogue in some sample types [168], and may be formed from TTX by metabolism in vivo. For this reason, 11-oxo-TTX needs to be considered when determining sample toxicity and robust toxicological data is needed.

Table 2. Acute toxicities of TTX, and analogues, when administered to mice by intraperitoneal injection

Analogue	**LD_{50}** (µg/kg)	**Source**
STX.2HCl	10.3 (9.2 – 11.6)[a]	[169]
NEO.2HCl	3.5 (3.2 – 4.2)[a]	[169]
TTX	8-10.7	[159-162]
4-epi-TTX	64[c]	[2, 30]
4,9-anhydro-TTX	490[c]	[2, 30]
6-epi-TTX	60	[170]
5-deoxy-TTX	>320[d]	[171]
11-deoxy-TTX	71	[170]
8,11-dideoxy-TTX[b]	>700[d]	[172]
5,11-dideoxy-TTX	>550[d]	[173]
6,11-dideoxy-TTX	420	[174]
11-norTTX-6(R)-ol	70[e,g]	[175]
11-norTTX-6(S)-ol	54[g]	[176]
11-oxo-TTX	120[f,g], 16[e]	[166, 177]
5,6,11-trideoxy-TTX	750[f]	[178]
Tetrodonic acid	30,000	[159]

Brackets indicate 95% confidence

a di-hydrochloride salt
b Synthetic analogue (has not been reported to occur naturally)
c Value calculated based on published MU/mg [2]
d LD_{50} not determined as material available was insufficient to kill the mice
e LD_{99} value
f Minimum lethal dose
g Acetate salt

3.2.2. Acute toxicity by oral administration

There is very little information available on the acute toxicity of TTX and its analogues by oral administration [2]. Symptoms following oral administration were the same as those observed by intraperitoneal injection, although the time to death was extended [179]. The LD_{50} of TTX by oral administration has been reported as 332 µg/kg [180], 333.5 µg/kg [161], and 435 µg/kg (380-495, 95% confidence) [181] in ddY mice, and 532 µg/kg in Kunming mice [162]. By comparison the LD_{50} of STX.2HCl in mice is 263 µg/kg (251-267, 95% confidence) [33, 163] and 443 µg/kg (379-484, 95% confidence)[169]. Comparison of the acute toxicities of TTX and STX by intraperitoneal and oral administration to mice is summarized in Table 3.
Oral toxicity has been reported for 4,9-anhydro-TTX, 16,900 µg/kg (14,400-19,800, 95% confidence) in ddY mice [181]. This corresponds to a 39 fold lower toxicity compared to TTX by oral administration, which is similar to the 49 fold lower toxicity reported using intraperitoneal injection.

Table 3. Comparison of TTX and STX acute toxicities to mice by intraperitoneal and oral administration

	LD_{50}, i.p (µg/kg)	**LD_{50}, oral** (µg/kg)
TTX	8-10.7	332, 333.5, 532 435 (380-495)
STX[a]	10.3 (9.2-11.6)	263, 443 (379-484)

Brackets indicate 95% confidence
a di-hydrochloride salt

Based on the limited data available, toxicity of STX and TTX seem to be similar. However, the variability of values reported in literature was high and further work is required to confirm these observations.

3.3. Mechanism of tetrodotoxin toxicity

TTX, like STX, interacts with binding site 1 of voltage-gated sodium channels, which blocks conductance and results in inhibition of neuromuscular transmission [75]. Voltage-gated sodium channels are transmembrane proteins that are responsible for generation of action potentials in excitable cells and can be differentiated by their primary structure, kinetics, and relative sensitivity to TTX. They are composed of an α-subunit of approximately 260 kDa, which is associated with one or more regulatory β-subunits of approximately 35kDa each [182]. These channels are expressed in excitable cells, including nerve, muscle, and neuroendocrine cell types [183]. They are also expressed at low levels in non-excitable cells, where their physiological role is unclear.

Sodium channels in the adult central nervous system and heart contain β1 through β4 subunits, whereas sodium channels in adult skeletal muscle have only the β1 subunit [184].

At least six distinct receptor sites for neurotoxins and one receptor site for local anaesthetics and related drugs have been identified, shown in Table 4 [185].

Table 4. Classifications of binding sites and associated drugs on sodium channels

Receptor site	Toxins
Neurotoxin receptor site 1	Tetrodotoxins Saxitoxins μ-Conotoxins
Neurotoxin receptor site 2	Veratridine Batrachotoxin Grayanotoxin
Neurotoxin receptor site 3	α-Scorpion toxins Sea anemone toxins
Neurotoxin receptor site 4	β-Scorpion toxins
Neurotoxin receptor site 5	Brevetoxins Ciguatoxins
Neurotoxin receptor site 6	δ-Conotoxins
Local anesthetic receptor site	Local anesthetic drugs Antiarrhythmic drugs Antiepileptic drugs

Ten different mammalian α-subunit isoforms ($Na_V1.1$-$Na_V1.9$ and Na_X) have been characterized (Table 5). The different sodium channels are expressed in different regions of the body and vary greatly with sensitivity to TTX, with $Na_V1.5$, $Na_V1.8$ and $Na_V1.9$ resistant to TTX, and no information for Na_X . TTX and STX analogues have varying isoform specificity [77, 186], although relative binding affinities do not correlate with acute toxicities. 11-oxo-TTX was demonstrated to bind 4-5 times more strongly than TTX to skeletal muscle fiber [187], which does not correlate to the near equipotent acute toxicity of these substances by intraperitoneal administration [166, 177].

Table 5. Expression and EC_{50} of tetrodotoxin and saxitoxin to various mammalian sodium channel isoforms.

Isoform	Expression[a]	TTX IC_{50}[b] (nM)	STX IC_{50}[c] (nM)
$Na_V1.1$	Central neurons (Brain type I); cardiac myocytes	6	2.3
$Na_V1.2$	Central neurons (Brain type II), mainly localized to unmyelinated and premylinated axons	12	1.0
$Na_V1.3$	Central neurons (Brain type III), primarily expressed in embryonic/early prenatal life; cardiac myocytes	4	13.4
$Na_V1.4$	Skeletal muscle (high levels in adult muscle, low levels in neonatal muscle)	Rat: 5 Human: 25	1.88
$Na_V1.5$	Cardiac myocytes, immature and denervated skeletal muscle, certain brain neurons	1,000,000-2,000,000	212.6
$Na_V1.6$	Somatodendritic distribution in out-put neurons of cerebellum, cerebral cortex, hippocampus; Purkinje cells in cerebellar granule cell layer; astrocytes, and Schwann cells; dorsal root ganglion; nodes of Ranvier in peripheral nerve system and central nervous system; cardiac myocytes	Rat 1 Mouse: 6	1.09
$Na_V1.7$	Dorsal root ganglion, sympathetic neurons, Schwann cells, neuroendocrine cells	Rat: 4 Human: 25	408
$Na_V1.8$	Dorsal root ganglion neurons and their axons, human heart, and intracardiac neurons	60,000,000	-
$Na_V1.9$	c-type neurons in dorsal root ganglion nociception	40,000,000	-
Na_X	Dorsal root ganglion neurons; neurons of hippocampus, thalamus, and cerebellum, median preoptic nucleus, but maintly in the circumventricular organs; PNS; heart, skeletal muscle; uterus	-	-

a Expression of mammalian sodium channels [183, 188]
b TTX IC_{50} values reported in literature [183, 188, 189]
c PST IC_{50} values reported in literature [77]

3.4. Mutagenicity of tetrodotoxin

TTX shows no mutagenicity activity when assessed in a range of assays which comprised of a bacterial reverse-mutation assay (Ames test) [190], a human lymphocyte chromosome aberration assay, a mouse bone-marrow micronucleus assay and an in vivo rat-liver unscheduled DNA synthesis assay, suggesting that TTX does not pose a genotoxic risk.

3.5. Regulation of tetrodotoxin

Some Asian countries have policies around pufferfish to manage the risk of intoxication. This includes certification of chefs trained in handling the fish, and identification of those species known to contain high levels of toxicity, and those known to contain low levels of toxicity [191, 192]. Based on the assumption that the maximum portion size would be ≤1000 g, a TTX concentration of <10 MU/g (2 mg/kg) is considered non-toxic for assessment in determining which species of pufferfish are safe to consume [193, 194].
In other areas, such as Europe, fish belonging to families Tetraodontidae, Molidae, Diodontidae and Canthigasteridae or products derived from them, are prohibited for sale [195, 196].

4. METHODS OF ANALYSIS

4.1. Animal Bioassays

The TTX mouse bioassay has been used for many decades to determine pufferfish toxicity to assess which species were safe to eat [193]. It was first approved as an official method by the Food Hygienic Association of Japan and included in the Japanese Food Hygiene Examination Manual in 1978 and revised in 1991 [197]. The method is similar to the PSP mouse bioassay (AOAC 959.08) [198], although acetic acid is used for sample extraction instead of hydrochloric acid, and a larger dilution volume is used which reduces the sensitivity of the method compared to that of the PSP mouse bioassay. Aliquots of the extract are injected into male mice of a specified strain and size and median death time, judged by cessation of respiration, is used to calculate the toxicity (MU).
Positive TTX mouse bioassay results cannot be distinguished between STX or TTX as the causative toxin as they exhibit the same symptomology, and both toxin groups have been reported together in fish, crabs, and gastropods [81, 199-201]. Therefore, there is a possibility that some historical intoxications may have been incorrectly assigned to either TTX or PST.

4.2. Biomolecular Methods

4.2.1. Functional methods

In the presence of ouabain, veratridine enhances sodium influx in the mouse neuroblastoma cell line Neuro-2A, causing cellular swelling and subsequent death. TTX, which blocks the sodium channel of excitable membranes, antagonizes this effect, enabling cell growth to continue. This phenomenon was used to develop a tissue culture assay for TTX and STX [202]. The method underwent refinement using

a water-soluble tetrazolium salt to enable measurement from a microplate reader instead of being based on subjective labour-intensive cell-counts [203, 204].

A receptor binding assay for determination of STX and TTX by their displacement of ^{3}H-STX from its receptor in rat brain membranes was described in 1984 [205]. The measurement of the radioactivity of the remaining beta-emitting ^{3}H-STX is inversely proportional toxicity of the extract. This approach has also been modified to use ^{3}H-TTX as the competitive radiolabel [206]. This method has undergone validation for STX in shellfish, although TTX has not been validated [207].

4.2.2. Immunoassay methods

The ELISA is the most common immunoassay format reported for TTX detection. The production of a monoclonal antibody has been reported which enabled the development of a direct TTX detection method using alkaline phosphatase-labelled antibody [208]. Several other ELISA-based methods have also been developed: these methods require complex preparation of antibodies and other reagents [209-211].

Commercial immunoassay kits for TTX are available [212-214], although variability of performance and availability could hinder monitoring programmes relying on the assay for regular high-throughput testing [215].

In 1998, tissue biosensors were developed for determination of both STXs and TTXs [216]. The sensor showed a linear response against TTX and had good correlation with the mouse bioassay. Other biosensors using indirect surface plasmon resonance (SPR) have been reported, [217, 218], as well as surface-enhanced Raman scattering (SERS) [219] and Fluidic force discrimination (FFD) immunoassays [220]. The analysis with biosensors requires expensive instrumentation and more validation to determine if they are suitable for use in a monitoring programme [215].

4.3. Chemical methods

4.3.1. High performance liquid chromatography

In 1982, the configuration of a continuous TTX analyser was published [147]. The system involved high performance liquid chromatography separation followed by a post-column derivatization and fluorescence detection. This method has undergone several improvements over time [221, 222]. This methodology is similar in principle to the post-column oxidation fluorescence detection method used for paralytic shellfish toxins (PCOX) [223, 224]. TTX analogues are separated on a reverse phase column using ion pairing reagents and then chemically converted to fluorescent 2-amino-quinazoline derivatives (C_9 base) by alkali treatment (Figure 9) [225, 226]. The analysis of the C_9-base is not specific to TTX, and toxicity may be overestimated, since less toxic analogues are also converted, and the accurate measurement of TTX

in biological samples is somewhat hindered by naturally fluorescing contaminants [227].

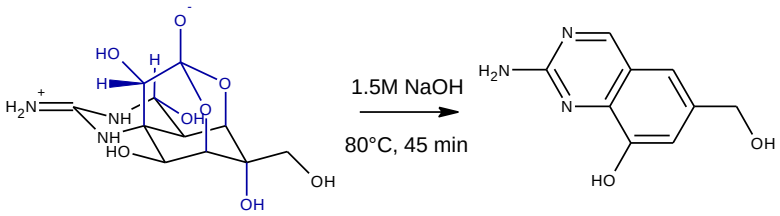


Figure 9. Derivatisation of TTX to C9 base under alkaline conditions [227]

The use of liquid chromatography with UV detection for TTX analysis has also been described [137, 228, 229]. However, both HPLC with fluorescent and UV detection methods are non-specific for TTX and have led to false positives [148].

4.3.2. Gas chromatography-Mass spectrometry

The analysis of TTX by gas chromatography-mass spectrometry (GC-MS) requires derivatisation prior to introduction of the sample into the instrument [227]. Similar to the liquid chromatography-fluorescence method described above, TTX analogues are derivatised to the C_9-base using alkali treatment. As the C_9-base is not volatile this analogue cannot be analysed directly by GC, and must undergo a second derivatization step using bis(trimethylsilyl)acetamide to form a more stable and volatile trimethylsilane (TMS) derivative. Analysis by GC-MS of the TTX-derivative formed yields several chromatographic peaks, including partially converted di-TMS-C_9-base, and the target analyte (tri-TMS-C_9-base). This can be measured with acquisition of the following ions: m/z 407, 392, and 376 [227]. Analysis of samples from biological sources requires additional clean-up steps to remove impurities prior to derivatisation. As with the fluorescence and UV-based methods the analysis is not specific to TTX, often confounding toxin assignment, and less toxic analogues will also be converted to the C_9-base, which will result in an overestimation of the amount of TTX present.

4.3.3. Liquid chromatography-Mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) is a well-established tool for the analysis of marine toxins, and has been used for over a decade for regulatory monitoring of lipophilic shellfish toxins [230-234]. With the advancements of hydrophilic interaction liquid chromatography (HILIC), analysis of polar analytes has become achievable and is becoming a practical tool for routine analytical laboratories.

LC-MS has been demonstrated to be suitable for analysis of TTX and its analogues [19, 83, 235-244]. Significant benefits of the approach are that the toxin can be monitored directly without derivatisation with an excellent level of specificity. It is also possible to analyse the C9-base using this approach [19]. This allows alternative selectivity which can be used as confirmation of the presence of TTX in samples. This

approach can also allow detection of a range of compounds related to TTX, if related uncharacterized analogues are suspected [110].

For quantitative analysis of TTX derivatives, there is a requirement for certified reference material for each analogue monitored. Certified reference material is currently available only for TTX itself (Cifga, Spain). Semi-quantitation of other analogues is possible assuming a relative response factor of 1 compared to TTX. To make an approximation of sample toxicity in TTX equivalents, it is necessary to have robust toxicological information to allow toxin equivalency factors to be generated and subsequently applied.

4.3.4. Toxicity equivalency factors

In the past, testing has largely relied on the mouse bioassay. This involves injecting an extract of a sample of shellfish into the peritoneum of a group of mice, and recording the time that the mice take to die. This test gives an estimate of the total toxicity of the extract, i.e. the sum of the toxicities of the various congeners present in the sample [245]. Although the mouse bioassay is still and accepted method, its ongoing use has raised ethical concerns. Furthermore, this route of administration is not relevant to the human situation. The mouse bioassay is an assay based on time of death, not a toxicological parameter such as toxin dose. It has been shown that there is no correlation between acute toxicity (LD_{50}) and the results obtained in the mouse bioassay [169].

The development of new analytical techniques, particularly LC-MS, allows individual concentrations of toxin congeners in an extract to be determined with accuracy and precision. For risk assessment and management, however, knowledge of the amount of toxin congeners is not sufficient, but there is also the need to know the relative toxicity of each of the congeners, so that the total toxicity of the material can be estimated. This requires the determination of Toxicity Equivalency Factors (TEFs), the toxicity ratio of a compound to a reference compound from the same chemical group and sharing the same mode of action.

To determine approximate TEFs for several of the TTX analogues, we used the acute toxicity values reported in literature by intraperitoneal injection (Table 2). The LD_{50} values were first converted into molar units (nmol/kg) to generate molar toxicity factors in line with those used for the STX group [224]. LD_{50} is inversely related to the toxic dose, where a lower LD_{50} indicates a more potent analogue, and a higher LD_{50} indicates a less toxic analogue. Although it is desirable to have TEFs based on oral administration, these factors are the best that are presently available. As the LD_{50} was not determined for 5-deoxyTTX, 8,11-dideoxyTTX and 6,11-dideoxyTTX as the dose used was insufficient to result in death, the TEF for these analogues were generated from the maximum dose to give a conservative value. Other analogues such as

5,6,11-trideoxy-TTX, only the minimum lethal dose was determined, and the TEF generated from these is also an overestimation. The calculated molar toxin equivalency factors (TEF) values are shown in Table 6.

Table 6. Calculated molar toxicity factors for TTX analogues based on published LD_{50} by intraperitoneal administration

	LD_{50} (mouse, i.p.) (µg/kg)	**Mr** (g/mol)	**LD_{50} (mouse, i.p.)** (nmol/kg)	**TEF**
TTX	10	319.3	31	1
4-epi-TTX	64	319.3	200	0.156
4,9-anhydro-TTX	490	301.3	1626	0.019
6-epi-TTX	60	319.3	188	0.167
5-deoxy-TTX	>320[a]	303.3	>1055	0.030[e]
11-deoxy-TTX	71	303.3	234	0.134
8,11-dideoxy-TTX[b]	>700[a]	287.3	>2436	0.013[e]
5,11-dideoxy-TTX	>550[a]	287.3	>1914	0.016[e]
6,11-dideoxy-TTX	420	287.3	1462	0.021
11-norTTX-6(R)-ol	70	289.3	242	0.129
11-norTTX-6(S)-ol	54	289.3	187	0.168
11-oxo-TTX	16[c]	335.3	48	0.656
5,6,11-trideoxy-TTX	750[d]	271.3	2764	0.011[e]
Tetrodonic acid	30000	319.3	93956	0.0003

a LD_{50} not determined as material available was insufficient to kill the mice
b Synthetic analogue (has not been reported to occur naturally)
c Worst case value, based on the different reported values (16 and 120 µg/kg).
d Minimum lethal dose
e Calculated based on either minimum lethal dose, or toxicity not determined, TEF values are worst-case overestimates

4.3.4.1. Toxicity in pufferfish profiles

To assess the appropriateness of applying the TEF values for results generated by analytical chemistry methods to approximate total toxicity for toxicological and regulatory relevance, we applied the TEF values determined in Table 6 to analytical results presented in literature along with mouse bioassay results.

One publication presented both quantitative results of individual TTX analogues, alongside total toxicities by mouse bioassay [235]. The reported values of the individual TTX analogues measured by LC-MS were re-calculated to toxin equivalents, and then summed to give an estimated total toxicity by LC-MS. Overall the comparison between the recalculated LC-MS and mouse bioassay results were very good, except for the gastrointestinal tract samples, which showed higher mouse toxicity. This may have been due to the exclusion of oxidised analogues such as 11-oxo-TTX which may have been present in these tissues. Comparison of total toxicity by mouse bioassay and LC-MS are shown in Figure 10.

In these samples TTX made up 21-73% of the total toxicity, indicating the importance of considering the other TTX analogues.

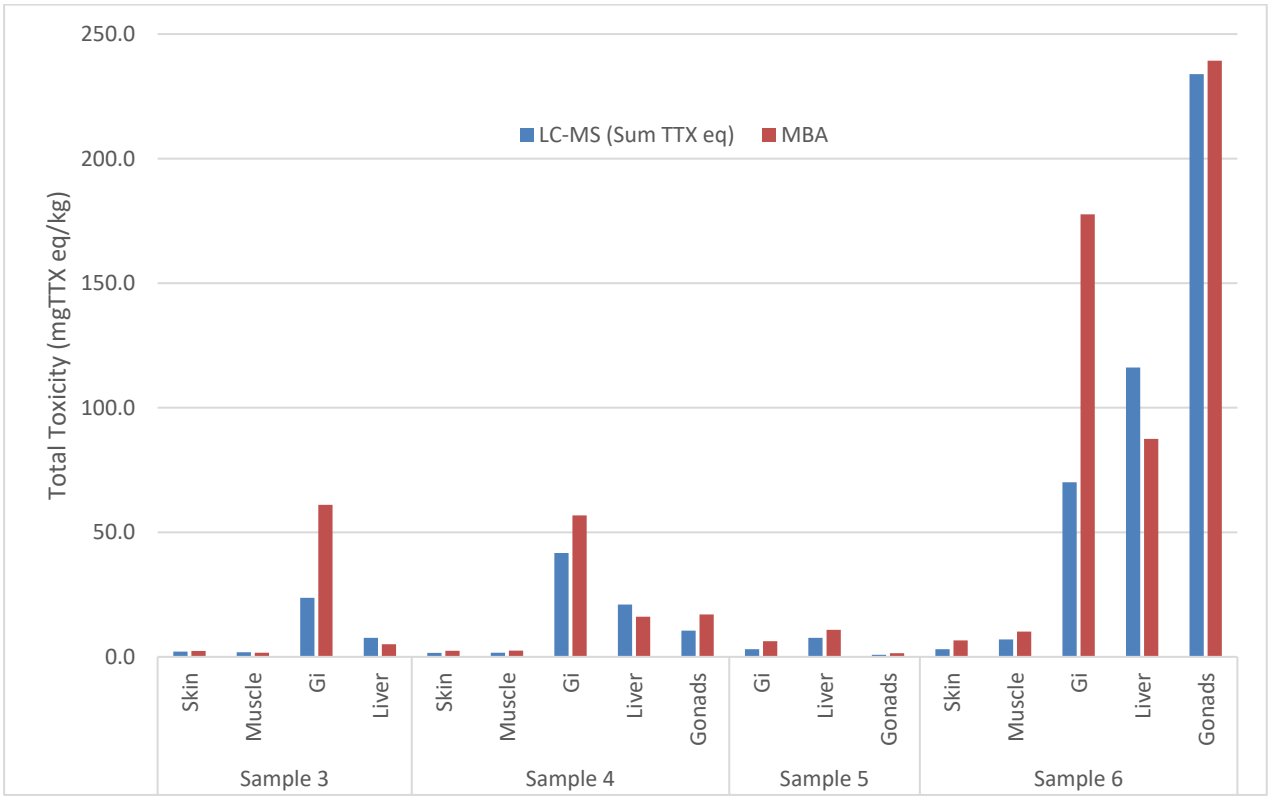


Figure 10. Comparison of total toxicity determined by LC-MS/MS recalculated using TEF factors from Table 6 and mouse bioassay [235]

4.3.4.2. Toxicity in New Zealand shellfish samples

The New Zealand shellfish sample that had the highest TTX concentration during the monitoring period (see 2.1.2) was subjected to additional analysis. A targeted TTX acquisition method was used to allow monitoring of a range of TTX analogues. The concentration of each analogue was semi-quantified using an assumed relative response factor of 1 for SIR acquisition. These results were converted to TTX equivalents (mg TTX eq/kg) using the TEFs developed in Table 6, with the results presented in Table 7. TTX was clearly the most abundant analogue observed in the shellfish sample, with minor contributions of several of the other analogues. The TTX analogue known as 4-epi-5,6,11-trideoxyTTX was assumed to have the same toxicity as 5,6,11-trideoxyTTX in these calculations.

Table 7. TTX analogue profile for Greenshell mussel sample from SC032D

TTX analogue	mg TTX eq/kg[a,b]
TTX	1.6
4-epi-TTX	0.00004
11-oxo-TTX	ND
5-deoxy-TTX	0.00017
11-deoxy-TTX	0.00024
4,9-anhydro-TTX	0.00026
11-norTTX-6(R)-ol	0.00004
11-norTTX-6(S)-ol	0.00007
5,11-dideoxy-TTX	0.00001
6,11-dideoxy-TTX	ND
4-epi-5,6,11-trideoxy-TTX	0.0059
5,6,11-trideoxy-TTX	0.00017

a RRF assumed to be 1

b TEF applied approximated from published acute toxicity data (Table 6)

In other samples analysed, the TTX profiles were different, although TTX still made up >90% of total toxicity. For grey side-gilled sea slugs (*Pleurobranchaea maculata*), and flatworms, 11-norTTX-6(S)-ol attributed 4-7% of the total toxicity, and in flat worms 11-oxo-TTX and 11-deoxy-TTX made up 1-2% of the total toxicity.

5. DISCUSSION

TTX is a widely reported and remarkably distributed toxin with a fascinating history, particularly with respect to pufferfish. It has been reported in many terrestrial and marine species, although its origin still remains unclear. Accumulation from the diet, whether from bacteria or from other sources, is an attractive hypothesis and is supported by cultured pufferfish being non-toxic [246]. However, levels found in bacteria and marine sediments are low [247], and production of TTX by bacterial species is still questionable [148, 248]. Bacterial cultures have been reported to contain low TTX concentrations and are suggested to be the ultimate biosynthetic origin of the toxin [154]. However, these results remain controversial and are disputed due to poor specificity of the methods of analysis used, and negative results obtained when using more specific methods of analysis [148, 248]. Several literature articles recommend that a re-examination is required of the potential production of TTX by bacteria using specific chemical analysis. However, due to the large number of species which have been claimed to produce TTX, this would involve a significant amount of work to eliminate possible false-positives, and to confirm potential TTX-producing bacterial cultures. It has been suggested that development of metagenomics sequencing and heterologous expression systems, which have allowed the biosynthetic gene clusters from unculturable bacteria to be investigated in other fields may prove useful in helping understanding TTX's origin and biosynthesis, although this is hindered due to the unique nature of the TTX backbone structure

[154]. Some animals can accumulate milligram quantities of TTX in their tissues, and before the dietary hypothesis can be accepted, it will be necessary to show that such quantities can be derived from the dietary sources that are available to the animals [2].

Understanding the mechanism of TTX accumulation in marine foodstuffs is important for managing risk. In New Zealand, the grey side-gilled sea slug is the most well-known TTX-containing organism, and can lay highly toxic eggs [249]. The presence of these organisms in shellfish aquaculture areas could be a possible vector of the toxin. Many of the worm species demonstrated to contain high concentrations of TTX are sufficient for ingestion of individual worms to result in poisoning and it is plausible that a single specimen could contaminate foodstuffs. Such a contamination could not be managed by routine monitoring programmes, and this needs to be further evaluated as to whether there is a risk of this occurring and if it poses a food safety concern. Because flatworms (*Stylochoplana sp.*) are relatively simple anatomically, occupy a low trophic level, and contain very high concentrations of TTX, they may prove to be a useful model organism for ongoing studies investigating the origin and function of TTX [114, 250]. However, the worms are rare and mobile, making them difficult to obtain in sufficient quantities for study. Although the concentrations of TTX observed in shellfish are orders of magnitude lower than other species present in the marine environment, as stationary filter feeders they may prove a suitable model which may assist in elucidating the origin of TTX.

Existing data on TTX toxicity by oral administration is limited, with variable results reported in the literature. Extensive recent work has been performed on the paralytic shellfish toxin group to determine the acute oral toxicity of individual analogues [169]. Toxicity via intraperitoneal injection and oral administration are quite different, with oral administration being more relevant to human food consumption. For this reason, improved oral toxicity data for TTX is desired. Other TTX analogues thought to be highly toxic, such as 11-oxo-TTX, may also be made a priority for toxicological assessment. Although intoxications induced by pufferfish appear to be largely due to TTX itself, 11-oxo-TTX may play a significant role in the toxicity of certain crabs, in which it has been reported at a higher concentration than TTX [168]. It is feasible to prepare 11-oxo-TTX by direct oxidation of TTX, and should be investigated [187]. Other analogues are not readily available, and although it would be desirable to obtain robust toxicological information for these analogues as well, they would need to first be obtained in sufficient quantities. Although total synthesis has been reported for some TTX analogues[251-261], this is a long and complex process with low yield. Obtaining purified TTX analogues for reference material and toxicology will likely come from naturally contaminated material[171, 175-177, 262-265]. Further review of toxin profiles found in samples will provide useful information on which analogues are of significance, and practical for isolation, purification and toxicological evaluation.

While it is known that TTX and STX can competitively bind to voltage-gated sodium channels [206], it is unknown if TTX and PST have additive or competitive

toxicological effects. When assessing sample toxicity, co-occurrence of TTX with PST needs to be considered; the mouse bioassay, which is used for regulatory monitoring of PST in some countries, is not able to distinguish TTX from PST. The presence of PST in shellfish is regulated whereas the presence of TTX, currently, is not. Shellfish containing PST below the regulatory limit of 0.8 mg STX.2HCl eq/kg could be found to be above the regulatory threshold for this toxin class if TTX is also present. This situation has been observed in a non-commercial New Zealand shellfish sample. Analysis by LC-MS showed this mussel sample contained TTX at a concentration of 1.6 mg/kg and PST below the regulatory limit at 0.4 mg STX.2HCl. When it was subjected to the PSP mouse bioassay, the toxicity of the sample was determined to be 7278 MU/kg or 1.3 mg STX.2HCl eq/kg, which is above the regulatory limit of 0.8 mg STX.2HCl eq/kg. This information is relevant because it is important that commercially-produced New Zealand shellfish is safe to eat and is able to enter export markets where marine toxins are monitored using methods such as the PSP mouse bioassay. Modern multi-toxin LC-MS methods can simultaneously quantify paralytic shellfish toxins and TTX and are used in New Zealand for routine regulatory monitoring of regulated toxin classes.

6. CONCLUSION

TTX is a ubiquitous natural toxin found in a variety of animals at different trophic levels. Its presence in bivalve molluscs has not been heavily investigated and many aspects of its origin and distribution remain unclear. The key information resulting from this literature review is summarized below.

- There have been reports of TTX in bivalve shellfish, including recent ones from the United Kingdom, Greece, China, the Netherlands and non-commercial samples from New Zealand. There have been no confirmed reports of illness attributed to the consumption of New Zealand shellfish containing TTX.

- The levels observed in shellfish are typically low, considerably lower than found in other marine organisms known to cause human intoxication (e.g. pufferfish). The levels reported for shellfish to date are all below 10 MU/g (2 mg/kg), which represents the level used to classify non-toxic species of pufferfish in Japan. If these levels are representative of TTX levels generally present in bivalve molluscs consumed by humans, this could indicate that TTX may be regarded as a low food safety risk. Contrary to this is the report of high TTX levels in Japanese scallops in the 1990s (up to 40 MU/g, or ~8mg/kg). If real and accurate, this indicates the potential for TTX to accumulate in bivalves to levels that would pose a genuine food safety concern.

- Toxicity information for TTX does exist and shows that is has similar acute toxicity to saxitoxin. Most of the information available relates to acute toxicity thro1ugh intraperitoneal injection to mice, this route of administration is not relevant to how

seafood is consumed. There currently is no robust toxicity information via oral administration (gavage and voluntary feeding), and existing data are limited with variable results reported in the literature. 11-oxo-TTX has been shown to be an important analogue, potentially equipotent with TTX, and has been documented to be a dominant analogue in some crabs.

- The source of TTX in shellfish, and indeed all animals, remains unresolved making it a difficult issue to manage should regulation be considered to be required and enforced. Accumulation in bivalves is unknown, as they are filter feeders and do not feed on known TTX-containing specimens.

- The possibility of regulation for TTX in shellfish is currently under debate internationally, with an expert EFSA panel currently preparing a scientific opinion on the evaluation of the toxicity of TTX and analogues in bivalve molluscs and marine gastropods. If regulation is deemed necessary, New Zealand regulatory authorities, scientists and shellfish producers need to be aware of the maximum permissible level allowed, what TTX analogues require monitoring, and whether TTX is to be regulated on its own, or together with the other paralytic shellfish toxins (e.g. STX).

Recommendations for Future work:

- To ensure that any regulatory decisions are evidence based, it is essential that robust toxicological data via the oral route is generated for TTX and its analogues. To do this purified material will be required. TTX itself is readily available, although it is insufficiently pure for toxicological studies, and other analogues are not available at this time.

- To help evaluate this risk of TTX in bivalve shellfish, monitoring of TTX in non-commercial New Zealand samples is ongoing. This will provide data on seasonal and regional trends should they exist, and to determine if there are any at-risk areas warranting further investigation.

- Should TTX be regulated internationally, management of TTX in at-risk areas would be needed. Therefore it remains necessary to identify the source, and mechanism of TTX production (which up to this point has been unsuccessful).

7. ACKNOWLEDGEMENTS

The Safe New Zealand Seafood programme (CAWX1317) for supporting the analysis of tetrodotoxin in non-commercial shellfish. Dr. Rex Munday is also acknowledged for his assistance with recovering literature on tetrodotoxin, which has provided the backbone for this review.

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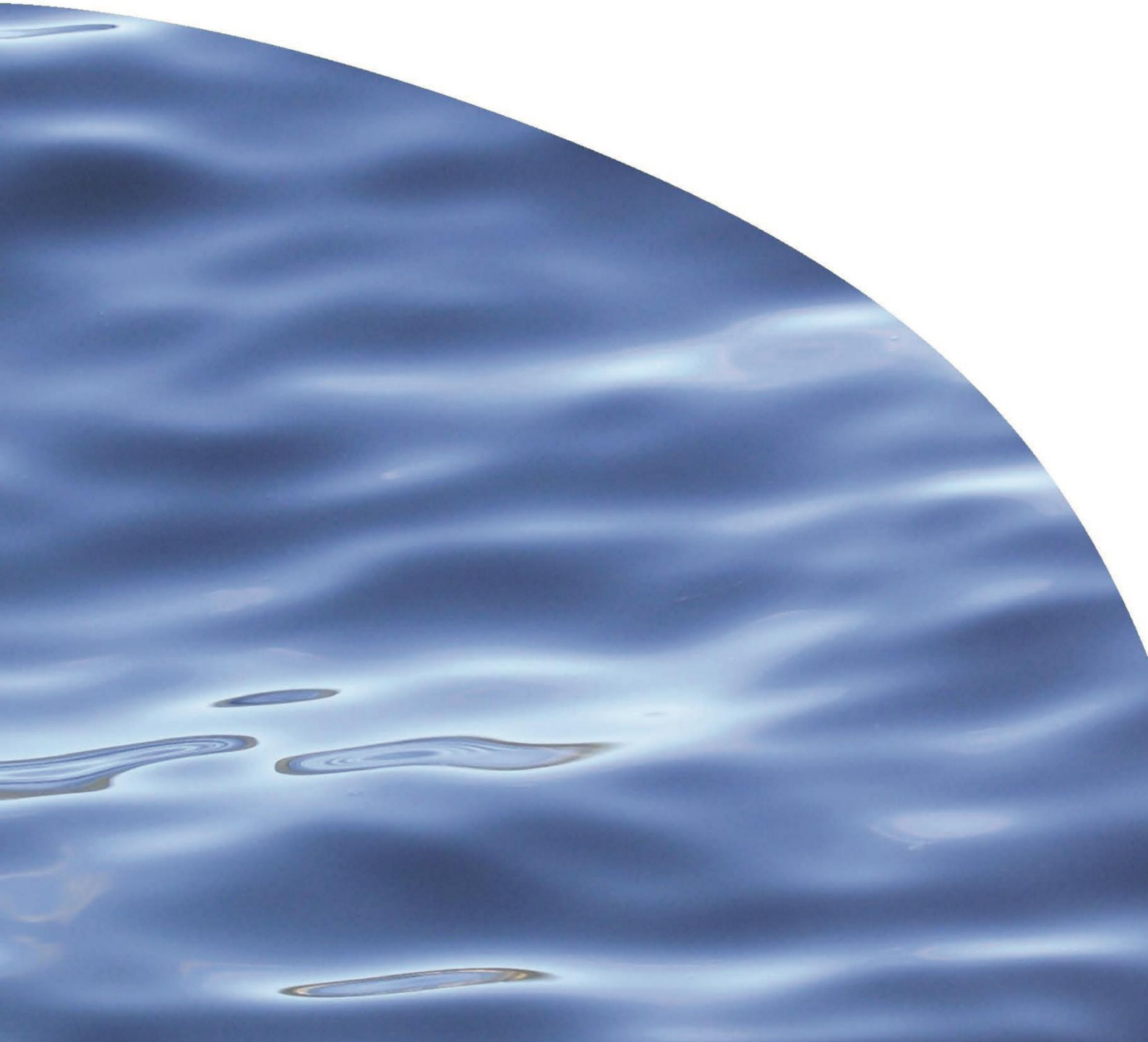
TETRODOTOXIN IN NON-COMMERCIAL NEW ZEALAND BIVALVE SHELLFISH



TETRODOTOXIN IN NON-COMMERCIAL NEW ZEALAND BIVALVE SHELLFISH

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Prepared for the Ministry for Primary Industries

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ISSUE DATE: 30 March 2020

RECOMMENDED CITATION: Harwood DT, Boundy MJ 2020.Tetrodotoxin in non-commercial New Zealand bivalve shellfish. Prepared for the Ministry for Primary Industries. Cawthron Report No. 3173A. 18p.

1. EXECUTIVE SUMMARY

- Tetrodotoxin (TTX), a neurotoxin typically associated with pufferfish intoxications, has been detected in bivalve shellfish from Japan, the United Kingdom, Greece, China, the Netherlands and New Zealand. Typically, the levels reported are low and the potential risk to shellfish consumers was unclear.

- A recent EFSA scientific opinion concluded that a level of <0.044 mg TTX/kg, based on a 400 g portion size, does not result in adverse effects in humans. At this time it is uncertain whether this guideline will be adopted as an enforceable level in the EU.

- To obtain a greater understanding of the risks associated with the presence of TTX in seafood, in 2017 the New Zealand Ministry for Primary Industries commissioned Cawthron to prepare a review on this topic from available literature: Boundy MJ, Harwood DT 2017. Review of literature to help identify risks associated with tetrodotoxin in seafood, including bivalve molluscs. Prepared for MPI. Cawthron Report No. 2986. 45 pages.

- MPI also contracted the Cawthron Institute to undertake a survey of non-commercial New Zealand shellfish for TTX over a period of 15 months (Dec 2016 – Mar 2018). During this period 766 samples were analysed from 8 different bivalve matrices. TTX levels were found to be low and similar to those observed in other countries, except for pipi. The levels observed were considerably lower than those reported in other marine organisms that contain TTX above the EFSA safe guidance level and are known to cause human intoxication (e.g., pufferfish).

- All pipi (*Paphies australis*) samples analysed as part of the New Zealand survey were found to contain detectable levels of TTX, and pipi from one sampling site in the Hokianga Harbour contained TTX consistently above the recommended EFSA safe guidance level. In contrast, TTX was not observed in cockles from this same sampling area.

- There have been no reports of human illness attributed to the consumption of New Zealand shellfish containing TTX.

- The source of TTX in shellfish, and indeed all animals, remains unresolved making it a difficult issue to manage should regulation be required and enforced. Accumulation in bivalves is a particular mystery, as they are filter feeders and do not feed on specimens presently known to contain TTX.

2. INTRODUCTION

Tetrodotoxin (TTX; Figure 1) is a potent neurotoxin that has been responsible for many human intoxications and deaths around the world, particularly from consumption of pufferfish (fugu). The distribution of TTX and its analogues in the environment is remarkably diverse, being found in a variety of organisms from both marine and terrestrial environments. The source of TTX is still controversial and not definitively proven, although it is likely of microbial origin. Increasing reports of the detection of TTX in farmed aquaculture species such as bivalve molluscs has drawn considerable recent attention to the toxin, reinvigorating scientific interest and questioning the need for new regulation to be introduced.

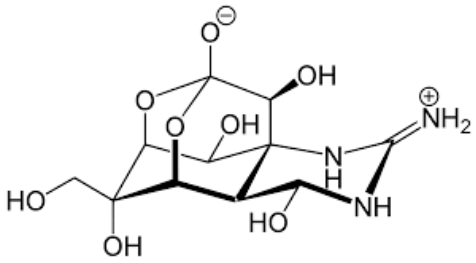

Figure 1. Molecular structure of tetrodotoxin.

Due to recent interest surrounding the presence of TTX in bivalve molluscs, the European Commission requested the European Food Safety Authority for an opinion on the risks to public health related to the presence of TTX and analogues in marine bivalves and gastropods. In a Scientific Opinion published March 2017, it was concluded that a concentration below 44 µg TTX equivalents/kg shellfish meat (0.044 mg TTX eq/kg), based on a large portion size of 400 g, was considered not to result in adverse effects in humans (Knutsen et al. 2017). It also suggested that liquid chromatography with tandem mass spectroscopy (LC–MS/MS) methods are the most suitable for the identification and quantification of TTX and its analogues, with limits of quantitation ranging between 0.001 and 0.025 mg/kg. TTX has similar sodium channel blocking action and potency to saxitoxin, but is structurally dissimilar. The regulatory level adopted in New Zealand, and in many other countries, for the saxitoxin group toxins (collectively refered to as paralytic shellfish toxins) in bivalve molluscs is 0.8 mg STX·2HCl eq/kg, based on a 100 g portion size.

There have been reports of TTX in bivalve molluscs from Japan, New Zealand, the United Kingdom, Greece, China, and the Netherlands. Briefly, in the early 1990s TTX was reported at concentrations of up to 40 MU/g (~8 mg TTX/kg) in the digestive glands of Japanese scallop (*Patinopecten yessoensis*). TTX was confirmed as being present using a variety of analytical techniques that included high performance liquid chromatography with fluorescence detection and fast atom bombardment-mass spectrometry (FAB-MS) after the toxin was partially purified from the scallop tissue. In New Zealand, TTX was first reported in pipi (*Paphies australis*) collected from Whangapoua on the Coromandel Peninsula during April 2011. Concentrations up to 0.8 mg/kg (McNabb et al. 2014) were reported in these samples. Analyses were performed using two separate methods that employed LC-MS/MS.

The first analysed the intact toxin, and the second monitored a TTX-C_9 base derivatisation product generated by TTX dehydration under highly alkaline conditions. In Europe, TTX was first reported in bivalve molluscs in 2014 (Turner et al. 2015a). Mussels (*Mytilus edulis*) and Pacific oysters (*Crassostrea gigas*), harvested from two sites on the south coast of England were screened for TTX using a HILIC-MS/MS method and the presence of TTX was confirmed using LC-MS/MS after derivatisation under alkaline conditions to the TTX-C_9 base. In China during 2015, as part of the validation for a new LC-MS method, manila clams (*Ruditapes philippinarum*) purchased from markets in China were analysed for the presence of TTX and trace levels were observed (Zhang et al. 2015). Dutch researchers have also reported the presence of TTX in bivalve molluscs during an Emerging Toxins workshop held in September 2016 in Baiona, Spain. They reported TTX in mussels (*Mytilus edulis*) during 2015 with a maximum level of 0.124 mg/kg, and samples analysed in 2016 had a maximum level of 0.253 mg/kg. The Baiona data suggested seasonal and regional variation.

To help determine if the presence of TTX in non-commercial New Zealand bivalve shellfish represents a risk to consumers, MPI contracted Cawthron Institute (Agreement number 405359) in Dec 2016 to undertake a survey of New Zealand shellfish for TTX. The samples analysed were collected as part of the non-commercial marine biotoxin monitoring programme. It was intended that this research would identify any potential food safety risks associated with TTX, so that MPI can develop appropriate risk management strategies, if needed.

3. METHODS

Shellfish samples received weekly (Dec 2016 - Mar 2018) at Cawthron for paralytic shellfish toxin testing as part of the MPI administered non-commercial marine toxin shellfish monitoring programme were also analysed for TTX. A HILIC-MS/MS method was used that was developed at Cawthron for routine regulatory monitoring of the paralytic shellfish toxin group (Boundy et al. 2015, Turner et al. 2015b), which could also be expanded to monitor TTX. The limit of reporting for the method was 0.002 mg TTX/kg (Boundy and Harwood 2016). When TTX was observed in shellfish samples it was possible to re-analyse the sample extract using a targeted TTX acquisition method to allow monitoring of a range of TTX analogues. As no reference material is currently available for the various known TTX analogues it was not possible to accurately quantify them. Therefore, the concentration of each analogue was semi-quantified using an assumed relative response factor of 1. This will introduce a source of error, but in the absence of reference material it is the only option currently available to allow semi-quantification of TTX analogues.

Briefly, 5.0 ± 0.1 g of homogenised shellfish tissue was weighed into a centrifuge tube followed by the addition of 5 mL of 1% acetic acid. The mixture was vortex-mixed before being placed into a boiling water bath for 5 min. Samples were then cooled for 5 min in an ice slurry, before further vortex mixing. Samples were centrifuged at 3,200 × g for 10 min before pipetting a 1 mL aliquot into a 5 mL polypropylene tube and adding 5 µL of 25% ammonia. For sample cleanup, Supelclean ENVI-Carb 250 mg/3 mL SPE cartridges (Sigma-Aldrich, St. Louis, MO) were conditioned at 6 mL/min using 3 mL of 20% acetonitrile + 0.25% acetic acid, before the addition of 3 mL of 0.025% ammonia. A 400 µL aliquot of the acetic acid extract was loaded onto the cartridge, followed by washing with 700 µL of deionized water. Sample extracts were eluted with the addition of 2 mL of 20% acetonitrile + 0.25% acetic acid and collected. SPE eluents were vortex-mixed prior to dilution of 100 µL aliquots with 300 µL acetonitrile.

During the survey, pipi (*Paphies australis*) sourced from the Hokianga Harbour (SF021) were found to contain TTX levels well above levels observed in other areas. As this site is not routinely monitored for marine toxins, MPI was requested to increase the frequency of sampling for the duration of this study. From March 2017, fortnightly sampling of the Koutu Point pipi bed was performed. Also, individual pipi (n=12) from one Koutu Point sampling event were tested, in addition to a pooled sample, to determine TTX variability between individuals.

In addition, to assess whether the presence of TTX in New Zealand shellfish is a recent phenomenon a subset of 27 archived (2001-2003) shellfish homogenate samples were retrieved from frozen storage and analysed for the presence of TTX. Samples that are in frozen storage are typically from routine monitoring activities and contain detectable levels of regulated marine toxins. Very few pipi samples were in the archive.

4. RESULTS

Survey results (Dec 2016 – Mar 2018)

In total, 766 samples were analysed during this time period. The sample matrices analysed comprised greenshell mussels (63%), tuatua (28%) and pipi (6%), with less than 10 samples in total of each of blue mussels, clams, cockles, Pacific oysters and rock oysters. No TTX was detected in the majority of samples (69%). Another 27% of samples had detectable TTX levels but these were below the recommended safe guidance level reported in the EFSA scientific opinion (0.002 - 0.044 mg/kg). A further 4% of samples had TTX levels greater than this guidance level (≥0.044 mg/kg), with all of these shellfish being pipi. All pipi tested as part of this study contained detectable levels of TTX. These percentages are likely to be biased by the unequal numbers of samplings for each species but provide a valuable insight into TTX levels in many recreationally harvested shellfish species. See Table 1 for a breakdown of the shellfish species tested and TTX levels observed.

Table 1. Summary of non-commercial shellfish samples analysed for TTX (Dec 2016-Mar 2018).

	TTX level (mg/kg)			
Shellfish species	**<0.002**	**0.002-0.044**	**≥0.044**	**Total**
Blue mussel	4	3	0	7
Clams	1	0	0	1
Cockle	7	1	0	8
Greenshell mussel	318	162	0	480
Pacific oyster	0	1	0	1
Pipi	0	15	29	44
Rock oyster	3	6	0	9
Tuatua	194	22	0	216
	527	210	29	**766**

There were 56 sampling sites in total. Information regarding the sampling site, total number of samples tested and additional detail about the TTX levels observed in the shellfish analysed is shown in Table 2. The majority of samples were taken from sites in the North Island. This is based on historical information that shows these sites represent the most at-risk areas for harmful algal bloom events, and hence why there is monitoring for regulated marine toxins.

Table 2. Sampling sites and summary of TTX levels observed in non-commercial shellfish samples tested during the survey period (Dec 2016-Mar 2018).

		TTX level (mg/kg)					
Site	Site code	Average	Maximum	<0.002	0.002-0.044	>=0.044	Total sample #
MANGONUI HARBOUR	SA006	0.007	0.025	2	3	0	5
THE BLUFF-90 MILE BEACH	SA025	0.000	0.007	33	3	0	36
WAIPAPAKAURI	SA027	0.001	0.006	27	4	0	31
TAPEKA POINT	SA030	0.015	0.023	0	2	0	2
TAURUA - REEF POINT	SA036	0.000	0.000	1	0	0	1
BLACK ROCKS (Bay of Islands)	SA040	0.000	0.000	3	0	0	3
HOUHORA WHARF	SA129	0.013	0.023	0	2	0	2
OAKURA	SB001	0.001	0.002	1	1	0	2
PARUA BAY	SB007	0.000	0.000	1	0	0	1
PATAUA	SB008	0.038	0.045	0	1	1	2
WHANANAKI	SB032	0.028	0.045	0	3	1	4
BROWNS BAY	SC032D	0.009	0.033	0	11	0	11
WHANGAPARAOA PENINSULA	SC032F	0.010	0.010	0	1	0	1
TAIRUA HARBOUR	SD012	0.000	0.000	1	0	0	1
WAIHI BEACH	SD017	0.001	0.010	25	8	0	33
TAURANGA HARBOUR - UPPER	SD018	0.000	0.000	1	0	0	1
TAURANGA HARBOUR - LOWER	SD021	0.048	0.048	0	0	1	1
PAPAMOA BEACH	SD025	0.001	0.008	29	5	0	34
PUKEHINA BEACH	SD028	0.000	0.000	36	0	0	36
BOWENTOWN	SD030	0.013	0.013	0	1	0	1
KATIKATI - TAURANGA HARBOUR	SD031	0.041	0.041	0	1	0	1
KATIKATI - TAURANGA HARBOUR	SD031P	0.150	0.150	0	0	1	1
KAURI POINT	SD031S	0.038	0.038	0	1	0	1
WHAKATANE HEADS	SD032	0.001	0.002	5	2	0	7
WAIOTAHI	SD036	0.007	0.012	1	5	0	6
OHOPE BEACH	SD037	0.000	0.004	35	3	0	38
WHANGAPARAOA	SD041	0.001	0.007	25	6	0	31
THORNTON	SD042	0.000	0.000	5	0	0	5
TE KAHA	SD050	0.002	0.002	0	1	0	1
TOLAGA BAY WHARF	SE001	0.005	0.017	2	2	0	4
MAHIA, OPOUTAMA	SE006	0.003	0.018	14	20	0	34
PANIA REEF	SE007	0.002	0.015	18	17	0	35
TAIKORAI ROCKS - PORANGAHAU	SE010A	0.002	0.013	19	11	0	30
LOTTIN POINT	SE019	0.003	0.003	0	1	0	1
GISBORNE WHARF	SE028	0.004	0.004	0	1	0	1
MURIWAI, WEST COAST	SF009	0.006	0.015	1	3	0	4
CORNWALLIS (MANUKAU HBR)	SF015	0.004	0.022	11	28	0	39
RAGLAN	SF016	0.001	0.007	27	13	0	40
KAWHIA	SF017	0.006	0.024	6	32	0	38
MOHAKATINO	SF018	0.000	0.003	39	2	0	41
OAKURA BEACH	SF020	0.000	0.000	13	0	0	13
KOUTU POINT (HOKIANGA HBR)	SF021	0.134	0.470	5	0	25	30
TINOPAI (KAIPARA HBR)	SF026	0.006	0.010	0	4	0	4
MAUNGANUI BLUFF	SF029	0.001	0.007	29	5	0	34
MITIMITI	SF031	0.000	0.002	4	1	0	5
AOTEA HARBOUR	SF033	0.000	0.000	1	0	0	1
BAYLEYS BEACH	SF156	0.000	0.000	2	0	0	2
TAPU BAY - TASMAN BAY	SG006	0.000	0.000	1	0	0	1
WEDGE POINT	SG023	0.000	0.000	1	0	0	1
ONAPUA BAY	SG123	0.000	0.000	3	0	0	3
POHARA	SG313	0.000	0.000	1	0	0	1
OHAWE BEACH	SH001	0.000	0.002	33	1	0	34
FOXTON	SH002	0.000	0.003	34	1	0	35
LOWER KINA ROAD	SH023	0.000	0.000	1	0	0	1
THE KAIK	SI004	0.002	0.002	0	1	0	1
CAPE FOULWIND	SJ004	0.000	0.006	31	3	0	34

Figure 1 shows a map of New Zealand overlaid with the location and number of shellfish samples tested for TTX from each site. The size of the circle corresponds to the number of samples collected from the site over the duration of the survey (maximum = 41 samples). Colouration within each circle shows the proportion of samples that fall within each of the three defined TTX levels.

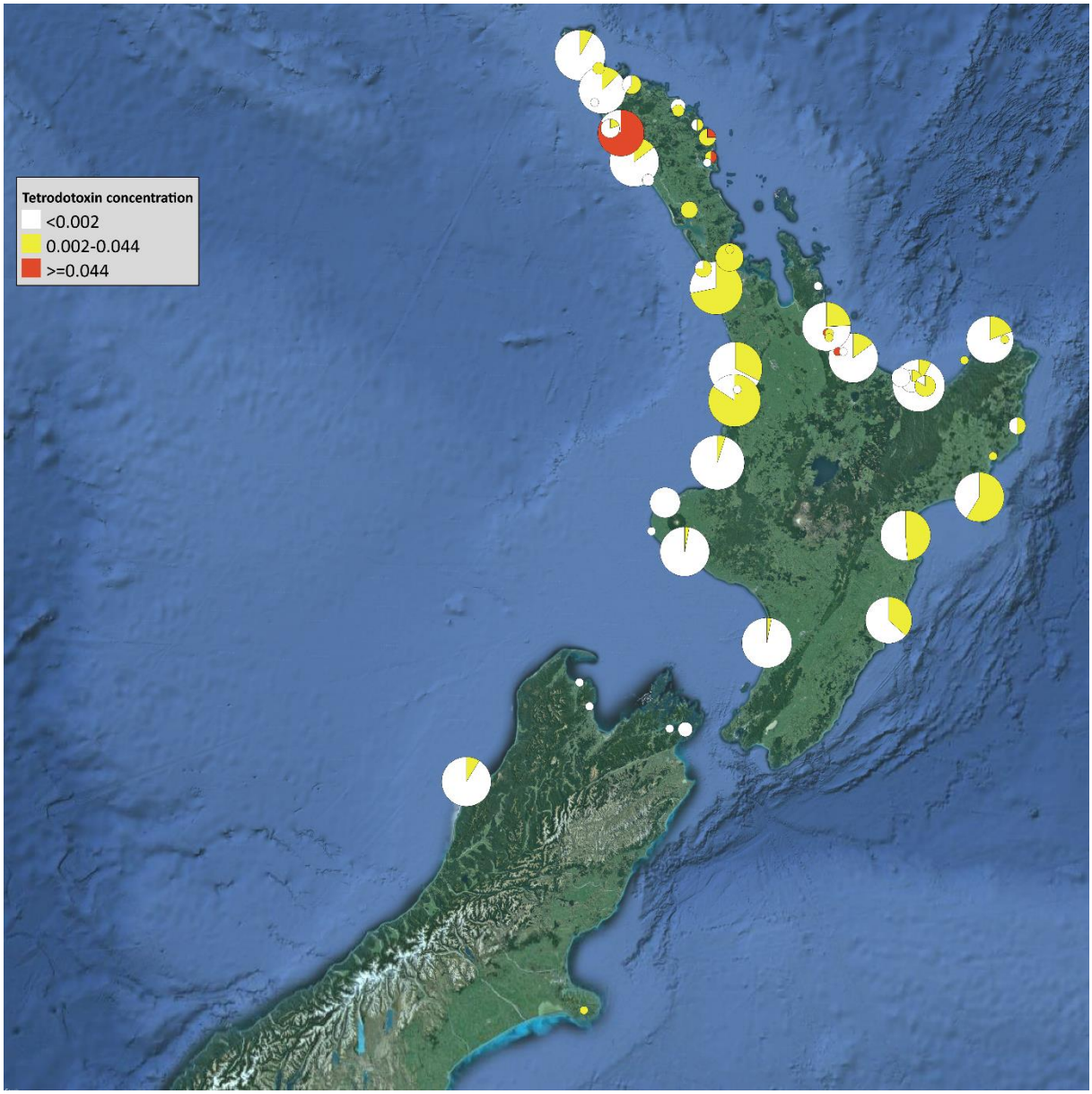

Figure 1. Location of sampling sites and level of TTX observed in non-commercial samples taken between Dec 2016 – Mar 2018. Circle area corresponds to number of samples (max = 41) from a particular site. No samples were taken for the area of New Zealand not shown in the map.

Most pipi samples included in the survey came from Koutu Point (SF021) in the Hokianga Habour, where consistently elevated TTX levels were observed (Figure 2; blue dots). In contrast, cockles collected from the same site during the first five sampling events did not contain detectable TTX (Figure 2; black dots).

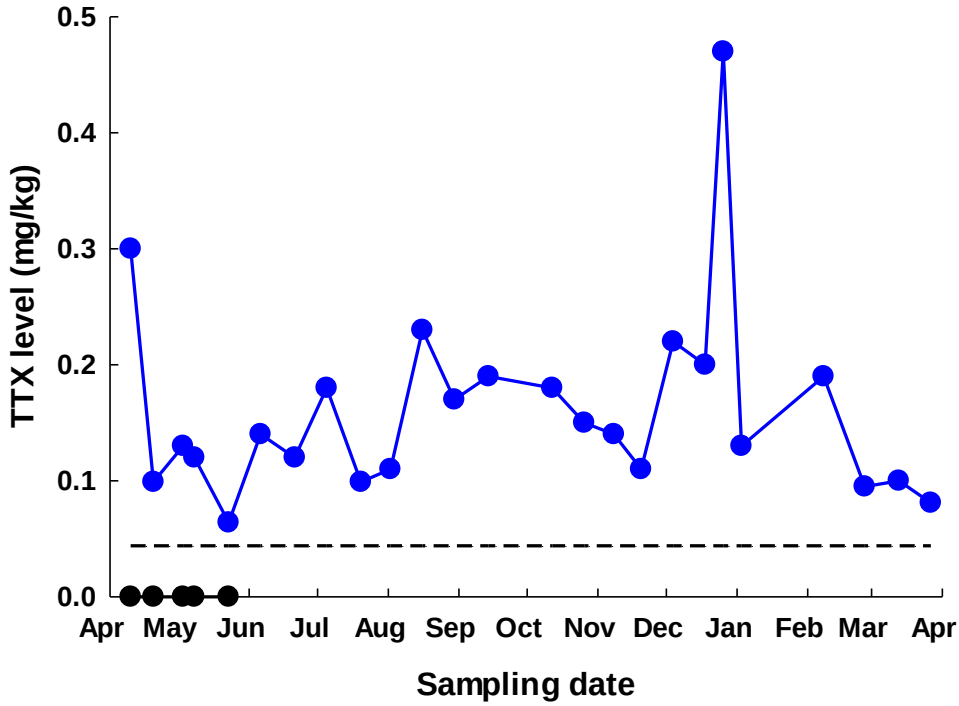

Figure 2. TTX levels in pipi and cockles sourced from Koutu Point (SF021) in the Hokianga Harbour (Apr 2017 – Mar 2018). Blue dots = pipi; black dots = cockles. Dotted line represents EFSA safe guidance level (0.044 mg/kg).

Other shellfish samples were sourced from the Hokianga Harbour as part of a PhD project (collected on 27/10/2017). These were collected outside of the MPI-administered survey and were also tested for the presence of TTX. Samples included juvenile mussels and oysters, snails, pipi and cockles from areas close to the Koutu Point sampling site and from the harbour entrance >5 km away. Low levels of TTX were observed in all of the samples, ranging from 0.003-0.04 mg/kg. None of these additional samples exceeded the safe guidance level reported in the EFSA scientific opinion.

Analysis of archive shellfish samples

To determine if the presence of TTX in New Zealand shellfish is a recent phenomenon, archived samples (2001-2003 n=18, 2007-2009 n=9) were obtained from frozen storage and analysed. Of these samples, 8 contained detectable TTX levels (30% of the total analysed). The highest TTX concentrations in the samples taken between 2001 and 2003 was 0.019 mg/kg, and the highest concentration in the samples from 2007-2009 was 0.021 mg/kg. No archive samples were pipi. The detection rate of TTX in the library samples was consistent with the detection rate observed in the survey samples taken and analysed fresh from Dec 2016 – Mar 2018 (31% TTX detections).

Presence of TTX analogues

Selected shellfish samples that contained TTX above a threshold level of 0.02 mg TTX/kg were re-analysed for the presence of known TTX analogues using a targeted TTX acquisition method. In all cases TTX was found to be the most abundant analogue, accounting for >98% of the total TTX analogues present. Other TTX analogues were observed but in most cases were present at too low a concentration to allow accurate quantitation. As a representative example, see Figure 3 showing the presence of TTX (0.19 mg TTX/kg) in a pipi sample and trace detections of structurally-related analogues. The assignment of the TTX analogues was made based on comparison of retention time against a naturally contaminated and well characterised flatworm quality control sample.

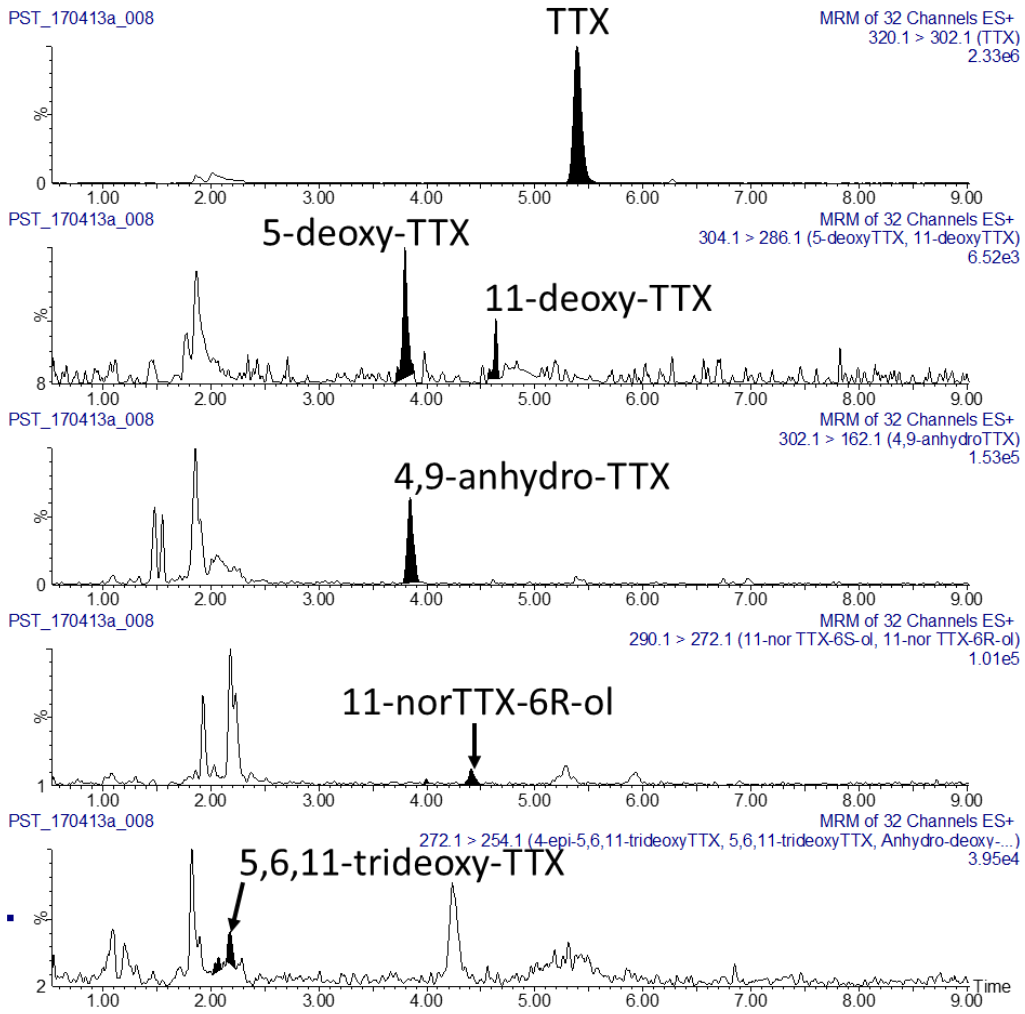

Figure 3. Observed TTX analogues from a Koutu point pipi sample (0.19 mg TTX/kg).

TTX variation in individual pipi

To assess variability of TTX in pipi samples growing within close proximity, 12 individuals sourced from Koutu Point were analysed for their TTX content. Similar TTX levels were observed between the individuals, with variability <20%RSD (Figure 4). A pooled result, generated from a homogenate of 12 individual pipi collected from the same location at the same time, gave a result of 0.14 mg TTX/kg. This is in line with the median level from the analysis of individuals.

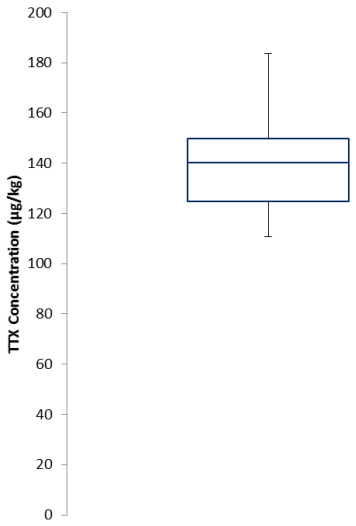

Figure 4. Box and whisker plot showing TTX levels measured in 12 individual pipi sourced from Koutu Point (SF021). Shown is the median, interquartile range and 5th and 95th percentiles.

Pre-survey results (Dec 2015 – Nov 2016)

Because the LC-MS/MS PST method acquires data for TTX which is not routinely processed, it was possible to extract TTX concentration information from non-commercial shellfish samples analysed between Dec 2015 – Nov 2016 (prior to the MPI survey being initiated). In total, TTX concentration information for an additional 697 non-commercial shellfish samples was able to be generated. As with the main survey, the majority of samples were greenshell mussels (66%) and tuatua (27%) with the remainder made up of blue mussels (2%), Pacific oysters (2%) and cockles, abalone and pipi (<10 samples each in total). No TTX was detected in 51% of the samples, lower than the proportion observed in the main survey (69% non-detections). This resulted in a higher percentage (48%) of pre-survey samples having detectable TTX levels than in the main survey (27%). A further 1% of samples had TTX levels greater than the recommended EFSA safe guidance level (≥0.044 mg/kg). These samples were greenshell mussels (n=6) and pipi (n=2). The greenshell mussel samples came from the Manukau Hbr (SF015) and Kawhia Hbr (SF017), with another sample from Browns Bay (SC032D). These greenshell mussel results contrast with the findings of the main survey, where none of the analysed mussels exceeded the recommended EFSA guidance level. As for pipi, only 2 samples were included for analysis during this time period and the levels observed were in line with the main survey findings. See Table 3 for a breakdown of the shellfish species tested and TTX levels observed.

Table 3. Summary of non-commercial shellfish samples analysed for TTX during the pre-survey period between Dec 2015-Nov 2016.

	TTX level (mg/kg)			
Shellfish	**<0.002**	**0.002-0.044**	**>=0.044**	**Total**
Blue mussel	12	4	0	16
Cockle	3	1	0	4
Greenshell mussel	205	249	6	460
Pacific oyster	4	12	0	16
Abalone	5	1	0	6
Pipi	1	1	2	4
Tuatua	127	64	0	191
	357	332	8	**697**

Outlier sample

One of the pre-survey samples was a greenshell mussel sample from Browns Bay in the Hauraki Gulf (SC032D). It was found to contain 1.6 mg TTX/kg, as detailed in (Boundy and Harwood 2017). This site had been sampled for another purpose, to determine the spatial extent of a reasonably large paralytic shellfish toxin bloom event that occurred within the Mahurangi inlet north of Auckland city. Paralytic shellfish toxins were also present in this sample at a level of 0.4 mg STX·2HCl eq/kg, which represents half the regulatory limit for this toxin class. To assess the contribution of TTX to the total toxicity of the sample, the sample was subjected to the PSP mouse bioassay (AOAC959.08) and was found to be above regulatory limit at 1.3 mg STX·2HCl eq/kg. A greenshell mussel sample taken 2 weeks prior from the same site contained only trace TTX levels, demonstrating the rapid appearance of this toxin in shellfish at that site (Table 4).

Table 4. TTX levels in samples taken from SC032D and nearby sites from May-July 2016

Date Sampled	Matrix	Site	Distance from SC032D (km)	TTX level (mg/kg)
11 May 2016	Pacific oyster	SC032F	11	0.002
1 June 2016	Greenshell mussel	SC032F	11	0.02
6 June 2016	Greenshell mussel	SC032F	11	0.01
6 June 2016	Greenshell mussel	SC032	5	<LOQ
19 June 2016	Greenshell mussel	SC032D	-	0.004
3 July 2016	Greenshell mussel	SC032D	-	1.6
11 July 2016	Pacific oyster	SC032F	11	0.004
17 July 2016	Rock oyster	SC032F	11	0.003

5.DISCUSSION

This survey of non-commercial New Zealand bivalve shellfish conducted over a period of 15 months has identified low levels of TTX in some shellfish species, with the majority of samples being below the reporting limit of the method. In total, 31% of all shellfish tested during the main MPI-funded survey (Dec 2016 - Mar 2018) contained detectable levels of TTX (>0.002 mg/kg). No commonly harvested shellfish species tested, including mussels, oysters, clams and tuatua, contained TTX levels above the recommended EFSA safe guidance level of 0.044 mg/kg. However, TTX was observed in all pipi tested and levels exceeded the recommended EFSA guidance level at times, including all samples taken from the Hokianga Harbour site. The reason why pipi contain TTX at sites where other filter feeding shellfish do not remains unclear and warrants further investigation. In addition, pipi from the Hokianga Harbour were consistently higher than pipi from other sites, which again is unexplained at this time.

Other relevant observations from this survey include;

- the presence of TTX in New Zealand shellfish appears more likely in northen parts of the country, which may be linked with their warmer waters.
- the observation of TTX in New Zealand shellfish does not represent a new phenomenon as archive samples contained TTX at a similar frequency and level to shellfish included in the main survey (Dec 2016 – Mar 2018).
- no seasonal trends in TTX levels were observed at sites sampled multiple times over the period of this study. This observation contrasts with a previous temporal finding in greenshell mussels from two sites on the west-coast North Island - Cornwallis, Manukau Harbour (SF015) and Kawhia Harbour (SF017). In samples collected from these sites between April 2016 – June 2016 (prior to the main survey), there appeared to be an increase in TTX concentrations (see Figure 5 Cawthron Report 2986 (Boundy and Harwood 2017)). This seasonal trend was not repeated during the current survey and the reasons for this are currently not clear.
- in shellfish found to contain TTX, other analogues were observed only at low levels relative to TTX itself.

TTX has been reported in many terrestrial and marine species, including bivalve shellfish, although its origin remains unclear. Accumulation from the diet, whether from bacteria or another source, is an attractive hypothesis supported by the observation that cultured pufferfish are found to be non-toxic. However, TTX levels found in bacteria and marine sediments are low, and production by bacterial species has still not been demonstrated. Many scientists believe bacteria are the source of TTX in shellfish, and this opinion is reflected in the abstract of the recent EFSA opinion on the presence of TTX in bivalve shellfish, which starts “TTX and its analogues are produced by marine bacteria and have been detected in marine bivalves and gastropods from European waters”. Bacterial cultures have been reported to contain low TTX concentrations and are suggested to be the ultimate biosynthetic origin of the toxin. However, these results remain controversial and are disputed

due to poor specificity of the methods of analysis used, and negative results obtained when more specific methods of analysis are employed.

Having an accurate assessment of TTX toxicity, and understanding the mechanism of TTX accumulation in marine foodstuffs, is important for managing the potential risk to consumers. Existing data on TTX toxicity by oral administration is limited, with a wide range of results reported in the literature. Most of the information available relates to acute toxicity through intraperitoneal injection of mice, and this route of administration is of questionable relevance, given that seafood is consumed orally rather than injected. There is currently no robust toxicity information via oral administration (gavage and voluntary feeding). In addition, toxicity for other TTX analogues (such as 11-oxo-TTX) is needed, as they are potentially equipotent with TTX and have been documented to be dominant analogues in some species of crab. In New Zealand, the grey side-gilled sea slug is the most well-known TTX-containing organism, and they can lay highly toxic eggs. The presence of these organisms in shellfish harvesting areas could make them a possible vector of the toxin. In addition, many marine worm species also contain high TTX concentrations and they could potentially contaminate bivalve shellfish. This mechanism of toxin transfer represents a plausible explanation for the elevated TTX levels observed in the outlier mussel sample from Browns Bay. The likelihood of this possibility is heightened by the fact that the Browns Bay site is close to where toxic slugs have been found in the past.

While it is known that TTX and STX both bind to voltage-gated sodium channels, it is unknown if TTX and the paralytic shellfish toxins have additive or competitive toxicological effects. When assessing sample toxicity, co-occurrence of TTX with paralytic shellfish toxins needs to be considered. This is because the PSP mouse bioassay, which is used for regulatory monitoring in some countries, is not able to distinguish TTX from paralytic shellfish toxins. The presence of paralytic shellfish toxins in shellfish is regulated whereas the presence of TTX is not. Shellfish containing paralytic shellfish toxins below the regulatory limit of 0.8 mg STX·2HCl eq/kg could be found to be above the regulatory threshold for this toxin class if TTX is also present. This situation, although likely to be a rare occurrance, has been observed in a non-commercial New Zealand shellfish sample. Analysis showed a single mussel sample in 2016 from a site close to Auckland city contained 1.6 mg of TTX/kg and paralytic shellfish toxins at 0.4 mg STX·2HCl eq/kg (half the regulatory limit). When subjected to the PSP mouse bioassay, the toxicity of the sample was determined to be 7278 MU/kg or 1.3 mg STX·2HCl eq/kg, which is above the regulatory limit of 0.8 mg STX.2HCl eq/kg. This information is relevant because it shows that even though the levels of TTX observed in New Zealand shellfish are typically low, there is potential for higher levels to be present. Also, factors that precipitate accumulation of high levels of TTX in shellfish are unknown. If found in commercial shellfish these higher levels could result in trade issues and adverse effects on human health. It also demonstrates that if other toxins are present, and they exhibit the same mechanism of action as TTX, there is potential for the total toxicity of the sample to exceed the regulatory level applied when the PSP mouse bioassay is used as this bioassay is not able to distinguish the toxin(s) responsible for the observed toxicity. In New Zealand we use chemical analytical methods for all routine regulatory monitoring of marine biotoxins in

shellfish. For paralytic shellfish toxins a LC-MS/MS method is used that was developed and validated at Cawthron. This method is currently employed as a screening method, with AOAC 2005.06 (Anon 2005) being the approved method for this toxin class. The LC-MS/MS method allows specific identification of the various paralytic shellfish toxin analogues present in contaminated samples and also has the ability to simultaneously quantify TTX, if present. Therefore, methodology is readily available should TTX be required to be monitored in New Zealand shellfish in addition to paralytic shellfish toxins.

Recommendations for future work:

- **To ensure that any future regulatory decisions are based on sound science, robust toxicological data is needed for TTX, including via the oral route of administration.** Findings from this study demonstrate that determining the toxicity of TTX in the presence of other co-occuring paralytic shellfish toxins (e.g. TTX + STX) is of high importance. It is intended that this new information will support the case that TTX could and should be monitored as part of the paralytic shellfish toxin group, which would ultimately improve the risk assessment of hydrophilic marine toxins in shellfish. Analytically this is possible using LC-MS/MS methods, such as the one used at Cawthron. To address this need the Safe New Zealand Seafood research programme is supporting a project to improve the toxicological information available for TTX.

  Note: In recent years MPI has supported extensive toxicological work on the paralytic shellfish toxin group and this has resulted in updated toxicological information and improved monitoring of this toxin class.

- **Toxicological information is needed for TTX analogues that are observed in marine organisms.** Although not found at high levels in shellfish, relative to TTX, they are present in some types of seafood (e.g., types of crab). Obtaining sufficient quantities of the various TTX analogues for toxicological evaluation will be challenging. Nevertheless, investigations are warranted.

- **It remains important to determine the source and mechanism of TTX found in bivalve shellfish**. Should TTX be regulated internationally based on the EFSA scientific opinion, management of shellfish in at-risk areas would be needed. Although the concentrations of TTX observed in shellfish are orders of magnitude lower than other species present in the marine environment (e.g., sea slugs), it is important to determine how TTX accumulates in them. As stationary filter feeders, bivalve shellfish may prove a suitable model to assist with elucidating the origin of TTX. To explore this possibility, Laura Biessy from the Cawthron Institute is undertaking a PhD project titled ‘Elucidating the source and transmission of tetrodotoxin in New Zealand bivalves’. This research is being co-ordinated through the Safe NZ Seafood research programme. It is anticipated that work in this area will lead to better prediction of TTX accumulation in shellfish.

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