Elucidating the Origin of Tetrodotoxin in *Pleurobranchaea maculata* and *Stylochoplana* sp.

A thesis

submitted in fulfilment

of the requirements for the degree

of

Doctor of Philosophy in Biological Sciences

at

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by

Lauren R. Salvitti

THE UNIVERSITY OF
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ABSTRACT

Tetrodotoxin (TTX) is an extremely potent neurotoxin that acts by selectively targeting voltage gated sodium channels blocking propagation of action potentials. Long believed to be present only in pufferfish, TTX has now been detected in a wide range of phylogenetically unrelated terrestrial and aquatic taxa. Despite decades of research the exact origin of TTX remains a mystery. Current literature supports three hypotheses: endogenous, symbiotic bacteria, or bioaccumulation through a dietary source.

In 2009, the opisthobranch *Pleurobranchaea maculata* (grey side-gilled sea slug) was found to contain high concentrations of TTX in New Zealand. A large collaborative project, of which my research was a major part of, was initiated to explore the origin of TTX in *P. maculata*. During extensive benthic surveys conducted to identify possible dietary sources of TTX, high concentrations (ave. 380 mg kg^{-1}) were detected in *Stylochoplana* sp. (Platyhelminthes) from Pilot Bay (Tauranga, New Zealand). Tetrodotoxin concentrations were found to vary temporally, peaking between June and August. The co-occurrence of *Stylochoplana* sp. and *P. maculata* in Pilot Bay raised the possibility that *Stylochoplana* sp. could be a dietary source of TTX for *P. maculata*. A real-time PCR assay was developed, and detected *Stylochoplana* sp. in seven out of nineteen *P. maculata* foreguts.

Symbiotic bacterial production of TTX in the tissues of *P. maculata* and *Stylochoplana* sp. was also explored. Isolated strains (102; 17 unique strains - identified using 16S rRNA gene analysis) were analyzed using a recently developed method to detect the C9 base of TTX. In addition to enhanced sensitivity, this method has the advantage that it might detect precursor and degradation products. To explore the possibility that TTX is produced by a consortium of bacteria, experiments were undertaken where homogenized tissue was spiked into marine broth and samples were collected over two weeks for toxin and molecular analysis. No C9 base or TTX production was detected in isolates or from bacterial communities, suggesting that a symbiotic microbial source of TTX is unlikely in these organisms.

The ability of non-toxic *P. maculata* to sequester TTX from an environment known to contain toxic populations of the same species was also assessed. Sixteen non-toxic specimens were kept in mesh cages (eight anchored to

the benthos and eight suspended 0.5 m above it) for eight weeks and fed a non-toxic food source. Toxin analysis revealed that more 'benthic' specimens (4 verses the 2 from suspended specimens) sequestered TTX and were shown to retain higher concentrations (max. 0.79 versus 0.43 mg kg^{-1}). These data suggest a localized microbial source of TTX that is more readily available from the benthos. Diet analysis, utilizing next generation sequencing of toxic and non-toxic *P. maculata* identified their diet comprised a wide array of organisms, with *Thelepus* sp. and *Plumularia* sp. being prevalent in toxic individuals, and further testing of these organisms is suggested.

Lastly, immunohistological methods, employing a monoclonal antibody targeting TTX, were conducted with tissues from *P. maculata* and *Stylochoplana* sp.. Strong TTX signals were detected in the mantle and oocytes of *P. maculata* and the ova and pharynx of the *Stylochoplana* sp.. These data suggest ecological roles for TTX including: defense in adults, protection in progeny, and prey capture in *Stylochoplana* sp..

A synthesis of the studies presented in this thesis, and those that were conducted as part of the larger project, are also presented and future studies to elucidate the origin of TTX in New Zealand taxa are suggested.

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Postgraduate Studies Office
Student and Academic Services Division
Wahanga Ratonga Matauranga Akonga
The University of Waikato
Private Bag 3105
Hamilton 3240, New Zealand
Phone +64 7 858 5096
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Nature of contribution by PhD candidate	I aided in collections of all specimens and processed all samples for toxin and molecular analysis. I wrote this chapter with input and edits as detailed below.
Extent of contribution by PhD candidate (%)	90%

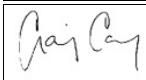
CO-AUTHORS

Name	Nature of Contribution
Susanna A. Wood	Co-Advisor and main point of contact from Cawthron Institute. Aided in development of Real-time PCR primers and assays. Helped with manuscript development and editing.
David I. Taylor	Conducted sample collections from the South Island and advised on Platyhelminthes ecology.
Paul McNabb	Preformed all liquid chromatography-mass spectrometry analysis and was consulted regarding data analysis.
S. Craig Cary	Advisor and main point of contact at Waikato University. Aided in overall study direction and manuscript preparation.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

Name	Signature	Date
Susanna A. Wood		6/7/2014
David I. Taylor		6/7/2014
Paul McNabb		6/7/2014
S. Craig Cary		6/7/2014

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Nature of contribution by PhD candidate	I aided in collection of all specimens. Lab work was organized and mostly conducted by myself. Writing and data analysis were conducted by myself with assistance as detailed below.
Extent of contribution by PhD candidate (%)	90%

CO-AUTHORS

Name	Nature of Contribution
Susanna A. Wood	Co-Advisor and main point of contact from Cawthron Institute. Aided in development of lab experiments and with lab work. Helped with manuscript development and editing.
Paul McNabb	Preformed all liquid chromatography-mass spectrometry analysis and was consulted regarding data analysis.
S. Craig Cary	Advisor and main point of contact at Waikato University. Aided in overall study direction and manuscript preparation.

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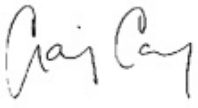
Susanna A. Wood		6/7/2014
Paul McNabb		6/7/2014
S. Craig Cary		6/7/2014

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Nature of contribution by PhD candidate	I aided in experimental design, initial deployment and collection of cages. I prepared samples for toxin and molecular analysis. Writing and editing of the manuscript was conducted with assistance as detailed below.
Extent of contribution by PhD candidate (%)	80%

CO-AUTHORS

Name	Nature of Contribution
Susanna A. Wood	Co-Advisor and main point of contact from Cawthron Institute. Aided in development of lab experiments and preparation of next generation sequencing samples. Susie helped with data analysis, manuscript development, and editing.
Rex Fairweather	Conducted pilot study for this experiment. Aided in development of cage setup and was responsible for weekly feedings of deployed slugs.
David Culliford	Aided in development of cage setup and was responsible for weekly feedings of deployed slugs.
Paul McNabb	Preformed all liquid chromatography-mass spectrometry analysis and was consulted regarding data analysis.
S. Craig Cary	Advisor and main point of contact at Waikato University. Aided in overall study direction and manuscript preparation.

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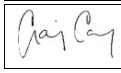
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

Name	Signature	Date
Susanna A. Wood		6/7/2014
Rex Fairweather		18/6/14
David Culliford		18/6/2014
Paul McNabb		6/7/2014
S. Craig. Cary		6/7/2014

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Nature of contribution by PhD candidate	I participated in all specimen collections. I conducted the method development and sample preparation for toxin analysis. Writing and editing of the manuscript was conducted with assistance as detailed below.
Extent of contribution by PhD candidate (%)	90%

CO-AUTHORS

Name	Nature of Contribution
Susanna A. Wood	Co-Advisor and main point of contact from Cawthron Institute. Aided in development of lab experiments, data analysis, manuscript development, and editing.
Leigh Winsor	Consulted on histological analysis and conducted some of the histological staining of sections. Aided in editing of manuscript.
S. Craig Cary	Advisor and main point of contact at Waikato University. Aided in overall study direction and manuscript preparation.

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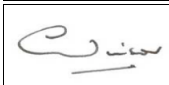
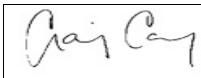
Susanna A. Wood		6/6/2014
Leigh Winsor		6/6/2014
S. Craig Cary		6/6/2014

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CHAPTER I. LITERATURE REVIEW

1. Tetrodotoxin

Tetrodotoxin (TTX) is a potent neurotoxin whose effects have been cited in literature as far back as 2500 B.C. in hieroglyphics of ancient Egypt and in Oriental literature which warned of the dangers of eating pufferfish (Clark et al. 1999). The logbook entry on September 7, 1774 of Captain James Cook details the consumption of a local tropical fish from New Caledonia, now thought to be a pufferfish, and the resulting symptoms of weakness, numbness, and vomiting of his crew (Isbister et al. 2002). In 1880 the toxin in pufferfish was named tetrodotoxin by Professor Yoshizumi Tahara after the order of fish tetraodontidae, which encompasses most of the known pufferfish species (Tahara 1910). Tarichatoxin, originally isolated from the newt *Taricha torosa* (Brown & Mosher 1963; Mosher et al. 1964) and maculotoxin, isolated from the blue-ringed octopus *Hapalochlaena maculosa* (Sheumack et al. 1978) are now recognized as being identical to TTX.

1.1. Structure

Tetrodotoxin has a non-protein structure with a low molecular weight (319.27 g mol^{-1}) (Tsuda et al. 1964). Although its toxic properties have been long known it was not isolated until 1950 (Yokoo 1950) and in 1964 its structure (Figure 1; $C_{11}H_{17}N_3O_8$) was determined independently by three groups (Tsuda et al. 1964; Woodward & Gougoutas 1964; Goto et al. 1965). The highly unique structure contains a hemilactal anion and single positively charged guanidinium group attached to an oxygenated carbon backbone. Although the structure of TTX has been confirmed, its biosynthetic pathway *in vivo* is still unknown. Several proposed synthetic pathways have been published but have yet to be verified (reviewed in Chau et al. 2011; Hinman & Du Bois 2003; Kishi et al. 1972b; Kotaki & Shimizu 1993).

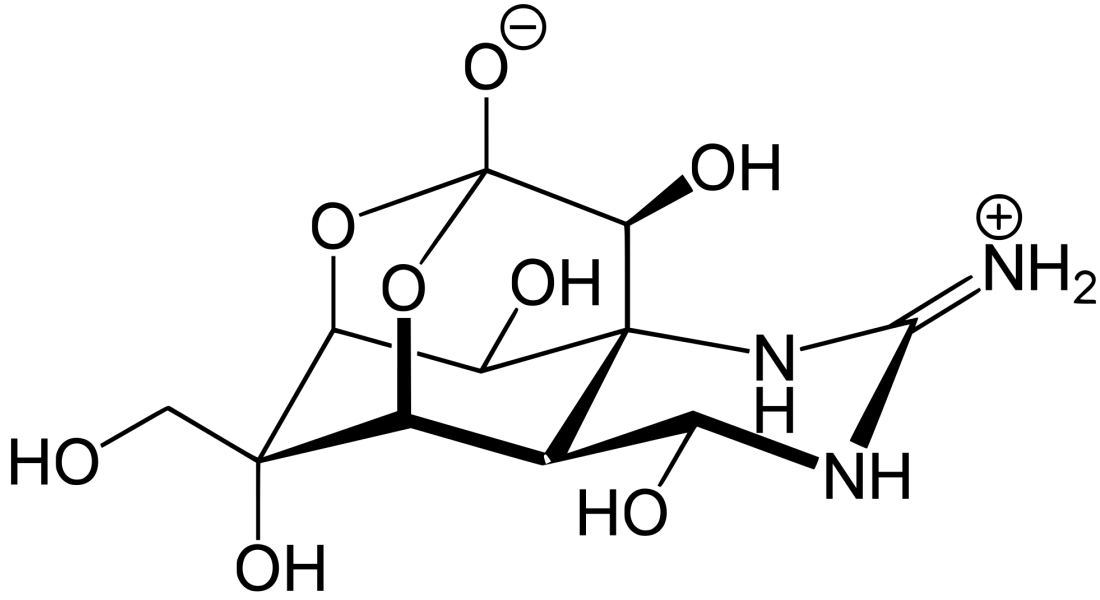

Figure 1. Structure of tetrodotoxin.

1.2. Activity

Tetrodotoxin selectively binds to and physically blocks voltage-gated sodium channels (Na_v) thus inhibiting action potential propagation, resulting in paralysis of nerves and muscle (reviewed in Narahashi 2001). Pivotal to its activity and accurate binding is the distinctive guanidinium moiety, which forms an ion-pair with negatively charged functional groups on the sodium channel (Lee & Ruben 2008). Voltage gated sodium channels are a class of transmembrane proteins comprised of a pore forming α-subunit and associated β-subunits (Soong & Venkatesh 2006; Lee & Ruben 2008). The α-subunit consists of four repeated domains (DI – DIV) each with six α-helical segments connected by P-loop regions. It is these P-loop regions to which TTX binds, blocking the flow of sodium ions through the pore (Narahashi 2001).

The highly selective nature of TTX renders it extremely potent with concentrations as low as 10000 Mouse Units (MU; ca. 2 mg) enough to be fatal to an adult human (wt. 50 kg) when ingested (Noguchi & Ebesu 2001). Tetrodotoxin poisoning often results from the consumption of pufferfish, regarded as a delicacy in Japan and consumed in many Asian countries, when it is improperly prepared. Commercial preparation of pufferfish is strictly regulated and only conducted by licensed chefs who thoroughly remove the highly toxic liver tissue. Treatment with heat (i.e., cooking) does not inactivate the toxin and numerous poisonings have occurred when TTX-bearing organisms are caught and consumed at home (Wan et al. 2007; Noguchi & Arakawa 2008). The onset of symptoms (Table 1) can be rapid and both the progression and the severity depends on the amount of TTX ingested and the health and age of the victim (Kaku & Meier 1995; Noguchi & Ebesu 2001). In Japan, there have been 378 incidents of pufferfish poisonings involving 546 patients and 35 deaths between 1995 and 2007 (Noguchi & Arakawa 2008).

Presently there are no known antidotes or antitoxins for TTX poisoning in humans. However, several studies have described successful treatments in mice and guinea pigs. A monoclonal antibody administered intravenously was shown to neutralize the effect of oral TTX poisoning in mice with a direct correlation between antibody concentration and recovery (Rivera et al. 1995). Additionally, significant improvements in cardiac and respiratory function were observed in

guinea pigs when 4-aminopyridine, a potassium channel blocker, was administered 30 minutes post intoxication (Chang et al. 1997). Currently emergency care for human patients consists of supportive care with treatment continuing until the body can naturally purge itself of the toxin.

Table 1. Stages of tetrodotoxin poisoning and related symptoms.

Stage	Characteristic Symptoms
First	Numbness or paralysis of the mouth, lips, tongue and pharynx. Gastrointestinal symptoms, diarrhea, abdominal pain, nausea, headache, dizziness, vomiting.
Second	Advanced paralysis of extremities, slurred speech and incoordination.
Third	Neuromuscular symptoms: cranial nerve palsies, muscular twitches, slurred speech, difficulty swallowing, widespread paralysis Additional symptoms include cardiac arrhythmias, shortness of breath, hypotension or hypertension, fixed or dilated pupils.
Fourth	Unconsciousness, severe paralysis, asphyxiation due to respiratory muscle paralysis, loss of reflexes, seizures, cardiac arrhythmias

1.3. Production

Due to its highly selective nature, TTX has become a valuable tool in ion-channel medical studies. Tetrodotoxin is also utilized for its medicinal benefits including the alleviation of heroin withdrawal symptoms and pain relief in cancer patients (Hagen et al. 2008, 2011; Song et al. 2011). Currently there are two main processes by which TTX is produced commercially. First is the isolation and purification of TTX from the ovary tissues of pufferfish. Initial methods of TTX isolation reported low yields: 13 mg crystalline TTX from 20 kg of pufferfish ovaries (Yokoo 1950). Novel methodology reported by Goto et al. (1964) simplified the process, yielding 1-2 g of TTX from 100 kg of pufferfish ovaries. Tetrodotoxin yields have recently been improved in a patented method by Zhou and Shum (2003) resulting in 6 g of toxin per 100 kg of ovaries. The second method of TTX production is by *de novo* synthesis. In 1972, Kishi et al. (1972a, 1972b) reported the first synthesis of TTX via two different routes, utillizing stereospecific conversions from dihydrofurancetamide into DL-tetrodotoxin. Additional attempts to synthesize tetrodotoxin have been successful (Hinman & Du Bois 2003; Ohyabu et al. 2003), the most recent reporting a 0.34% overall yield (Sato et al. 2008). However, both extraction of TTX from pufferfish tissues and synthesis of TTX involve lengthy steps, are expensive, and relativity inefficient. Thus the demands for a cheap and sustainable source of TTX have driven researchers to persist in their search to identify the ultimate origin of TTX and its biosynthetic pathway.

1.4. Origin

Tetrodotoxin was initially thought to only occur in the tissues of pufferfish of the Tetraodontidae family. It was not until 1964 when TTX, originally labeled as tarichatoxin, was also discovered in the eggs of the California newt *Taricha torosa* (Twitty & Johnson 1934; Mosher et al. 1964). Tetrodotoxin has since been identified in organisms across eight different phyla, excluding bacteria, inhabiting a diverse range environments (Table 2). The large genetic diversity of TTX bearing organisms suggests that it is highly unlikely they possess a convergent pathway coding for TTX biosynthesis and instead it has been suggested that they obtain TTX from an alternative source. Thus several alternative methods of TTX accumulation (and thus the source), have been presented including: bioaccumulation of TTX or its precursors through the food chain (Noguchi et al.

1982; Daly et al. 1997; Noguchi et al. 2006b; Noguchi & Arakawa 2008; Gall et al. 2012b), bacterial (symbiotic or free living) production of TTX (e.g., Yasumoto et al. 1986; Yu et al. 2004; Wang et al. 2008), and endogenous production of TTX (e.g., Hanifin et al. 2002; Lehman et al. 2004). These are detailed in the following sections.

Table 2. Organisms reported to contain tetrodotoxin (excluding bacteria).

Phylum	Class	Order	Family	Binomial name	Reference
Chordata	Amphibia	Anura	Dendrobatidae	*Colostethus inguinalis*	Daly et al. (1994)
			Brachycephalidae	*Brachycephalus pernix*	Pires et al. (2005)
				Brachycephalus ephippium	Pires et al. (2002)
			Bufonidae	*Atelopus oxyrhynchus*	Mebs and Schmidt (1989)
				Atelopus ignescens	Daly et al. (1994)
				Atelopus peruensis	Mebs et al. (1995)
				Atelopus spumarius	Daly et al. (1994)
				Atelopus spurelli	Daly et al. (1994)
				Atelopus subornatus	Mebs et al. (1995)
				Atelopus varius	Daly et al. (1994, 1997), Kim et al. (1975), Yotsu-Yamashita and Tateki (2010)
			Rhacophoridae	*Polypedates* spp.	Tanu et al. (2001)
		Caudata	Ambystomatidae	*Ambystoma tigrinum*	Yotsu et al. (1990)
			Salamandridae	*Notophthalmus viridescens*	Yotsu-Yamashita and Mebs (2003)
				Taricha torosa	Brown and Mosher (1963)
				Taricha granulosa	Brodie et al. (1974), Cardall et al. (2004), Hanfin et al. (2002), Yotsu et al. (1990)
				Taricha rivularis	Brodie et al. (1974), Wakely et al. (1966)
				Cynopsis ensicauda	Yasumoto et al. (1988)
				Cynops pyrrhogaster	Tsuruda et al. (2001)
				Paramesotriton hongkongensis	Brodie et al. (1974), Yotsu et al. (1990)

				Triturus sp.	Mosher (1964), Wakely et al. (1966), Yotsu et al. (1990), Yotsu-Yamashita et al. (2007)
	Actinopterygii	Tetraodontiformes	Tetraodontidae *	*Takifugu xanthopterus*	Nagashima et al. (2001)
				Takifugu niphobles	Yu et al. (2004)
				Fugu rubripesa	Wu et al. (2005a,b)
				Fugu vermicularisa	Lee et al. (2000), Noguchi et al. (1987)
				Fugu pardalisa	Yasumoto et al. (1986)
				Fugu poecilonotusa	Yasumoto et al. (1986), Yotsu et al. (1987)
				Lagocephalus sceleratus	Bentur et al. (2008), Katikou et al. (2009)
				Takifugu rubripes	Saito et al. (1984), Tani (1945)
		Perciformes	Gobiidae	*Gobius criniger*	Noguchi and Hashimoto (1973)
Mollusca (gastropods)	Cephalopoda	Octopoda	Octopodidae	*Hapalochlaena* cf. *lunulata*	Hwang et al. (1989)
				Hapalochlaena maculosa	Sheumack et al. (1984)
				Hapalochlaena fasciata	Williams and Caldwell (2009)
	Gastropoda	Neogastropoda	Nassariidae	*Niotha clathrata*	Jeon et al. (1984)
				Niotha lineata	Hwang et al. (1990)
				Nassarius semiplicatusa	Wang et al. (2008)
			Muricidae	*Rapana rapiformis*	Hwang et al. (1991)
				Rapana venosa venosa	Hwang et al. (1991)
		Sorbeoconcha	Ranellidae	*Charonia sauliae*	Narita et al. (1981)
			Buccinidae	*Babylonia japonica*	Noguchi et al. (1981)
		Neotaeniogloss	Naticidae	*Polinices didyma*	Shiu et al. (2003)
			Bursidae	*Tutufa lissostoma*	Noguchi et al. (1984)
		Littorinimorpha	Ranellidae	*Charonia lampas lampas*	Rodriguez et al. (2008)

		Notaspidea	Pleurobranchidae	*Pleurobranchaea maculata*	McNabb et al. (2010)
		Vetigastropoda	Trochidae	*Gibbula umbilicalis*	Silva et al. (2012)
				Monodonta lineata	Silva et al. (2012)
Nemertea (nematodes)	Anopla	Paleonemertea	Cephalothricidae	*Cephalothrix rufifronsa*	Carroll et al. (2003)
		Heteronemertea	Lineidae	*Lineus longissimusa*	Carroll et al. (2003)
Echinodermata (starfish)	Stelleroidea	Paxillosida	Astropectinidae	*Astropecten latespinosus*	Maruyama et al. (1984)
				Astropecten polyacanthusa	Miyazawa et al. (1985)
	Asteroidea	Paxillosida	Astropectinidae	*Astropecten scoparius*	Lin et al. (1998)
Chaetognatha (arrow worms)	Sagittoidea	Aphragmophora	Sagittidae	*Flaccisagitta enflata*	Thuesen and Kogure (1989)
				Parasagitta elegans	Thuesen and Kogure (1989)
			Pterosagittidae	*Zonosagitta nagae*	Thuesen and Kogure (1989)
		Phragmophora	Eukrohniidae	*Eukrohnia hamata*	Thuesen and Kogure (1989)
Arthropoda	Merostomata	Xiphosura	Cleroidea	*Carcinoscorpius rotundicauda*	Dao et al. (2009), Kungsuwan et al. (1987)
	Malacostraca	Decapoda	Carpiliidae	*Lophozozymus pictor*	Tsai et al. (1995)
			Xanthidae	*Atergatis floridus*	Noguchi et al. (1983)
				Zosimus aeneus	Yasumura et al. (1986)
	Maxillopoda	Siphonostomatoida	Caligidae	*Pseudocaligus fugu*	Ikeda et al. (2006)
Platyhelminthes (flatworms)	Turbellaria	Polycladida	Planoceridae	*Planocera multitentaculata*	Miyazawa et al. (1987)
				planocerid sp.	Ritson-Williams et al. (2006)
Dinoflagellata	Dinophyceae	Gonyaulacales	Goniodomataceae	*Alexandrium tamarense*	Kodama et al. (1996)

*See Noguchi et al. (2008) for comprehensice list of all Tetraodontidae reported to contain TTX.

1.4.1. Bioaccumulation

In 1982 Noguchi et al. (1982) reported the observation of frequent debris from the starfish *Astropecten polyacanthus* in the digestive gland of the toxic trumpet shell *Bablyonia japonica*. Upon further investigation it was found that *A. polyacanthus* contained TTX and was most likely the source of this toxin for *B. japonica*, presenting researchers with the first evidence of TTX bioaccumulation. Subsequent examples include a study by Noguchi et al. (2006b) where 5,000 specimens of *Takifugu rupripes* were held in net-cages at sea or in land aquaria, isolated away from any TTX-bearing organisms, for between 1 to 3 years. Tissues tested for toxin via the traditional mouse bioassay, and in some cases liquid chromatography – mass spectrometry (LC-MS), were all non-toxic indicating this species of pufferfish are most likely acquiring TTX through the food chain. Additionally, when non-toxic *T. rubripes* held in aquaria were fed toxic diets of TTX-containing wild caught pufferfish liver, they themselves became toxic, further supporting the hypothesis of an exogenous source of TTX in *T. rubripes* (Noguchi et al. 2006a). Daly et al. (1997) described similar results in the Panamanian frog *Atelopus varius*. Captive specimens raised for 2-3 years on non-toxic diets had no detectable TTX-like compounds when tested via inhibition of [^{3}H] saxitoxin binding, compared to wild caught specimens at 0.84 μg TTX equivalents per 100 mg of skin (Daly et al. 1994). It can be concluded that, at least for these organisms, diet plays a significant role in the concentrations of TTX sequestered in their tissues.

Acquisition of TTX from diet, and without harmful effects to host tissues, can be partially explained by the presence of TTX-binding proteins in some organisms. For example, these have been found in the blood plasma from the pufferfish *T. niphobles* (Matsui et al. 2000), hemolymph of the horseshoe crab *Carcinoscorpius rotundicauda* (Ho et al. 1994) and from the muscle tissue of several gastropod species (Hwang et al. 2007). In addition, preferential accumulation of TTX, compared to paralytic shellfish toxin (PST), in the liver of *T. rubripes* has been shown (Matsumoto et al. 2005) and carrier mediated uptake of TTX elucidated (Matsumoto et al. 2007).

1.4.2. Bacterial source

Ubiquitous throughout the marine environment, bacteria have been shown on numerous occasions to live symbiotically with marine organisms (Lee et al. 2001; Dubilier et al. 2008). In 1986, TTX production from a *Pseudomonas* sp., isolated from a red calcareous alga, *Jania* sp., was reported (Yasumoto et al. 1986). Since then evidence for bacterial-associated TTX production, either ingested, or through symbiosis with a range of marine host species, has been suggested (see Chapter III, Table 1).

Pufferfish are one of the most extensively studied TTX-containing organisms with multiple groups reporting the isolation of TTX-producing bacterial strains from their tissues (see Chapter III, Table 1). However, low concentrations of TTX produced by isolated bacterium do not account for the high TTX measured from host tissues (Wu et al. 2005a, b; Wang et al. 2008). It must be noted however that the relatively low concentrations of TTX produced could be due to the altered conditions when grown *in vitro* and are not necessarily indicative of wild production levels. Noguchi and Arakawa (2008) suggest that the amount of TTX produced by bacterium found in the gut of *Fugu* species is a minor contributor, and that TTX is most likely bioaccumulated from the environment. Concerns were also raised when a false positive TTX trace was detected in the culture medium used to isolate bacteria when measured by high-performance liquid chromatography (HP-LC) and gas chromatography–mass spectrometry (GC-MS) indicating a lack of specificity (Matsumura 1995a). In contrast, it has been suggested that TTX-producing bacteria might require a specific inducer molecule to produce elevated concentrations of TTX *in vitro*, explaining the low concentrations seen when cultured in a laboratory setting (Proksch et al. 2002). Alternatively, it has been proposed that symbiotic bacteria synthesize TTX precursors, which are subsequently metabolized and accumulated by host species (Chau et al. 2011). Further studies addressing the metabolic pathways and associated genes involved in TTX production will be crucial to understanding the origins of TTX across all phyla.

1.4.3. Endogenous production

To date, evidence for endogenous production of TTX has primarily been gathered from terrestrial organisms (Shimizu & Kobayashi 1983; Hanifin et al.

2002; Cardall et al. 2004; Gall et al. 2012a). A study by Hanifin et al. (2002) using captive rough-skin newts (*Tarachia granulosa*) showed a mean increase in TTX concentrations of 21% when specimens were raised on a TTX-free diet over one year. Furthermore, Cardall et al. (2004) observed the significant regeneration of TTX in *T. granulosa* after 9 months, following electrical stimulation used to induce the release of toxin from the skin. It was also shown that female newts continue to deposit TTX into eggs after three years in captivity (Gall et al. 2012a). Additionally, no molecular (16S gene) evidence of bacteria associated with TTX-rich organs of *T. granulosa* was detected (Lehman et al. 2004). Based on this evidence endogenous production is given as the most likely the source of TTX in *T. granulosa*.

1.5. Ecological roles

As suggested with other secondary metabolites, TTX production or sequestration is presumably metabolically expensive and thus must present an evolutionary advantage (Pawlik 1993). Benefits of containing TTX, or being resistant to TTX, are multifaceted: allowing for protection from predators, attracting mates, or consumption of toxic prey. Tetrodotoxin is primarily thought to function as a chemical defense (Mahmud et al. 2003a, b; Hanifin 2010). A protective role in offspring has also been suggested in pufferfish (Itoi et al. 2014), horseshoe crabs (Kanchanapongkul 2008), newts (Mosher et al. 1964), and the blue-ringed octopus (Sheumack et al. 1984), where TTX has been detected in reproductive organs as well as larvae and eggs. The use of TTX as a male-attracting pheromone was shown in the pufferfish *Fugu niphobles* via traditional y-maze experiments, where 15 pM of TTX was shown to effectively attract specimens (Matsumura 1995b).

Resistance to TTX has been observed in several taxa including garter snakes, newts, and pufferfish (Geffeney et al. 2005; Venkatesh et al. 2005a; Soong & Venkatesh 2006; Maruta et al. 2008; Feldman et al. 2012; McGlothlin et al. 2014). The basis of this resistance is due to specific mutations to the amino acid sequence of the pore region of voltage gated sodium channels leading to conformational changes which impart various levels of resistance to TTX binding. An example of TTX resistance has been observed between garter snakes (*Thamnophis sirtalis*) and their toxic prey the rough-skinned newt, *T. granulosa*

(Brodie III & Brodie Jr 1999; Brodie Jr et al. 2002; Geffeney et al. 2005). Mutations in the voltage-gated sodium channel gene ($Na_v1.4$) have altered the P-loop region of the protein, reducing its affinity for TTX (Geffeney & Ruben 2006; Feldman et al. 2009, 2010; McGlothlin et al. 2014). Tetrodotoxin resistance has also occurred in related species of garter snake (*Thamnophis couchii*) and their prey toxic (*Taricha torosa*) suggesting that the presence of TTX in prey is a driving force behind various sodium channel mutations conferring TTX resistance in predator species (Brodie III et al. 2005; Feldman et al. 2012). The trade off for TTX resistance seems to have resulted in a reduction of crawl speed in garter snakes (Brodie III & Brodie Jr 1999; Brodie III et al. 2005). This is presumably due to decreases in the propagation of action potentials; a consequence of sodium channel conformational changes (Lee et al. 2011). Changes to sodium channel amino acid sequences have also been observed in the toxic pufferfish *Takifugu rupries* and *T. nigroviridis* allowing for consumption of TTX bearing prey, and presumably the sequestration of the toxin in their own tissues (Venkatesh et al. 2005b; Jost et al. 2008).

2. Pleurobranchaea maculata

First described in 1832 by Quoy and Gaimard, *Pleurobranchaea maculata* (the grey side-gilled sea slug) is a marine gastropod found in the coastal regions of New Zealand, southeastern Australia, Japan, China, and Sri Lanka (Figure 2; Gibson 2003; Willan 1983). It belongs to the Opisthobranchia, meaning gills behind and to the right of the heart, group of gastropods that can be found worldwide. Opisthobranchs are characterized by their reduced or non-existent shell, single gill, and rhinophores (Wagele & Klussmann-Kolb 2005; Wagele et al. 2006). As slow soft-bodied organisms lacking the protection of a shell, opisthobranchs have developed a number of unique feeding and defensive strategies. For example the acquisition of chemical defenses such as toxic secondary metabolites or functional cnidocysts from cnidarian prey has been observed in several species (e.g., Putz et al. 2010). In addition nutrient supplementation by assimilation of functioning chloroplasts from grazed algae has been reported in species from the order Ascoglossa (reviewed in Rumpho et al. 2000). *Pleurobranchaea maculata* are voracious scavengers known to prey on a range of invertebrates, including sea anemones such as *Actinia tenebrosa*, and can grow to ca. 100 mm in length (Ottaway 1977; Gibson 2003). They have an

elongate body covered dorsally by an acidic (pH = 1–2) puckered mantle with a foot projecting the entire circumference (Willan 1983). The mantle can range in color from a molted pale grey to a dark brown allowing *P. maculata* to easily conceal itself within its benthic environment (Willan 1983). There are currently 10 species listed in the Pleurobranchaea genus, with *P. maculata* being the only species to occur in the waters of New Zealand (Willan 1983; Roskov et al. 2015). Cytochrome oxidase subunit I (*COI*) sequences collected from populations from both the North and South Island were found to have <1% sequence variability verifying that these populations are the same species (Wood et al. 2012b).

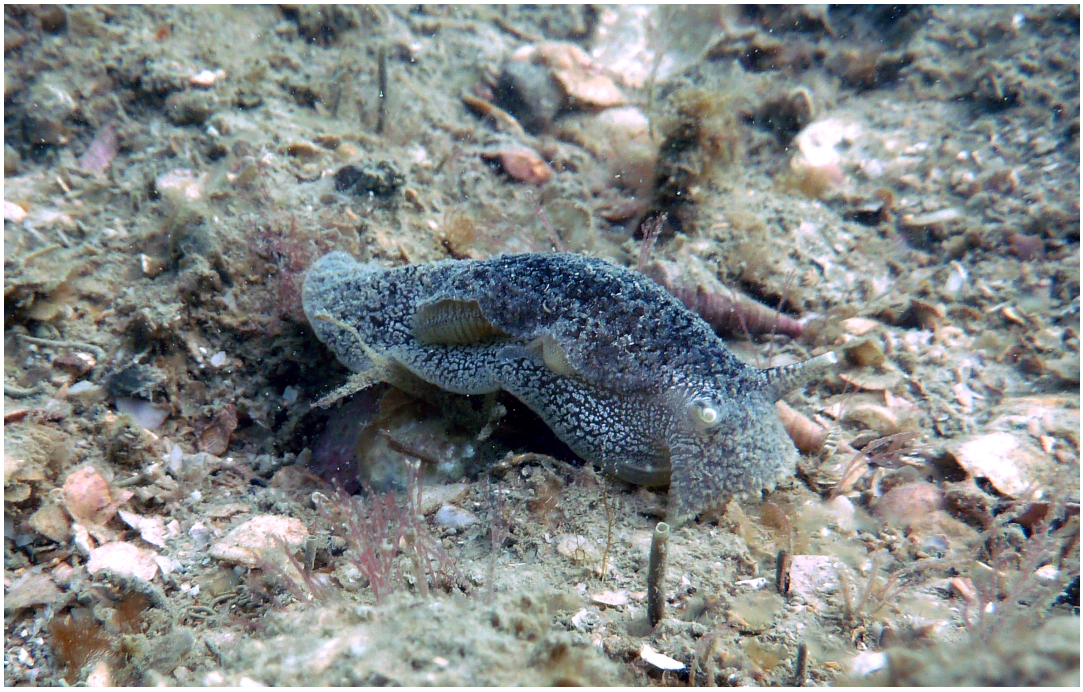

Figure 2. Adult *Pleurobranchaea maculata* from Auckland Harbor, New Zealand. The gills can clearly been seen under the mantle on the right side of the body. (Photo by Mike McMurtry).

In 2009 a series of dog poisonings were reported in the Auckland region of New Zealand along beaches of the Hauraki Gulf, from Whangaparoa to Coromandel (McNabb et al. 2009). In-depth surveys of the biota collected from intertidal zones of affected regions were conducted and accompanied by mouse bioassays (McNabb et al. 2010). Initial information suggested that a number of dogs came in contact with beach-cast *P. maculata*. Further toxin analysis via liquid chromatography – mass spectrometry (LC-MS) of *P. maculata* sampled from the Hauraki Gulf revealed high concentrations of TTX (ave. 385 mg kg^{-1}), which was also identified in the vomit of one of the stricken dogs (McNabb et al. 2009, 2010). This was the first ever report of TTX in an opisthobranch and the first account of the toxin in New Zealand (McNabb et al. 2009).

Subsequent studies on the *P. maculata* from New Zealand have revealed significant variability in TTX concentrations between individuals, populations, and across seasons. In 2010, Wood et al. (2012b) collected *P. maculata* from ten sites around New Zealand and analyzed TTX concentrations via LC-MS. They reported concentrations ranging from undetectable to 1,414 mg kg^{-1} in an individual from Illiomama Rock (Auckland) with no correlations between TTX and mass. Spatial variability was revealed when TTX was undetected in individuals collected from the two South Island sites - Tasman Bay and Kaikoura, indicating a clear separation of toxic and non-toxic populations across the Cook Straight. Comparison of the cytochrome oxidase subunit I (*COI*) gene from both toxic and non-toxic specimens showed < 1% sequence variability between them, indicating that the two populations are the same species. Concentrations of TTX measured over a six-month period from June to December of 2010 in *P. maculata* from Narrow Neck Beach (Auckland) significantly declined from 181.9 (± 48.4) mg kg^{-1} to 17.5 (±5.4) mg kg^{-1}. This extreme variability in toxin concentrations between individuals follows trends shown in other TTX-containing organisms such as the blue-ringed octopuses *Hapalochlaena fasciata* and *H. lunulata* (Williams & Caldwell 2009), the horseshoe crab *Carcinoscorpius rotundicauda* (Dao et al. 2009), the gastropods *Rapana rapiformis* and *R. venosa venosa* (Hwang et al. 1991), and the newt *T. granulosa* (Hanifin et al. 1999). Wood et al. (2012b) suggested a protective role of TTX in *P. maculata* as the highest concentrations of toxin were seen during the egg-laying season (June – August).

Aquaria based studies have been conducted in an effort to elucidate the origin of TTX in *P. maculata* (Wood et al. 2012a; Khor et al. 2013). First, twenty-five toxic specimens from Illiomama Rock were kept in aquaria for 126 days and maintained on a non-toxic diet of Greenshell^{TM} mussel, *Perna canaliculus* (Wood et al. 2012a). Total tetrodotoxin concentrations significantly decreased during the study, with concentrations in the gonad tissue depurating the slowest. Egg masses and fecal pellets were also collected and analyzed. No TTX was detected in any of the fecal pellets, however, egg masses were found to contain TTX, decreasing in toxicity with each subsequent laying, suggesting that *P. maculata* invest their TTX in offspring (Wood et al. 2012a). Khor et al. (2013) maintained twelve non-toxic *P. maculata*, sourced from Tasman Bay, in aquaria for up to 39 days while fed a TTX diet produced from toxic *P. maculata* tissues. Non-toxic specimens were found to sequester TTX in as little as one hour, with one egg mass laid 24 hours after the first feeding also testing positive for TTX (Khor et al. 2013). Subsequent egg masses laid progressively increased in TTX concentration through the experiment. The highest concentrations of TTX were found in either the digestive or mantle tissues suggesting a dietary source and a protective role in adults. This study demonstrated that despite the lack of TTX in South Island populations, non-toxic specimens are able to resist the toxic effects of TTX as well as sequester it within their tissues, similar to their North Island counterparts. A dietary supply of TTX was further explored and benthic surveys were conducted in areas of high densities of *P. maculata*. Toxin analysis of organisms collected revealed trace levels of TTX in a crab (*Macrothalamus hirtipes*), snail (*Turbo smaragdus*), coralline algae (*Corallina officinalis*), and low concentrations (0.25 mg kg^{-1}) in the sand dollar *Arachnoides zelandiae* (Khor et al. 2013). However, these concentrations are not high enough to account for those seen in toxic *P. maculata*.

3. Main objectives

The overarching aim of this doctoral research was to elucidate the origin of TTX in *P. maculata* through histochemical, molecular, and classical bacterial isolation methods. During a routine collection for *P. maculata* specimens at Pilot Bay, Tauranga, *Stylochoplana* sp. (flatworms) were also collected and processed for TTX (Figure 3). Significant concentrations of TTX were discovered in their tissues and therefore this organism was included in the following studies. Chapter

II describes the first detection of TTX in the *Stylochoplana* sp. from New Zealand and shows TTX concentrations, accessed via LC-MS, over a nine month period. The 18s rRNA and mitochondrial *COI* genes of *Stylochoplana* sp. were sequenced. Additionally, a dietary link was explored after a *P. maculata* specimen was observed ingesting a *Stylochoplana* sp. individual. A real-time PCR assay was developed to detect the *COI* gene of *Stylochoplana* sp. from *P. maculata* gut contents.

Chapter III details attempts to isolate a TTX-producing bacterium from the tissues of *P. maculata* and *Stylochoplana* sp. Standard published isolation techniques were employed and several media types (Marine, TCBS, ORI), previously successful at isolating TTX producing bacteria, were utilized to isolate bacterial strains (Lee et al. 2000; Yan et al. 2005). Due to suggestions that TTX production in bacteria could be influenced by external factors, marine broth was also spiked with homogenized tissues from *P. maculata* and *Stylochoplana* sp. and bacterial community effects on TTX-production were assessed. Cultures were tested for TTX over a fourteen day period and community composition analyzed using Automated Ribosomal Intergenic Spacer Analysis (ARISA).

Chapter IV investigates the potential for a dietary or environmental (exogenous) source of TTX in *P. maculata* through relocation of non-toxic South Island specimens into an environment known to contain toxic North Island *P. maculata*. Dietary habits of toxic *P. maculata* were also explored through Illumina™ next generation sequencing of gut contents collected from native populations of Pilot Bay, Tauranga (North Island).

Chapter V describes the development of immunohistochemical methods for the localization of TTX in the tissues of *P. maculata* and *Stylochoplana* sp. using an anti-TTX monoclonal antibody. Additional staining was conducted using Alcian Blue-periodic Acid-Schiff (AB-PAS) to differentiate neutral and acidic mucins.

A summary of conclusions and future directions for the study of the New Zealand *P. maculata* and *Stylochoplana* sp. are detailed Chapter VI.

A map showing the different sampling sites used in the following chapters is shown in Figure 4.

Figure 3. *Stylochoplana sp*. collected from Pilot Bay, Tauranga, New Zealand and photographed in captivity. Bar = 2 mm. (Photo by Barry O'Brien).

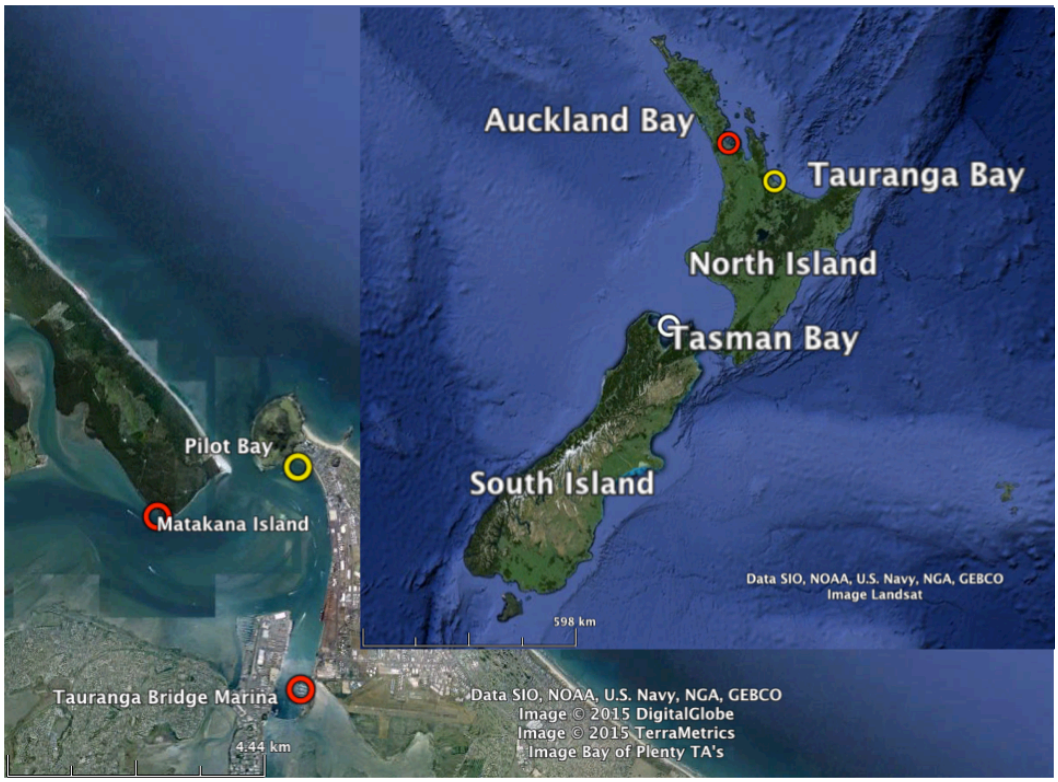

Figure 4. Map of New Zealand sampling sites for *Pleurobranchaea maculata* and *Stylochoplana* sp. (Google Earth). Larger map shows Taranga Bay in detail. Icons: white = non-toxic *P. maculata*, red = toxic *P. maculata*, yellow = toxic *P. maculata* and *Stylochoplana* sp.

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CHAPTER II. First identification of tetrodotoxin in the flatworm *Stylochoplana* sp.; a source of tetrodotoxin for *Pleurobranchaea maculata*?

Preface

This chapter describes the first detection of tetrodotoxin (TTX) in the flatworm *Stylochoplana* sp. from the Pilot Bay study site in Tauranga Harbor, New Zealand. Molecular techniques were employed to determine if *Pleurobranchaea maculata* preys on *Stylochoplana* sp., a potential source of TTX. As primary author I assisted with all collections and prepared all samples for TTX analysis. I also conducted all of the molecular analysis, unless otherwise stated, and wrote the manuscript. Susie Wood helped with designing the experiments, developing the real-time PCR assay, and editing of the manuscript. David Taylor assisted with species identification, Platyhelminthes ecology, and sampling. Paul McNabb conducted all liquid chromatography-mass spectrometry analysis. Craig Cary advised on experimental design, data analysis, and editing of the manuscript. Sanger sequencing was conducted by Waikato DNA Sequencing Facilities. All authors reviewed and edited manuscript content. This thesis chapter has been published in Toxicon with the following citation:

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ABSTRACT

High concentrations of the neurotoxin tetrodotoxin (TTX) were detected by liquid chromatography-mass spectrometry (LC-MS) in the Platyhelminthes *Stylochoplana* sp. from Pilot Bay (Tauranga, New Zealand). This is the first detection of TTX in this genus. Concentrations were monitored from March to November (2013) and found to significantly decrease from a peak in July (avg. 551 mg kg^{-1}) to November (avg. 140 mg kg^{-1}). *Stylochoplana* sp. co-occurred with TTX-containing *Pleurobranchaea maculata* (Opisthobranchia). A *Stylochoplana* sp.-specific real-time PCR assay was developed targeting the mitochondrial cytochrome c oxidase subunit *I* (CO*I*) gene to determine if *P. maculata* consumed *Stylochoplana* sp.. Positive *Stylochoplana* sp. signals were obtained for 7 of 19 *P. maculata* tested. Mass calculations suggest that *Stylochoplana* sp. could supply Pilot Bay *P. maculata* with the TTX required to account for the concentrations reported in previous studies (ca. 1.04 mg TTX per individual) based on an ingestion rate of one individual every 2 – 3 days throughout their lifetime. However, due to the lack of *Stylochoplana* sp. in other areas with dense *P. maculata* populations, and high concentration (up to ca. 1420 mg kg^{-1}) of TTX detected in some individuals, it is unlikely that *Stylochoplana* sp. represent the sole source of TTX in *P. maculata*.

INTRODUCTION

Tetrodotoxin (TTX) is an extremely potent neurotoxin that selectively targets voltage gated sodium channels by blocking the exchange of ions across cellular membranes (Narahashi et al. 1967; Narahashi 2001). Doses as low at 1 – 2 mg can be fatal to humans, leading to the rapid onset of paralysis often with fatal consequences (Noguchi & Arakawa 2008). Due to the popularity of TTX-containing puffer fish as a delicacy in many Asian countries, multiple deaths are still attributed to TTX intoxication each year (Hwang & Noguchi 2007; Arakawa et al. 2010). Prior to 1964, TTX had only been identified in puffer fish. Since then it has also been found in a range of marine and terrestrial organisms including: molluscs, diatoms, newts, frogs, octopuses, gastropods, flatworms (see the following for a review: Chau et al. 2011; Hanifin 2010; Miyazawa & Noguchi 2001; Noguchi & Arakawa 2008) and recently the opisthobranch *Pleurobranchaea maculata* (McNabb et al. 2010). The widespread occurrence of TTX across so many phylogenetically diverse organisms has led researchers to hypothesize a bacterial origin for TTX, either from symbiotic relationships with hosts or due to bioaccumulation through the food chain (Lin & Hwang 2001; Noguchi et al. 2006a, b). In contrast, studies on terrestrial newts (*Taricha granulosa*) have found that TTX concentrations increase in captivity when fed a TTX-free diet (Hanifin et al. 2002). The newts also have the ability to regenerate TTX after the release of toxin, suggesting an endogenous source (Cardall et al. 2004). However, studies to date have been unable to conclusively determine the source of TTX in any organism. This has been complicated by the difficulties in describing its biochemical pathway, preventing typical isotopic feeding studies, and the inconsistencies in methodologies used to detect TTX (Matsumura 2001; Chau et al. 2011).

In 2009, the presence of TTX in New Zealand was discovered when extensive surveys of beaches in Auckland were conducted due to a series of dog illnesses and deaths. Tetrodotoxin was detected in *P. maculata* at concentrations ranging from 91 to 850 mg kg^{-1} when measured by liquid chromatography–mass spectrophotometry (LC-MS) (McNabb et al. 2010). It was concluded that the affected dogs had come in contact with beach-cast *P. maculata*. Since then, populations of *P. maculata* from various sites on both the North and South Island have been screened for the presence of TTX with distinct spatial and temporal

patterns revealed (Wood et al. 2012a). These studies detected no TTX in specimens collected from two sites in the South Island, in comparison to significantly varied concentrations in North Island specimens. Seasonal analysis revealed an increase in TTX concentrations during the egg laying period (June to August) and aquarium studies have shown a strong correlation between TTX concentrations in the first laid egg mass and total TTX in the corresponding adult (Wood et al. 2012a, b). Collectively these studies suggest that adults invest TTX into their offspring, presumably to function as a chemical defense.

The origin of TTX in *P. maculata* has recently been explored. Khor et al. (2013) investigated the ability of *P. maculata* to obtain TTX through their diet. Non-toxic specimens sourced from the South Island were maintained in aquariums, fed a TTX-containing diet, and harvested at different time points over 39 days. Using LC-MS they detected TTX in all tissue types tested after just one hour post consumption. All specimens survived and continued to sequester TTX throughout the experiment without any observable negative effects to their health. As part of this study two extensive environmental surveys were conducted at sites (Narrow Neck Beach and Illiomama Rock, Auckland) known to contain high densities of toxic *P. maculata* (Taylor et al. 2011; Wood et al. 2012a). Only very low TTX concentrations were detected in a sand dollar (0.25 mg kg^{-1}; *Arachnoides zelandiae*) and trace levels of TTX in the mollusc *Turbo smaragdus*, crab *Macrophthalmus hirtipes,* and coralline turf algae *Corallina officinalis*. Thus it was deemed highly unlikely that these organisms were the source of TTX in *P. maculata*. The authors acknowledged that a TTX-containing food source could have been overlooked during the surveys. However, based on the short life span (<1 year), the high concentrations of TTX, and abundance of *P. maculata* at these sites, they suggested that it was unlikely that diet is the only source of TTX for this organism.

While sampling *P. maculata* populations at Pilot Bay, Tauranga (North Island) we noticed a high abundance of a small flatworm, *Stylochoplana* sp. (De et al. 2009), occupying the shallow subtidal area where *P. maculata* occur in high abundance. Due to the similarity of habitat, the potential for these species to consume comparable benthic organisms, and that previous studies have reported TTX in other flatworm species (Jeon et al. 1986; Miyazawa et al. 1986, 1987;

Stokes et al. 2014), samples were collected and tested for TTX. An initial collection of five individuals (7 June 2012) were tested and found to contain significant concentrations of TTX (38 – 155 mg kg^{-1} freeze dried weight). We describe the identification and quantification of TTX in *Stylochoplana* sp. using LC-MS. Selected samples were also screened using a recently developed method to detect the carbon backbone (C9 base - 2-amino-6-(hydroxymethyl)quinazolin-8-ol) of TTX (McNabb et al. 2014), thus potentially detecting precursors or degradation products of TTX (see Appendix 1). An analysis of the variability of TTX across nine months, and molecular characterisation of the 18S rRNA and mitochondrial cytochrome c oxidase subunit I (CO*I*) genes of *Stylochoplana* sp. were also undertaken. To investigate the possibility that *Stylochoplana* sp. may be a source of TTX for *P. maculata*, a *Stylochoplana* sp.-specific real-time PCR assay was developed and used to examine the foregut contents of 19 *P. maculata*.

MATERIALS AND METHODS

Specimen collection

Three to six *Stylochoplana* sp. were collected by scuba divers from Pilot Bay (37°63'5"S, 176°17'6"E), Tauranga, New Zealand approximately once a month between March and November of 2013. Each flatworm was placed in a separate plastic bottle with seawater (100 mL) and transported to the laboratory in an insulated container. Specimens were carefully transferred using a small brush, weighed and frozen (-20 °C) before analysis. *Pleurobranchaea maculata* (19) were collected by scuba divers from several sites in Tauranga Bay, New Zealand between September 2012 and March 2014. Sites included Pilot Bay, Matakana Island (37°64'S, 176°1'E), and Tauranga Bridge Marina (37°67'S, 176°19'E). Specimens were transported back to the laboratory in chilled seawater (300 mL) and either dissected immediately, or frozen (-20 °C) and dissected at a later date. To determine if the real-time PCR assay could detect *Stylochoplana* sp. in the *P. maculata* foregut matrix, a *P. maculata* was fed a live *Stylochoplana* sp. and the foregut was sampled 30 min after feeding. Prior to sacrifice, a small section was cut from the anterior end of the *Stylochoplana* sp. and extracted for TTX analysis. Foregut contents from all individuals were aseptically removed and DNA extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA) according to the manufacturer's instructions. Small portions (ca. 250

mg) of digestive tissue were removed from each *P. maculata* specimen and analysed for TTX.

DNA Extraction and Molecular Analysis of Platyhelminthes

DNA was extracted from ca. 0.25 mg of *Stylochoplana* sp. (n=2) using the E.Z.N.A. Mollusc DNA Kit (Omega, USA) according to the manufacturer's protocol. PCR reactions were performed in 25 μL volumes with the reaction mixture containing: 10× PCR buffer (Invitrogen, USA), 1.5 mM $MgCl_2$ (Invitrogen, USA), 0.02 mg mL^{-1} bovine serum albumin (BSA, Sigma, USA), 0.2mM (each) de-oxynucleoside triphosphate (Bioline, UK), 0.5 μM (each) primer (IDT, USA), 0.04 U of Platinum *Taq* DNA polymerase (Invitrogen, USA), and 10-20 ng of template DNA.

The PCR protocol for the 18S rRNA primers (18S_1F/18S_701R; Pochon et al. 2013) employed the following conditions: 3 min at 95 °C, 20 cycles of 94 °C for 30 s, 62 °C for 30 s (decreased by 0.5 °C at each cycle), 72 °C for 1 min, and followed by 12 additional cycles with an annealing temperature set at 52 °C, and a final extension of 72 °C for 7 min. For the CO*I* primers (jgLCO1490/jgHCO2198, Geller et al. 2013) the PCR reaction mixture was held at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and with a final extension of 72 °C for 7 min.

PCR reactions were run on an iCycler thermal cycler (Biorad, USA). PCR products were visualized on 1% agarose gel and then purified using a QuickClean II PCR Extraction Kit (Genescript, USA). Bi-directional sequencing was undertaken using the BigDye Terminator v3.1 Cycle Sequencing Kit on a ABI 3130x1 Genetic Sequencer (Applied Biosystems, USA). Sequences were compared with submissions from the NCBI GenBank database using MegaBlast (18S rRNA gene; Benson et al. 2012) and the publically available databases using the BOLD Identification System (COI; Ratnasingham & Hebert 2007). Sequences obtained in this study were deposited in the NCBI GenBank database under accession numbers KP259873 and KP259874.

Toxin Analysis

Sub-samples (ca. 0.25 g) of *Stylochoplana* sp. tissue and *P. maculata* digestive tissue were extracted with 2.25 mL of Milli-Q containing 0.1% v/v acetic acid or a *pro rata* volume if the starting mass was less than 0.25 g. Each sample was manually homogenized with a glass pestle and vortexed to ensure complete disruption of tissues. Samples were centrifuged (3000 × *g*, 10 min) and an aliquot of the supernatant (100 µL) added to 900 µL of 100% methanol containing 0.1% v/v acetic acid and frozen (-20 °C) for at least 1 hr. Samples were centrifuged (3000 × *g,* 10 min) and diluted 1:4 or 1:200 with 100% methanol containing 0.1% v/v acetic acid. Samples were analysed for TTX and all known analogues using LC-MS as described in McNabb et al. (2010) (see Appendix 2).

Three additional *Stylochoplana* sp. specimens were collected on 25 October 2013 from Pilot Bay. Specimens were pooled, and tissues were homogenized and centrifuged (3000 × *g*, 10 min). An aliquot of the supernatant (100 µL) was extracted and tested for TTX as described above. An additional extraction was undertaken on this pooled sample where an aliquot (100 µL) of the supernatant was dehydrated with 10M NaOH (11 µL, 1M final concentration). Samples were then boiled (45 min), cooled, and neutralized (pH 4 – 6) with acetic acid and solid phase extraction undertaken (Phenomonex Strata X - 60 mg/3 mL). Samples were eluted using 20% methanol/1% formic acid and analysed for the C9 base (2-amino-6-(hydroxymethyl)quinazolin-8-ol) using the methods described in McNabb et al. (2014) (see Appendix 1). This method detects TTX precursor or degradation products that form the C9 base (2-amino-6-(hydroxymethyl)quinazolin-8-ol) of TTX under the reaction conditions described. The method will not detect all potential molecules related to TTX and will exclude some newly discovered analogues (Kudo et al. 2014), however as the C9 base reaction is the basis of HPLC detection this method will at least detect anything previously assigned to TTX by HPLC.

Real-Time PCR Analyses for Inhibition in DNA Samples

Pleurobranchaea maculata foregut DNA samples were initially tested for PCR inhibition utilizing the exogenous positive control salmon DNA assay as described in Haugland et al. (2005). Briefly, reactions (12.5 µL) were prepared with the following components: 2× Rotor-Gene Probe PCR master mix (6.25 µL,

Qiagen), BSA (0.2 µg, Sigma, USA), primers targeting the internal transcribed spacer region 2 of the rRNA gene operon of chum salmon, *Oncorhynchus keta* (0.4 µM; Sketa F2 and Sketa R3, IDT DNA, USA), TaqMan probe (SketaP2) synthesized with a CAL fluor red 610 reporter dye at the 5′-end and a Black Hole Quencher 2 at the 3′-end (0.2 µM, Biosearch Technologies, USA), salmon testes DNA (15 ng, Sigma, USA), and 1 µL of sample DNA (ca. 10 ng). Reactions were analysed on a Rotor-Gene 6000 real-time rotary analyser (Qiagen, Netherlands) with the following cycling conditions: 95 °C for 3 min, 50 cycles of 95 °C for 3 s and 57 °C for 10 s. Samples were run in triplicate and inhibition was inferred when standard deviation of cycle threshold (C_t) values were more than 0.5 units different than positive controls. In the case of inhibition, template DNA was diluted and re-analysed.

Primer Design and Optimization for Real-Time PCR Analyses

Primers sites for detection of *Stylochoplana* sp. using real-time PCR were identified using a multiple sequence alignment (ClustalW; Thompson et al., 1994) of a ca. 650 bp region of the CO*I* gene from 221 closely related marine organisms obtained from GenBank (www.ncbi.nlm.nih.gov). Primers were designed (PrimerQuest, IDT) to amplify a 92 bp region of the *Stylochoplana* sp. CO*I* gene: Stylo 333F: 5′-CCCTCCGTTGTCTGGAAATATAG for the forward primer and Stylo 425R: 5′-CTAAGATAGATCTAACACCGGCTAAA for the reverse primer. Primer sequences were checked *in silico* for potential cross-reactivity in GenBank using the BLAST online software (http://blast.ncbi.nlm.gov/Blast.cgi) and then synthesized by IDT DNA (USA). Primers were optimized for use with the Rotor-Gene 6000 real-time rotary analyser (Qiagen, Netherlands) using *Stylochoplana* sp. genomic DNA and the limits of detection determined using serially diluted DNA (1 fg – 10 ng). The optimized assay consisted of a 20 µL reaction including: 10 µL of PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, USA), 1µM of each primer, and 10 ng of DNA template. Cycling parameters were 2 min at 95 °C followed by 40 cycles of 95 °C for 15s and 60 °C for 30 s, followed by a melt step from 50-90 °C rising by 0.5 °C. Reactions were run in triplicate and no-template controls were included in all runs. The threshold for positive samples was set at 0.001 pg. Positive real-time PCR triplicate reactions from *P. maculata* foregut DNA samples were pooled and sent to Waikato DNA Sequencing Facility for bi-direction sequencing using the Stylo

333F and 425F primers. Briefly, samples were prepared using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA) according to the manufactures instructions and sequences were resolved on an ABI 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA) fitted with 50 cm capillary arrays.

Statistical Analysis

Statistical analyses were conducted on temporal TTX concentrations in *Stylochoplana* sp. using R (http://www.r-project.org/; Team 2013). A distance based permutational ANOVA was used to assess differences in TTX concentrations across time using 4,999 permutations. This method is preferable to traditional ANOVA, because permutational ANOVA calculates P-values using permutations, rather than relying on tabled P-values, which has stricter assumptions in terms of normality and heterogeneity of variances (Anderson 2001a, b). A Posteriori pair-wise t-test comparison with 999 permutations were undertaken to investigate where significant differences between time points occurred. A linear regression was used to compare mass and TTX concentration of *Stylochoplana* sp. specimens collected over the 9 month period.

RESULTS

Polyclad flatworms from New Zealand waters, including *Stylochoplana* sp., are poorly described, with the last taxonomic study conducted in the late 1800s (Cheeseman 1983; De et al. 2009). *Stylochoplana* sp. in this study were usually no more than 1 cm in length (Fig. 1). Specimens were primarily found on *Ulva lactuca* (sea lettuce) and occasionally on the shallow sandy bottom at Pilot Bay. They were characterized by their translucent body with grey and white specks, two nuchal tentacles with eyes clustered at the base, and a ruffled pharynx (Morton & Miller 1973; De et al. 2009).

Partial sequences of the 18S rRNA gene (473 bp) and of the CO*I* gene (677 bp) were obtained. Using MegaBlast and the BOLD Identification System the highest sequence similarities to these gene segments were: *Paraplanocera oligoglena* (Platyhelminthes; KC869796, 99% similarity) for the 18S rRNA gene segment, and *Pseudostylochus intermedius* (Platyhelminthes; AB049114, 78.5% similarity) for the CO*I* gene segment.

Tetrodotoxin was confirmed by LC-MS analysis in all *Stylochoplana* sp. specimens (Fig. 2 and 3), and no other TTX analogs were identified. The pooled (n=3) *Stylochoplana* sp. sample contained 640 mg kg^{-1} of TTX, and 3100 mg kg^{-1} of the C9 base, indicating the presence of possible TTX precursors or degradation products.

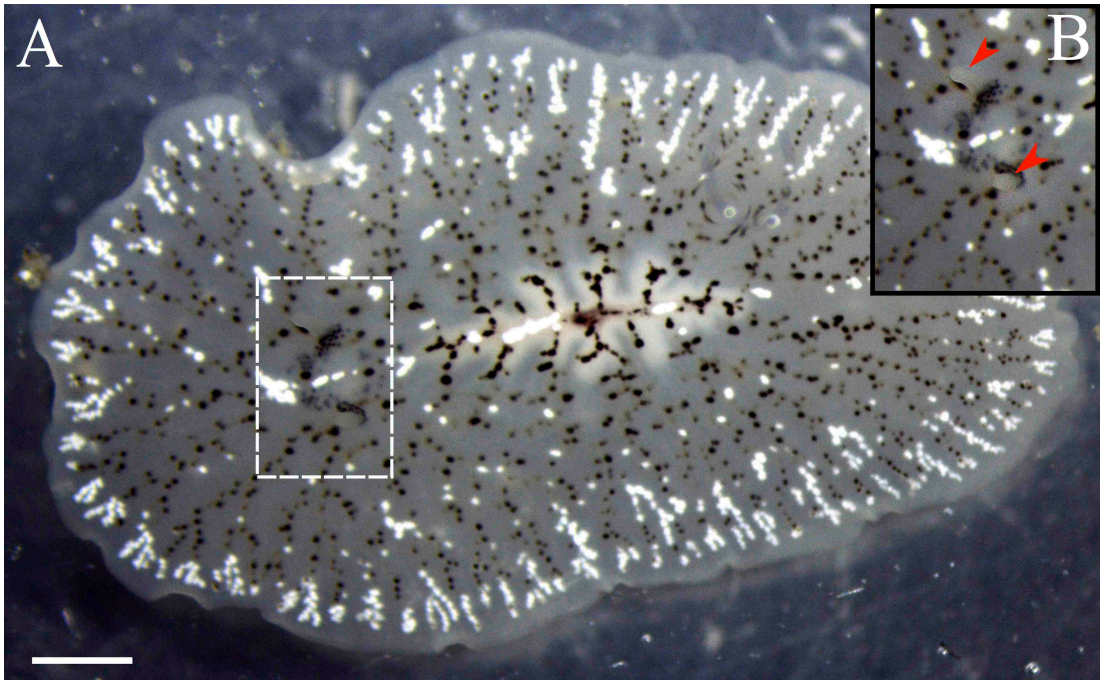

Fig. 1. (A) *Stylochoplana sp.* collected from Pilot Bay, Tauranga, New Zealand (B) Detailed view of anterior region (white box) highlighting nuchal tentacles. Bar = 0.2 cm.

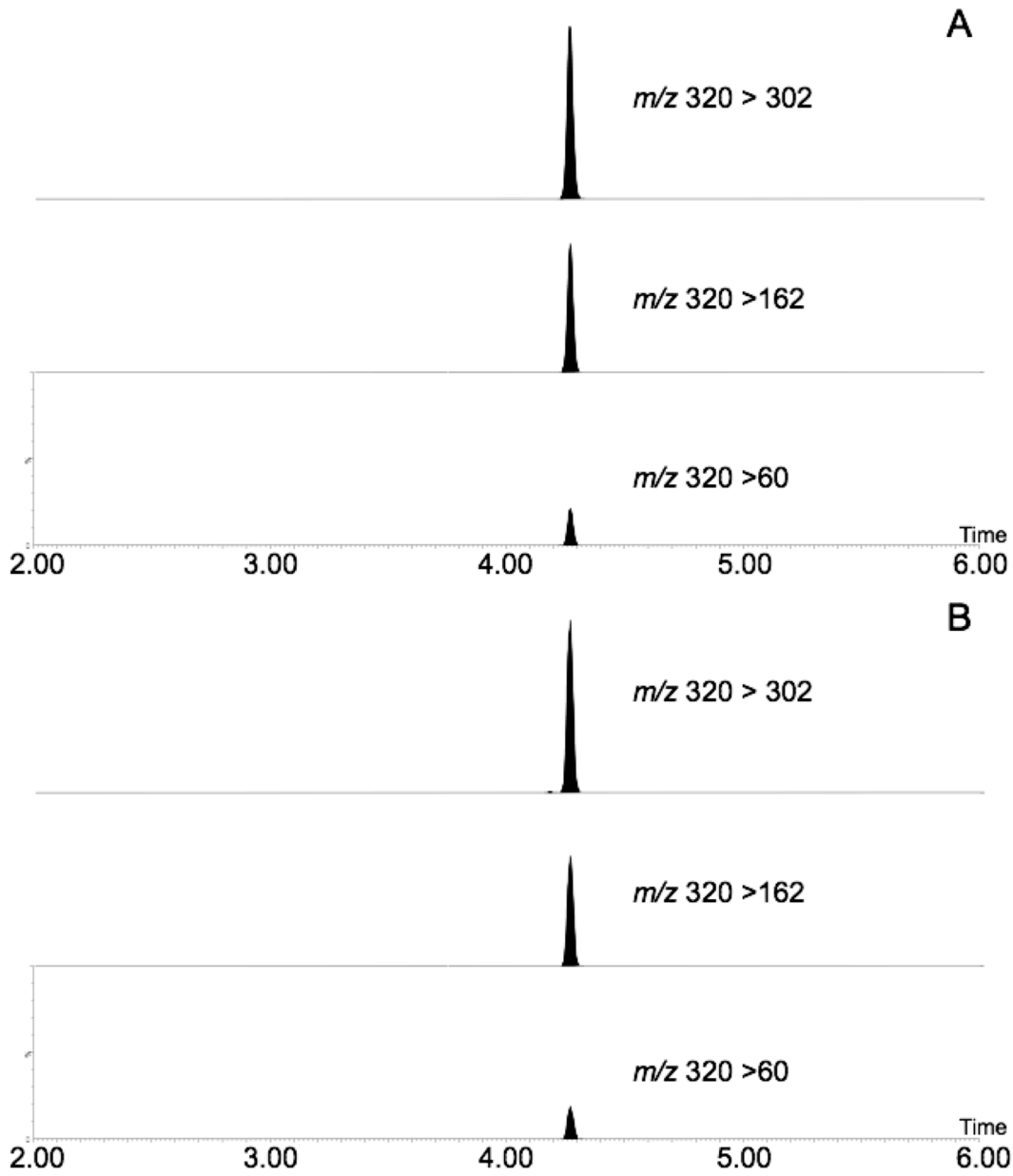

Fig. 2. Liquid chromatography-mass spectrometry chromatogram of (A) *Stylochoplana* sp. and (B) authentic TTX standard at 10 ng mL^{-1}. Retention times (4.27 min) were identical and ion ratios for the samples matched the standards within 5%.

In general TTX concentrations declined in the *Stylochoplana* sp. sampled between August to November 2013 (Fig. 3), ranging from an average of 460 mg kg^{-1} of all individuals collected from March to July to an average of 260 mg kg^{-1} from all individuals from August to November. These differences were found to be statistically significant ($F = 3.01$, $df = 9$, $P = 0.01$). The pairwise analysis showed that samples collected in June were significantly different from those collected in September, October, and November, and the July samples were significantly different from those collected in November ($P < 0.01$). Within sampling periods there was considerable variability in TTX concentrations among individuals. This was most pronounced in early March, where the minimum concentration was 0.22 mg kg^{-1} and the maximum 814 mg kg^{-1} (Fig. 3), a 3700 fold-difference.

There was no relationship between concentrations of TTX in *Stylochoplana* sp. and their body mass over the 9 month period ($R^2 = 0.044$, Fig. 4). No significant correlation was seen when data was separated by month (data not shown). The highest concentrations (1070 mg kg^{-1}) were measured in an individual that weighted 66 mg, but low concentrations (105 mg kg^{-1}) were measured in a *Stylochoplana* of similar body mass (75 mg, Fig. 4).

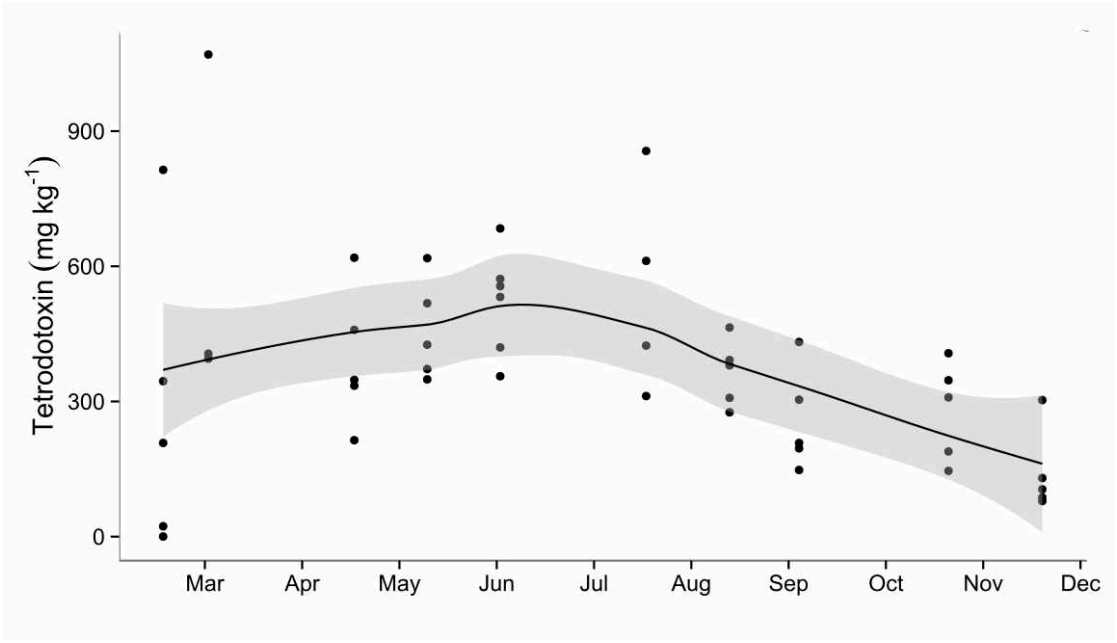

Fig. 3. Tetrodotoxin (TTX) concentrations in *Stylochoplana* sp. collected between March and November 2013 from Pilot Bay (Tauranga, New Zealand). The black line is a loess smoothed local polynomial regression and grey shading represents one standard error.

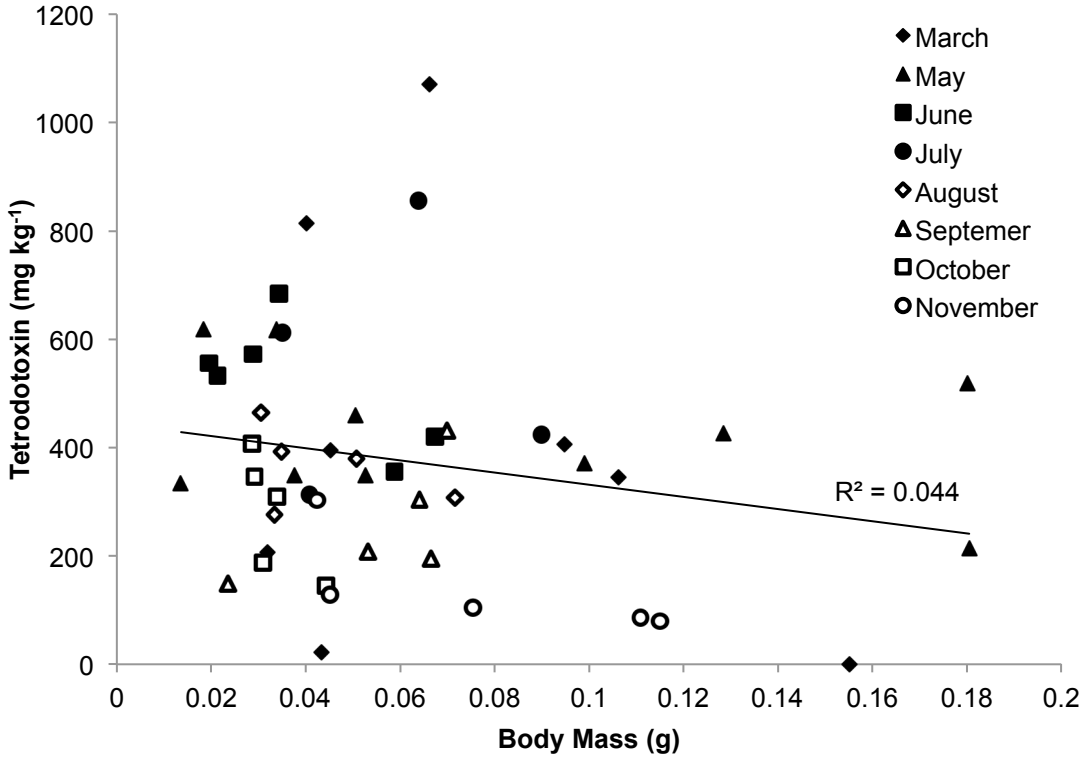

Fig. 4. Tetrodotoxin concentrations in individual *Stylochoplana* sp. relative to body mass. *Stylochoplana* sp. were collected from Pilot Bay (Tauranga, New Zealand) between March and November 2013.

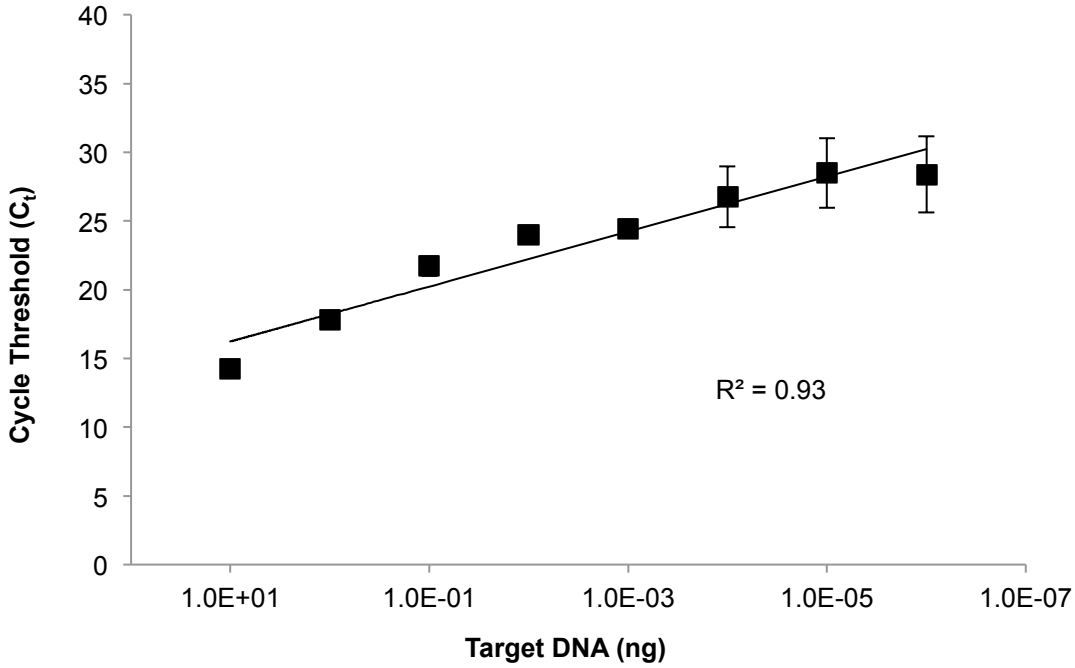

Fig. 5. Sensitivity of the *Stylochoplana* sp. real-time PCR assay. Dilutions were made from genomic DNA and mean values of cycle threshold (C_t) plotted against $\log_{10}$-transformed DNA concentrations. Results are averages of triplicate samples, error bars = 1 std.

Real-time PCR primers specific for, and amplifying a 92 bp region of, the *Stylochoplana* sp. CO*I* gene were designed and tested. The *in silico* analysis using NCBI BLAST showed that primers did not match any other related marine organisms (such as annelids, molluscs, echinoderms, and ascidians). The assay had a linear dynamic range of detection over at least eight orders of magnitude with concentrations of template DNA ranging from 1 fg to 10 ng (Fig. 5). The amplification efficiency of the assay was 0.93 and there was little variation among replicates (Fig. 5). The melt curve temperature for *Stylochoplana* sp. was 77.2 °C (± 0.14 °C).

Fifteen of the 19 samples from the digestive tissue of *P. maculata* contained TTX (Table 1). Seven of these samples were positive for the *Stylochoplana* sp. CO*I* gene using the real-time PCR assay (Table 1). There were no correlations between TTX concentrations of *P. maculata* tissue and the presence of the *Stylochoplana* sp. CO*I* gene. Real-time PCR analysis of the *P. maculata* individual (digestive tissue, 1780 mg kg^{-1} TTX) fed a *Stylochoplana* sp. (323 mg kg^{-1} TTX) also gave a positive detection. The sequenced products of all positive real-time PCR products were identical to the corresponding region in the *Stylochoplana* sp. CO*I* sequence.

Table 1. Tetrodotoxin (TTX) concentrations in digestive tissue from *Pleurobranchaea maculata* collected between September 2012 and March 2014, and results of *Stylochoplana* sp. real-time PCR assay. P.B. = Pilot Bay, M.I. = Matakana Island, T.B. = Tauranga Bridge Marina. ND not detected. *not all three reactions showed a positive result.

Date	Location	(TTX mg kg^{-1})	Real-Time PCR
Oct 27, 2012	P.B.	ND	-
	P.B.	46.6	-
	P.B.	ND	-
	P.B.	ND	-
Mar 13, 2013	M.I	35.1	-
May 28, 2013	M.I	4.0	-
	M.I	3.7	-
Sept 2, 2013	P.B.	ND	+
	P.B.	0.2	-
Sept 12, 2013	M.I	1.8	-
	M.I	0.2	-
Mar 21, 2014	T.B.	24.8	+
	T.B.	30.6	-
	T.B.	41.8	+*
	T.B.	23.0	+
	T.B.	8.6	+*
	T.B.	100	-
	T.B.	10.5	+
	P.B.	1680	+

DISCUSSION

This is the first report of TTX in *Stylochoplana* sp. (Phylum: Platyhelminthes; Class: Turbellaria; Family: Leptoplanidae). Concentrations of TTX ranged from 0.22 to 1070 mg kg^{-1} with a combined average across this study (n = 48) of 380 mg kg^{-1}. Tetrodotoxin was identified for the first time in New Zealand in *P. maculata* in 2009, and very high concentrations (max. ca. 1420 mg kg^{-1}) were found in some populations (McNabb et al. 2010; Wood et al. 2012b). Since then low concentrations of TTX have been found in two other New Zealand organisms: the sand dollar *Arachnoides zelandiae* (0.25 mg kg^{-1}) and the bivalve *Paphies australis* (0.8 mg kg^{-1}) (Khor et al. 2013; McNabb et al. 2014). Trace levels have also been identified in the mollusc *Turbo smaragdus*, crab *Macrophthalmus hirtipes,* and coralline turf algae *Corallina officinalis* (Khor et al. 2013). It is plausible that this recent spate in identification of TTX-containing organisms in New Zealand is due to a southern spread of a TTX-containing microorganism, a trend now documented for multiple marine microalgae and potentially linked to global warming (Nagai et al. 2011). However, it is more likely that these new identifications are due to concerted sampling efforts, including extensive benthic surveys, to identify the source of TTX in *P. maculata* and significant advances in the sensitivity and specificity of methods used to detect TTX i.e., LC-MS (Khor et al. 2013; McNabb et al. 2014).

Tetrodotoxin has been identified in Platyhelminthes previously (Miyazawa et al. 1986, 1987; Tanu et al. 2004; Ritson-Williams et al. 2006; Stokes et al. 2014), and in species from several other similar phyla including free-living marine nematodes (Kogure et al. 1996) and nemerteans (Ali et al. 1990; Kajihara et al. 2013). At this time TTX has not been reported from any other *Stylochoplana* sp. Assuming 10,000 mouse units (MU) is equivalent to 2 mg TTX (Noguchi & Ebesu 2001), the average concentrations of TTX in the *Stylochoplana* sp. described in this study are within the same range as those reported from other flatworms, for example *Planocera multientaculata* at 60 mg kg^{-1} (Miyazawa et al. 1986) and a *Planocerid* sp. max. ca. 1000 mg kg^{-1} in the pharynx (Ritson-Williams et al. 2006). The TTX concentrations of *Stylochoplana* sp. in this study are very similar to those found in upper North Island *P. maculata* populations (Wood et al. 2012a), potentially indicating a geographic influence on TTX concentrations in these organisms. To date, only one population of *Stylochoplana*

sp. has been monitored for TTX, and concentrations from other populations around New Zealand are required to confirm this hypothesis.

Tetrodotoxin concentrations in *Stylochoplana* sp. were tested over a nine-month period in 2013. Significant differences in TTX concentrations were measured among samples collected from June and July compared to those collected in November ($P < 0.01$). Average concentrations were higher (460 mg kg^{-1}) between March and July, before decreasing from August to November (260 mg kg^{-1}). Similar seasonal trends were observed in *P. maculata* and the decrease was correlated with the onset of the egg-laying season (Wood et al. 2012a). Although little is known about the reproductive cycle of *Stylochoplana* sp. a preliminary captivity study was undertaken with individuals collected from Pilot Bay on 7 June 2012 (data not shown). One individual laid eggs two weeks after capture, suggesting that the start of the egg laying season may also correspond to the peak TTX concentrations observed in *Stylochoplana* sp.. Seasonal differences in TTX concentrations have also been observed in ribbon worms (Cephalothrix sp.; Asakawa et al. 2003). Toxicity concentrations ranged from 420 to 656 mg kg^{-1} in specimens collected from 10 samples, each consisting of 10 worms, from November to March. Average TTX concentrations then sharply decreased to 199 mg kg^{-1} in April (n=10), before reaching a maximum of 1169 mg kg^{-1} in June (n=10). Seasonal fluctuations in TTX concentrations in the pufferfish *Takifugu poecilonotus* have been linked to different sexual maturity stages (Ikeda et al. 2010). Toxicity levels were highest in the female ovaries during the "maturation period" from December to March, but were highest in the liver during the "ordinary period" (just after spawning). Tetrodotoxin concentrations were high in male and females during the year, but declined sharply in both sexes after the April spawning season.

During the preliminary captive study of *Stylochoplana* sp., TTX was detected in the egg masses and concentrations were higher than in adults suggesting they invest TTX into their offspring, presumably to function as a chemical defense. The same trend was observed in *P. maculata* (Wood et al. 2012b), and the terrestrial flat worms *Bipalium adventitium* and *Bipalium kewense* (Stokes et al. 2014). Miyazawa et al. (1987) also suggested a protective role of TTX in the flatworm *Planocera multitentaculata*. Secreted mucus was found to

contain TTX, and extremely high concentrations were found in the oviduct and egg masses. Similarly, Ritson-Williams et al. (2006) found high concentrations of TTX in the egg masses of a *Planocerid* sp. from Guam. However, they argue that if TTX was utilized as a defence mechanism, higher concentrations should have been distributed throughout the body. They presented live *Planocerid* sp. to an assemblage of reef fish *in situ*, and found that the fish ate the toxic flatworms, suggesting that TTX was not a deterrent compound. Further characterization of TTX distribution in the tissues of the *Planocerid* sp. showed high concentrations of the TTX analog, 11-nortetrodotoxin-6(S)-ol, in the pharynx suggesting that the toxin is more likely utilized for capturing prey. Tetrodotoxin has also been implicated in the capture of planktonic prey by the flatworms *Mesostoma* cf. *lingua* (Dumont & Carels 1987) and *Stenostomum* cf. *leucops* (Nandini et al. 2011) and in the subduing of earthworms by the terrestrial flatworms *B. adventitium* and *B. kewense* (Stokes et al. 2014). Further studies exploring the fine scale distribution of TTX at the cellular level in *Stylochoplana* sp. are required to explore its ecological function in this organism.

In contrast to the variability in TTX observed in *P. maculata* populations (10 to 51 fold-differences; Wood et al. 2012a), the variability of TTX in *Stylochoplana* sp. within sampling periods was generally less than a 4 fold-difference. The one exception was the 13 March 2013 when a 3700 fold-difference among the highest and lowest individual was measured. The more consistent concentrations of TTX in *Stylochoplana* sp. potentially indicate that their source of TTX is continuous or more readily available than that of *P. maculata* despite occupying the same habitat. There are no direct observations of the eating habits of the New Zealand *Stylochoplana* sp., however Turbellarians in general are carnivorous, preying mostly on invertebrates with some also shown to ingest algae (Kozloff 1972; Reynoldson & Sefton 1976; Barnes 1987). The co-occurrence of TTX-containing *P. maculata* and *Stylochoplana* sp. at the Pilot Bay sites potentially suggests a dietary source of TTX.

The average amount of TTX per individual *Stylochoplana* sp. was ca. 23 µg in this study. If we assume that it takes ca. 6 months to reach 60 mg (ave weight of *Stylochoplana* sp. collected in this study) then an individual would need to consume 128 ng of TTX every day of their life, assuming there is no loss of

TTX overtime, to reach 23 μg of TTX. Yu et al. (2004) isolated the TTX producing *Microbacterium arabinogalactanolyticum* (ca. 0.53 pg TTX cell^{-1}) from the ovaries of *Takifugu niphobles*. If *Stylochoplana* sp. were to obtain their TTX from this strain they would have to ingest ca. 250,000 cells day^{-1}. Based on the cell dimensions provided in Yu et al. (2004) the volume of one cell is ca. 0.5 μm^3. If we conservatively presume that 1 μm^3 of bacterial cell equals 2 pg (i.e., twice the weight of water), then one cell equates to a mass of 1 pg. *Stylochoplana* would thus be ingesting 2.5×10^{-4} mg of bacteria per day, or ca. 0.0004% of their own body weight, suggesting this is a plausible source of TTX. However, it is highly unlikely that half of each cell is comprised of TTX, and we suggest that limitations in the specificity of the mouse bioassay may have resulted in a marked over estimation of TTX concentrations produced by this strain. In contrast Wang et al. (2008) used an enzyme-linked immunosorbent assay (ELISA) to measure TTX concentrations in a *Vibrio* sp. strain (max. 184 ng TTX g^{-1} cells). Based on these values, *Stylochoplana* sp. would need to consume ca. 0.7 g per day of this bacterium, clearly not possible for these small organisms. Another possibility is that bacteria living symbiotically within *Stylochoplana* sp. continually produced TTX or precursor molecules. Further investigation is required to explore this scenario.

No correlation between body mass and TTX concentrations was observed (Fig. 4). Studies on other marine organisms, such as the sea stars *Astropecten polyacanthus* and *A. scoparius* (Miyazawa et al. 1985), the ribbon worm *Cephalothrix* sp. (Asakawa et al. 2003), and *P. maculata* (Wood et al. 2012a) have also observed no correlation in mass and TTX concentrations in specimens collected at the same location and time. Variable concentrations are most likely due to a food source of TTX that is inconsistent or patchy in the environment.

We suggest that *Stylochoplana* sp. may be a source of TTX for *P. maculata* and an aquarium feeding experiment demonstrated that *P. maculata* readily consumed *Stylochoplana* sp. (Supplementary video). To investigate this a *Stylochoplana* sp.-specific real-time PCR assay targeting a segment of the CO*I* gene was developed and used to probe the foregut contents of 19 *P. maculata*, of which 7 were positive. One of the positive detections came from the foregut of a *P. maculata* sample which had no detectable TTX in its gut. It is possible that the

Stylochoplana sp. ingested was non-toxic or below the detection limits. Although there was no correlation between TTX concentrations and positive detections, a negative result does not necessarily signify that *P. maculata* have never ingested *Stylochoplana* sp., as the assay relies on DNA still being present in the foregut and therefore only represents a 'snap-shot' of their dietary habits. Nevertheless, the positive detection in multiple *P. maculata* suggests that for some populations *Stylochoplana* sp. may be an important source of food and TTX. However, based on the following calculations and assumptions it seems unlikely to be the sole source.

Eight *P. maculata* collected 8 June 2011 from Pilot Bay had average TTX concentrations of ca. 1.04 mg TTX per individual and weights of 11.6 g (Wood et al. 2012a). Growth of one individual in captivity monitored from birth was 20 g in 175 days (Wood et al. 2012a). If it were assumed that *P. maculata* exhibit the same growth rate in the wild then 10.2 μg of TTX must be ingested per day. Therefore, if *P. maculata* were to ingest one *Stylochoplana* sp. individual (23 μg) every ca. 2 to 3 days for its entire life, this could possibly account for the total TTX measured in each adult. While this number is plausible, it seems unlikely that *P. maculata* would consume this quantity during their juvenile life stages suggesting that it is unlikely that they represent their sole TTX source.

Detection of C9 base was shown by LC-MS to be five times higher than TTX in the pooled *Stylochoplana* sp. sample (25 October 2013), possibly indicating marked quantities of TTX precursors or degradation products in their tissues. It is plausible that *P. maculata* are able to take a pre-cursor compound and synthesize it into TTX, thus explaining how some individuals can contain extremely high concentrations of TTX. Further research is required to investigate differences among C9 base and TTX concentrations. Characterisation of the compounds accounting for these differences may provide valuable insights into the biosynthesis of TTX.

In summary this study documents the first detection of TTX in the flatworm *Stylochoplana* sp. from New Zealand. The temporal data collected showed a significant difference in TTX concentrations among seasons and this, in concert with detection of TTX in egg masses, suggests that TTX may have a

protective function in this organism. We suggest that bacteria (or other micro-organisms) produce a precursor molecule that is converted to TTX within *Stylochoplana* sp., a theory supported by the difference in C9 base and TTX concentrations in the pooled *Stylochoplana* sp.. The presence of this as-yet unknown molecule could also explain why TTX has not been detected in the multiple environmental samples i.e., sediment and biofilm, we have collected to date (Khor et al. 2013). Using real-time PCR we demonstrated that *Stylochoplana* sp. are consumed by *P. maculata in situ*, and are one of the sources of TTX for this organism. This detection adds to the growing list of marine organisms now known to contain TTX. Because *Stylochoplana* sp. are relatively simple anatomically, occupy a low trophic level, and contain very high levels of TTX, they may prove to be a useful model organism for ongoing studies investigating the origin and function of TTX.

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CHAPTER III. No evidence for a culturable bacterial tetrodotoxin producer in *Pleurobranchaea maculata* (Gastropoda: Pleurobranchidae) and *Stylochoplana* sp. (Platyhelminthes: Polycladida)

Preface

This chapter describes the efforts to culture a tetrodotoxin-producing bacteria from the tissues of *Pleurobranchaea maculata* and *Stylochoplana* sp.. As primary author I was involved with all sampling of specimens as well as the laboratory-based work including culturing and sample preparation. I undertook the data analysis, and writing of the manuscript. Serena Khor (University of Waikato) and Susie Wood (Cawthron) provided assistance with the intensive bacterial isolation component of this study. Sanger sequencing was conducted by Waikato DNA Sequencing Facilities. Paul McNabb conducted all of the liquid chromatography-mass spectrometry analysis. Craig Cary advised on experimental design, data analysis, and editing of the manuscript. All authors reviewed and edited the manuscript. This thesis chapter has been published in Toxins with the following citation:

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ABSTRACT

Tetrodotoxin (TTX) is a potent neurotoxin found in the tissues of many taxonomically diverse organisms. Its origin has been the topic of much debate with suggestions including; endogenous production, acquisition through diet, and bacterial synthesis. Bacterial production of TTX has been reported in isolates from marine biota, but at lower than expected concentrations. In this study bacterial production of TTX in *Pleurobranchaea maculata* (Opisthobranchia) and *Stylochoplana* sp. (Platyhelminthes) was explored utilizing a recently developed sensitive method to detect the C9 base of TTX via liquid chromatography – mass spectrometry. To account for the possibility that TTX is produced by a consortium of bacteria, a series of experiments using marine broth spiked with various *P. maculata* tissues were undertaken. Bacterial strains were characterized by sequencing of a region of the 16S ribosomal RNA gene. Sixteen unique strains from *P. maculata* and one from *Stylochoplana* sp. were isolated, representing eight different genera; *Pseudomonadales*, *Actinomycetales*, *Oceanospirillales*, *Thiotrichales*, *Rhodobacterales*, *Sphingomonadales*, *Bacillales*, and *Vibrionales*. Molecular fingerprinting of bacterial communities from broth experiments showed little change over the first four days. No C9 base or TTX was detected in isolates or broth experiments (past day 0), suggesting the source of TTX in *P. maculata* and *Stylochoplana* sp. is unlikely the bacteria cultured here.

1. INTRODUCTION

Tetrodotoxin (TTX) is a small non-protein neurotoxin closely related to saxitoxin [1, 2]. It selectively targets voltage-gated sodium channels resulting in the inhibition of action potentials across neurons. Ingestion of quantities as little as 1-2 mg can be fatal to humans [3, 4]. Its highly selective nature has resulted in its frequent use in neurological medical studies, yet its biosynthetic pathway is still largely unknown [5, 6]. The name tetrodotoxin is derived from the *Tetrodontidae* order of pufferfish, in which TTX was first found. However, it has since been discovered globally in a wide range of organisms covering eight different phyla, excluding bacteria [5]. The source of TTX and its distribution among so many phylogenetically unrelated species remains a mystery. The most commonly cited hypothesis is that TTX has a bacterial origin (Table 1). In 1986 the first TTX-producing bacteria, a *Pseudomonas* species, was isolated from a red calcareous alga, *Jania* sp. [7]. Tetrodotoxin and the TTX analogue anhydro-tetrodotoxin were detected via high performance liquid chromatography (HPLC) and mouse bioassay [7].

Tetrodotoxin producing bacteria representing 22 genera have since been isolated from a range of host organisms including; puffer fish, octopi, sea stars, reef crabs, sea urchins, sea snails, gastropods, worms, and algae [5, 8, 9]. A summary of the bacterial genera, the concentrations of TTX they produce, the method of detection, and the organisms they were isolated from is provided in Table 1. The most common method of bacterial isolation among these studies involves homogenization of the host organism tissue followed by plating of aliquots onto non-selective medium. Individual bacterial strains are then selected and cultured in liquid media before harvesting and testing for TTX via various methods including: mouse bioassay, enzyme-linked immunosorbent assay (ELISA), gas chromatography-mass spectrometry (GC-MS), and HPLC [10-15]. However, the TTX concentrations in these bacterial cultures are significantly lower than the amounts contained in host organisms leading to doubt that they are the definitive source of TTX [15-18]. For example, Wang et al. [14] reported TTX concentrations of max. 184 ng g^{-1} from an isolated *Vibrio* sp. in comparison to 36 μg g^{-1} tissue in the host sea snail *Nassarius semiplicatus*.

Matsumura [19] provided additional uncertainty by demonstrating that the culture media used to isolate the TTX producing bacteria could produce false positives for TTX when analyzed by HPLC and GC-MS. Of the numerous studies demonstrating bacterial TTX-production, to our knowledge only one [20] has used liquid chromatography-mass spectrometry (LC-MS) to confirm the presence of TTX (Table 1). The use of non-disputable chemical methods as a means of quantifying TTX in bacterial isolates would greatly assist in dispelling the controversy surrounding the bacterial origin of TTX.

Research on terrestrial TTX-containing organisms has found limited evidence to support exogenous sources of TTX and endogenous production is commonly postulated. Lehman et al. [21] were unable to PCR amplify 16S ribosomal RNA (rRNA) genes using bacterial specific primers from toxic tissues of the rough skinned newt (*Taricha granulosa*), including the liver, gonads, and skin. Positive amplification was obtained from intestines, however, TTX concentrations in these tissues were consistently low. Additionally, when *T. granulosa* were induced via electrical stimulus to excrete TTX through their skin, TTX concentrations were found to regenerate after nine months in captivity, despite being maintained on a TTX-free diet [22]. It was also shown that female newts continue to deposit TTX into eggs after three years in captivity [23] Collectively these studies indicate that symbiotic bacteria are unlikely to be the source of TTX in this species.

In 2009, populations of the opisthobranch *Pleurobranchaea maculata* (grey side-gilled sea slug; Family: Pleurobranchidae; [24]) from Auckland, New Zealand, were found to contain significant concentrations of TTX [24]. Located in shallow sub-tidal areas they are known to be opportunistic scavengers with diets including algae, mussels and anemone [25]. Recent studies have revealed distinct spatial patterns in TTX concentrations among populations with specimens from the South Island containing no detectable TTX [26]. It has also been suggested that the high concentrations of TTX measured in adults during the egg laying season (June-August) and in eggs and early larval stages suggests that *P. maculata* utilize TTX for protection and to increase survival rates of their progeny [26]. In 2013, high concentrations of TTX were detected in a Platyhelminthes *Stylochoplana* species from Pilot Bay (Tauranga, New Zealand), a site where

toxic *P. maculata* occur [27]. Similar seasonal trends were shown in the *Stylochoplana* sp. population and preliminary studies on TTX in egg masses suggest that the toxin could also play a protective role in this species. Salvitti et al. [27] used molecular techniques to probe the foregut contents of *P. maculata* and demonstrated that they consume *Stylochoplana* sp.. However, based on the concentrations of TTX in *Stylochoplana* sp. and *P. maculata*, and probable growth and consumption rates it is unlikely that they are their only supply of TTX. The co-occurrence of these species may indicate that they are both sourcing TTX from the same dietary source. A microbial origin (either dietary or endosymbotic) of TTX (or a precursor molecule) is logical possibility, given that extensive environmental surveys of hundreds of organisms at sites with dense populations of highly toxic *P. maculata* only detected trace (<0.1 mg kg^{-1}) quantities of TTX in a few organisms [28].

Chau et al. [29] recently isolated a limited number (16 isolates, 9 strains) of bacteria from adult *P. maculata* and found no evidence of TTX production. Multiple researchers have suggested that microbial organisms may produce a precursor molecule which is then converted to TTX through a yet-to be identified biochemical pathway within the host organisms [30, 31]. This could explain why TTX was not detected in *P. maculata* isolates previously and/or why only low concentrations have been shown to be produced by other bacteria. McNabb et al. [32] recently developed an LC-MS method to detect the carbon backbone of TTX. This method detects TTX precursor or degradation products that form the C9 base (2-amino-6-(hydroxymethyl)quinazolin-8-ol) of TTX under the reaction conditions described. The method will not detect all potential molecules related to TTX and will exclude some newly discovered analogues [33], however as the C9 base reaction is the basis of HPLC detection this method will at least detect anything previously assigned to TTX by HPLC. This is the first study to utilized this method to screen bacterial isolates for the C9 base of TTX.

The aim of this study was to utilize standard microbiological methods, similar to those used in previous studies, to attempt to isolate TTX-producing bacteria from *P. maculata* and *Stylochoplana* sp. [15, 30, 34]. In 2013, three *P. maculata* and three *Stylochoplana* sp. from Pilot Bay, New Zealand were collected and aseptically dissected. Over 100 bacterial strains were isolated on

three different media types under aerobic conditions. To determine the diversity of the strains a region of the 16S rRNA gene was PCR amplified and the products were analyzed by restriction digest analysis. Representatives of each unique banding pattern were sequenced, grown in batch culture and analyzed for the C9 base using LC-MS. Researchers have suggested that the significantly lower concentrations of TTX produced by isolates *in vitro* may be due to the lack of an 'inducer' provided by either the host organism or associated bacterial community [5, 35]. To explore the possibility that TTX is produced by a consortium of bacteria or influenced by host tissues, a series of broth experiments were also undertaken. These involved inoculating marine broth with subsamples organs/tissue from *P. maculata* and *Stylochoplana* sp. and tracking TTX concentrations over a series of days.

Table 1. Bacteria reported to produce tetrodotoxin or TTX like compounds.

Ref	Source	Toxicity of host species/tissue*	Bacteria	Toxicity (TTX or related substances)**	Detection method*
[9]	*Takifugu niphobles* (pufferfish)	intestines: N/A	*Raoultella terrigena*	4.3 μg L^{-1}	ELISA
[36]	*Fugu obscurus* (pufferfish)	liver: 80 MU g^{-1}	*Lysinibacillus fusiformis*	23.9 MU in 200 mL broth	mouse bioassay
[13]		ovary:125 MU g^{-1}	*Bacillus* sp.	+	HPLC, EMI-MS
[15]	*Takifugu obscurus* (pufferfish)	ovary: N/A	*Aeromonas* sp.	1.88 μg L^{-1} cultured bacteria	ELISA
[20]	*Arothron hispidus* (pufferfish)	1 μg g^{-1}	*Vibrio harveyi*	0.05 - 1.57 ug mL^{-1}	LC-MS
[14]	*Nassarius semiplicatus* (sea snail)	2×10^2 MU g^{-1} tissue (3.6 mg in 100 g tissue)	*Vibrio* spp.	11-184 ng g^{-1}	competitive ELISA
			Marinomonas spp.	85-98 ng g^{-1}	competitive ELISA
			Tenacibaculum spp.	54 ng g^{-1}	competitive ELISA
[8]	*Pseudocaligus fugu* (copepod)	N/A	*Roseobacter* sp.	+	HPLC, GC-MS, LC-MS
[37]	*Chelonodon patoca* (pufferfish)	skin: N/A	*Serratia marcescens*	+	HPLC
[17, 18]	*Fugu rubripes* (pufferfish)	ovary: 120 ± 6.2 MU g^{-1}	*Bacillus* spp.	0.1-1.6 MU g^{-1} cells	mouse bioassay
			Nocardiopsis dassonvillei	0.5 MU g^{-1} cells	mouse bioassay
			Actinomycete spp.	0.1-1.6 MU g^{-1} cells	mouse bioassay
[16]	*Takifugu alboplumbeus* (pufferfish)	intestines: 24.9 ± 24.2 MU g^{-1} [38]	*Vibrio* spp.	78.3 MU in 500 mL broth (4×10^7 cells)	mouse bioassay
	Takifugu niphobles (pufferfish)	ovary - 100-1000 MU g^{-1} [38]	*Microbacterium arabinogalactanolyticum*	105.3 MU in 500 mL broth (4×10^7 cells)	mouse bioassay
[30]	Seven species of nemertean worms	N/A	*Vibrio* spp.	+	HPLC
[12]	*Fugu vermicularis radialis* (pufferfish)	70 ± 8 MU g^{-1}	*Vibrio* spp.	+	HPLC
[39]	*Meoma ventricosa* (sea urchin)	N/A	*Pseudoalteromonas* spp.	+	immunoassay
[10]	*Niotha clathrata* (marine gastropod)	2-50 MU g^{-1}	*Vibrio* spp.	+	HPLC
			Pseudomonas spp.	+	HPLC
			Aeromonas spp.	+	HPLC
			Plesiomonas spp.	+	HPLC
[40]	Freshwater sediment	+ HPLC, GC-MS	*Micrococcus* spp.	+	HPLC
			Bacillus spp.	+	HPLC
			Caulobacter spp.	+	HPLC
			Flavobacterium spp.	+	HPLC
[41]	Marine sediment	+ HPLC, GC-MS	*Streptomyces* spp.	+	HPLC

[42]	Deep sea sediment	25-90 ng TTX equivalents g^{-1} of mud [43]	*Vibrio* spp.	+	HPLC
			Bacillus spp.	+	HPLC
			Acinetobacter spp.	+	HPLC
			Alteromonas spp.	+	HPLC
			Aeromonas spp.	+	HPLC
			Micrococcus spp.	+	HPLC
[44]	Four species of Chaetognaths (arrowworms)	320 pg $individual^{-1}$ [45]	*Vibrio* spp.	280 - 790 pg μL^{-1} culture medium	cell culture bioassay
[11]	*Hapalochlaena* cf. *lunulata* (based on reported locality) (blue-ringed octopus)	140-174 MU $idividual^{-1}$	*Vibrio* spp.	+	HPLC, GC-MS
			Pseudomonas spp.	3 MU, +	mouse bioassay, HPLC, GC-MS
			Bacillus spp.	5 MU, +	mouse bioassay, HPLC, GC-MS
			Alteromonas spp.	+	HPLC, GC-MS
[46]	*Takifugu niphobles* (pufferfish)	intestine 3890 MU g^{-1}	*Shewanella putrefaciens*	15 MU in 250 mL culture broth, +	mouse bioassay, HPLC, GC-MS
[47]	*Fugu vermicularis vermicularis* (pufferfish)	178 MU g^{-1}	*Vibrio* spp.	3 MU, +, +	mouse bioassay, HPLC, GC-MS
[48]	*Astropecten polyacanthus* (comb seastar)	32 MU g^{-1}	*Vibrio* spp.	+	HPLC, GC-MS
[49]	*Fugu poecilonotus* (pufferfish)	N/A	*Pseudomonas* spp.	+	HPLC, GC-MS
[47]	*Atergatis floridus* (reef crab)	+ TLC, eletrophoresis	*Vibrio* spp.	+	HPLC, GC-MS
[7]	*Jania spp*. (red alga)	N/A	*Pseudomonas* spp.	+	HPLC, GC-MS

* MU: Mouse Units, HP-LC: high-performance liquid chromatography, GC-MS: gas chromatography–mass spectrometry, TLC: thin layer chromotography, EMI-MS: Electrospray ionization-mass spectrometry, ELISA: enzyme-linked immunosorbent assay, LC-MS: liquid chromatography-mass spectrometry

** '+' : Denotes positive detection but no quantitative information given

2. RESULTS AND DISCUSSION

2.1. Bacterial isolation and toxin analysis

All of the tissue samples from each *P. maculata* and *Stylochoplana* sp. tested positive for TTX via LC-MS (Table 2). Tetrodotoxin concentrations in *P. maculata* specimens from Matakana Island were uncharacteristically low when compared to those reported in populations from near-by Pilot Bay [ave. 90 mg kg^{-1}; 27, 26]. The individuals were collected in May, before known peaks in TTX occur (June – August), which may partially explain their unusually low TTX concentrations. Additionally, it is possible that the individuals used in this study had not consumed any *Stylochoplana* sp., a suggested dietary source of TTX for this species [27]. The concentrations detected still indicate that they may have accessed (or harbored) an alternative and possibly microbial source of TTX. Thus it was deemed reasonable to continue isolating bacteria from these individuals. In contrast, TTX concentrations of *Stylochoplana* sp. (ave. 174 mg kg^{-1}) were consistently in the range of previously sampled specimens [ave. 380 ± 210 mg kg^{-1}; 27]

Table 2. Concentrations (mg kg^{-1}) of tetrodotoxin (TTX) and number of bacterial strains isolated from pooled (n=3) tissues samples of *Pleurobranchaea maculata* and *Stylochoplana* sp. collected 7 May 2013 from Matakana Island (M.I.) and Pilot Bay (P.B), New Zealand, and TTX concentrations in pooled samples of *P. maculata* tissue used for broth experiments collected from Illiomama Rock (I.R) (Auckland), New Zealand collected 28 September 2011.

Sample	**Location**	**TTX (mg kg^{-1})**	**Bacterial Strains**
P. maculata; digestive	M.I.	2	16
P. maculata; gonad	M.I.	5	3
P. maculata; mantle	M.I.	7	21
P. maculata; 'rest'	M.I.	8	23
Stylochoplana sp.	M.I., P.B.	174	39
P. maculata; digestive	I.R.	771	-
P. maculata; gonad	I.R.	136	-
P. maculata; mantle	I.R.	97	-

A total of 102 bacterial strains were isolated from the 5 samples (*P. maculata* - 63; *Stylochoplana* sp. - 39) and their diversity was assessed by restriction fragment length polymorphism analysis (RFLP) of a region of the 16S rRNA gene (Table 2). This analysis identified 28 unique strains or operation taxonomic units (OTUs). Sequencing of the 16S rRNA gene from a representative isolate of each OTU yielded 16 unique strains from *P. maculata* tissues and one from *Stylochoplana* sp. tissues. Phylogenetic analyses revealed that the *P. maculata* 16S rRNA gene sequences grouped into eight distinct clades representing the orders: *Pseudomonadales*, *Actinomycetales*, *Oceanospirillales*, *Thiotrichales*, *Rhodobacterales*, *Sphingomonadales*, *Bacillales*, and *Vibrionales*, whereas the *Stylochoplana* sp. sequences grouped into one clade representing *Vibrionales* (Figure 1). One *P. maculata* isolate PRMR011, grouped phylogenetically with the isolates from *Stylochoplana* sp.. Chau et al. [29] sequenced the 16S rRNA gene from ten different bacterial strains isolated from the homogenized tissues of the gonad, digestive tract, and reproductive organs of adult *P. maculata* collected from Narrow Neck Beach (Auckland, New Zealand). Their sequences grouped into two different clades representing the orders *Alteromonadales* and *Vibrionales* (shown in red – Figure 1). In this study strains representing an additional seven clades have been isolated. These differences could possibly be due to the individuals having been collected from different geographic locations, or having consumed a different dietary source prior to sampling, and further sampling and bacterial isolation efforts from multiple populations are required to establish the degree of variability in microbial consortiums among sites.

Although many bacteria isolated grew from the *Stylochoplana* sp. inoculum, the molecular analysis showed that the diversity was very low (only one strain). This may indicate that this bacterial species (*Vibro* sp.) is very abundant, or alternatively that other bacterial strains could not grow on the media used in this study, possibly due to an antimicrobial interaction. Pryosequencing could be used to help elucidate the total bacterial diversity with this organism in future studies. The *Vibro* sp. strain isolated from *Stylochoplana* sp. was also detected in *P. maculata*, and although no TTX was identified, ‘shared’ strains may be good candidates for further investigation.

To date, eleven studies have isolated a TTX-producing *Vibrio* sp. [10-12, 14, 16, 30, 42, 44, 47, 48, 50] making this the most common genus to be associated with

TTX production. Other common groups associated with TTX production include *Bacillus* spp. [11, 17, 18, 36, 40, 42], *Pseudomonas* spp. [7, 10, 11, 49], *Aeromonas* spp. [10, 15, 42], and *Alteromonas* spp. [11, 42]. Although representatives of three of these genera were isolated in this study, phylogenetic comparisons are challenging as only three previous studies that have attempted to isolate TTX-producing bacteria have undertaken any molecular analysis and submitted these data to public databases [14, 29, 39].

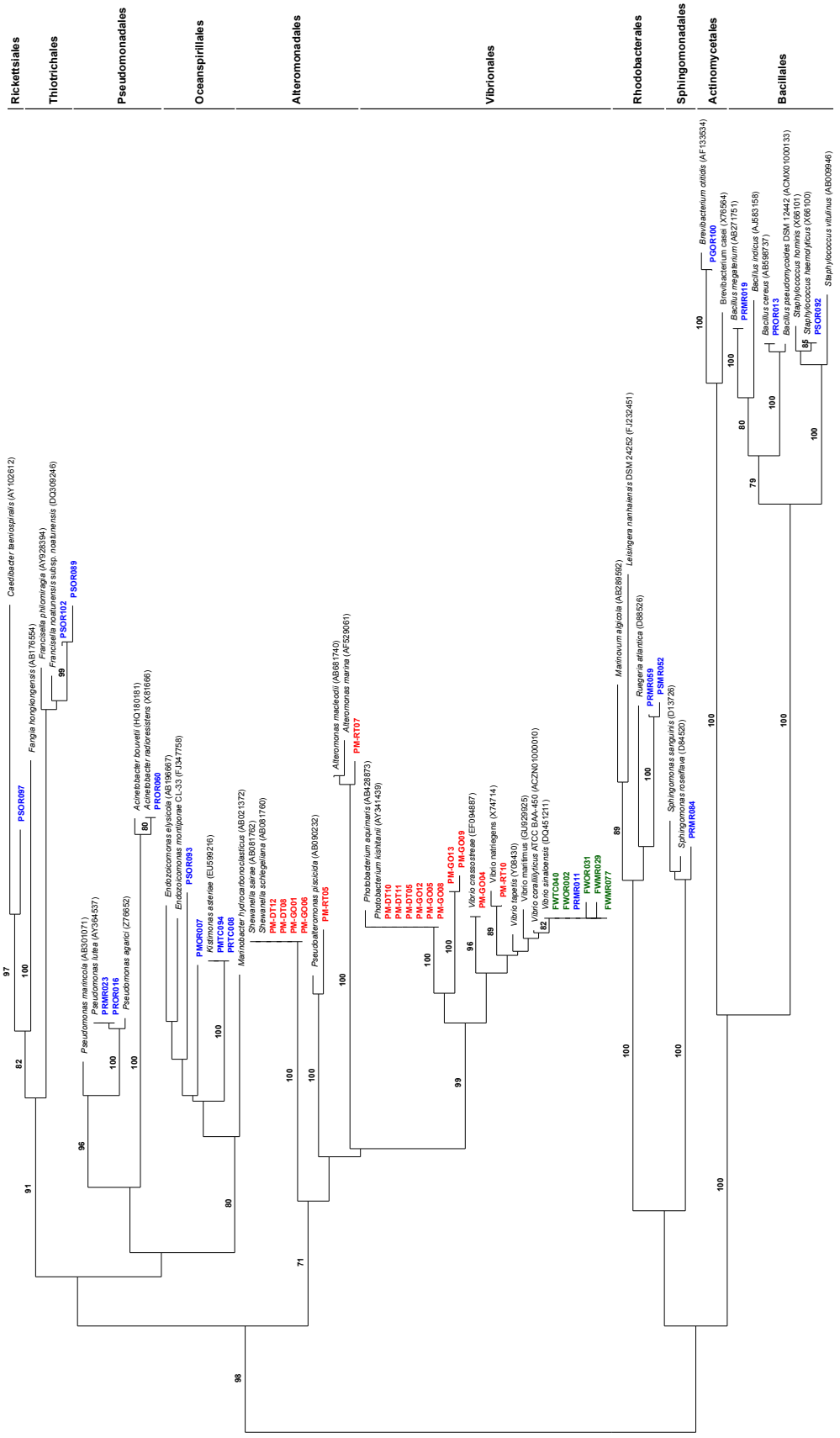
Rickettsiales
Thiotrichales
Pseudomonadales
Oceanspirillales
Alteromonadales
Vibrionales
Rhodobacterales
Sphingomonadales
Actinomycetales
Bacillales
Caedibacter taeniospiralis (AY102812)
PSOR097
Fangia hongkongensis (AB176554)
Francisella philomiragia (AY928394)
Francisella noatunensis subsp. noatunensis (DQ309245)
PSOR102
PSOR089
Pseudomonas marincola (AB301071)
Pseudomonas lutea (AY364537)
PRMR023
PROR016
Pseudomonas agarici (Z76652)
Acinetobacter bouvetii (HQ180181)
Acinetobacter radioresistens (X81666)
PROR060
Endozoicomonas elysicola (AB196667)
Endozoicomonas montiporae CL-33 (FJ347758)
PSOR093
PMOR007
Kistimonas asteriae (EU598216)
PMTC094
PRTC008
Marinobacter hydrocarbonoclasticus (AB021372)
Shewanella sairae (AB081762)
Shewanella schlegeliana (AB081760)
PM-DT12
PM-DT09
PM-GO01
PM-GO06
Pseudoalteromonas piscicida (AB090232)
PM-RT05
Alteromonas macleodii (AB681740)
Alteromonas marina (AF529061)
PM-RT07
Photobacterium aquimaris (AB428873)
Photobacterium kishitanii (AY341439)
PM-DT10
PM-DT11
PM-DT05
PM-GO12
PM-GO05
PM-GO08
PM-GO13
PM-GO09
Vibrio crassostreae (EF094587)
PM-GO04
Vibrio natriegens (X74714)
PM-RT10
Vibrio tapetis (Y08430)
Vibrio maritimus (GU929925)
Vibrio coralliilyticus ATCC BAA-450 (ACZN01000010)
Vibrio sinaloensis (DQ451211)
PWTC040
PWMR022
PRMR011
FWOR031
FWMR029
FWMR077
Marinovum algicola (AB289592)
Ruegeria atlantica (DB8526)
PWMR059
PRMR052
Sphingomonas sanguinis (D13726)
Sphingomonas rosaliae (D84520)
PRMR084
Brevibacterium otitidis (AF133534)
PSOR100
Brevibacterium casei (X76564)
Bacillus megaterium (AB271751)
PRMR019
Bacillus indicus (AJ583156)
Bacillus cereus (AB596737)
PROR013
Bacillus pseudomycoides DSM 12442 (ACMX01000133)
Staphylococcus hominis (X66101)
Staphylococcus haemolyticus (X66100)
PSOR092
Staphylococcus vitulinus (AB009946)
0.10

Figure 1. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences of isolates from this study and related bacteria. For comparison, isolates from a similar study [29] are included in the analysis. Isolates from different organisms are color coded as follows: green = *Stylochoplana* sp., blue = *Pleurobranchaea maculata* (this study), red = *P. maculata* [29]. (Bootstrap values < 70 are omitted).

To date, the majority of studies describing TTX-producing bacteria have only conducted qualitative detection of toxin from isolated strains, without measuring quantitative concentrations of TTX produced (Table 1). Of those that have provided quantitative concentrations, strains have been shown to produce very low TTX concentrations compared to host organisms. For example, Wu et al. [17, 18] isolated TTX-producing bacterial strains from the tissues of pufferfish *Fugu rubripes* including the ovaries (120 MU g^{-1}), liver (78.5 MU g^{-1}) and intestines (36.2 MU g^{-1}). In contrast, the toxicity concentrations in bacterial isolates were only 0.1 - 1.6 MU g^{-1} of cells. Researchers have suggested that the relatively low concentrations of TTX-producing bacterium are due to the altered conditions when grown *in vitro* or, alternatively, that strains are providing hosts with TTX precursors [8, 13, 14, 17, 18, 51, 52]. Thus in this study we tested bacterial strains for the C9 base of TTX using the methods described in McNabb et al. [32] as it may detect TTX precursors or degradation products. It also has the additional benefit of greater sensitivity with the limit of detection ca. 0.1 mg kg^{-1} compared to the standard TTX LC-MS–based method used by our research group of ca. 0.5 mg kg^{-1} [24]. Despite the additional benefits and sensitivity of this method no C9 base was detected in any samples (data not shown).

2.2. Bacterial community analysis

The *P. maculata* used to initiate the broth experiments all contained TTX although the concentrations varied considerably (Table 2). One limitation of this experiment is that specimens were maintained in aquarium for up to five days prior to dissection and inoculation of the broths. It is possible that TTX-producing bacteria may have expired or been expelled during this period. However, given the considerable concentration of TTX in the individuals (Table 1), we suggest that any TTX-producers would have been present in high concentration, and therefore it is unlikely that there would be none remaining. Tissue samples were pooled by type prior to initiation of the broth experiments. Marine broth samples from day 0 had trace levels of TTX (data not shown). No TTX was detected in the day 3, 6, and 10 samples. The multidimensional scaling (MDS) analysis of the bacterial communities as determined using ARISA showed a 40% similarity between those samples taken in the first four days indicating a smaller change in the community structure and abundance of each strain as compared to later time points (Figure 2). By day 4 the broths all contained dense bacterial assemblages

(as determined by the cloudy nature of the broth), thus, if a TTX- producing bacterium were present in the initial inoculum it should have had sufficient time to produce toxins before possibly being outcompeted by other bacteria within the community.

Using standard microbiological methods, and very similar media and conditions to studies describing the successful isolation of many TTX producing bacteria no TTX-producing isolates were identified from either *Stylochoplana* sp. or *P. maculata* in this study. By using a new method that detects the C9 base of TTX, we had anticipated that the possibility of detecting precursor of degradation molecules, would be increased, however, none was detected. The biosynthetic pathway of TTX is unknown, thus it is possible that not all precursors would be detected via this method. Another possibility is that an 'unculturable' bacterium might produce TTX. Many studies have now shown that less than 1% of bacteria within a particular community are culturable [53]. However, based on previously published research (Table 1), many of the TTX-producing strains are genera which can be easily cultured. Strains isolated in this study fall into four of these genera, yet no C9 base of TTX was detected in any of the isolates, or microbial communities, suggesting that further efforts to isolate TTX-producing bacterium might not be warranted. Among this literature extensive culturing efforts are not reported (i.e., generally less than 50 strains are isolated) to identify a TTX-producer and there are few [to our knowledge one; 29] that report unsuccessful attempts to isolate TTX-producing strains.

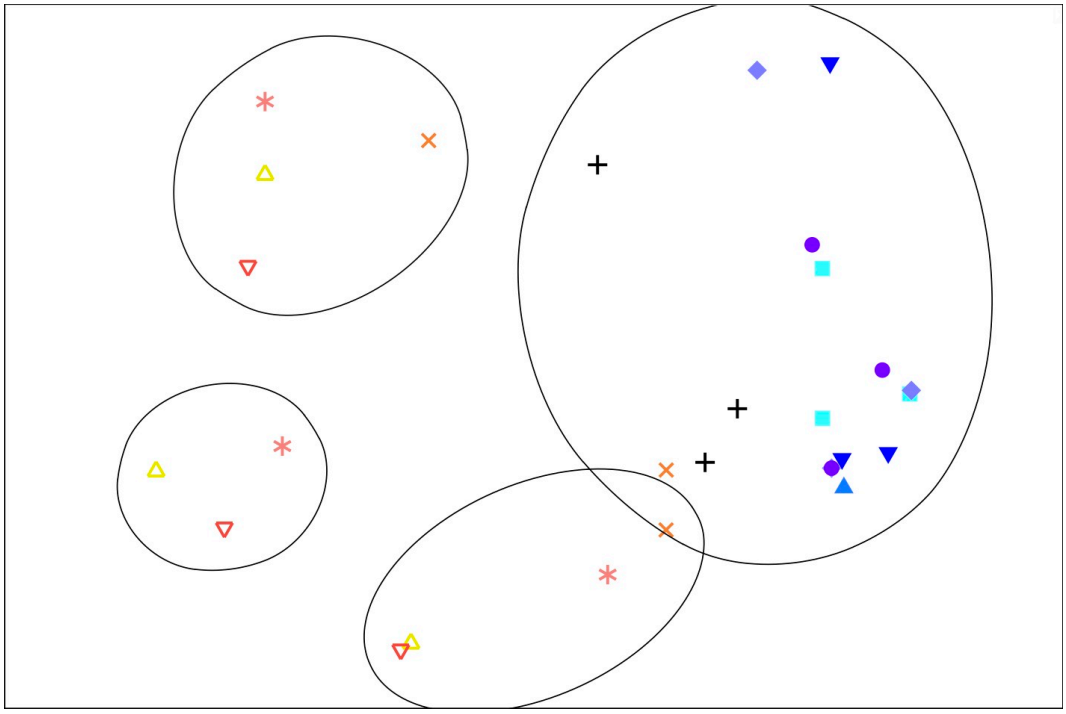

Figure 2. Two-dimensional non-metric multidimensional scaling ordination based on Bray-Curtis similarities of Automated Ribosomal Intergenic Spacer Analysis (ARISA) fingerprints of bacterial communities at different time points in broth experiments (stress = 0.1). ▲ 0 days, ▼ day 1, ■ day 2, ◆ day 3, ● day 4, + day 6, × day 8, ✱ day 10, △ day 12, ▽ day 14. Points enclosed by solid line cluster at 40% similarity.

3. EXPERIMENTAL SECTION

3.1. Bacterial strain isolation

3.1.1. Collection and inoculation

Collections took place (7 May 2013) from two sites in Tauranga Harbor, New Zealand. Three *P. maculata* and two *Stylochoplana* sp. were collected by divers from Matakana Island (37°38'38" S, 176°8'55" E) and an additional *Stylochoplana* sp. specimen from Pilot Bay (37°63'5" S, 176°17'6" E). Specimens were transported to the laboratory in insulated containers and placed in aerated aquaria overnight before being rinsed with deionized water. *Pleurobranchaea maculata*, were aseptically dissected and separated into four tissue types: gonad, digestive organs, mantle, and remaining tissues ('rest'). Each of the four tissue types from the three individuals were combined and homogenized to give four samples. The three *Stylochoplana* sp. specimens were combined and homogenized into one sample. Subsamples from each were frozen (-20 °C) for later toxin analysis. Aliquots of the five combined samples were diluted 1:10 (w/v) in marine broth (Difco), manually homogenized using a glass pestle, and centrifuged (1,000 × *g*, 1 min). The supernatant was then diluted (100, 1,000, and 10,000 fold) and 50 µL aliquots were used to inoculate three types of agar which have previously been used to isolate TTX-producing bacteria: marine agar 2216 (Difco), Thiosulfate Citrate Bile Salts Sucrose TCBS agar (Difco), and Ocean Research Institute (ORI) agar [9, 54]. Agar plates were placed in an incubator (ca. 20 °C) and grown for up to nine days. Multiple representatives of individual colonies that differed in morphology were selected. To ensure that each culture was comprised of a single strain, each was streaked again onto marine agar 2216 (Difco) and grown for 2-3 days at 27 °C. Single colonies were collected, grown overnight (27 °C) in marine broth 2216 (Difco), and stored frozen (-20 °C) after being split into two tubes with the following treatments: (1) preserved with 15% sterile glycerol and stored at -80 °C for later culturing, and (2) centrifuged (10,000 × *g*, 10 min) with the supernatant removed for later DNA extraction.

3.1.2. Molecular and phylogenetic analysis of bacterial strains

DNA was extracted from bacterial pellets using a prepGEM® DNA Bacterial Extraction Kit (Zygem) according to the manufactures instructions. The PCR of bacterial 16S rRNA genes was performed using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1518R (5'-

AAGGAGGTGATCCANCCRCA-3'). Reactions were carried out in 25 μL volumes with the reaction mixture containing; 2.5 μL of 10×PCR buffer (Invitrogen, USA), 1.3 mM $MgCl_2$ (Invitrogen, USA), 0.2 mM (each) deoxynucleoside triphosphate (Bioline, UK), 0.02 mg mL^{-1} bovine serum albumin (BSA, Sigma, USA), 0.25 μM of each primer (IDT, USA), 0.04 U of Platinum *Taq* DNA polymerase (Invitrogen, USA), and 20-30 ng of template DNA. The reaction mixture was held at 94 °C for 2 min followed by 30 cycles of 94 °C for 20 sec, 57 °C for 20 sec, 72 °C for 1 min, with a final extension of 72 °C for 7 min. The resulting PCR products were screened by restriction fragment length polymorphism (RFLP) patterns generated using the restriction endonuclease HaeIII (as per the manufactures instructions) and based on their banding patterns grouped into operational taxonomic units (OTUs). One representative of each OTU was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) on a ABI3100 (Applied Biosystems, CA, USA) using the 27F primer. Sequences obtained in this study were deposited in the NCBI GenBank database under accession numbers KJ995704 – KJ995726. Phylogenetic analysis of isolates was conducted by aligning their sequences, and those from Chau et al. [29], to closely matching sequences from the Greengenes [55] database of bacterial 16S sequences using ARB [56]. Aligned sequences were 399 bp in length and all gaps and ambiguities were excluded from the alignment to ensure reliability. Phylogenetic inferences were made using the PHYLIP package [57]. Pairwise evolutionary distances were computed from percent similarities by the correction of Jukes and Cantor [58] and the phylogenetic tree was constructed by the Neighbor-joining method [59]. The support for each node was determined by assembling a consensus tree of 1000 bootstrap replicates.

3.1.3 Bacterial culturing

One representative of each unique bacterial strain (as identified using RFLP) was retrieved from the cryopreserved stocks, inoculated into marine broth (400 mL) and grown at 30 °C with shaking (110 rpm) for 4 days. Cultures were centrifuged (6,000 × *g*, 20 min) and the supernatant removed and the pellets frozen (-20 °C) for later C9 analysis (Section 3.3).

3.2. Bacterial community broth experiments

3.2.1. Collection and inoculation

Three *P. maculata* were collected by divers (28 September 2011) from Illiomama Rock (36°48'44"S, 174°52'48"E), Auckland Bay, New Zealand. The specimens were transported to the laboratory in insulated containers and placed in aquaria for 5 days. *Pleurobranchaea maculata* were aseptically dissected and separated into three tissue types: gonads, digestive tract, and mantle. Each of the three tissue types from the three individuals were combined, homogenized (1 min, Heidolph Diax 600 Homogeniser, Heidolph, Germany) and diluted 1:10 (w/v) in marine broth (Difco), Samples were then homogenized (1 min, Heidolph Diax 600 Homogeniser, Heidolph, Germany) to ensure that bacteria from the tissues were dispersed throughout the media, and then centrifuged (1,000 × *g*, 1 min) to prevent tissue being inoculated into broths. Aliquots (200 μL) of each supernatant were added to separate 1-L marine broth (Difco) in Erlenmeyer flasks. Three control flasks were used. The first containing only marine broth, A second contained marine broth spiked with TTX (Tocris Bioscience, Cat. No: 1078) at a final concentration of 6.9 μg mL^{-1} (to ensure that there was no TTX degradation over the experimental period). This concentration was chosen as it could easily be detected allowing changes in TTX to be monitored throughout the experiment. A final control consisting of marine broth, mantle tissue supernatant, and sodium azide (0.02% final volume w/v) was used to ensure that any increase in TTX was not due to TTX-unbinding from tissue, or a similar scenario causing an increase in toxins. Flasks were placed in a thermally controlled shaker (120 rpm) set approximately at 25 °C. Sub-samples for TTX analysis (30 mL) and DNA extraction (1 mL) were collected on day 0, 1, 2, 3, 4, 6, 8, 10, 12, and 14. These were centrifuged (3,000 × *g*, 10 min) and the supernatant removed before the remaining pellets were stored frozen (-20 °C) for later TTX and molecular analysis.

3.2.2. Molecular analysis

DNA was extracted from the broth experiment pellets using hexadecyl trimethyl-ammonium bromide (commonly known as the CTAB method) as described in Barrett et al. [60]. PCR reactions for ARISA were preformed using the reaction mixture described above and the bacterial primers ITSF and ITSReub from Cardinale et al. [61]. Reactions were run on an DNAEngine® Peltier thermal

cycler (Biorad, USA) with the following cycling parameters: 94 °C for 2 min, 30 cycles of 94 °C for 45 sec, 55 °C for 60 sec, 72 °C for 2 min, and a final extension of 72 °C for 7 min. PCR products were visualized on 1% agarose gel and then diluted 20 fold using Milli-Q water. Intergenic spacer fragments were run on an ABI 3130 xI sequencer (PE Applied Biosystems, Foster City, USA) employing the GeneScan mode at 15 kV for a run time of 45 min according to the manufacturer's manual. The internal GS1200LIZ Zy Standard (0.25 μL;PE Applied Biosystems) was added to each sample to determine the size of fluorescently labelled fragments during analysis. PeakScanner™ software v1.0 (PE Applied Biosystems) and an in-house pipeline modified from Abdo et al. [62] written using Python 2.7.1 (Python Software Foundation) and R (http://www.r-project.org) were used to process ARISA profiles. Electropherogram analysis included all peaks that made up 0.1% of the entire signal, were between 100 and 1200 base pairs, and were over 30 relative fluorescence units (rfu). Peaks were binned to the nearest 1 base pair. ARISA fluorescence intensities data were fourth root transformed and analyzed with the PRIMER 6 software package (PRIMER-E, Ltd., UK) using nonmetric multidimensional scaling (MDS) based on Bray-Curtis similarities conducted with 100 random restarts. Results and agglomerative hierarchical clustering of similarities, executed using the CLUSTER function, were plotted onto two-dimensional plots.

3.3. Tetrodotoxin and C9 analysis

Tissue samples from *P. maculata*, *Stylochoplana* sp., and pellets from broth aliquots from day 0, 3, 6, 8, and 10 were extracted using a slightly modified method from McNabb et al. [24]. Milli-Q water containing 0.1% v/v acetic acid was added on a 1:10 w/v basis to sub-samples of tissue or cell pellet and homogenized (for tissue; Heidolph Diax 600 Homogeniser; Heidolph, Germany) or sonicated (for cell pellet; Misonix XL2020, Misonix Inc., USA). Samples were centrifuged (3,000 × *g*, 10 min) and an aliquot of the supernatant (1 mL) transferred into 9 mL of 100% methanol containing 0.1% v/v acetic acid and placed at -20 °C for at least 1 hr. After freezing, samples were centrifuged (3,000 × *g*, 10 min) and diluted 1:4 with 100% methanol containing 0.1% v/v acetic acid. Samples were analyzed for TTX using LC-MS as described in McNabb et al. [24].

Frozen bacterial isolate pellets for testing for the C9 base were extracted using methods from McNabb et al. [32]. Briefly, Milli-Q water with 0.1% acetic acid was added to ca. 1 g pellet on a 1:10 w/v basis. Samples were then homogenized using a sonicator (Heidolph Diax 600 Homogeniser; Heidolph, Germany) set at level 4 for 30 s and centrifuged (3,000 × *g*, 5 min). Supernatant was transferred to a new tube and sodium hydroxide was added to a final concentration of 1 M. Tubes were then placed in a boiling water bath (45 min), cooled, and neutralized with concentrated acetic acid to pH 4-6 using pH paper. Samples were purified and concentrated using an SPE cartridge (Phenomonex Strata X; 60 mg 3 mL^{-1}). These were conditioned with methanol (MeOH, 100%; 3 mL) followed by 50 mM ammonium acetate (3 mL). After samples were loaded, the filter was washed with 50 mM ammonium acetate (3 mL), followed by 5% MeOH in 50 mM ammonium acetate (3 mL). Samples were eluted (3 mL) using of 30% MeOH containing 1% formic acid (3 mL) and tested for the C9 base via LC-MS as described in McNabb et al. [32].

4. CONCLUSIONS

This study used a recently developed highly sensitive LC-MS based method to attempt to identify the C9 base or TTX precursor/degradation products in bacterial strains isolated from toxic *P. maculata* and *Stylochoplana* sp.. A total of 102 strains were isolated and sequencing of the 16S rRNA gene from a representative isolate of each OTU yielded 16 unique strains from *P. maculata* tissues and one from *Stylochoplana* sp. tissues. Despite these intensive culturing efforts, newly developed extremely sensitive TTX detection capabilities, and an experiment where we investigated whether a consortium of bacteria from *P. maculata* could produce TTX, no evidence was found to support a bacterial origin of TTX in *P. maculata* or *Stylochoplana* sp..

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CHAPTER IV. *In situ* accumulation of tetrodotoxin in non-toxic *Pleurobranchaea maculata* (Opisthobranchia)

Preface

This chapter describes the relocation of non-toxic *Pleurobranchaea maculata* from the Tasman Bay (South Island) to mesh cages deployed in Pilot Bay (North Island). Specimens were maintained for 8 weeks before collection and analysis, and native populations were also sampled during this time period. As primary author I was involved with the design of the cages, deployment and final collection of samples, sample preparation for tetrodotoxin and molecular analysis, data analysis, and wrote the manuscript. Susie Wood assisted with molecular sample preparation, data analysis, and manuscript editing. Rex Fairweather and David Culliford conducted the deployment and final collection of cages as well as the weekly feeding of *P. maculata*. Next generation sequencing was preformed by New Zealand Genomics Ltd., and initial data analysis was conducted by Louis Ranjard (University of Auckland). Paul McNabb undertook all liquid chromatography-mass spectrometry analysis. Craig Cary advised on experimental design, data analysis, and editing of the manuscript. All authors reviewed and edited manuscript content.

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ABSTRACT

Tetrodotoxin (TTX) is a highly potent neurotoxin targeting voltage gated sodium channels. It is found in numerous phyla, including both marine and terrestrial taxa, however, its origin is a topic of considerable debate. To investigate the origin of TTX in the Opisthobranch *Pleurobranchaea maculata* an experiment was conducted where verified non-toxic specimens were transplanted to a habitat with toxic populations of the same species. Sixteen individuals were kept in mesh net cages either, (1) anchored to the seafloor, or (2) deployed 0.5 m off the benthos. They were fed a non-toxic diet for eight weeks before being analysed for TTX via liquid chromatography-mass spectrometry. Four of the six remaining individuals from the cages on the benthos contained low concentrations of TTX (max. 0.79 mg kg^{-1}), whilst only two of the eight from the suspended cages contained TTX and the concentrations were lower (max. 0.43 mg kg^{-1}). Among the positive individuals the highest concentrations were always detected in the gonad tissues. These data, in concert with previous studies, provide compelling evidence for a dietary source of TTX for this species and suggests that this source may be more readily available from the benthos. Next-generation sequencing of the foregut contents from toxic and non-toxic individuals, utilizing the MiSeq Illumina™ platform and targeting the 18S rRNA gene, revealed high abundances of sequences of taxa belonging to the Cnidaria and Annelida phyla. Two *Patiriella regularis* (New Zealand cushion star) specimens were observed preying upon *Pleurobranchaea maculata* egg masses that were found attached to the outside of mesh cages. *Patiriella regularis* specimens, *Pleurobranchaea maculata* egg masses, as well as biofouling scrapings from mesh cages all contained trace levels of TTX.

1. INTRODUCTION

Tetrodotoxin (TTX) is a potent, and potentially lethal neurotoxin found worldwide in both marine and terrestrial organisms (reviewed in Chau et al. 2011). It is most notably found in pufferfish species in the Indo-Pacific region and, in 2009, was detected for the first time in the opisthobranch *Pleurobranchaea maculata* in New Zealand (McNabb et al. 2010). The definitive source of TTX has eluded researchers for many decades with suggestions of a bacterial source (e.g., Simidu et al. 1987; Noguchi et al. 1987; Hwang et al. 1989; Wu et al. 2005; Wang et al. 2008; Wang & Fan 2010), bioaccumulation through the food chain, (Noguchi et al. 1982; Daly et al. 1997; Noguchi et al. 2006; Noguchi & Arakawa 2008; Gall et al. 2012b) or the possibility that organisms produce it endogenously (Hanifin et al. 2002; Cardall et al. 2004; Lehman et al. 2004; Gall et al. 2014). Multiple marine bacteria isolated from pufferfish (Matsui et al. 1989; Lee et al. 2000; Yu et al. 2004; Wu et al. 2005; Wang & Fan 2010), gastropods (Cheng et al. 1995), red algae (Yasumoto et al. 1986), and various sediments (Do et al. 1991, 1993) have tested positive for low levels of TTX. In comparison, evidence to support a dietary source has been demonstrated in several studies. For example, no TTX was detected in the tissues of *Takifugu rubripes* (pufferfish) cultured in land aquaria and sea net-cages after being maintained on a TTX-free diet for 1 to 3 years (Noguchi et al. 2006). Matsui et al. (1981) and Honda et al. (2005) demonstrated that *T. rubripes* raised in captivity and fed toxic tissues of wild *Takifugu vermiculare porphyreum* and *Takifugu vermicularis*, respectively, accumulated TTX into their skin, liver, and ovaries. Additionally, Noguchi et al. (1982) concluded a dietary source when *Astropecten polyacanthus* (starfish) tissues were identified in the gut of the TTX-containing trumpet shell, *Charonia sauliae*. Whole specimens of the starfish were captured, tested, and also found to contain TTX. Examples of endogenous production of TTX are largely limited to studies on the rough-skinned newt *Taricha granulosa*. Hanifin et al. (2002) showed an overall increase in TTX levels of 29 captive *T. granulosa* specimens after one year in captivity where they were fed on a TTX-free diet. It was also shown that captive female newts continue to deposit TTX into eggs for up to three years (Gall et al. 2012a). Additionally, Cardall et al. (2004) demonstrated that when captive *T. granulosa* were electrically stimulated to release TTX, toxin concentrations recovered over a nine month period despite being fed a non-TTX diet. Symbiotic TTX-producing bacteria could explain these

increases in TTX over time; however, molecular techniques did not detect any bacterial signature (16S rRNA gene sequences) in toxic tissues, including the skin, gonads, liver, and eggs, suggesting that a bacterial source of TTX was unlikely (Lehman et al. 2004).

Toxic and non-toxic populations of *P. maculata* occur in New Zealand. The North and South Islands of New Zealand are geographically separated by an approximately 70 km wide body of water ('Cook Straight'). To date, no TTX has been detected in South Island *P. maculata* populations, whereas *P. maculata* from the North Island contain widely varying concentrations with extremely high TTX concentrations (ca. 1400 mg kg^{-1}) detected in populations from Tauranga, Auckland, and Whangarei (Wood et al. 2012a). Khor et al. (2013) showed that non-toxic *P. maculata* can accumulate TTX when fed a TTX-containing diet with no negative effects. Additionally, concentrations of TTX in North Island specimens were shown to depurate over 126 days when kept in aquaria and fed non-toxic diets (Wood et al. 2012b). A similar trend in temporal TTX depuration rates was observed in most organs in wild populations (Wood et al. 2012a). One exception to this pattern was the slow depuration rate of gonad tissue which, in conjunction with the observed decrease in TTX concentrations of wild populations during egg laying season (June – August) and high concentrations of TTX found in egg masses and larvae, suggests a larval protective role of TTX in *P. maculata*. Collectively these findings indicate that *P. maculata* are most likely acquiring TTX from an environmental source. Salvitti et al. (Salvitti et al. 2015) detected high concentrations of TTX in *Stylochoplana* sp. (Platyhelminthes) collected from Pilot Bay, Tauranga and, using molecular techniques, showed that this could be a source of TTX for *P. maculata*. However, given the significant variability among individuals, the absence of *Stylochoplana* sp. at some sites with toxic *P. maculata* populations, and based on expected life spans and TTX concentrations within the adults, they suggest that it is unlikely *Stylochoplana* represent their sole source of toxin.

The aim of this study was to investigate whether TTX could accumulate and be detected in the tissues of non-toxic *P. maculata* when maintained in an environment known to contain toxic populations. Sixteen non-toxic *P. maculata* from the South Island were transported to a North Island site previously shown to

contain toxic populations and maintained for eight weeks in net cages (Wood et al. 2012a). Cages were either anchored to the sea floor or suspended in the water column to assist in delineating the source of TTX i.e., sediment or water column. Wild *P. maculata* from the same area were collected over a three month period to provide a baseline indication of TTX concentrations. *Pleurobranchaea maculata* are known as opportunistic scavengers, potentially consuming a wide range of organisms including mussels, anemones, and marine worms (Willan 1983). To gain insights into the range of organisms consumed, foregut contents were collected from and additional 11 toxic and non-toxic *P. maculata* from the North Island study region and genetically surveyed for prey items.

2. MATERIAL AND METHODS

2.1. Pleurobranchaea maculata collection

Pleurobranchaea maculata (n=26) were collected by scuba divers from Tasman Bay, New Zealand (41°05'S, 173°06'E) on 16 July 2013 and transported in chilled seawater to the laboratory within 4 h. Ten individuals were frozen (-20 °C) immediately to determine base-line TTX concentrations. The remaining 16 were placed in plastic bags filled with seawater (ca. 300 mL) and shipped overnight to a laboratory based at the study site in Tauranga (37°67' S, 176°17' E), New Zealand. Upon arrival at the laboratory, specimens were placed in plastic aquariums (14 L) containing 13.5 L of seawater and maintained at ambient temperatures (ca. 18 °C) for 1 week before deployment. Throughout this study *P. maculata* were fed *Perna canaliculus* (Greenshell™ mussel) collected from Tasman Bay (16 July 2013) and stored frozen (-20 °C). Previous testing of *P. canaliculus* has shown that they do not contain TTX in their tissues and are readily consumed by *P. maculata* (Khor et al. 2013).

2.2. Deployment and collection

Each *Pleurobranchaea maculata* was weighed immediately prior to deployment and after collection. The experiment was undertaken in Pilot Bay, Tauranga, New Zealand (37°63' S, 176°17' E) a site known to support toxic *P. maculata* (Wood et al. 2012a). Sixteen Jarvis Walker collapsible bait traps consisting of a fine nylon mesh (2 × 2 mm) around a metal frame (480 × 250 mm) were deployed. Eight of these were modified by replacing the bottom mesh with wider plastic netting (6 × 6 mm) to enable *P. maculata* greater access to benthos.

Eight cages were suspended 0.5 m above the benthos with cork floats and the remaining eight modified cages were anchored approximately 2 cm into the soft substrate using metal pegs. All cages were anchored to the seafloor in an alternating pattern of suspended and benthic type using galvanized 20 mm chain secured with anchors at either end. The chain was laid parallel to the shoreline to maintain cages at approximately the same depth (~3 m). A single *P. maculata* was added to each cage on 23 July 2013.

Pleurobranchaea maculata were fed at least once a week with frozen pieces of *Perna canaliculus* placed in cages by divers. Cages were collected after 8 weeks (18 September 2013). Scrapings of bio-fouling and collection of biota from the cages were conducted twice during deployment (23 August and 9 September 2013).

2.3. Seasonal tetrodotoxin analysis

To determine if the concentration of accumulated TTX in the caged specimens was comparable to those in nearby wild populations four *P. maculata* were collected every three weeks from Matakana Island (37°64' S, 176°15' E), New Zealand (ca. 2.5 km from the experiment site) between 27 March and 19 June 2013. These were transported chilled in seawater to the laboratory and frozen (-20 °C) for later TTX analysis.

2.4. Molecular analysis of stomach contents and bioinformatics

Eleven adult *P. maculata* were collected from Pilot Bay and Matakana Island, New Zealand, between September 2012 and September 2013. *Pleurobranchaea maculata* were dissected using sterile techniques and DNA from foregut contents extracted using the PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc.) according to the manufacturers protocols. PCR reactions were performed in 50 μL reactions with the following reaction mixture: 45 μL Platinum® PCR SuperMix High Fidelity (Life Technologies), 0.4 μg BSA, 0.5 μM (each) primer (18S 1F and 400R modified to include an Illumina™ adaptor; Pochon et al. 2013), and 10-30 ng of template DNA. PCR reaction mixtures were held at 94 °C for 3 min followed by 27 cycles of 94 °C for 30 s, 55 °C for 45 s, 68 °C for 1 min, and with a final extension of 68 °C for 8 min. Products were visualized on a 1% agarose gel and purified using SPRIselect reagent kit

(Beckman Coulter, USA) according to the manufacture's protocol. Purified products were quantified using a Qbit (Invitrogen), diluted to 1 ng μL^{-1} using Milli-Q water, and sent to New Zealand Genomics Ltd. (Auckland, New Zealand) for library preparation and sequencing on the MiSeq Illumina™ platform.

Illumina™ datasets were demultiplexed using MiSeq Reporter v2.0. All further analysis were performed using MOTHUR version 1.33.3 (Schloss et al. 2009). The sequences corresponding to the forward and reverse primers were trimmed, and merged into single contigs (max. length 452 nucleotides). Contigs were aligned to the SILVA reference alignment (Guillou et al. 2013) at a 50% threshold and chimera removal performed using the UCHIME algorithm. Sequences were first grouped at the Order level before operational taxonomic units (OTUs) were created using a 0.02 pairwise sequence distance cut-off value. OTUs represented by less than 10 reads across all samples were removed. OTUs were then classified to identify taxonomic annotation using the Protist Ribosomal Reference Database (Guillou et al. 2013), using a threshold of 80% similarity. Unknown sequences (92) were removed.

2.5. Tetrodotoxin analysis

The wild *P. maculata* specimens (entire organism) were homogenised and prepared for TTX analysis. The caged specimens were dissected and small portions of the mantle, gonad, and digestive tissue were aseptically removed and stored (-20 °C). The remaining material was homogenised and prepared for TTX analysis. The individual organs were only analysed when results from combined organs were positive. The digestive tissue was aseptically removed from the eleven *P. maculata* collected for molecular gut content analysis and a portion prepared for TTX-analysis. For all others sub-samples (ca. 2 g) from the above whole organisms or organs were extracted with 18 mL of Milli-Q water containing 0.1% v/v acetic acid or a *pro-rata* volume if the starting mass differed. Tissues were then disrupted with a homogenizer for ca. 1 min (Heidolph Diax 600 Homogeniser, Heidolph, Germany) and centrifuged (3000 × *g*, 10 min). Supernatant was removed and 100% methanol containing 0.1% v/v acetic acid was added at a ratio of 10:1. Samples were then frozen (-20 °C) for 1 hr, centrifuged (3000 × *g,* 10 min), and diluted 1:4 with 100% methanol containing

0.1% v/v acetic acid. Samples were analysed for TTX via liquid chromatography – mass spectrometry (LC-MS) as described in McNabb et al. (2010).

3. RESULTS

3.1. Tetrodotoxin analysis

No TTX was detected in the ten *P. maculata* collected from Tasman Bay (South Island) for TTX baseline analysis. Two of the benthic caged *P. maculata*, specimens were lost during deployment. Of those remaining, the average weight gain was 8.34 g, with only one specimen losing weight (1.76 g). Average weight gain for suspended specimens was 14.42 g (Table 1).

Four of the six remaining benthic specimens tested positive for TTX, with concentrations of the whole *P. maculata* ranging from 0.05 to 0.79 mg kg^{-1} (ave. 0.30 mg kg^{-1}, Table 1). Tetrodotoxin concentrations of individual organs from benthic specimens were always highest in the gonad tissue (0.26 to 4.53 mg kg^{-1}, ave. 1.56 mg kg^{-1}) and lowest in the mantle tissue, (0.07 to 0.32 mg kg^{-1}, ave. 0.15; Table 1).

Only two of the eight suspended specimens tested positive for TTX with an average of 0.33 mg kg^{-1} (Table 1). Gonad tissue contained the highest concentration of TTX in both positive suspended samples (ave. 2.94 mg kg^{-1}). The lowest TTX concentration (0.2 mg kg^{-1}) in specimen #14 was in the mantle, while the digestive tissue from specimen #12 contained the lowest concentration of TTX (0.29 mg kg^{-1}).

Fine sediment was found covering all cages along with numerous *Obelia* sp., a hydrozoan, growing on the netting. Additionally, two egg masses from wild *P. maculata* were observed entangled in the *Obelia* sp. and *Patiriella regularis* (New Zealand cushion star) were observed preying upon the masses. One of the cage scrapping samples contained trace concentrations of TTX (0.04 mg kg^{-1}). Both egg masses contained TTX (0.59 and 1.98 mg kg^{-1}), as did the *P. regularis* (0.09 and 0.14 mg kg^{-1}) that were preying upon them (Table 2). The *Obelia* sp. associated with egg mass #2 also contained low concentrations of TTX (0.08 mg kg^{-1}).

Tetrodotoxin concentrations in *P. maculata* collected during 2013 from Matakana Island were extremely variable ranging from 0.37 mg kg^{-1} collected 27 March 2013 to 487 mg kg^{-1} on 8 May 2013 (Table 3).

Table 1

Weight gain and tetrodotoxin (TTX) concentrations of *Pleurobranchaea maculata* after eight weeks in cages deployed on the sediment (specimens #1-8) or suspended (0.5 m from sediment, specimens #9-16) in Pilot Bay, Tauranga, New Zealand from July to September 2013. Individuals 3 and 7 escaped during the experiment. Method limit of detection was 0.05 mg kg^{-1}. ND = not detected or < 0.05 mg kg^{-1}.

Specimen #	Starting weight (g)	Weight gain (g)	TTX (mg kg^{-1})			
			Total	Mantle	Digestive	Gonad
1	6.36	9.61	0.05	ND	0.14	0.26
2	5.85	7.38	0.21	0.07	0.13	0.67
4	9.06	10.06	0.79	0.32	0.97	4.53
5	4.97	13.05	ND			
6	3.05	-1.76	ND			
8	5.11	11.73	0.10	0.07	0.39	0.80
9	3.21	13.76	ND			
10	4.10	16.42	ND			
11	2.19	16.42	ND			
12	2.34	13.41	0.43	2.37	0.29	3.14
13	1.88	10.77	ND			
14	1.59	17.54	0.23	0.20	1.23	2.73
15	1.33	13.42	ND			
16	1.40	13.61	ND			

Table 2

Tetrodotoxin (TTX) concentrations of cage scrapings, associated organisms, and egg masses from wild *Pleurobranchaea maculata* collected from 23 August and 12 September 2013. ND = not detected.

Sample	**TTX (mg kg^{-1})**
Egg mass #1 (23 Aug)	0.59
Egg mass #2 (23 Aug)	1.98
Patiriella regularis preying on egg mass #1 (23 Aug)	0.09
Patiriella regularis preying on egg mass #2 (23 Aug)	0.14
Obelia sp. on cage (23 Aug; associated with egg mass #2)	0.08
Cage scraping (23 Aug)	Trace
Cage scraping (12 Sept)	ND
Cage scraping (12 Sept)	ND
Cage scraping (12 Sept)	ND
Cage scraping (12 Sept)	ND

Table 3

Tetrodotoxin (TTX) concentrations and average mass (± one standard deviation) of wild *Pleurobranchea maculata* (n=4 at each sample date) from Matakana Island collected between 27 March and 19 June 2013.

Date	**Mass (g)**	**TTX (mg kg^{-1})**			
		Ave.	**Min.**	**Max.**	**Std. Deviation**
27 Mar 2013	5.8 ± 2.8	55	0.37	217	108
17 Apr 2013	7.1 ± 2.9	90	0.38	304	144
08 May 2013	13.2 ± 1.2	127	2.81	487	239
29 May 2013	15.2 ± 3	2.8	1.00	3.8	1.3
19 June 2013	23.5 ± 3.3	122	9.12	236	110

3.2. Pleurobranchaea maculata diet analysis

Digestive tissue from seven of the eleven *P. maculata* collected for gut analysis, two from Pilot Bay and five from Matakana Island, tested positive for TTX (Table 4) with concentrations ranging from 0.23 to 46.6 mg kg^{-1} (ave. 13.01 mg kg^{-1}). The raw sequencing data produced 723,602 assembled sequences and 522,107 after alignment, filtering, and chimera removal. Bioinformatics analysis resulted in the clustering of sequences into 116 OTUs. Removal of sequences belonging to the Pleurobranchia clade, most likely a result of inefficient dissection incorporating *P. maculata* tissue into the sample, reduced the total number of sequences to 234,163. Samples 5 and 9 were removed from further analysis due to more than 99% of their sequences belonging to the Pleurobranchia clade. Four (samples 11, 10, 7, 6) of the five samples with positive TTX detection had greater than 86% of their sequences consist of Cnidarian species, with the most toxic sample (#2) having 94% of its sequences consist of an Annelid species of the *Thelepus* genus (Figure 1). Of the sequences falling into the Cnidaria phyla, more than 99% aligned with those from the Hydrozoan class, with >85% of these more specifically belonging to the genus *Plumularia*. Across all the non-toxic specimens (1, 3, 4, 8) the most abundant phyla present were also the Cnidaria (>60%). However, sequences belonging to the Echinodermata phyla made up the majority (>95%) of the sequences from specimen #4 and sequences from the Arthopoda phylum, all belonging to the Maxillopoda class, made up the majority (>76%) of the sequences in sample #3. Six samples here were shown to have low numbers of sequences belonging to the Platyhelminthes phylum. However, no correlation between TTX concentrations and sequence abundances were apparent.

Table 4

Tetrodotoxin (TTX) results of digestive tissue from *Pleurobranchaea maculata* collected between September 2012 and September 2013. P.B. = Pilot Bay, M.I. = Matakana Island. ND = not detected.

	Date	**Location**	**(TTX mg kg^{-1})**
1	27 Sept 2012	P.B.	ND
2	27 Sept 2012	P.B.	46.60
3	27 Sept 2012	P.B.	ND
4	27 Sept 2012	P.B.	ND
5	13 Mar 2013	M.I	35.11
6	28 May 2013	M.I	4.06
7	28 May 2013	M.I	3.74
8	23 Aug 2013	P.B.	ND
9	02 Sept 2013	P.B.	0.23
10	12 Sept 2013	M.I	1.82
11	12 Sept 2013	M.I	0.15

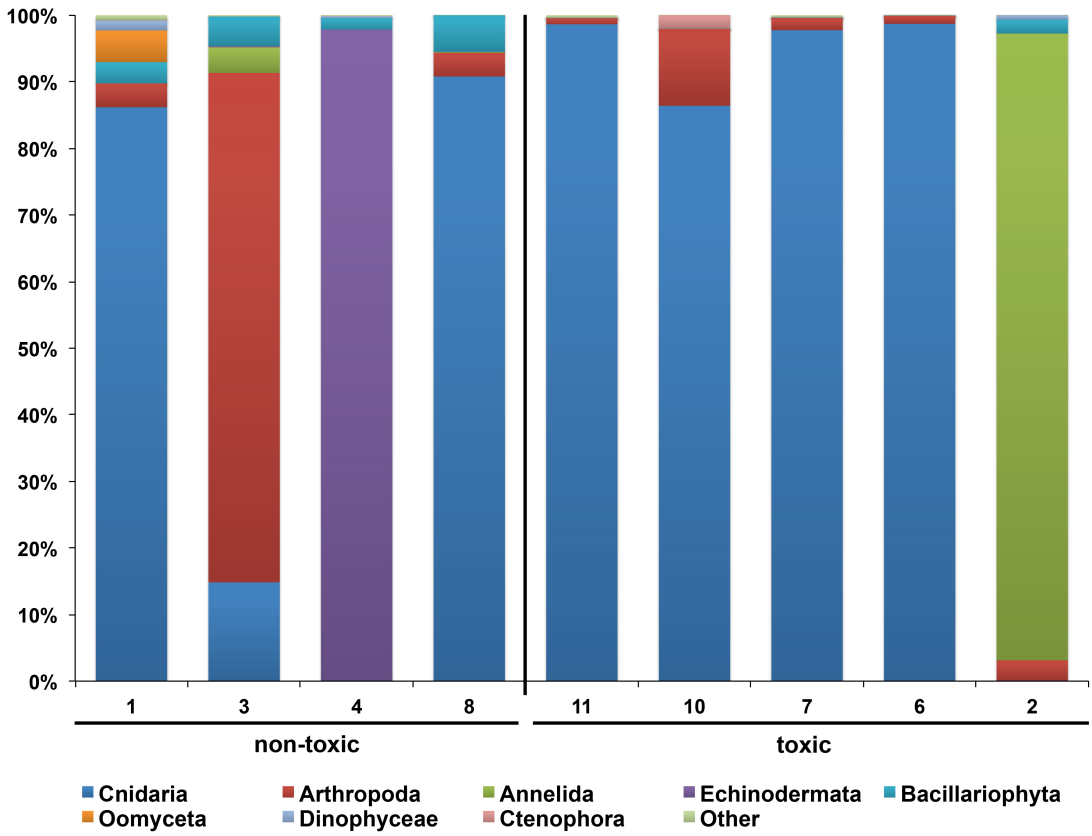

Figure 1. Stacked column showing the relative distribution of the dominant (>1% across all samples) phyla from wild *Pleurobranchaea maculata* gut content ordered from least toxic to most toxic. X-axis labels correspond to samples in Table 4.

4. DISCUSSION

This study demonstrates that when non-toxic *P. maculata* are kept in an environment (for 8 weeks) known to harbour TTX-containing populations, they can sequester TTX into their tissues (0.05 to 0.79 mg kg^{-1}). The TTX concentrations detected in the individuals in cages were markedly lower than those of wild individuals collected in 2011 (ave. 90 mg kg^{-1}, Pilot Bay, Wood et al. 2012a) and 2013 (ave. 80 mg kg^{-1}, Matakana Island, this study). However, the samples collected as part of this study (Table 3) showed marked variability and the minimum concentrations on each sampling date were similar to those in the caged *P. maculata*.

Other factors influencing TTX concentrations in the caged specimens may include: (1) Seasonal variability - it has been shown that North Island populations of *P. maculata* exhibit seasonal changes in TTX concentrations, with peaks occurring from June to August before decreasing throughout the rest of the year. The caged specimens in this study were deployed from the end of July to September, thus they may have missed the period when the TTX source is most abundant in the environment. (2) Restricted access to larger dietary sources - high TTX concentrations were recently detected in *Stylochoplana* sp. from Pilot Bay and identified as a source of TTX for *P. maculata* (Salvitti et al. 2015). In this study six of the wild specimens analysed using NGS were also found to contain sequences belonging to the Platyhelminthes phylum (data not shown). The size of the mesh used in the cages in this experiment would have restricted or prevented transplanted specimens access to *Stylochoplana* sp. and, (3) When maintaining multiple *P. maculata* in aquaria we observed that they were cannibalistic (Wood and McNabb unpub. data), potentially providing another avenue for acquisition of high quantities of TTX. This mechanism of TTX accumulation would not have been available to the transplanted specimens.

Average TTX concentrations between benthic and suspended specimens were similar (0.30 and 0.33 mg kg^{-1}, respectively), however, more benthic specimens (4 out of 6), acquired TTX in comparison to the suspended specimens (2 out of 8). This suggests a benthic origin of TTX, which may have been limitedly available to suspended *P. maculata* only when currents and/or weather conditions enabled suspension of benthic material/organisms into the water column. Trace levels of TTX were detected in scrapings of fine sediment off of

the cages taken on 23 August 2013 possibly indicating a microbial source among this material. For example, *Acinetobacter* sp. and *Streptomyces* sp. isolated from marine sediments have been shown to produce low levels of TTX (Do et al. 1990, 1991). Further bacterial or algal isolation from sediment at sites with dense *P. maculata* populations could be conducted by fractionation of this material, with culturing efforts focused on those fractions with the highest TTX concentrations.

Two egg masses from wild *P. maculata* collected from the outside of the deployed cages on 28 August 2013 contained low TTX concentrations. The first (#1) was found directly adhered to the mesh of the cage, while the second (#2) was tangled in an *Obelia* sp. hydrozoan that had grown onto the mesh. Low concentrations of TTX were detected in the *Obelia* sp. specimen, however, this is most likely a result of residual deposits from the entangled egg mass. *Patiriella regularis* individuals were discovered ingesting each egg mass, and both also contained low levels of TTX in their tissues. This is the first detection of TTX in *P. regularis*, adding to the growing list of TTX-containing organisms in New Zealand including; the bivalve *Paphies australis* (0.80 mg kg^{-1};McNabb et al. 2014), the sand dollar *Arachnoides zelandiae* (0.25 mg kg^{-1}), and trace levels in the cats eye snail *Turbo smaragdus*, the crab *Macrophthalmus hirtipes*, and coralline turf algae *Corallina officinalis* (Khor et al. 2013). The extreme variability of TTX in so many coexisting organisms might suggest that they are exposed to similar sources of TTX but some organisms have greater sequestration abilities, or that TTX is possibly transferred from one to another (Salvitti et al. 2015). Further investigation, involving sampling more individuals, is required to explore whether the TTX detected in *Patiriella regularis* was due to accumulation in their tissues, indicating TTX resistance, or if it was a residual signal from consumption/contact with the *Pleurobranchaea maculata* egg masses. Resistance to TTX has been shown in the predator garner snake (*Thamnophis sirtalis*), which have altered amino acid residues on their sodium channel preventing TTX from binding and allowing them to prey upon TTX containing newts (*Taricha* sp.) (Brodie Jr et al. 2002; Geffeney et al. 2005). Changes to amino acid residues has also been shown to occur in pufferfish species (Yotsu-Yamashita et al. 2000; Venkatesh et al. 2005; Maruta et al. 2008) A similar mechanism may explain the ability of *Patiriella regularis* to ingest the toxic egg masses.

Distribution of TTX in the tissues of both benthic and suspended *P. maculata* were similar to those found in native populations prior to egg laying (June to August) with highest levels found in the gonad tissue (Wood et al. 2012b). Non-toxic *P. maculata* sourced from the South Island of New Zealand have previously been shown to sequester TTX rapidly in a laboratory-based experiment and also demonstrated that the highest TTX concentrations generally occurred in the gonads (Khor et al. 2013). These patterns, in concert with the detection of TTX in egg masses and larvae suggest that TTX plays a role in protecting *P. maculata* progeny (Wood et al. 2012a, b). However, observations in this study of the New Zealand cushion star (*Patiriella regularis*) preying upon egg masses conflict with this assumption. The mechanisms for acquisition and incorporation of TTX into the tissues of *P. maculata* are currently unknown. Tetrodotoxin-binding proteins have been isolated from marine pufferfish (Matsui et al. 2000; Yotsu-Yamashita et al. 2001), crab (Nagashima et al. 2002), and several gastropod species (Hwang et al. 2007). Additional analyses of *P. maculata* tissues are required to identify whether these TTX-binding proteins are present in this organism.

Diet analysis of the foregut of *Pleurobranchaea maculata*, revealed that the majority of specimens with TTX present in their digestive tissues contained Cnidarian sequences. Cnidarian are soft-bodied aquatic organisms that are known for harbouring nematocyst, or stinging cells, to aid in protection and prey capture (Turk & Kem 2009). Additionally, many species have also been found to contain various neurotoxins that target voltage gated sodium channels, similar to TTX (reviewed in Frazão et al. 2012; Moran et al. 2009; Wanke et al. 2009). These toxins target site 3 of the sodium channel α-subunit, in comparison to TTX, which selectively binds to site 1. In New Zealand there are several species belonging to the *Plumularia* genus, however, to date none have been reported to contain any toxins. The diet analysis of one *P. maculata* specimen (#2), with the highest TTX concentration (46.6 mg kg^{-1}) did not follow this pattern, with more than 93% of its sequences related to Annelid species of the *Thelepus* genus. Although there are no studies detailing neurotoxins in this specific genus, toxins from Annelids are not unprecedented. For example, the neurotoxin glycerotoxin has been isolated from the venom of the sea worm *Glycera convoluta* (Meunier et al. 2002). The foregut samples only represent a ‘snap shot’ of the dietary habits of *P. maculata* and

because TTX accumulates, the dietary item responsible for the TTX in each individual might not have been consumed just prior to capture. Thus, conclusions relating TTX concentrations to abundance of sequences, must be interpreted with caution. Never the less, this is the first study to explore the dietary preferences of this organism and these data provide new insights and support prior studies that suggest *P. maculata* is a carnivorous scavenger (Ottaway 1977; Willan 1983) with the ability to acquire TTX from a variety of dietary sources.

It is also conceivable that *P. maculata* are producing TTX themselves, i.e., endogenous production, as suggested for the terrestrial newts *Taricha granulosa* (Hanifin et al. 2002; Cardall et al. 2004). Under this scenario the genes involved in TTX production could be environmentally triggered. We cannot rule out this possibility; however, because TTX was not detected in all of the transplanted specimens, it seems an unlikely scenario. Further transcriptomic studies may help provide insights into this concept, for example Le et al. (2007) showed a linear correlation in transcripts of fibrinogen-like proteins with TTX concentrations in pufferfish. Comparisons between gene transcripts of toxic and non-toxic populations of *P. maculata* could possibly identify those genes associated with TTX production. Additionally, it must be acknowledged that transplanted specimens could have been infected by a symbiotic TTX-producing bacterium upon deployment into the new environment, that were not present in their original environment. Comparisons of bacterial communities from tissues of both populations could identify symbionts associated only with toxic populations.

This study is the first to show that non-toxic *P. maculata* can accumulate, or start producing, TTX when maintained in the wild at a site known to contain toxic individuals. Although we can not eliminate the possibility that something environmental causes *P. maculata* to start producing TTX endogenously, the variability among individuals in concert with previous studies suggest that this is unlikely. The mesh cages used in this experiment prevented large organisms being consumed, thus we postulate a microbial origin of TTX is the most likely scenario. However, whilst this could account for the TTX concentrations detected in the caged specimens, it would only partially explain the high TTX concentrations detected in some individuals collected in this study as part of the monthly surveys. Salvitti et al. (Salvitti et al. 2015) has already demonstrated that

the flatworm *Stylochoplana* sp. provides a dietary source of TTX for *P. maculata* and we suggest that other prey items may also contain TTX. The high abundances of sequences of taxa belonging to the Cnidaria and Annelida in toxic *P. maculata* is of interest as these organisms have not been specifically targeted in previous environmental surveys of TTX in New Zealand. These organisms should be investigated in future studies aimed at elucidating the origin of TTX in *P. maculata*.

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Supplementary

Figure 1S. Photo of *Patiriella regularis* preying on egg mass #1 taken on August 28, 2013 from Pilot Bay, New Zealand.

Chapter V: Intracellular immunohistochemical detection of tetrodotoxin in *Pleurobranchaea maculata* (Gastropoda) and *Stylochoplana* sp. (Turbellaria)

Preface

This chapter describes the immunohistological detection of tetrodotoxin in the tissues of *Pleurobranchaea maculata* and *Stylochoplana* sp. and the implications for ecological roles of TTX. As primary author I was involved with all sample collections, tissue preparation, immunohistological methodology development, and writing of the manuscript. Barry O'Brien, Pawel Olszewski, Linda Peters, and Mark Poli advised on development of immunohistological techniques and microscopy. Leigh Winsor advised on histological techniques and conducted several of the staining techniques. Craig Cary and Susie Wood advised on experimental design, data analysis, and editing of the manuscript. All authors reviewed and edited the manuscript. This thesis chapter has been published in Marine Drugs with the following citation:

Salvitti LR, Wood SA, Winsor L, Cary SC (2015) Intracellular immunohistochemical detection of tetrodotoxin in *Pleurobranchaea maculata* (Gastropoda) and *Stylochoplana* sp. (Turbellaria). Marine Drugs. 13(2): 756-769.

ABSTRACT

Tetrodotoxin (TTX), a potent neurotoxin targeting sodium channels, has been identified in multiple marine and terrestrial organisms. It was recently detected in the Opisthobranch *Pleurobranchaea maculata* and a Platyhelminthes *Stylochoplana* sp. from New Zealand. Knowledge on the distribution of TTX within these organisms is important to assist in elucidating the origin and ecological role of this toxin. Intracellular micro-distribution of TTX was investigated using a monoclonal antibody-based immunoenzymatic technique.. Tetrodotoxin was strongly localized in neutral mucin cells and the basement membrane of the mantle, the oocytes and follicles of the gonad tissue, and in the digestive gland of *P. maculata*. The ova and pharaynx were the only two structures to contain TTX in the *Stylochoplana* sp.. Using liquid chromatography – mass spectrometry, TTX was also identified in the larvae and eggs, but not the gelatinous egg cases of *P. maculata*. Tetrodotoxin was present in egg masses of *Stylochoplana* sp..These data suggest that TTX has a defensive function in adult *P. maculata*, who then invest this in their progeny for protection. Localization in the digestive gland of *P. maculata* potentially indicates a dietary source of TTX. *Stylochoplana* sp. may use TTX in prey capture and for the protection of offspring.

1. INTRODUCTION

Tetrodotoxin (TTX) is a potent non-protein neurotoxin that selectively targets and blocks voltage-gated sodium channels. It is most notably found in the tissues of pufferfish species from the Tetraodontidae family [1, 2]. Tetrodotoxin is fatal to humans (wt. 50 kg) at levels of just 1 – 2 mg [3]. Tetrodotoxin was initially thought to only occur in pufferfish, but has since been discovered in a growing number of organisms including frogs, newts, gastropods, crabs, an algae species, arrow worms, and land planarians among others [2, 4-6].

Numerous researchers have suggested that the incidence of TTX in so many genetically unrelated organisms is due to an exogenous source such as symbiotic bacterial production or bioaccumulation through diet [2, 7-9]. Bioaccumulation of TTX has been implicated in several instances in which prey animals have also been shown to contain TTX, or during captive studies where organisms removed from their natural environments lose their toxicity [1, 10-12]. In contrast, bacterial production of TTX has also been reported in several marine organisms including the gastropod *Niotha clathrata* [13], the blue-ringed octopus *Octopus maculosus* (i.e., *Hapalochlaena maculosa*, but may be *Hapalochlaena* cf. *lunulata* based on locality) [14], and the pufferfish *Fugu vermicularis vermicularis* [15]. However, concentrations of toxin produced by isolated bacterial strains are generally orders of magnitude lower than host organisms, suggesting bacterial production is unlikely the sole source of toxin [7, 8, 16]. Alternatively, studies on terrestrial newts (*Taricha granulosa*) showed an increase in toxin concentrations when kept in captivity and the ability to regenerate TTX overtime after the release of toxin through the skin, suggesting an endogenous source in this instance [17, 18]. Currently the definitive origin of TTX remains debated, and the strategies for acquisition most likely vary among species.

Studies using chemical methods to detect TTX have shown sequestration of toxin varies among tissue types in many organisms [2]. For example, in the pufferfish *Takifugu niphobles* high concentrations of TTX were present in the liver, ovaries, and intestines, while skin and muscle tissues only had low concentrations of toxin [1]. Micro-distribution of TTX has been demonstrated using TTX specific monoclonal antibody (mAB) immunoenzymatic techniques in newts [19, 20], a ribbon worm and flat worm [21], pufferfish [22-24] and

octopuses [25]. Understanding the accumulation and sequestration of TTX at the cellular level provides additional information regarding the ecological functions of TTX. For example, in predator-prey trials conducted by Williams et al. [26] using the rough-skinned newts, *Taricha granulosa* and their natural predator the garter snake *Thamnophis sirtalis*, it was shown that rejected newts possessed significantly higher concentrations of TTX in the skin compared to those that were consumed.

In 2009, the opisthobranch *Pleurobranchaea maculata* was found to contain high concentrations of TTX when a number of dogs became ill after consuming beach-cast individuals in New Zealand [27]. Subsequent studies using liquid chromatography-mass spectrometry (LC-MS) revealed that only the TTX variant was present. The highest concentrations of TTX were in the mantle, gonad, and digestive tissue, with total TTX concentrations (highest average (ave.). 369 mg kg^{-1}) varying significantly between individuals and season [12, 28]. In a series of aquaria based studies, the egg-laying season was shown to coincide with seasonal peaks in TTX concentrations (June – August) [28]. The high concentrations of TTX detected in egg masses, and subsequent depuration of TTX from adults after spawning, suggest that TTX plays a protective role in offspring of *P. maculata*. In 2013, high concentrations (ave. 376 mg kg^{-1}) of TTX were detected in *Stylochoplana* sp. (a marine flatworm), collected from Tauranga, New Zealand [29]. Concentrations of TTX were less variable than in *P. maculata*, but also decreased from winter (June – August) to spring (September to November). The small size of *Stylochoplana* sp. (ca. 60 mg) have prohibited dissection and LC-MS analysis of TTX concentrations in various tissues, thus to date there is no information on how TTX is distributed within this organism. In this study immunohistological techniques, in conjunction with the T20G10 anti-TTX monoclonal antibody (mAB) [30], were used to investigate the micro-distributions of TTX within each organism at the cellular level. These data may provide insights on ecological function and the source of TTX in these organisms.

2. RESULTS AND DISCUSSION

2.1. Pleurobranchaea maculata

2.1.1. Mantle

Species from the group Opisthobranchia have extremely reduced, or in some cases have completely lost, their protective shell, resulting in a diverse range of alternative defensive strategies [31-33]. These include the acidification of the mantle [33-35], incorporation of nematocysts from cnidarian prey [31], development of spicules [36], secretion of ink [37], and acquisition of secondary metabolites [32, 38]. The mantle, or dorsal body wall, of *P. maculata* consists of multiple folds or puckering of the epidermis, which has previously been reported to be extremely acidic (pH = 1-2)[39]. In the immunostained section of the mantle, TTX, visualized as brown color deposits, was most strongly localized in the basement membrane layer as well as in tear-shaped membrane bound cells (Fig. 1A). This is similar to immunohistochemical studies on the pufferfish *Tetraodon nigroviridis* [22], *Tetraodon steindachneri* [24], and Takifugu niphobles [40] where TTX was shown to be sequestered in both basal cells and succiform cells of the epidermis. The pink color of the tear-shaped cells in both the Hematoxylin & Eosin (H&E) and the Alcian Blue – Periodic Acid Schiff (AB-PAS) stained sections reveal that these erythrophil cells secrete neutral mucin, suggesting that cells responsible for the acidity of the mantle and sequestration of TTX are separate. Sequestration of TTX in the skin has been reported in a number of other organisms including the pufferfish *Takifugu vermicularis* and *Chelonodon patoca*, [23], the California newt *Taricha torosa* [41], the red-spotted newt *Notophthalmus viridescens* [19], the Japanese newt *Cynops pyrrhogaster* [20], the rough-skin newt *Taricha granulosa* [18], the octopus *Hapalochlaena lunulata* [25, 42], and the frog *Brachycephalus ephippium* [43], and a possible defensive mechanism is suggested.

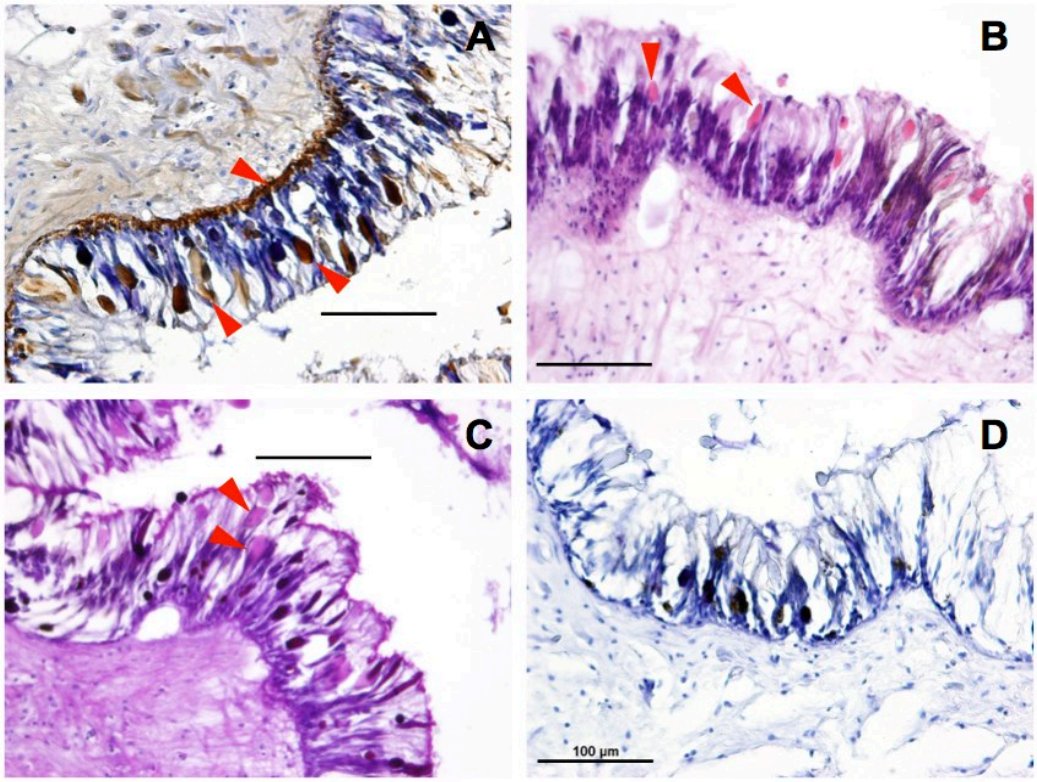

Figure 1. *Pleurobranchaea maculata* mantle tissue sectioned at 10 µm. Red arrows indicate tetrodotoxin (TTX) containing cells. (A) TTX-specific monoclonal antibody (mAB) immunohistological staining (TTX identified by the brown color deposits) (B) Hematoxylin and Eosin (H&E) staining, (C) Alcian Blue-Periodic Acid Schiff (AB-PAS) staining and, (D) mAB negative control. Black bars = 100 µm.

2.1.2. Reproductive and digestive tissue

Analysis of the mAB incubation of the gonad and digestive tissue of *P. maculata* showed the strongest antigen- antibody reaction was in the oocytes and their surrounding follicles, (Fig. 2A). Positive staining occurred to a lesser degree in the digestive gland (Fig. 2A). Localization of TTX in the reproductive organs/tissues has been seen in several other organisms including the oocytes of the pufferfish *Takifugu niphobles* [40], Takifugu vermicularis and Chelonodon patoca [23], the ovaries, oviduct, and testis of the sword-tailed newt *Cynops ensicauda* [44], and the ovaries blue ringed octopuses *Hapalochlaena lunulata* and *Hapalochlaena fasciata* [45]. Researchers have suggested that TTX plays a protective role in host organisms, and the localization of TTX in reproductive organs imparts the toxin into offspring to increase their survival rates [45, 46].

The localization of TTX in the digestive gland tissue of *P. maculata* is suggestive of a dietary source of TTX in this species (Fig 2A). Previous reports addressing the origin of TTX in *P. maculata* have provided evidence suggesting that a dietary source is probable [Chp IV; 47, 29, 12]. A dietary source was suggested by Wood *et al*. [12] due to the depuration of TTX in *P. maculata* when kept in captivity and fed a non-toxic diet. Khor *et al*. [47] demonstrated that non-toxic *P. maculata* have the ability to sequester TTX into their tissues when fed an artificial toxic food source. Additionally, non-toxic *P. maculata* sequestered low concentrations of TTX when transplanted to a known habitat of toxic specimens for 8 weeks (Chp IV). Further evidence was provided when real-time PCR assays revealed *P. maculata* ingested the co-existing TTX-containing *Stylochoplana* sp. [29]. Collectively these studies suggest that TTX in *P. maculata* is most likely obtained from a dietary source.

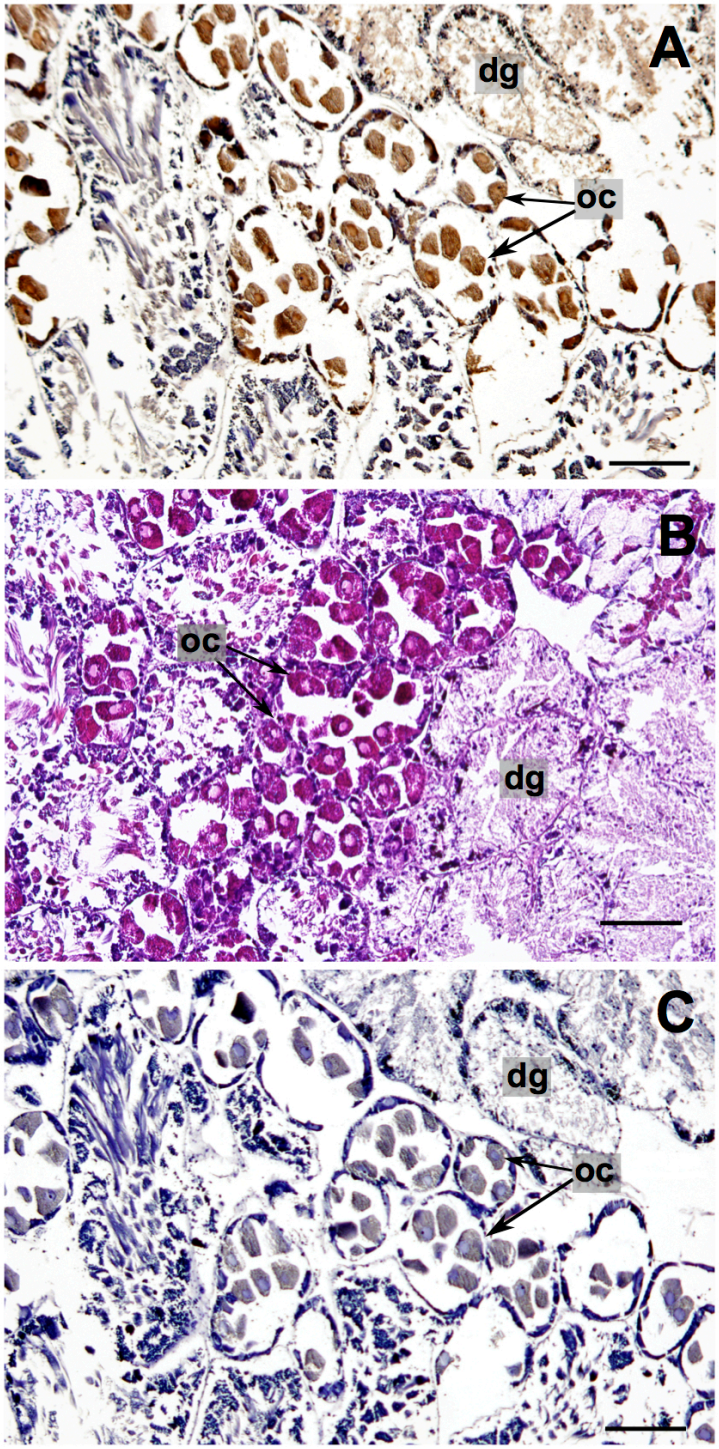

Figure 2. *Pleurobranchaea maculata* gonad/digestive tissue sectioned at 10 μm. (A) Tetrodotoxin (TTX)-specific monoclonal antibody (mAB) immunohistological staining (TTX identified by the brown color deposits), (B) Hematoxylin and Eosin staining (H&E), and (C) mAB negative control. dg = digestive gland, oc = oocyte. Black bars = 200 μm.

2.2. Stylochoplana sp.

Stylochoplana sp. were fixed flat and sectioned dorsoventrally. Tetrodotoxin, identified by the brown color deposits, is contained in the ova as well as portions of the pharynx (Fig 3). The localization of TTX in the reproductive and digestive organs has also been reported in other flatworm species. Tanu *et al*. [21] used immunohistological techniques to show that TTX was contained in the ovum of the flatworm *Planocera reticulata*. Miyazawa *et al*. [48] showed the oviduct and digestive organs, including the pharynx and intestines, to be the most toxic tissues in the flatworm *Planocera multitentaculata* via mouse bioassay. A study on a planocerid species found on a reef in Guam demonstrated the highest concentrations of TTX were in the pharynx and through a series of feeding studies the researchers suggested that TTX was utilized in prey capture [49]. The sequestration of TTX in the pharynx of *Stylochoplana* sp. suggests this species may also use it to capture prey. The detection of TTX in the ova corroborates the observation of TTX in reproductive structures of *Pleurobranchaea maculata*, as well as many TTX-containing organisms, and most likely acts as a protective mechanism in offspring (see earlier discussion).

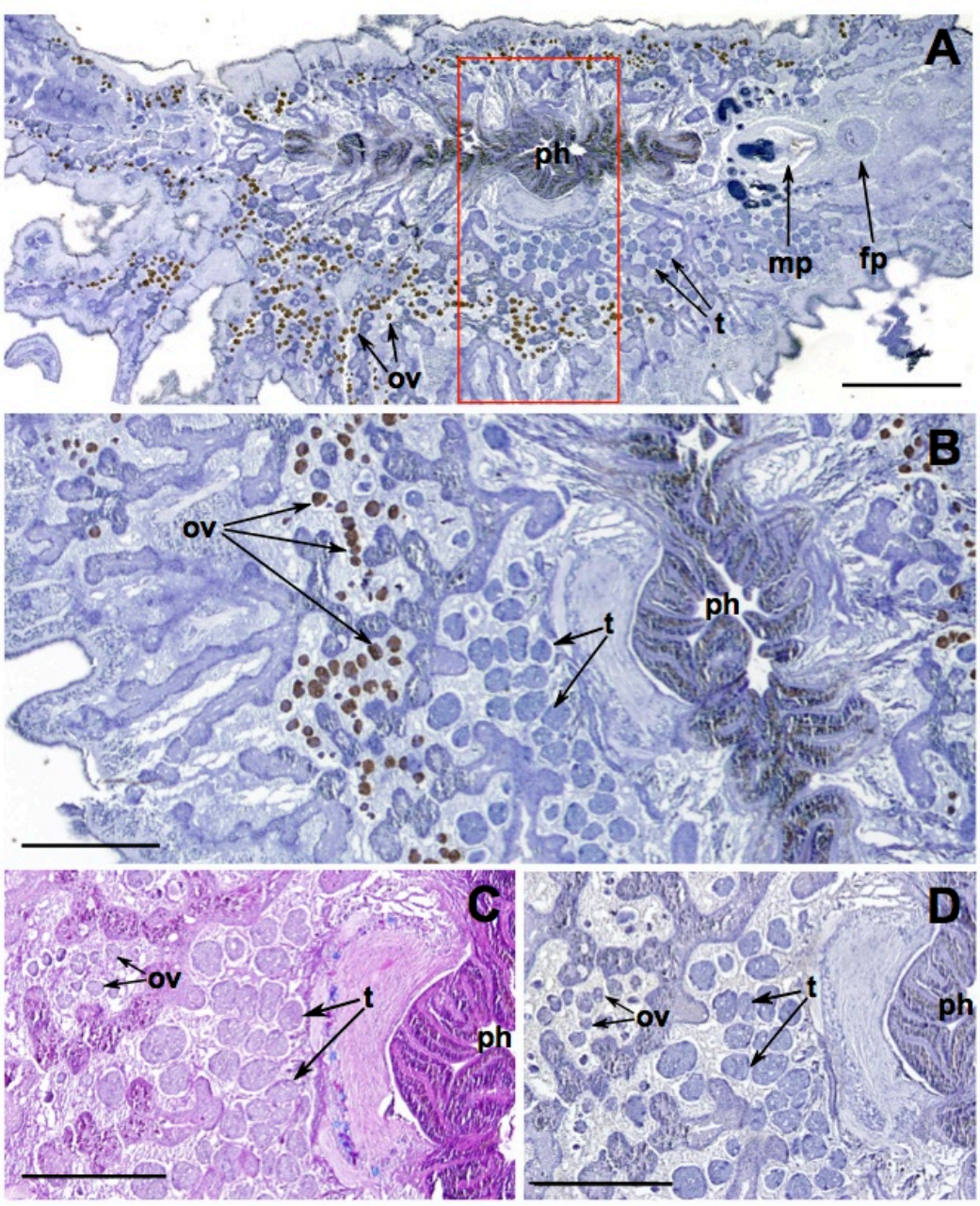

Figure 3. *Stylochoplana* sp. dorsoventral view sectioned at 7 µm. (A) Tetrodotoxin (TTX)-specific monoclonal antibody (mAB) immunohistological staining, (TTX identified by the brown color deposits) (B) Enlargement and 90° rotation of red box on A to show detailed view of ova, testes, and pharynx, (C) Alcian Blue-Periodic Acid Schiff (AB-PAS) staining, and (D) mAB negative control. ph = pharynx (see supplemental data for additional figures), ov = ova, t = testes, mp = male pore, fp = female pore. Black bar = 1 mm (A), 500 µm (B–D).

2.3. Offspring

The high water and mucopolysaccharide content of the gelatinous matrix of the egg masses prevented histological studies on these samples. However, the egg masses of both *P. maculata* and *Stylochoplana* sp., and hatched larvae of *P. maculata*, were tested for TTX utilizing LC-MS (Table 1). To ascertain where TTX is localized in the egg masses of *P. maculata*, the outer gelatinous matrix, with and without the eggs, was tested. No TTX was detected in the gelatinous matrix with the eggs removed, thus it is assumed that TTX is invested only into the eggs. While this would be beneficial for the larvae once hatched, the lack of TTX in the gelatinous coating surrounding the eggs is at odds with the suggestion that TTX has a protective function in the egg masses [12]. This, in conjunction with the observation of the sea star *Patiriella regularis* consuming *Pleurobranchaea maculata* egg masses (Chp IV), suggests that TTX may not act as a predator deterrent at this life stage.

Tetrodotoxin concentrations of egg masses from a *Stylochoplana* sp. kept in aquaria were higher than those *P. maculata* egg masses tested in this study, but still in the range of previously recorded TTX concentrations of *P. maculata* egg masses (max. 100 mg kg^{-1}) [12]. It was not possible to localize the TTX in the egg masses of *Stylochoplana* sp. as these were only *ca.* 75 mg making separation of the gelatinous matrix and eggs difficult. Tetrodotoxin has been shown to be sequestered in the eggs and egg masses of several other species including; the flatworm *Planocera multitentaculata* [48], the California newt *T. torosa* [41], the rough skin newt *T. granulosa* [50, 51], the horseshoe crab *Carcinoscorpius rotundicauda* [52, 53], the blue ring octopus *H. maculosa* (probably *H. fasciata* based on the authors' locality) [54], and *H. lunulata* [45], and the frog *A. chiriquiensis* [55]. The foremost ecological role suggested for TTX residing in the egg masses and offspring is for protection. A recent study by Itoi *et al*. [46] demonstrates this possibility by showing that several predatory fish species ingested toxic pufferfish (*T. rubripes* and *T. niphobles*) larvae, but always promptly spat them out. Immunohistological techniques revealed the localization of TTX in the outer layer of both pufferfish species. Several other studies have also shown the presence of TTX in pufferfish eggs and gonads signaling a potential role for the toxin in progeny protection [56, 57].

Table 1. Tetrodotoxin (TTX) concentrations of egg masses and hatched larvae of *Pleurobranchaea maculata* (P.M.) collected from Pilot Bay, New Zealand on 9 September 2013 (n=1) and average TTX concentrations in egg masses of *Stylochoplana* sp. (S.S.) kept in aquaria (n=2).

Sample	TTX
P.M egg mass (-eggs)	ND
P.M. egg mass (+ eggs)	3.7 mg kg^{-1}
P.M. larvae	48.3 pg $indivdual^{-1}$
S.S. egg masses	108 ± 2 mg kg^{-1}

3. EXPERIMENTAL SECTION

3.1. Sample collections

Toxic *P. maculata* and *Stylochoplana* sp. specimens were collected from Pilot Bay, Tauranga, New Zealand (37°63'5" S, 176°17'6" E) [28, 29]. *Pleurobranchaea maculata* individuals were collected on 27 September 2012 and *Stylochoplana* sp. specimens on 12 September and 25 October 2013. Non-toxic *P. maculata* specimens were collected from Tasman Bay, New Zealand (41°05' S, 173°06' E) on 7 August 2012 [28].

Egg masses from *P. maculata* were collected on 9 September 2013 from Pilot Bay and kept in an aerated aquarium for 3 days before freezing (-20 °C) sections for TTX analysis. Egg masses were tested in their entirety and in addition small sections were carefully scrapped clean of egg capsules leaving only the gelatinous casing for testing. The remaining egg masses were left in the aquarium until hatching (day 7). A sample of larvae (50 mL) was centrifuged (3,000 × *g*, 10 min), seawater removed, and frozen (-20 °C) for TTX analysis. Additional subsamples (50 mL) of larvae were collected, fixed with ethanol, and used for enumeration to determine the number of individuals in each sample. Counts were conducted using a 5 mL chamber and a dissecting microscope (Olympus SZ60).

An additional twenty-three *Stylochoplana* sp. specimens were collected from Pilot Bay (7 June 2012) and transported to laboratory aquaria in individual small plastic containers with 50 mL of seawater. Specimens were maintained in aerated aquariums (19L) with 14L of filtered seawater (0.22 μm). One individual laid two egg masses fourteen days after collection. Egg masses were removed from tanks and frozen (-20 °C) for TTX analysis. The adults were not included as part of this study.

3.2. Histochemistry

Pleurobranchea maculata were aseptically dissected into tissue types including the mantle, gonad, and digestive tissues. Small sections of the mantle tissue from *P. maculata* were removed, while the gonad and digestive tissues were kept intact due to the fragility of theses tissues. *Stylochoplana* sp. specimens were left whole due to their small size (ave. 60 mg) and fixed flat using the techniques described in Newman and Cannon [58]. Briefly flatworm specimens

were transferred using a small artist brush to a piece of filter paper dampened with ambient seawater in order to encourage them to lie flat. Filter paper was then transferred into a container with frozen fixative (2% glutaraldehyde/4% paraformaldehyde), which was left to melt. Once fixative was melted, filter paper was removed from the solution and specimens were stored for 24 hours (4 °C).

Tissues and specimens were fixed overnight in 2% glutaraldehyde/4% paraformaldehyde, dehydrated through increasing concentrations of ethanol to xylene, embedded in paraffin, and sectioned at 7 to 10 μm thickness on a microtome (Leica RM 2055, Leica Biosystems, Germany). Immunohistological sections were deparaffinized and rehydrated in ethanol before treatment with 3% H_2O_2/10% methanol to remove endogenous peroxidase activity followed by incubation with normal goat serum (VectorLabs) to prevent non-specific binding. Both the H_2O_2/methanol mixture and normal goat serum (VectorLabs; component of VECTASTAIN® ABC kit, diluted according to manufacturer's instructions) were diluted with 1× phosphate buffered saline (1×PBS, pH 7.2). Slides were then incubated with a TTX-specific monoclonal antibody (mAb) T20G10 diluted to 0.5 ug mL^{-1} [30, 59] in concert with VECTASTAIN® ABC kit (VectorLabs) according to the manufacturer's instructions (Table 2). As a negative control, isolated tissue sections on each slide were incubating with solely with PBS in place of the anti-TTX mAB. Visualization of the antigen-antibody complex was conducted using 3,3′-diaminobenzidine (DAB) substrate solution resulting in a brown color deposit. Sections were counterstained with Gill's II Hematoxylin (Surgipath®, Leica Biosystems, Germany), mounted, and observed under a light microscope (Leica DMRE with plan fluorite lenses, Leica Biosystems, Germany).

Table 2. Immunohistological incubation scheme. Steps were undertaken at room temperature unless otherwise specified. PBS = phosphate buffered saline, anti-TTX mAB = monoclonal antibody, DAB = 3,3′-diaminobenzidine.

Step	Solution	Time (min)
1.	3% H_2O_2/10% methanol	10
2.	1×PBS	10 × 3
3.	Normal Goat Serum (Vector Labs)	20
4.	1×PBS	10 × 3
5.	anti-TTX mAB T20G10*	Overnight @ 4°C
6.	1×PBS	10 × 3
7.	Biotinylated secondary antibody (anti-rabbit IgG)*	60
8.	1×PBS	10 × 3
9.	VECTASTAIN® ABC reagent*	60
10.	1×PBS	10 × 3
11.	DAB	2 - 5
12.	Deionized H_2O	5
13.	Counterstain (Gill's II Hematoxylin)	2

* reagents diluted with 1× PBS, pH 7.2, modified with 0.5% Triton X-100 and 0.25% m/v type B gelatin.

Some sections of *Stylochoplana* sp. and *P. maculata* mantle tissue were also stained with Gills II hematoxylin (Surgipath®; Leica Biosystems, Germany) and eosin (Surgipath®; Leica Biosystems, Germany), and the Alcian Blue-Periodic Acid-Schiff (AB-PAS) method to differentiate between neutral and acidic mucins [60]. For AB-PAS staining, paraffin sections were rehydrated with water and stained with alcian blue (5 min), rinsed with distilled water, and flooded with 1% periodic acid (2 min) and rinsed again. Slides were then immersed in Schiff's reagent (8 min) and washed in running water (10 min). Sections were lightly counterstained with Mayer's hematoxylin (2 min) before a final rinse with water. Sections were then dehydrated in an ascending ethanol series, cleared in xylene, mounted in D.P.X. (Merck Millipore, MA, USA), and observed under a light microscope (Leica DMRE with plan fluorite lenses, Leica Biosystems, Germany).

3.3. Tetrodotoxin analysis

Entire egg masses of *Stylochoplana* sp. specimens and sub-samples of egg masses from *P. maculata*, both with and without eggs, and hatched larvae were extracted for TTX. Samples (ca. 0.1 g) were first diluted 1:10 (w:v) with Milli-Q containing 0.1% v/v acetic acid. Each sample was manually homogenized with a glass pestle and vortexed to ensure complete disruption of tissues. Samples were centrifuged (3,000 × *g*, 10 min) and an aliquot of the supernatant was removed. This was diluted 1:10 with 100% methanol containing 0.1% v/v acetic acid and frozen (-20 °C) for at least 1 hr. Samples were then centrifuged (3,000 × *g,* 10 min) and diluted 1:4 with 100% methanol containing 0.1% v/v acetic acid and analyzed for TTX using LC-MS as described in McNabb *et al*. [27].

4. CONCLUSIONS

Tetrodotoxin was found to be sequestered in the mantle, reproductive tissues, eggs, and larvae of *P. maculata*. Definitive characterization of the type of TTX-containing cells in the mantle of *P. maculata* is difficult to ascertain with paraffin techniques, and electron microscopy would greatly aid in identifying cell types and elucidating their potential functions. Tetrodotoxin localization in the digestive tissue could be indicative of a dietary source of TTX in this species. The *de novo* synthesis or sequestration of secondary metabolites from prey for use as a defense mechanism in Opisthobranchs is a well-known phenomenon [reviewed in

31]. The sequestration of TTX in the mantle, eggs, and larvae may be suggestive of a defensive role in *P. maculata*.

Localization of TTX in the pharynx, ova, and egg masses of *Stylochoplana* sp. could be indicative of ecological roles including aiding in capturing prey and protection of offspring. Feeding studies are required to confirm if TTX contributes to prey capture, and additional collection of TTX-positive egg masses and localization of TTX would further support the suggestion of protection in offspring.

The methods by which *P. maculata* and *Stylochoplana* sp. sequester TTX are unknown. Immunohistologically-stained sections of *P. maculata* show that low concentrations of TTX are present throughout most tissues, while TTX is exclusively localized in the ova and pharynx of the *Stylochoplana* sp.. This could be a product of differing anatomy (coelomate verses acoelomate) or that sequestration techniques differ between the two species. Tetrodotoxin-binding proteins have been isolated from a number of invertebrates including horseshoe crabs [61], xanthid crabs [62], and several gastropods [63] as well as several pufferfish species including *Takifugu niphobles* [64] and *Fugu pardalis* [65]. Determining if these proteins are present in *P. maculata* or *Stylochoplana* sp. would assist in understanding the transfer and transport of TTX in these organisms.

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Supplimentary

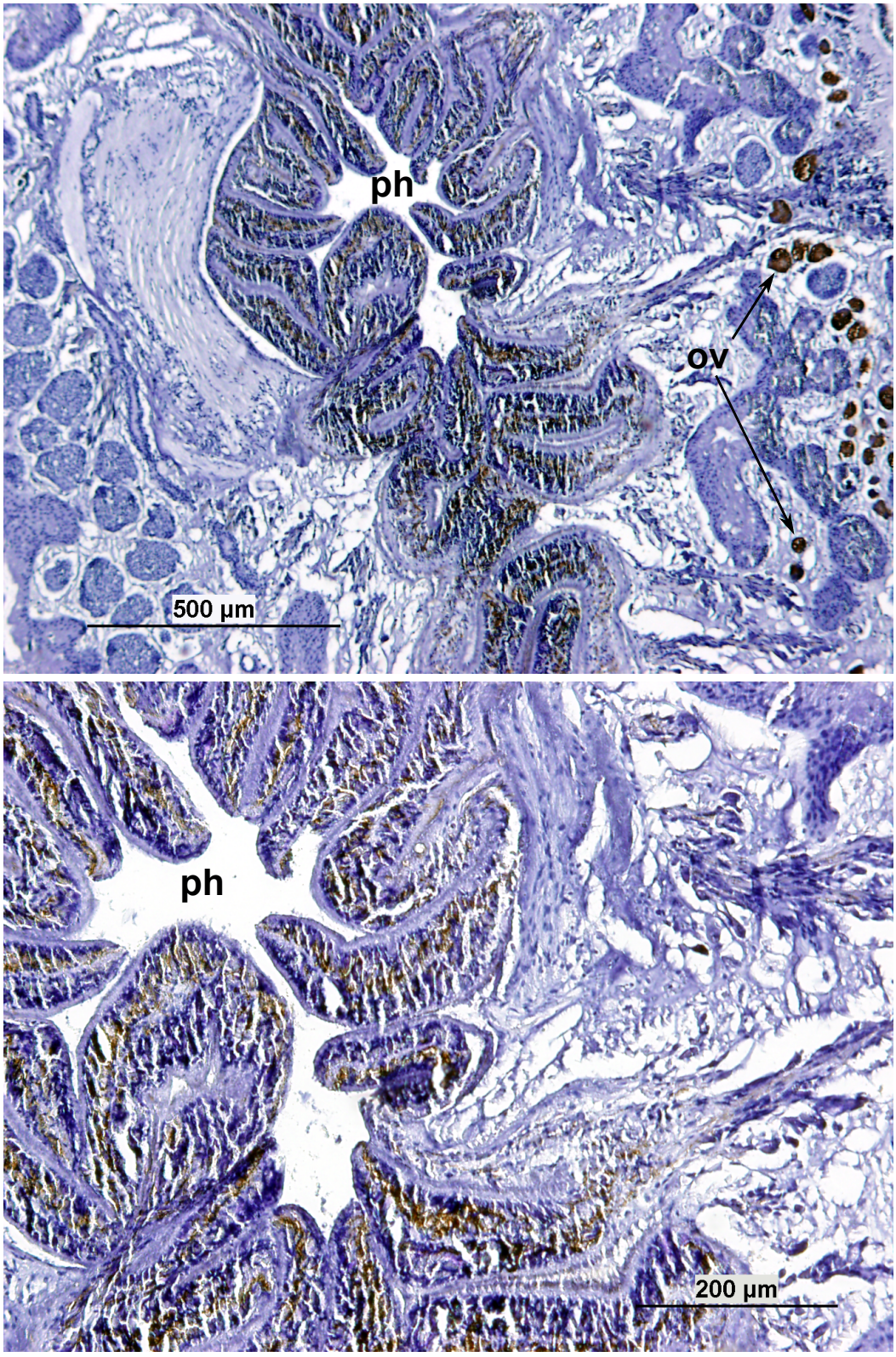


Figure 1S. *Stylochoplana* sp. dorsoventral view sectioned at 7 µm. Tetrodotoxin specific monoclonal antibody immunohistological staining (TTX identified by brown color deposits). Panels show pharynx (ph) under increasing magnification. ov = ova.

VI. CONCLUSIONS

The ultimate origin of tetrodotoxin (TTX) remains one of the greatest mysteries in marine natural toxins research. The phylogenetic diversity of organisms containing TTX suggests an exogenous, and most likely microbial, source of TTX. This thesis is part of a larger research initiative aimed at elucidating the origin of TTX in *Pleurobranchaea maculata*. A multifaceted approach was taken involving aquaria based studies, isolation of bacteria, *in situ* experiments, and surveys of natural populations. This chapter provides a synthesis of the research undertaken in my thesis but also draws on the results of the larger project in order to gain insights into the origin, ecological roles, and distribution of TTX in New Zealand marine organisms.

1. Summary of studies

The following sections provide short summaries and key findings of the individual studies resulting from the overarching research project. Sections 1.1 – 1.7 are summaries of current literature and Sections 1.8 – 1.11 are summaries of data generated directly from this thesis.

1.1 Identification of TTX in Pleurobranchaea maculata*; McNabb et al.* (2010)

Tetrodotoxin was first detected in the tissues and egg masses of *Pleurobranchaea maculata* when they were included in surveys conducted as a result of dog illnesses and deaths linked to visits to beaches in and around the Auckland region. This was the first detection of TTX in an opisthobranch and the first detection of TTX in New Zealand. This discovery provided the starting point for the research conducted in this thesis.

1.2 Geographic and within population variation of tetrodotoxin in Pleurobranchaea maculata*; Wood et al.* (2012a)

The key findings of this study were the distinct geographical separation of toxic and non-toxic populations. Significant variability of TTX concentrations within and among toxic populations was also reported. These data suggest a spatially variable exogenous source of TTX that is only available to North Island populations. The observation of extremely high concentrations of TTX, coinciding with dense populations of *P. maculata*, also suggests that there might

be some within population recycling of TTX due to cannibalism. Further evidence for this was provided from observations that multiple *P. maculata* maintained in aquarium, ate each other. Furthermore, one 2nd generation *P. maculata,* grown in captivity from an egg, contained no TTX indicating that *P. maculata* cannot produce TTX endogenously. It was also proposed that TTX plays a protective role in the progeny due to the detection of significant concentrations of TTX in egg masses and hatched larvae.

1.3 Depuration rates of tetrodotoxin in Pleurobranchaea maculata *in captivity; Wood et al.* (2012b)

The key findings of this study were the depuration of TTX from adult *P. maculata* when kept in captivity. Although *P. maculat*a might stop producing TTX in the aquarium environment, is seems most likely that this is indicative of an external source of TTX. Strong correlations between TTX concentrations in adults and their first laid egg masses indicated that adults invest TTX their progeny, presumably as a protective measure. Large differences in TTX concentrations in *P. maculata* tissues were not matched by differences in their associated bacterial community structure, suggesting that TTX in *P. maculata* is not a product of a microbial symbiont.

1.4 Sequestration rates of tetrodotoxin in Pleurobranchaea maculata *and environmental studies; Khor et al.* (2013a).

During an aquarium based study where non-toxic *P. maculata* were fed TTX-containing food, TTX was rapidly sequestered into all tissues types (highest in mantle tissue) within one hour and no negative behavioral effects were observed. Several environmental surveys at sites with high abundances of *P. maculata* revealed low concentrations of TTX in the sand dollar *Arachnoides zelandiae* and trace levels in the stalk-eyed mud crab *Macrothalamus hirtipes*, the cat's eye snail *Turbo smaragdus*, and the coralline algae *Corallina officinalis*. The ability to sequester TTX orally, and the existence of TTX at low concentrations in co-existing organisms, provided further evidence for an exogenous source of TTX in *P. maculata*.

1.5 Culturable bacteria from the tissues of Pleurobranchaea maculata*; Chau et al.* (2013)

This study attempted to isolate a TTX-producing bacterium from the tissues of *P. maculata* and their egg masses. Twenty-two bacterial strains were isolated and, using molecular techniques, shown to be from five different orders. None produced TTX, providing some evidence to suggest that a symbiotic microbial producer of TTX in *P. maculata* is unlikely, or that culturing of these organisms is not as straight forward as previous studies have suggested.

1.6 Development of a sensitive technique to detect the C9 backbone of tetrodotoxin and detection of tetrodotoxin in Paphies australis*; McNabb et al.* (2014).

The need for a more sensitive and specific method to detect potential precursor or degradation products of TTX led to the development of a novel methodology to detect the C9 base of TTX after reaction with a strong base. In addition to the successful development of the method, an important finding of this study was the detection of TTX in the marine bivalve *Paphies australis* at concentrations up to 0.8 mg kg^{-1}. As *P. australis* are filter feeders, these data indicate that TTX is most likely accumulated from an environmental source in this organism, or given that there were some *P. maculata* populations within the region it is plausible that they could be filtering a bi-product from *P. maculata* i.e., fragments from egg masses.

1.7 Identification of tetrodotoxin in other fauna at sites harboring toxic Pleurobranchaea maculata*; Ogilvie et al. (2012)*

This report details the detection of low concentrations of TTX in several additional marine organisms including the seven-armed starfish *Astrostole scabra*, the New Zealand rock oyster *Saccostrea glomerata*, and the Pacific oyster *Crassostrea gigas*. However, only one specimen of each tested positive TTX, while other individuals of these species tested were below detection limits. This suggests that there are most likely more organisms in New Zealand marine environments that have the ability to sequester/be resistant to TTX but that the source of TTX is either extremely patchy (which is less likely for a microbial source), or that these detections are, as suggested above, related to the consumption of bi-products of *P. maculata* or *Stylochoplana* sp. (see below).

1.8 *Chapter II – Identification of tetrodotoxin in* Stylochoplana *sp.; Salvitti et al. (2015a).*

The major finding of this study was the detection of TTX for the first time in the flatworm *Stylochoplana* sp. from Pilot Bay, which had, on average, TTX concentrations of 380 ± 220 mg kg^{-1}. Tetrodotoxin concentrations increased from March to a peak during the June to August period, and declined during the remainder of the sampling period ending in November. Patterns were similar to those seen in *P. maculata*, however, there was considerably less variability in TTX concentrations. This, along with the presence of markedly higher quantities of C9 base compared to TTX, suggests that *Stylochoplana* sp. acquires a precursor compound from a dietary source, which is converted to TTX in the organism. A real-time PCR genetic assay detected *Stylochoplana* sp. DNA in the foregut of *P. maculata* and this, in concert with TTX mass calculations indicates that *Stylochoplana* sp. could represent a major source of TTX in *P. maculata*. However, this does not explain the extremely high TTX concentrations observed in some populations. The presence of TTX in *Stylochoplana* sp. egg masses and the similarity of temporal patterns to *P. maculata* suggest that the toxin could also play a protect role in progeny of *Stylchoplana* sp..

1.9 *Chapter III – No evidence for a culturable bacterial tetrodotoxin producer in* Pleurobranchaea maculata *and* Stylochoplana *sp. Salvitti et al. (2015b)*

The possibility of a microbial producer of TTX for *P. maculata* and *Stylochoplana* sp. was explored in this chapter. Bacterial strains (102) were isolated from tissues of both organisms and analyzed for the C9 base using the sensitive methodology reported in McNabb et al. (2014). To take into account the possibility that a consortium of bacteria are required to produce TTX a series experiments were undertaken which involved inoculating broth with homogenized tissue of *P. maculata*. No culturable bacteria from the tissues of *P. maculata* or *Stylochoplana* sp. were found to produce the C9 base of TTX, nor did the presence of host tissues influence TTX production by culturable bacteria. These results, taken in conjunction with previously published literature, suggest that the origin of TTX in *P. maculata* and *Stylochoplana* sp. is exogenous or that TTX-producing bacteria are unculturable.

1.10 Chapter IV – In situ accumulation of tetrodotoxin in non-toxic Pleurobranchaea maculata*.*

The key finding of this chapter was the ability of non-toxic *P. maculata* to sequester TTX *in situ* when placed in exclusion cages in an environment known to support toxic populations of the same species. This further supports the hypothesis of an exogenous source of TTX. Specimens placed in cages directly on the benthos sequestered more TTX than those in cages suspended 0.5 m above the surface suggesting that TTX (or a precursor compound) is more readily available from the benthos. Diet analysis of the foregut contents of *P. maculata* native populations, using next generation sequencing, revealed relatively high abundances of sequences belonging to hydroids of the *Plumularia* genus (Cnidaria) and segments worms of the genus *Thelepus* (Annelida), highlighting suspect organisms that could be investigated further during ongoing studies exploring the origin of TTX.

1.11 Chapter V – Intracellular immunoenzymatic detection of tetrodotoxin in Pleurobranchaea maculata *and* Stylochoplana *sp.. Salvitti et al. (2015c)*

The most significant findings of this study include the observations of TTX in the neutral mucin cells of the epidermis and the basement membrane of the mantle tissue of *P. maculata* specimens, supporting earlier hypotheses that TTX plays a defensive role in this organisms. Additionally, strong sequestration of TTX was seen in the oocytes and their surrounding follicles in the gonad tissues. Using LC-MS, TTX was found only in the eggs and larvae of *P. maculata*, not the gelatinous egg mass. The presence of TTX in specialized reproductive tissues, as well as eggs and larvae, clearly indicates that TTX is imparted to progeny. Tetrodotoxin was also visualized in the digestive tissue, supporting a dietary source of toxin.

Tetrodotoxin sequestration in the *Stylochoplana* sp. was considerably more localized than *P. maculata*, and was only detected in the ova and the pharynx. Tetrodotoxin in the pharynx could aid in prey capture and its presence in the ova and egg masses (measured by LC-MS), are indicative of a protective function for offspring, similar to *P. maculata*.

2. Synthesis of studies

The ultimate goal of the larger project, of which this thesis was a part of, was to elucidate the origin of TTX in *P. maculata*. A triangulated approach was used to investigate the source of TTX, including aquaria studies detailing the depuration and uptake of TTX, *in situ* experiments to explore whether TTX sequestration was a product of the environment, culturing attempts aimed at isolating a TTX-producer, and histological techniques to explore the micro-distribution of TTX within tissue types. Based on the key results from my thesis, in concert with the parallel studies I hypothesize that *P. maculata* are most likely obtaining TTX from dietary source through several different pathways including; (1) the consumption of the flatworm *Stylochoplana* sp., (2) ingestion of microbial organisms from the benthos that produce a precursor compound which is converted to TTX with the organism, and (3) when abundances of *P. maculata* are dense, cannibalism (Figure 1).

Since the initial identification of TTX *in P. maculata* in 2009, TTX has been identified in New Zealand in 10 additional organisms (Table 1). The increase in TTX- containing organisms in most likely a consequence of more sensitive detection techniques and more extensive testing (McNabb et al. 2010, 2014), and/or possibly, due to the increases in ocean temperature, and the migration of a microbial TTX (or precursor)-producer southwards to New Zealand waters. Figure 1 is a schematic detailing the various suggested avenues of TTX sequestration in these organisms. Organisms contained within the dotted box in Figure 1 (*Arachnoides zelandiae, Turbo smaragdus, Macrophthalmus hirtipes, Patiriella regularis, and Astrostole scabra*) were sampled at sites containing high abundances of toxic *P. maculata* and only very low concentrations of TTX were detected in their tissues. I suggest that the detection of TTX in *Arachnoides zelandiae* is from a dietary source, most likely originating from mucus trails of *P. maculata* (Khor et al. 2013b) detected trace to 1.2 mg kg^{-1} concentrations of TTX in *P. maculata* mucus). As detailed in Chp IV, *Patiriella regularis* were witnessed ingesting the toxic egg masses of *Pleurobranchaea maculata* and TTX concentrations found in their tissues are most likely a result of this dietary choice. *Turbo smaragdus, M. hirtipes*, and *Astrostole scabra* specimens were also collected at sites with high abundances of *Pleurobranchaea maculata*, however, given their relatively small size, it is unlikely they ingested adult *P. maculata*, but

like *Patiriella regularis*, could have ingested their toxic egg masses. The sea squirt *Styela clava* (found recently to contain TTX ca. 0.2 mg kg^{-1}, unpublished data, Cawthron Institute), *Crassostrea gigas*, *Saccostrea commercialis*, and *Paphies australis* are all filter feeders and could potentially obtain their TTX from resuspended benthic micro-organisms, explaining the low concentrations found in their tissues. Based on the data from my studies I suggest that the microorganism may not produce TTX, but rather a precursor compound, and that these filter feeders would therefore have to contain the biochemical pathway to convert this compound to TTX. Alternatively, they may be acquiring TTX by filtering *Pleurobranchaea maculata* larvae or fragments of egg masses. Given that only a few individuals of the many tested contained TTX, this may be the more likely scenario.

Among these additional organisms, the flatworm *Stylochoplana* sp. had the highest, and most consistent, concentrations of TTX. The disparity between the concentrations of the C9 base and TTX provides compelling evidence that they obtain (from a dietary source), or produce, a precursor compound that is then converted to TTX. The TTX concentrations were generally very consistent among individuals, which could support either an endogenous or dietary source of this compound. A bacterial symbiont source of TTX for *Stylochoplana* sp. is unlikely because no TTX-producing bacteria were cultured from their tissues (Salvitti et al. 2015b).

The wealth of information generated during these collective studies has also provided clues on the ecological roles of TTX in *P. maculata* and *Stylochoplana* sp.. The sequestration of TTX in the mantle tissues of *P. maculata* most likely aids in the protection and defense against predators, while its presence in the oocytes of the gonad tissue, egg masses, and larvae demonstrate that they impart this protection onto their progeny. The micro-distribution of TTX in the ova and pharynx of *Stylochoplana* sp. is suggestive of several possible ecological roles. Firstly, TTX in the ova, as well as egg masses, suggests that it also plays a protective role in offspring. Secondly, TTX in the pharynx is most likely utilized to help subdue prey, as seen in other flatworms e.g., *Planocerid* sp. (Ritson-Williams et al. 2006)

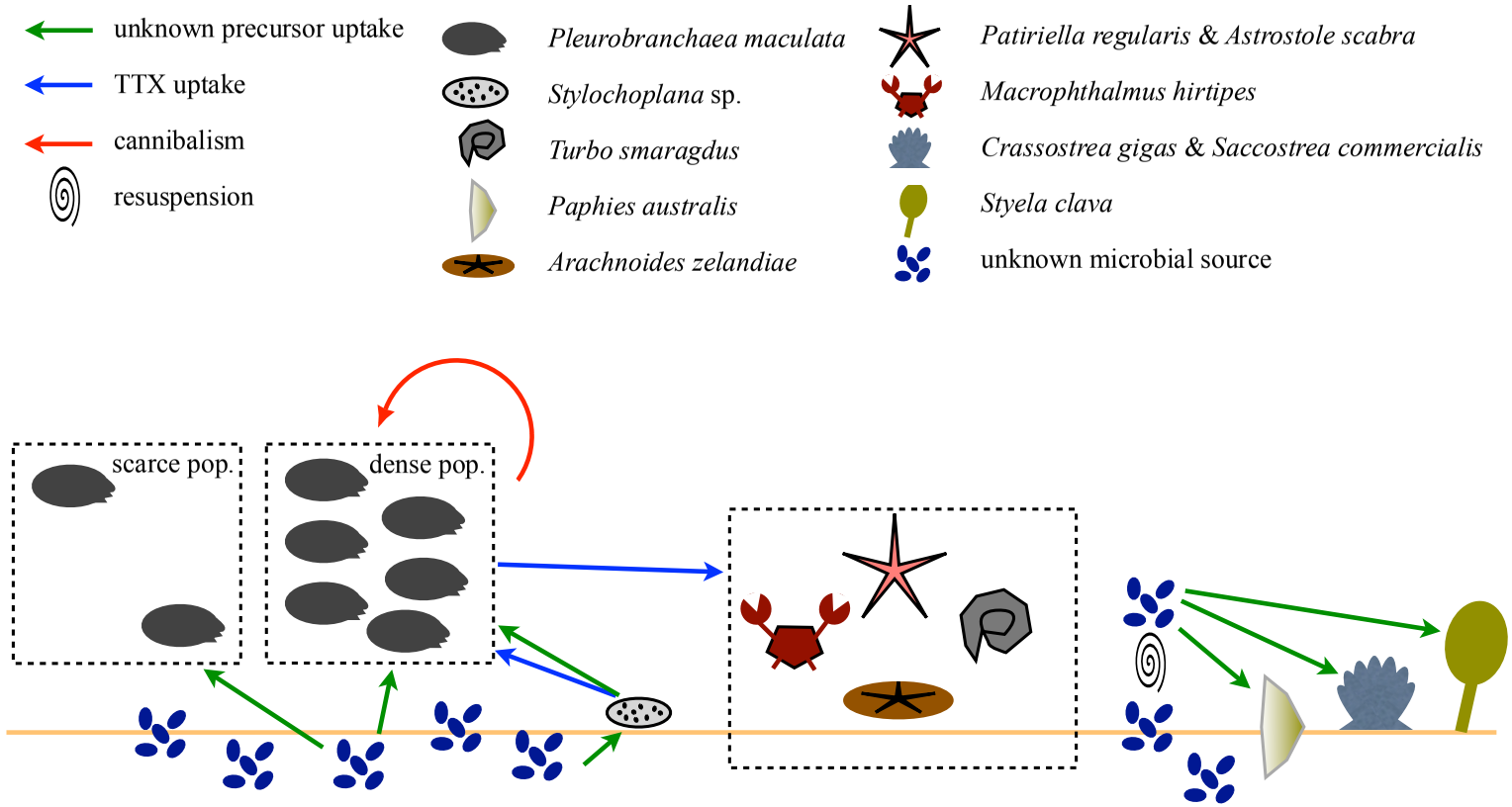
Table 1. Species from New Zealand waters found to contain tetrodotoxin within their tissues.

Species	Phylum	Feeding type	Max. mg kg^{-1}	TTX Variability	# tested	Reference	Detection of TTX in similar organisms
Pleurobranchaea maculata	Mollusca	Scavenger	1414	Extreme	>100	McNabb et al. (2010); Wood et al. (2012a)	Molluscs: *Nassarius* sp. (Huang et al. 2008); *Natica lineata* (Hwang et al., 1990); Tutufa lissostoma (Noguchi et al., 1984)
Stylochoplana sp.	Platyhelminthes	Scavenger	1070	Moderate	48	Salvitti et al. (2015)	Ribbon worm: *Cephalothrix linearis* (Ali et al., 1990); Flatworm: *Planocera multitentaculata* (Miyazawa et al., 1986); *Planocerid* sp. (Ritson-Williams et al., 2006)
Patiriella regularis	Echinodermata	Scavenger	0.14	N/A	2	Salvitti et al. (submitted)	Starfish: *Astropecten* sp. (Lin et al., 1998; Maruyama et al., 1984; Miyazawa et al., 1985; Noguchi et al., 1982)
Paphies australis	Bivalvia	Filter	0.8	Low	>100	McNabb et al. (2014)	Molluscs: *Nassarius* sp. (Huang et al. 2008); *Natica lineata* (Hwang et al., 1990); Tutufa lissostoma (Noguchi et al., 1984)
Arachnoides zelandiae	Echinodermata	Scavenger	0.25	N/A	≤ 5	Khor et al.(2013a)	Starfish: *Astropecten* sp. (Lin et al., 1998; Maruyama et al., 1984; Miyazawa et al., 1985; Noguchi et al., 1982)
Turbo smaragdus	Mollusca	Scavenger	Trace	N/A	≤ 5	Khor et al.(2013a)	Molluscs: *Nassarius* sp. (Huang et al. 2008); *Natica lineata* (Hwang et al., 1990); Tutufa lissostoma (Noguchi et al., 1984)

Table 1 cont.

Species	Phylum	Feeding type	Max. mg kg^{-1}	TTX Variability	# tested	Reference	Detection of TTX in similar organisms
Macrophthalmus hirtipes	Arthropoda	Scavenger	Trace	N/A	≤ 5	Khor et al.(2013a)	Horseshoe crab: *Carcinoscorpius rotundicauda (*Dao et al., 2009; Kungsuwan et al., 1987); Crab: *Lophozozymus pictor* (Tsai et al., 1995)
Corallina officinalis	Rhodophyta	Photosynthetic	Trace	N/A	≤ 5	Khor et al.(2013a)	Red alga: *Janis* spp. (Yasumoto et al., 1986)
Styela clava (sea squirts)	Tunicata	Filter	1.8	N/A	2	Unpub. data	Tunicate: *Phallusia nigra* (Freitas et al., 1996)
Crassostrea gigas (Pacific oyster)	Mollusca	Filter	0.08	N/A	26	Ogilvie et al. (2012)	Scallop: *Patinopecten yessoensis* (Kodama et al., 1996)
Saccostrea commercialis (rock oyster)	Mollusca	Filter	0.14	N/A	1	Ogilvie et al. (2012)	Scallop: *Patinopecten yessoensis* (Kodama et al., 1996)
Astrostole scabra (Seven-armed starfish)	Echinodermata	Scavenger	0.17	N/A	10	Ogilvie et al. (2012)	Starfish: *Astropecten* sp. (Lin et al., 1998; Maruyama et al., 1984; Miyazawa et al., 1985; Noguchi et al., 1982}

Figure 1. Proposed mechanism of tetrodotoxin (TTX) accumulation and/or an unknown precursor of TTX in New Zealand marine organisms. Filter feeders, including *Paphies australis*, *Crassostrea gigas*, *Saccostrea commercialis*, and *Styela clava* and the Platyhelminthes *Stylochoplana* sp. most likely obtain TTX from a benthic, or resuspended, microbial source. *Pleurobranchaea maculata* may obtain TTX from a variety of sources, including a benthic microbial source, ingestion of *Stylochoplana* sp., or through cannibalism when populations are high. The invertebrates *Turbo smaragdus*, *Arachnoides zelandiae*, *Patiriella regularis*, *Astrostole scabra*, and *Macrophthalmus hirtipes* have been found in areas of high *Pleurobranchaea maculata* abundance and likely gained their low levels of TTX by ingesting *P. maculata* egg masses or larvae.



3. Future

The origin of TTX remains one of the most contentious mysteries in marine natural toxins research. Its presence in many marine organisms (i.e., pufferfish) consumed by humans, and its potential medicinal benefits, has made it the focus of numerous studies. A large number of studies have targeted the potential for bacterial production of TTX, with some studies claiming to have isolated TTX-producing strains from host tissues. However, the use of inappropriate techniques and the lack of reproducibility have called previous results into question. Until research begins to implement the now more widely available sensitive analytical techniques, such as LC-MS, to convincingly show the production of TTX in microorganisms, this will continue to be an area of speculation. Additionally, most research groups have focused only on TTX in individual organisms, or groups of phylogenetically similar organisms. I suggest that solving this mystery will require combining their collective knowledge, and exploring commonalities and differences among organisms. The most significant advancement may occur when researchers working on terrestrial and aquatic organisms combine forces, as these groups tend to have contrasting views regarding the origin of TTX.

Further research initiatives, guided by the results stemming from this thesis, include several possible studies. The differences observed in the concentrations of the C9 base and TTX in the *Stylochoplana* sp. warrants additional investigation. Mass spectrometry could be used to screen *Stylochoplana* sp. to try and identify compounds containing the C9 base. If these can be elucidated, bacterial isolates and environmental samples could be screened for this compound to potentially identify its ultimate source. Its identification could also aid in the characterization of the currently unknown biosynthetic pathway of TTX. Additionally, surveys for *Stylochoplana* sp. populations at other sites around New Zealand, and analysis of their TTX concentrations in comparison to *P. maculata* populations, may provide new knowledge that could help in identifying the source of TTX in New Zealand. For example, if no TTX-containing *Stylochoplana* sp. were identified in the South Island, this would support the suggestion of a geographically limited source of TTX or TTX precursors in these organisms. The monitoring of temporal TTX concentrations in captive *Stylochoplana* sp. may also indicate whether these organisms obtain their

toxin from an exogenous source, or if TTX concentrations are maintained, this could suggest that they possibly produce TTX themselves, and are a primary origin.

Although a TTX-producing microbial symbiont was not isolated in this study, there is still the possibility that one exists within the tissues of *P. maculata*. Another possible way to test for this would be to 'co-house' toxic and non-toxic specimens together in the same aquarium, with a mesh barrier preventing contact but allowing free exchange of water (and bacteria) for 4 to 6 weeks. If the source of TTX is bacterial we would expect that over time this strain would be transferred to the non-toxic specimens. Biopsy samples using the technique developed in Khor et al. (2013b), could be used to monitor TTX concentrations among individuals throughout the experiment.

As observed in this study, the sequestration of TTX from the environment by non-toxic *P. maculata* was suggestive of a possible benthic microbial source of TTX (or precursor compound). The deployment of sediment traps at these sites would allow for a more direct analysis of the bacterial and algal communities present at these sites. These samples should be size sorted and tested for both TTX and the C9 base. If C9 base, (or TTX) positive fractions are identified, these could be the target of culturing efforts or molecular analysis. Next generation sequencing of the gut contents of *P. maculata* from this study showed an abundance of annelids from the *Thelepus* genus and hydroids from the *Plumularia* genus. These organisms should also be targets of future environmental surveys, and assayed for both TTX and its C9 base.

If no exogenous source can be located, then transcriptomic studies of non-toxic populations versus toxic populations may assist in identifying those genes responsible for TTX production. Although technically challenging, this study would be most useful if it was undertaken on multiple TTX-containing organisms (i.e., pufferfish, newts) simultaneously.

In conclusion, the studies conducted here have collectively shown that TTX is more widespread in the New Zealand marine environment than previously thought. The consistent detection of TTX in the tissues of *P. maculata* and

Stylochoplana sp. in North Island populations, and that data generated during this project, strongly indicate a dietary source, most likely from a benthic micro-organism. We have been unable to definitively identify this organism, but our data suggests we may need to explore currently unknown pre-cursor molecules rather than TTX. Studies that span multiple organisms and environments may help reveal the true identity/source of TTX, and should be the focus of future large-scale projects aimed at identifying TTX producers.

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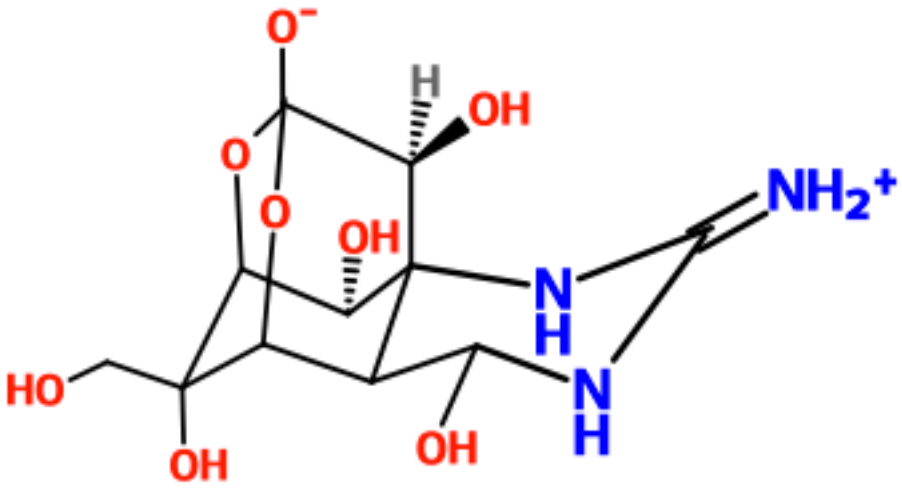
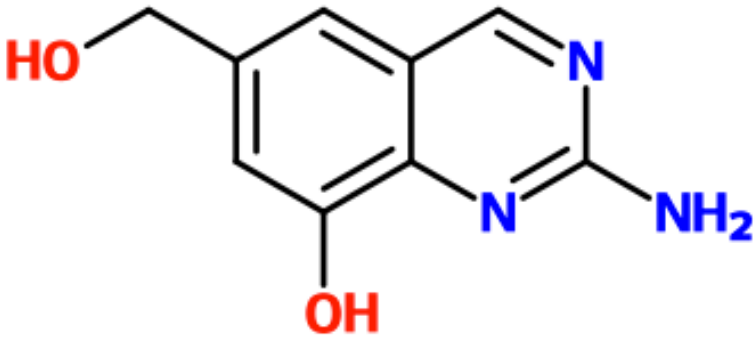
APPENDIX 1.



Tetrodotoxin ($C_{11}H_{17}N_3O_8$)
Mass = 319.1



C9 base ($C_9H_9N_3O_2$)
Mass = 191.1

Figure 1. Dehydration of TTX, with NaOH, to the C9 base - 2-amino-6-(hydroxymethyl)quinazolin-8-ol.

The following liquid chromatography-mass spectrometry (LC-MS) methods for the C9 (2-amino-6-(hydroxymethyl)quinazolin-8-ol) base of TTX are paraphrased from McNabb et al. (2014).

Liquid chromatography-mass spectrometry analysis was conducted using Waters Premier mass spectrometer coupled to a Waters Acquity UPLC system. A Thermo GOLD aQ 50 × 2 mm column (Thermo Fisher Scientific Australia Pty Ltd.) was used for reversed phase separation. Mobile phase A was 1% (v/v) formic acid and mobile phase B was 1% (v/v) formic acid in methanol. For the LC mobile phase parameters see the table below (Table 1).

Table 1. LC mobile phase parameters

Time, min	Flow, mL/min	Mobile phase A,	Mobile phase B,
0	0.25	100	0
0.5	0.25	100	0
0.75	0.25	75	25
1.5	0.25	75	25
1.55	0.25	25	75
2.0*	0.25	100	0

* 100% A is used from 2.0 min.

Ions monitored were: m/z 192.1 > 92.0 and 192.1 > 175.0 with a dwell time of 50 ms. The total run time was 4.0 min. The method was calibrated by spiking blank matrix-matched samples with TTX stock solution prior to extraction, base dehydration, and SPE cleanup. Spike levels were 10, 2, 1, 0.5, and 0.1 mg TTX/kg. A solvent blank was prepared from 1 mL water spiked with TTX equivalent to the 1 mg/kg matrix spike.

MassLynx software (Version 4.1, Waters MicroMass) was used to integrate the peaks. The concentrations of TTX in samples (mg/kg) were calculated directly from the area responses using a linear six-point calibration produced from the spiked blank matrix matched TTX standards (0.1–10 mg/kg) processed in the same way as samples.

McNabb PS, Taylor DI, Ogilvie SC, Wilkinson L, Anderson A, Hamon D, Wood SA, Peake BM (2014) First detection of tetrodotoxin in the bivalve *Paphies australis* by liquid chromatography coupled to triple quadrupole mass spectrometry with and without precolumn reaction. Journal of AOAC International. 97: 325-333.

APPENDIX 2.

The following liquid chromatography-mass spectrometry (LC-MS) methods for TTX analysis are paraphrased from McNabb et al. (2010):

A liquid chromatography-mass spectrometric (LC-MS) analysis method was set up to analyze for TTX utilising a Waters Acquity^{TM} UPLC and Waters Premier^{TM} triple quadruple mass spectrometer with electrospray source. A TosoHaas (Japan) TSK-GEL amide 80, 5 mL, 2.0 × 250 mm column was used for hydrophilic interaction liquid chromatography (HILIC) separation. Mobile Phase A was 10% acetonitrile –90% 50 mM formic acid, 2 mM ammonium formate in water. Mobile phase B was 90% acetonitrile –10% 50 mM formic acid, 2 mM ammonium formate in water. A gradient elution was used with a flow rate of 0.2 mL min^{-1} and linear gradient steps; T = 0, A = 0%, B = 100%; T = 10 min, A = 100%, B = 0%; t = 15min, A = 100%, B = 0% followed by a return to initial conditions.

Daughter ion spectra were collected by scanning all products from the m/z 320.1 parent mass from m/z 50 to m/z 330 at a collision energy of 35 eV whilst eluting samples from the UPLC using the gradient specified. Quantitative analysis was undertaken using mass channels monitored at: 320.1 > 162.1 (quantitative) and 320.1 > 60.0 (confirmative) as optimized by infusion of pure TTX and fragmentation using argon at a pressure of 3.5–4.0 10^{-3} mbar. Additional mass channels were included for known TTX analogues: 318.1 > 162.1 (11- oxo TTX), 304.1 > 162.1 (11-deoxy TTX), 302.1 > 162.1 (anhydro TTX) and 290.1 > 162.1 (11-nor TTX). The method was calibrated using a pure reference material (Tocris Bioscience, Cat. No: 1078). A stock solution was prepared by dissolving the entire contents of a single vial (1 mg) in 10% acetonitrile-water with 0.01% acetic acid to a final volume of 5 mL, which was stored at –20 °C for a maximum of 3 months. Calibration solutions were prepared by dilution from the stock with 80% methanol to give concentrations between 5 and 250 ng mL^{-1}. Data were collected and results calculated using MassLynx software with TargetLynx used for automation of calibration and quantitation. All calibrations were linear up to 250 ng mL^{-1} (R^2 > 0.98).

McNabb P, Selwood AI, Munday R, Wood SA, Taylor DI, MacKenzie LA, van Ginkel R, Rhodes LL, Cornelisen C, Heasman K, Holland PT, King C (2010) Detection of tetrodotoxin from the grey side-gilled sea slug - *Pleurobranchaea maculata*, and associated dog neurotoxicosis on beaches adjacent to the Hauraki Gulf, Auckland, New Zealand. Toxicon. 56(3): 466-473.