INVESTIGATING DIET AS THE SOURCE OF TETRODOTOXIN IN THE GREY SIDE-GILLED SEA SLUG, *PLEUROBRANCHAEA MACULATA*

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Abstract

Pleurobranchaea maculata (grey-side gilled sea slug) was discovered to contain the neurotoxin tetrodotoxin (TTX) in 2009 after a spate of dog poisoning cases on the beaches of Auckland, New Zealand. One of the great mysteries of TTX is the lack of conclusive evidence about its ultimate origin. Possible sources postulated have included both endogenous and exogenous. Additionally, within a species both toxic and non-toxic strains exist. For example, in New Zealand, *P. maculata* from the North Island are toxic, whereas *P. maculata* from the South Island are not. The overarching hypothesis of this Master's project is that TTX has a dietary origin in *P. maculata* and that they will preferentially feed on TTX-containing food. This Master's thesis aimed to test this hypothesis through three distinct aims: (1) To develop a non-lethal biopsy method and determine the feasibility for future research concerning TTX in *P. maculata*; (2) To investigate whether non-toxic *P. maculata* can accumulate TTX from a dietary source, and how TTX is distributed through the organism; and (3) To investigate whether *P. maculata* are attracted to TTX and if this varies depending on the TTX content within *P. maculata*.

A biopsy method was developed for taking approximately 200 mg tissue biopsies using a TemnoEvolution 18G × 11 cm Biopsy Needle inserted transversely into the foot. Six *P. maculata* were biopsied twice (nine days apart) and each individual was frozen immediately following the second sampling. Tetrodotoxin concentrations in biopsy samples, gonad, stomach, mantle and the remaining combined tissues and fluids were measured using liquid chromatography-mass spectrometry (LC-MS). Based on the proportional weight of the organs/tissues a total TTX concentration for each individual was calculated. There were strong correlations between biopsy TTX concentrations and the total ($r^2 = 0.88$), stomach ($r^2 = 0.92$) and gonad ($r^2 = 0.83$) TTX concentrations.

To investigate the accumulation of TTX, eighteen non-toxic *P. maculata* were maintained in aquariums and twelve were fed a TTX-containing diet. Three *P. maculata* were harvested after 1 hr, 24 hrs, 17 days and 39 days and TTX concentrations in their stomach, gonad, mantle and remaining tissue/fluids determined using LC-MS. Tetrodotoxin was detected in all organs/tissue after 1 hr

with an average uptake of 32%. This decreased throughout the experiment (21%, 15% and 9%, respectively). This study demonstrated that *P. maculata* can accumulate TTX from a dietary source.

To explore whether *P. maculata* were attracted to TTX, three preference experiments were undertaken; (1) an aquarium zonation experiment, (2) a toxic/non-toxic agar trail experiment and, (3) a direct choice experiment using combinations of toxic/non-toxic agar blocks. A statistically significant preference for TTX was found for toxic *P. maculata* in the agar trails ($P < 0.001$) and direct choice experiments ($P < 0.001$). For the non-toxic *P. maculata*, a statistically significant preference was only found for the direct choice experiment ($P = 0.002$).

Collectively these studies demonstrate that diet is a possible source in *P. maculata*. However, given the absence of identifiable TTX sources in environments where *P. maculata* are prevalent, in concert with their extremely high TTX concentrations and short life spans, it is unlikely to be the sole source for this species.

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Chapter 1 – Review of the Literature

1.1 Introduction to tetrodotoxin

Tetrodotoxin (TTX) is a naturally occurring, low molecular weight neurotoxin which causes death in humans upon the consumption of only 1-2 mg. It is most famous for causing deaths associated with eating puffer fish (Noguchi & Arakawa, 2008) and is named after the puffer fish family, *Tetraodontidae* (Ito et al., 2006) from which it was first isolated (Tahara, 1910; Yokoo, 1950). The toxin was named tetrodotoxin by Tahara (1910) but isolation of the crystalline compound was not achieved until Yokoo (1950) named the compound "spheroidine". The name "tetrodotoxin" was confirmed when it was later isolated by Tsuda and Kawamura (1952).

Originally, it was thought TTX was only found in puffer fish, however, TTX was soon discovered in terrestrial organisms, the first being the Californian newt (*Taricha torosa*) (Fuhrman, 1986; Mosher, Fuhrman, Buchwald, & Fischer, 1964). Since then, it has been identified in a range of other organisms, both marine (e.g., blue ringed octopus, horseshoe crabs, trumpet shells) and terrestrial (e.g., common garter snake, atelopid frogs) (Dao, Takata, Sato, Fukuyo, & Kodama, 2009; Mosher et al., 1964; Noguchi & Arakawa, 2008).

Puffer fish is a delicacy in Japan. The muscle of puffer fish is usually eaten as raw slices called "sashimi" (Noguchi & Ebesu, 2001). Puffer fish connoisseurs particularly enjoy the liver ("kimo") due the feeling of numbness around their lips but unfortunately it can contain TTX in high concentration and this has led to over 32 human deaths in Japan from 1987-96 and sporadic cases in other countries (Noguchi & Ebesu, 2001). Tetrodotoxin is heat-resistant and therefore, even after being cooked, there is no degradation of the toxin (Noguchi & Ebesu, 2001). In addition to Japan, other countries that report incidences of puffer fish poisoning are Singapore, Malaysia, Taiwan, Hong Kong, Fiji, China, Thailand, Kiribati, Australia, Bangladesh, U.S.A. and Papua New Guinea (Noguchi & Ebesu, 2001).

The Japanese Ministry of Health and Welfare enacted a guideline for edible species and parts of a puffer fish in 1983 prohibiting the sale of puffer fish liver (Noguchi & Ebesu, 2001). This has aided the decrease in number of fatalities due

to puffer fish poisoning (Narahashi, 2008). Tetrodotoxin poisoning is not only limited to the ingestion of puffer fish but has also occurred due to consumption of the digestive glands of the gastropod *Charonia sauliae* (1979, 1982, 1987) (Noguchi & Ebesu, 2001).

Symptoms of TTX poisoning often appear quickly and the victim's demise can occur within hours (Noguchi & Arakawa, 2008). Victims go through four stages of tetrodotoxication although the actual symptoms experienced as well as the severity depend on a variety of factors including; age, health and amount of toxin ingested (Noguchi & Ebesu, 2001). The first stage involves mainly neuromuscular (dizziness, paresthesia of lips, pupillary constriction) and gastrointestinal symptoms (nausea, vomiting and abdominal pain) (Noguchi & Ebesu, 2001). The second and third phases involve additional neuromuscular (paralysis of phalanges and extremities), cardiovascular/pulmonary (cardiac arrhythmias, dyspnea) and dermatologic symptoms (blistering) (Noguchi & Ebesu, 2001). The fourth stage involves respiratory failure, impaired mental faculties and seizures (Noguchi & Ebesu, 2001).

As yet, there are no antidotes or antitoxins to TTX. The only treatment available is supportive such as artificial respiration until recovery or death (Noguchi & Arakawa, 2008). Emetics and gastric lavage followed by activated charcoal may also be administered if vomiting has not already occurred in order to decrease the amount of TTX circulating within the body (Noguchi & Ebesu, 2001). In addition, fluid and electrolyte replacement therapy is performed to decrease fluid loss (Noguchi & Ebesu, 2001).

1.1.1 Function and distribution of tetrodotoxin

Tetrodotoxin functions by binding to voltage-gated sodium channels (Nakajima, Iwasaki, & Obata, 1962; Narahashi, Deguchi, Urakawa, & Ohkubo, 1960), in particular, the P-loop regions which are responsible for the regulation of action potentials (Lu, Zheng, Xu, Chen, & Zhang, 2011). The knowledge of this mode of action began with studies by Takahashi and Inoko (1889a, 1889b) who found that TTX was not an enzyme or organic base and it did not elicit a response in frog motor neurons. This was in contrast to the frog muscle itself, which

continued to respond to direct stimulation. Studies by Ishihara (1918) demonstrated that, not only were the motor neurons affected, but the sensory neurons were also impaired, however, only the portion of the neuron that had been exposed was affected. Experiments performed by Narahashi et al. (1960) used intracellular microelectrodes and showed that TTX did not affect resting membrane resistance or potential. Their research suggested that TTX blocked the increase in conductance by inhibiting movement of sodium ions across the neuronal membrane. Narahashi, Moore, and Scott (1964) demonstrated that although TTX blocked the mechanism for sodium conductance, it did not block the mechanisms for potassium conductance. This is a unique property as other chemicals, such as procaine, block both sodium and potassium channels (Blaustein & Goldman, 1966; Shanes, Grundfest, & Freygang, 1953; Taylor, 1959).

A study by Narahashi, Anderson, and Moore (1966) demonstrated that TTX did not have the same blocking effect internally compared to externally. This study was performed on the giant axon of a squid. An internal perfusion of TTX at the concentration of 1×10^{-5} M or 1×10^{-6} M had no effect whereas an external perfusion at the concentration of 1×10^{-7} M blocked action potentials for 3 to 6 min (Narahashi et al., 1966). Narahashi et al. (1966) inferred that the gate for the sodium channel was located externally as TTX would not be able to diffuse through due to its lipophilicity.

Voltage-gated sodium channels comprise of an α-subunit and four accessory β-subunits (Soong & Venkatesh, 2006). The α-subunit has four repeat domains (I - IV) which each have six membrane spanning segments (S1 - S6) (Narahashi, 2001). This subunit is the pore-forming subunit (Figure 1-1). Pores consist of two regions – an internal region and an external region formed by the P-loop regions between S5 and S6 (Narahashi, 2001). P-loop regions regulate the selectivity of sodium ions and are the site of TTX binding. The guanidinium group on TTX can fit the external orifice of sodium channels but the rest of the molecule cannot. This results in a blockage (Narahashi, 2008) which prevents sodium ions from entering and stops the propagation of an action potential (Lu et al., 2011).

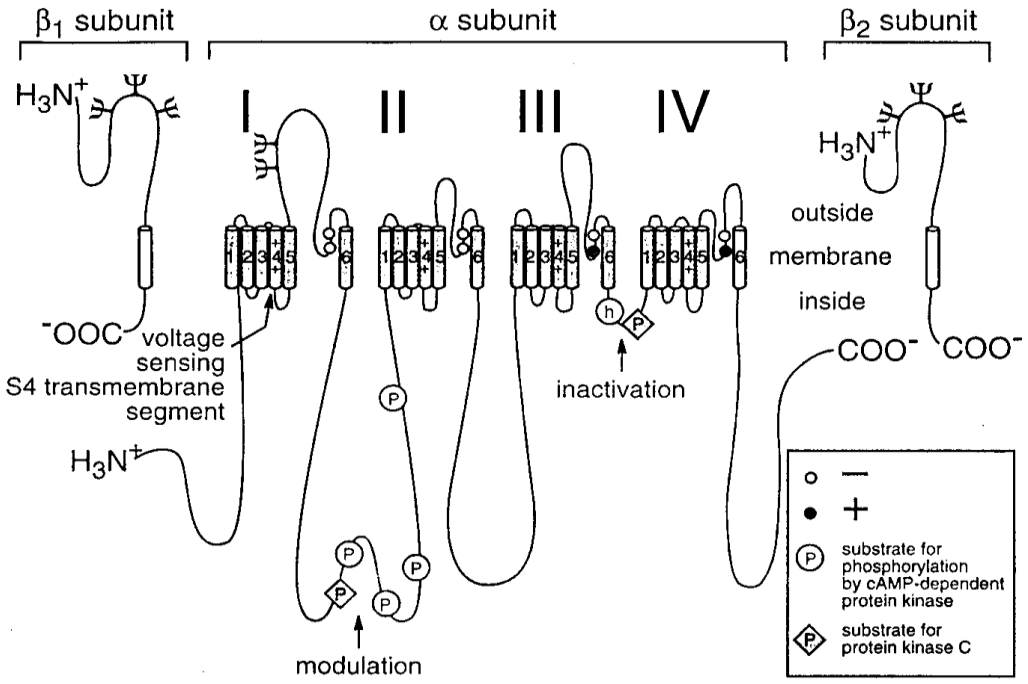

Figure 1-1. Diagram showing the tetrodotoxin binding site of Na_V channel from Narahashi (2001).

Ogura (1958) studied TTX distribution within the body and showed that concentrations of crystalline TTX administered subcutaneously were detected in the rat's blood, brain, kidneys, liver, heart, lungs and intestine peaking in concentration at 20 min with TTX concentrations highest in the kidneys and heart and lowest in the blood and brain. Based on the study by Ogura (1958), the half-life of TTX in the body is about 30 min in the heart and 3-4 hrs in the liver and kidney. Kao (1966) speculated that this indicated that TTX was excreted in an unchanged form.

1.1.2 Resistance to tetrodotoxin

In most mammals, the skeletal muscle sodium channels are TTX-sensitive (Lu et al., 2011). The substitution of amino acids in the P-loop regions of voltage-gated sodium channels confers varying levels of resistance to TTX (Lu et al., 2011; Soong & Venkatesh, 2006). These changes eliminate the interaction between the sodium channel and specific polar groups on the TTX molecule affecting the binding of TTX to the channel (Williams, 2010). Changes at multiple sites of the P-loop lead to cumulative resistance. Feldman, Brodie, Brodie, and

Pfrender (2010) found that allelic variation in one particular voltage-gated sodium channel gene ($Na_v1.4$) correlated strongly with TTX resistance in garter snakes (*Thamnophis sirtalis*).

Tetrodotoxin resistance has been shown in puffer fish and North American garter snakes. Both species consume a diet that contains TTX and it has been suggested that the reason why other mammals lack this resistance is due to the lack of TTX-bearing prey in their diet (Lu et al., 2011). Lee, Jones, Ahern, Sarhan, and Ruben (2011) found that TTX resistance came with biophysical costs such as decreasing the permeability of all permeable cations. This is costly to the performance of voltage-gated sodium channels.

A study by Saito et al. (1985) compared TTX resistibility in toxic and non-toxic species of puffer fish and found that the minimum lethal dose (MLD) for the three toxic species of puffer fish (*Fugu niphobles, F. pardalis* and *F. rubripes rubripes*) was higher (300-700 mouse units (MU)/20 g body weight) than the four non-toxic species of puffer fish (*Lagocephalus wheeleri, L. gloveri, Liosaccus cutaneus* and *Ostracion tuberculatus*; 0.9-20 MU/20 g body weight). It is interesting to note that the non-toxic species of puffer fish had slightly higher resistibility to TTX than the other fish species tested (*Girella punctata,Oplegnathus fasciatus, O. punctatus, Stephanolepis cirrhifer, Navodon modestus* and *Acanthogobius flavimanus*; 0.3-4.2 MU/20 g body weight) (Saito et al., 1985).

A similar study was undertaken by Koyama, Noguchi, Uzu, and Hashimoto (1983) on crabs and their resistibility to paralytic shellfish poisoning (PSP) and TTX. The toxic crab *Atergatis floridus* was found to be resistant to both PSP (MLD = 5000-10000 MU/20 g body weight) and TTX (MLD = 1000-2000 MU/20 g body weight) whereas non-toxic crab species (*Pilumnopeus indica, Pachgrapsus crassipes, Leptodius exaratus, Eriphia laevimona* and *Gaillardiellus orientalis*) had very low resistibility (0.5-2 MU/20 g body weight) (Koyama et al., 1983).

An alternative method of TTX resistance has been identified in the shorecrab (*Hemigrapsus sanguineus*) which contain TTX-binding proteins in their hemolymph (Williams, 2010). Yamamori, Yamaguchi, Maehara, and Matsui

(1992) studied non-toxic shorecrabs which had TTX-sensitive nerves and found that they had different levels of resistance to TTX but toxic shorecrabs were found to have much higher resistance levels. Yamamori et al. (1992) also found that body fluid of shore crabs had an effect of increasing TTX-resistance by up to 40-fold by reducing the effect of TTX on the nerves. Shiomi, Yamaguchi, Kikuchi, Yamamori, and Matsui (1992) then demonstrated the presence of TTX-binding high molecular weight substances in the body fluid that neutralised the effect of TTX. It is postulated that by binding to TTX, these proteins prevent TTX from binding to voltage-gated sodium channels (Hwang, Tsai, Lin, & Hwang, 2007a).

Tetrodotoxin resistance in puffer fish is not only explained by mutation of Na_v (Soong & Venkatesh, 2006) but also by TTX-binding proteins (Matsumoto et al., 2010; Yotsu-Yamashita et al., 2013; Yotsu-Yamashita et al., 2001). Yotsu-Yamashita et al. (2013) suggested that the puffer fish PSP and TTX binding protein (PSTBP) was a carrier protein that transferred TTX among their tissues. Lee et al. (2007) discovered genes (*flp-1, flp-2* and *flp-3*) in *T. chrysops* and *T. niphobles* that displayed a linear correlation with the toxicity of the liver of the two puffer fish. Tetrodotoxin-binding proteins have also been isolated from TTX-containing gastropods (Hwang et al., 2007a).

1.1.3 Attraction to tetrodotoxin

Some TTX-bearing organisms have been shown to have a particular preference for, and are attracted to, TTX (Hwang, Noguchi, & Hwang, 2004; Saito, Kageyu, Goto, Murakami, & Noguchi, 2000). Additionally, parasites have been shown to be attracted to TTX (Ito et al., 2006).

An experiment with puffer fish was undertaken where a choice of two different food sources was presented – food containing TTX versus TTX-free food (Saito et al., 2000). It was found that puffer fish made movements towards the food containing TTX and some even pecked at the food. However, they did not show preference for non-TTX containing food.

Similarly, an experiment was undertaken involving toxic (*Polinices didyma, Natica lineata, Natica vitellus, Zeuxis sufflatus, Niotha clathrata, Oliva miniacea, Oliva mustelina* and *Oliva hirasei*) and non-toxic (*Pomacea*

canaliculata and *Satsuma bairdi*) aquatic snail species (Hwang et al., 2004). The results showed that higher toxicity snails were attracted to TTX. Non-toxic snails, however, did not have this preference.

1.2 Detection of tetrodotoxin

Traditionally, mouse bioassays have been the most widely used tool for determining the TTX concentrations within organisms (Noguchi & Mahmud, 2001). This method involves an intraperitoneal injection of TTX and monitoring the symptoms (Noguchi & Mahmud, 2001) The potency of TTX is often measured in mouse units (MU) which is defined as "the amount of toxin required to kill a 20-g male mouse within 30 min after intraperitoneal administration" (Noguchi & Arakawa, 2008, p. 221). An unknown TTX extract is diluted with 0.1% acetic acid and aliquots are injected into a group of mice. The median value of the time till death at each dilution level is calculated and a dose-death time curve constructed (Noguchi & Mahmud, 2001). This method, although historically used, has several drawbacks such as low accuracy due to individual variation, lack of specificity, inconvenience and animal ethics and this has led to the development of alternative techniques such as high-performance liquid chromatography (HPLC), thin-layer chromatography, electrophoresis, enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-mass spectrometry (LC-MS) (Noguchi & Mahmud, 2001). Nowadays, both ELISA and LC-MS are the most common ways of detecting TTX.

High-performance liquid chromatography (HPLC) involves separating the toxin from contaminants and then converting the toxin into fluorescent compounds which get passed through a fluoromonitor (Noguchi & Mahmud, 2001). The retention time and fluorescent intensity are recorded by a chromatorecorder, leading to the production of a chromatogram (Noguchi & Mahmud, 2001). The toxins are then identified based on their retention time as compared to authentic TTX (Noguchi & Mahmud, 2001).

Thin-layer chromatography is a cheaper alternative method used where costly analytical systems are not available. It involves spotting TTX onto a silica gel-60 F_{254} pre-coated plate which is then developed in a sealed container with pyridine-

ethyl acetate-acetic acid-water (15:5:3: 4), 3-butanol-acetic acid-water (2:1:1) or 1-butanol-acetic acid-water (12:3:5) solvent (Noguchi & Mahmud, 2001). The solvent rises and the plate is then sprayed with 10% KOH and heated at 100°C for 10 minutes (Noguchi & Mahmud, 2001). The toxin is then visualised under UV light.

Electrophoresis is simple and rapid and involves application of 1 μL of TTX onto a cellulose acetate membrane. Ion molecules of TTX move towards the cathode with a mobility (R_m) smaller than that of STX (Noguchi & Mahmud, 2001). This is performed for 30 min in an electrolytic buffer solution with an applied electric field passed through it. This is then visualised under UV light (Noguchi & Mahmud, 2001).

Enzyme-linked immunosorbent assay (ELISA) is an immunoassay technique that uses antibodies and colour change to identify a substance (Zhou, Li, Pan, Liu, & Wang, 2007). Running an ELISA is relatively inexpensive and has the added advantages of high selectivity and sensitivity (Noguchi & Mahmud, 2001). Firstly, antigens are used to coat the surface of a microtiter plate. After this, the monoclonal antibody is added to bind with the antigen (Zhou et al., 2007). The antibody is then conjugated with an enzyme and finally, the enzyme's substrate is added and a colour is produced (Zhou et al., 2007). One disadvantage of this method is that cross-reactivity with non-TTX compounds can occur.

Liquid chromatography-mass spectrometry (LC-MS) involves combined HPLC coupled with a mass spectrometer. The HPLC is equipped with a column and a mobile solvent is used (Noguchi & Mahmud, 2001). The solution that comes out of the column is then split in order to provide flow to the ion-spray interface (Noguchi & Mahmud, 2001). Using LC-MS is advantageous because the exact mass of the compound is identified and it is a very sensitive technique.

1.3 Occurrence of tetrodotoxin – distribution and variability

Tetrodotoxin was first discovered in puffer fish but it has since been found in a wide variety of marine and terrestrial organisms from diverse phylogenetic backgrounds (Ito et al., 2006). Tetrodotoxin concentrations have been found to

vary among individuals in the same species, with season and with location (Table 1-1).

Table 1-1. A summary of the different organisms (excluding bacteria) that contain tetrodotoxin and their average toxicity. MU, mouse unit.

Animals	**Average Toxicity** (1 MU ≈ 0.22 μg of TTX) (Noguchi & Arakawa, 2008; Pires et al., 2002)	**Citation**
Puffer fish		
Takifugu niphobles	10 - >1000 MU/g tissue	Tani (1945)
Takifugu poecilonotus	10 - >1000 MU/g tissue	Tani (1945)
Takifugu pardalis	10 - >1000 MU/g tissue	Tani (1945)
Takifugu snyderi	10 - >1000 MU/g tissue	Tani (1945)
Takifugu porphyreus	100 - >1000 MU/g tissue	Tani (1945)
Takifugu chinensis	>1000 MU/g tissue	Tani (1945)
Takifugu obscurus	100 - >1000 MU/g tissue	Tani (1945)
Takifugu exascurus	100 - >1000 MU/g tissue	Tani (1945)
Takifugu pseudommus	10 - >1000 MU/g tissue	Tani (1945)
Takifugu chrysops	10 - 1000 MU/g tissue	Tani (1945)
Takifugu vermicularis	10 - 1000 MU/g tissue	Tani (1945)
Takifugu rubripes	10 - 1000 MU/g tissue	Tani (1945)
Takifugu xanthopterus	10 - 1000 MU/g tissue	Tani (1945)
Takifugu stictonotus	10 - 1000 MU/g tissue	Tani (1945)
Tetraodon alboreticulatus	10 - >1000 MU/g tissue	Kanoh (1988)
Pleuranacanthus sceleratus	10 - >1000 MU/g tissue	Kanoh (1988)
Chelonodon patoca	100 - >1000 MU/g tissue	Fuchi et al. (1991)
Arothron firmamentum	10 - 1000 MU/g tissue	Khora, Isa, and Yasumoto (1991)
Canthigaster rivulata	10 - 1000 MU/g tissue	Khora et al. (1991)
Lagocephalus lunaris	100 - >1000 MU/g tissue	Khora et al. (1991)
Lagocephalus inermis	100 – 1000 MU/g tissue	Khora et al. (1991)
Tajifugu flavidus	10 - >1000 MU/g tissue	Khora et al. (1991)
Tetraodon nigroviridis	10 – 1000 MU/g tissue	Mahmud, Yamamori, and Noguchi (1999a)
Tetraodon steindachneri	100 – 1000 MU/g tissue	Mahmud, Yamamori, and Noguchi (1999b)
Fish		
Oncorhynchus keta	Trace	Sato, Ogata, and Kodama (1998)
Flatworms		
Planocera multitentaculata	300 - >1000 MU/g tissue	Miyazawa et al. (1986)

Animals	Average Toxicity (1 MU ≈ 0.22 μg of TTX) (Noguchi & Arakawa, 2008; Pires et al., 2002)	Citation
Ribbonworms		
Lineus fuscoviridis,	15 – 503 MU/g tissue	Miyazawa et al. (1988)
Tubulanus punctatus,	<10 – 540 MU/g tissue	Miyazawa et al. (1988)
Cephalothrix linearis	<10 - >1000 MU/g tissue	Ali et al. (1990)
Arrowworms		
Parasagitta elegans	~320 MU/g tissue	Thuesen, Kogure, Hashimoto, and Nemoto (1988)
Flaccisagitta scrippsae	~290 MU/g tissue	Thuesen et al. (1988)
Flaccisagitta enflata	~75 MU/g tissue	Thuesen et al. (1988)
Aidanosagitta crassa	~30 MU/g tissue	Thuesen et al. (1988)
Spadella angulate	~60 MU/g tissue	Thuesen et al. (1988)
Eukrohnia hamata	~140 MU/g tissue	Thuesen et al. (1988)
Polychaetes		
Pseudopolamilla occelata	10 – 100 MU/g tissue	Yasumoto, Yotsu, Endo, Murata, and Kao (1989)
Xanthidae crabs		
Atergatis floridus	<10 – 237 MU/g tissue	Saito, Kohama, Ui, and Watabe (2006)
Zosimus aeneus	20 MU/g tissue	Yasumura, Oshima, Yasumoto, Alcala, and Alcala (1986)
Lophozozyzmus pictor	5 – 180 MU/g tissue	Tsai, Hwang, Chai, and Jeng (1995)
Horseshoe crab		
Carcinoscorpius rotundicauda	17 – 138 MU/g tissue	Dao et al. (2009)
Starfish		
Astropecten polyacanthus	<10 – 520 MU/g tissue	Miyazawa et al. (1985)
Astropecten scoparius	<10 – 46 MU/g tissue	Miyazawa et al. (1985)
Astropecten latespinosus	>1000 MU/g tissue	Maruyama, Noguchi, Jeon, Harada, and Hashimoto (1984)
Goby		
Yongeichthys criniger	20 – 120 MU/g tissue	Noguchi, Kao, and Hashimoto (1971)
Newts		
Taricha torosa	<10 – 500 MU/g tissue	Wakely, Fuhrman, Fuhrman, Fischer, and Mosher (1966)
Taricha rivularis	<10 – 350 MU/g tissue	Wakely et al. (1966)
Taricha granulosa	<10 – 160 MU/g tissue	Wakely et al. (1966)
Notophthalmus viridescens	50 – 100 MU/g tissue	Yotsu-Yamashita and Mebs (2003)
Cynopsis pyrrhogaster	<10 – >1000 MU/g tissue	Tsuruda et al. (2002)
Cynopsis ensicauda	<10 – 100 MU/g tissue	Wakely et al. (1966)

Animals	Average Toxicity (1 MU ≈ 0.22 μg of TTX) (Noguchi & Arakawa, 2008; Pires et al., 2002)	Citation
Triturus alpestris	<10 MU/g tissue	Yotsu-Yamashita, Mebs, Kwet, and Schneider (2007)
Triturus cristatus	41 MU/g tissue	Yotsu-Yamashita et al. (2007)
Triturus helveticus	6 – 40 MU/g tissue	Yotsu-Yamashita et al. (2007)
Triturus vulgaris	0.5 MU/g tissue	Yotsu-Yamashita et al. (2007)
Triturus marmoratus	<0.5 MU/g tissue	Wakely et al. (1966)
Frogs		
Atelopus varius varius	100 MU/g tissue	Kim, Brown, and Mosher (1975)
Atelopus varius ambulatorius	120 MU/g tissue	Kim et al. (1975)
Atelopus chiriquiensis	350 MU/g tissue	Kim et al. (1975)
Atelopus varius zeteki	1200 MU/g tissue	Kim et al. (1975).
Atelopus oxyrhynchus	50 – 161 MU/g tissue	Yotsu-Yamashita, Mebs, and Yasumoto (1992)
Colostethus inguinalis	100 – 1000 MU/g tissue	Daly, Gusovsky, Myers, Yotsu-Yamashita, and Yasumoto (1994)
Polypedates sp.	<10 – 923 MU/g tissue	Tanu, Mahmud, Tsuruda, Arakawa, and Noguchi (2001)
Brachycephalus pernix	340 MU/g tissue	Pires Jr et al. (2005)
Brachycephalus ephippium	78 - 280 MU/g tissue	Pires et al. (2002)
Cephalopoda		
Haplochlaena maculosa	>1000 MU/g tissue	Sheumack, Howden, Spence, and Quinn (1978)
Gastropods		
Charonia sauliae	<10 - >1000 MU/g tissue	Narita (1981)
Babylonia japonica	32 - 180 MU/g tissue	Yasumoto, Oshima, Hosaka, and Miyakoshi (1981)
Tutufa lissostoma	<700 MU/g tissue	Noguchi, Maruyama, Narita, and Kanehisa (1984)
Zeuxis siquijorensis	<10 MU/g tissue	Narita, Nara, Noguchi, Maruyama, and Hashimoto (1984)
Niotha clathrata	<10 – 35 MU/g tissue	Jeon (1984)
Natica lineata	230 – 720 MU/g tissue	Hwang, Chueh, and Jeng (1990)
Cymatium echo	10 – 100 MU/g tissue	Narita (1991)
Pugilina ternotoma	10 – 100 MU/g tissue	Narita (1991)
Gibbula umbilicalis	<10 MU/g tissue	Silva et al. (2012)
Monodonta lineata	<10 MU/g tissue	Silva et al. (2012)
Charonia lampas	<100 MU/g tissue	Silva et al. (2012)
Rapana rapiformis	3 – 140 MU/g tissue	Hwang, Lu, and Jeng (1991)
Rapana venosa venosa	9 – 76 MU/g tissue	Hwang, Lu, et al. (1991)

Animals	Average Toxicity (1 MU ≈ 0.22 μg of TTX) (Noguchi & Arakawa, 2008; Pires et al., 2002)	Citation
Nassarius semiplicatus	10 – 200 MU/g tissue	Wang, Yu, Luo, Zhou, and Lin (2008)
Polinices didyma	<500 MU/g tissue	Hwang et al. (2004)
Natica lineata	>1000 MU/g tissue	Hwang et al. (2004)
Natica vitellus	<100 MU/g tissue	Hwang et al. (2004)
Zeuxis sufflatus	>500 MU/g tissue	Hwang et al. (2004)
Niotha clathrata	>300 MU/g tissue	Hwang et al. (2004)
Oliva miniacea	>100 MU/g tissue	Hwang et al. (2004)
Oliva mustelina	<30 MU/g tissue	Hwang et al. (2004)
Oliva hirasei	<50 MU/g tissue	Hwang et al. (2004)
Zeuxis scalaris	2 – 140 MU/g tissue	Hwang, Lin, and Jeng (1992)
Zeuxis castus-like specimen	2 – 13 MU/g tissue	Hwang et al. (1992)
Zeuxis samiplicutus	4 – 186 MU/g tissue	Sui, Chen, Hwang, and Hwang (2002)
Nassarius glans	>1000 MU/g tissue	Hwang et al. (2005)
Nassarius papillosus	170 - >500 MU/g tissue	Jen et al. (2007)
Nassarius succinctus	200 - >500 MU/g tissue	Yu et al. (2007)
Nassarius nitidus	<10 MU/g tissue	Huang, Lin, and Lin (2008)
Pleurobranchaea maculata	<10 - >1000 MU/g tissue	McNabb et al. (2010)

Further description of TTX in selected species is provided below. This literature review focuses on puffer fish and newts, as these are two well described model species for studying TTX, and on the molluscs as this is the phylum that my study organism, *P. maculata*, is found.

1.3.1 Tetrodotoxin in puffer fish

Puffer fish are well known for containing TTX, particularly in the liver and ovaries, dating back to the beginning of recorded history (Fuhrman, 1986). Gaillard (1923) reported hieroglyphics of *Tetraodon lineatus* on ancient Egyptian tombs of the Fifth Dynasty (*c.* 2500 B.C.). In Asian countries, puffer fish were listed among the drugs in Chinese material medica with the earliest dating back to 2838-2698 B.C. by Emperor Shun Nung and also in the *Pen-T'so Kang Mu (Great Herbal)* of Li Shih-Chen in 1596 (Fuhrman, 1986). Li wrote about regional variability in TTX concentrations where some puffer fish were not poisonous but the puffer fish found in other regions were (Kao, 1966). It was also reported by Li

that people who soaked puffer fish eggs overnight found that it removed the toxin. Tetrodotoxin is water soluble and therefore, by soaking the eggs, the TTX would likely have leached out. In European countries, it was not until the publication of *History of Japan* by Engelbert Kaempfer in 1727 that the existence of poisonous puffer fish became known (Fuhrman, 1986).

Although it was thought that all species of *Tetraodon* fish contained the same toxin, it has now been shown that TTX is only found in about 6 out of 40 species (Kao, 1966; Shiomi, Inaoka, Yamanaka, & Kikuchi, 1985). A study by Nakamura and Yasumoto (1985) discovered tetrodonic acid, 4-epitetrodotoxin and anhydrotetrodotoxin in *Takifugu (Fugu) pardalis* and in *T. poecilonotus.* Not only has TTX analogues been found in puffer fish, paralytic shellfish poisoning (PSP) or saxitoxins (STX), have also been identified in puffer fish (Kodama, Noguchi, Maruyama, Ogata, & Hashimoto, 1983a; Kodama, Ogata, Noguchi, Maruyama, & Hashimoto, 1983b; Nakamura, Oshima, & Yasumoto, 1984).

Studies have also shown that toxic species of puffer fish when reared in captivity (Matsui, Sato, Hamada, & Shimizu, 1982) or held in net cages (Noguchi, Arakawa, & Takatani, 2006) became non-toxic. In another study by Kono et al. (2008), intramuscular injections of TTX and its analogs (4-epi-TTX, 4,9-anhydro-TTX, and 11-oxo-TTX) were administered to cultured non-toxic juvenile puffer fish in order to investigate why TTX is the most major analog in puffer fish when 4,9-anhydro-TTX is more stable and whether there is transformation of analogs within the puffer fish's body. Kono et al. (2008) found that accumulation rates were between 39 - 64% on the fourth day and decreased to 34 - 40% on the sixteenth day which suggested that part of the administered toxins was not accumulated. The authors discovered no transformation of TTX, 4-epi-TTX, 4,9-anhydro-TTX, and 11-oxo-TTX into chemically non-equilibrium analogs such as deoxy-TTXs and 11-nor-TTX-6-ol. However, as 4,9-anhydro-TTX was shown to be the major toxin on the sixteenth day in all cases after separate administration of TTX and three of its analogs, it was suggested that absorption of TTX via digestive glands is important for efficient accumulation of TTX and retention as TTX rather than as an analog of TTX.

In terms of toxicity, there is varying levels of toxicity with *Spheroides poryphyreus* having the highest toxicity followed by *S. rubripes, S. pardalis, S. alboplumbeus, S. basilewskianus, S. vermicularis, S. xanthopterus, S. chrysops, S. oscellatus, S. pseudommus, S. niphobles, Lagocephalus inermis, S. stictonotus* and finally, *Canthigaster rivulatus* (Kao, 1966). There is also seasonal variation in toxicity with toxin concentrations highest in winter and lowest in summer and autumn (Kao, 1966). Ikeda et al. (2010) demonstrated that there were maturation-associated changes in toxicity in *T. poecilonotus* that differed depending on the sex of the puffer fish. In their study, the "maturation period" was between December to March in females and November to March in males, "just after spawning" was in April and all other months were dubbed "the ordinary period". Females had highest toxicity in their livers during the ordinary period and then during the maturation period, had highest toxicity in their ovaries (Ikeda et al., 2010). Males, however, had little maturation-associated change in toxin distribution. The percentage of TTX-binding to high molecular-weight substances also differed with low binding ratios during the ordinary period and high binding ratios during the maturation period (Ikeda et al., 2010).

Habitat also appears to play a role in the type of toxin found in puffer fish with those that inhabit marine and brackish environments having TTX as the dominant toxin (Fuchi et al., 1991; Kanoh, 1988; Khora et al., 1991; Tani, 1945) while freshwater puffer fish have been found to contain STX as the dominant toxin (Kungsuwan, Arakawa, Promdet, & Onoue, 1997; Sato et al., 1997).

1.3.2 Tetrodotoxin in newts and their predators

Although Ishihara (1918) found that newts, as well as puffer fish were resistant to TTX, the discovery of TTX in newts of the genus *Taricha* did not occur until 1932 when transplanted eye and limb rudiments from *Taricha* into *Ambystoma* caused the host amphibians to become paralysed for a period of two to three days. However, physical development would continue as normal during that period of time (Twitty & Elliott, 1934; Twitty & Johnson, 1934). It was also discovered that when *Taricha* and *Amystoma* were joined as parabiotic twins, until *Taricha* completely resorbed its yolk, *Amystoma* would not recover from its

paralysis (Twitty & Elliott, 1934). It was speculated that the substance causing the paralysis was located in the embryonic food reserve of *Taricha* because injections of filtered extracts of *Taricha* eggs caused paralysis in *Amystoma* larvae (Twitty & Johnson, 1934). As growth of the *Taricha* continued, the effect of the filtered extracts weakened (Twitty & Johnson, 1934). Early attempts at purifying the chemical present in *Taricha* were not successful, although it was soon discovered that the chemical had a low molecular weight and was a neurotoxin (Fuhrman, 1986). Further studies showed that the toxin blocked preganglionic cholinergic and somatic motor nerves, an effect which mirrored that of STX and TTX (Kao & Fuhrman, 1963). At first it was called tarichatoxin but eventually it was recognised that tarichatoxin and tetrodotoxin were the same compound (Mosher et al., 1964).

The intra-tissue distribution of TTX was studied in the Japanese newt, *C. pyrrhogaster*. This was conducted by using a highly specific and sensitive monoclonal anti-TTX antibody that had been developed by Kawatsu, Hamano, Yoda, Terano, and Shibata (1997). *Cynops pyrrhogaster* secretes both TTX and 6-epiTTX from their skin when handled and this demonstrated a possible defensive function of TTX (Tsuruda et al., 2002). There are also maturation-associated changes in TTX concentration with eggs of *C. pyrrhogaster* containing a small amount of TTX inherited from their parents but this disappears during the larval stage. During the juvenile stage, there is an abrupt accumulation of TTX again, mainly in the muscles and skin (Tsuruda et al., 2002). By comparison, in *T. granulosa*, there are high TTX concentrations in the eggs (Hanifin, Brodie, & Brodie, 2003) and retention of TTX throughout the life cycle (Gall et al., 2011).

Similar to *C. pyrrhogaster, N. viridescens* has been found to contain not only TTX but also the analogs, 11-oxo-TTX and 6-epi-TTX. Yotsu-Yamashita and Mebs (2003) showed that the concentration of 11-oxo-TTX was high in both adults and early stage development newts (efts), almost as high as that of TTX but 6-epiTTX was only a minor component. However, the level of 6-epi-TTX was significantly higher in efts (1.8 ± 1.3 mg/g, SD, $n = 10$) than in adults (0.51 ± 0.26 mg/g, SD, $n = 12$) but there was no significant difference between the level of TTX and 11-oxo-TTX in efts (13 ± 7.4 and 9.1 ± 5.6 mg/g respectively) or adults

(16 ± 6.3 and 13 ± 6.2 mg/g, respectively). Newts of the *Triturus* species have also been discovered to have both TTX and the analog 6-epi-TTX at varying concentrations (TTX: 0.11–9.0 mg/g; 6-epi-TTX: 0.05–17.0 mg/g) with 6-epi-TTX as the major component (Yotsu-Yamashita et al., 2007).

Taricha granulosa are often preyed upon by *Thamnophis sirtalis* (garter snakes). Predator-prey interactions are often marked by a parallel arms-race where the prey develops better defences and their predator must evolve adaptations that overcome the prey's defences. Such is the case for the rough skinned newt and the garter snake. In the predator-prey trials run by Williams, Hanifin, Brodie, and Brodie (2010), *T. granulosa* with greater concentrations of TTX (4.52 ± 3.49 mg TTX/g skin) were more likely to be rejected by *T. sirtalis* than individuals with lower concentrations of TTX (1.72 ± 1.53 mg TTX/g skin). As survival probability is linked to TTX concentrations, selection can drive the escalation of toxin concentrations within newts (Williams et al., 2010). It is interesting to note though, that in *N. viridescens*, TTX and 6-epi-TTX do not provide protection against internal parasites (Mebs, Yotsu-Yamashita, Seitz, & Arakawa, 2012).

The presence of a high concentration of TTX within *T. granulosa* has led to the development of TTX resistance in the *T. sirtalis* (Williams, Brodie Jr, & Brodie III, 2003) with toxicity within the newts and resistance in the snakes being variable but tightly matched (Geffeney, Brodie, & Ruben, 2002). Phylogeographic evidence has detected at least two independent evolutions of TTX resistance in *T. sirtalis* found in western North America (Feldman et al., 2010). *Thamnophis sirtalis* may be predisposed to having a greater TTX resistance as the entire genus of *Thamnophis* has slightly higher TTX resistance when compared to other snake lineages (Motychak, Brodie Jr, & Brodie III, 1999). In addition to this, when *T. sirtalis* is repeatedly injected with TTX every two weeks for six months, there is no detectable effect on growth or TTX resistance (measured by ratios of post-injection speed to base speed) (Ridenhour, Brodie Jr, & Brodie III, 1999).

Thamnophis sirtalis have also been shown to retain the TTX consumed in their livers for a month or more after the consumption of a single newt and for at least seven weeks after being kept on a diet of newts (Williams, Brodie, & Brodie, 2004). The retention of TTX in their bodies would be advantageous against the *T.*

sirtalis' many predators (e.g. heron, ravens, crows, raccoons, minks and badgers) and it was suggested that the snakes may harbour TTX in the range just below the lethal dose for their predators (Williams et al., 2004). There is, however, a cost to TTX resistance in *T. sirtalis*. This cost comes in the form of a trade-off between TTX resistance and locomotor performance with slower snakes having higher TTX resistance than faster snakes (Brodie III et al., 2005).

Thamnophis sirtalis are not the only organism that derives a benefit from preying on rough-skinned newts. Caddisfly larvae (*Limnophilus* spp.) are major predators of the eggs of *T. granulosa* and may consume up to 29 eggs within 14 days (Gall, Stokes, French, Brodie, & Brodie, 2012). As *T. granulosa* eggs contain high amounts of TTX, this means that caddisfly larvae are exposed to TTX. In the study by Gall et al. (2012), it was demonstrated that caddisfly larvae accumulated TTX after consumption of 5 newt eggs and this TTX was retained by the larvae through to adulthood (up to 134 days after collection). It was speculated that this served as an anti-predator defense from dragonfly nymphs found in freshwater pond communities (Gall et al., 2012).

1.3.3 Tetrodotoxin in molluscs

1.3.3.1 Blue-ringed octopus *Hapalochlaena* spp.

The identification of TTX in the blue-ringed octopus occurred when investigations into the cause of a number of human fatalities due to receiving a bite from this animal were undertaken. Investigations into the posterior salivary gland of *Hapalochlaena maculosa* revealed that the cause of the human fatalities was tetrodotoxin (Sheumack et al., 1978).

Williams and Caldwell (2009) studied the distribution of TTX in *Hapalochlaena fasciata* and *Hapalochlaena lunulata*. Although TTX was found in both the posterior salivary gland and mantle of both species, *H. fasciata* had TTX in its anterior salivary gland, digestive gland, testes, brachial heart, nephridia, gill and oviducal gland while *H. lunulata* did not.

Williams, Stark, and Caldwell (2012) used immunolabelling of TTX with monoclonal antibodies to investigate the distribution of TTX within their bodies and found fluorescence around the lumen of secretory tubules and in circulatory

channels such as the branchial heart, gills and nephridia. They inferred that the TTX was located in the secretory cells lining the tubules and suggested that blue-ringed octopuses have a transport mechanism for TTX. A small amount of TTX was also found in a specimen of *Octopus bocki* in its digestive gland at concentrations undetectable by high-performance liquid chromatography (HPLC) but detectable by immunofluorescence.

It has been speculated that the function of TTX in blue-ringed octopuses is venom (due to presence in their posterior salivary glands) and also defence (due to presence in their mantle). Williams, Hanifin, Brodie, and Caldwell (2011a) studied maternal investment and found that offspring TTX concentrations are correlated with female TTX concentrations which suggested either maternal control over TTX distribution or passive diffusion. In addition to this, Williams et al. (2011a) demonstrated total TTX concentrations in the eggs continue to increase after being laid. However, Williams, Lovenburg, Huffard, and Caldwell (2011b) suggest that the TTX in the pelagic marine larvae may not function solely in a defensive role. This is because although the larvae may be distasteful, when tested, predators would still consume food items that contained similar concentrations of TTX to the larvae, thus demonstrating that the presence of TTX in the larvae did not exempt them from predation.

1.3.3.2 Gastropods

Various cases of paralytic gastropod poisoning incidents have occurred around the world with the causative agent usually TTX and occasionally PSP (Hwang, Tsai, Lin, & Hwang, 2007b). These cases have mainly occurred in Taiwan and Mainland China but also in Japan and Cuba due to the consumption of gastropods as a traditional, cheap and nutritional food source. Between 1994 and 2006, there were a total of nine cases of gastropod poisoning incidents in Taiwan (Hwang et al., 2007b). The gastropods involved in these poisoning cases were usually found in warm areas with sandy soil and could be easily harvested (Hwang et al., 2007b).

The family Naticidae inhabit sandy bottoms and prey on bivalves however; they have comparatively low TTX concentrations (Hwang et al., 2007b). Within

the family, *Natica lineata* has the highest toxicity and *P. tumidus*, the lowest (Hwang et al., 2007b). *Natica lineata* and *N. vitellus* have also been found to contain PSP (Hwang et al., 2007b).

Hwang, Tai, Chueh, Lin, and Jeng (1991) found that in Naticidae (in particular *N. lineata*), TTX was localised in the muscle, whereas in other species of gastropods, TTX was mainly found in the digestive gland. *Natica lineata* have 720 MU/g in their muscle and lower amounts in other parts such as mouth organs (28 MU/g) and digestive gland (12 MU/g). Comparatively, the lethal potency of *N. vitellus* was 80 MU/g and for *P. didyma*, 234 MU/g (Hwang et al., 1991).

The family Nassariidae inhabit tropical and temperate waters, colonising sandy and muddy bottoms near the coast. They feed on dead organisms. In Taiwan, nine species of Nassariidae contain TTX. Of these nine species, *N. glans* has the highest toxicity (538 MU/g in digestive gland and 1,167 MU/g in muscle) (Hwang et al., 2005). *Nassarius clathrata* also contains minor PSP, in addition to TTX (Hwang et al., 2007b).

In a study by Li, Yu, Zhou, and Li (2008), they investigated TTX distribution in gastropod *Nassarius succinctus* and observed TTX and all its analogs (including anhydro-TTX, trideoxy-TTX, deoxy-TTX and oxo-TTX) were present in the gullet, muscle and viscera. The viscera contained the highest TTX concentrations and trideoxy-TTX was the highest analog in all other tissues. Li et al. (2008) suggested that the presence of all TTX analogs indicated that a biotransformation processes could occur within gastropods. By comparison, *N. glans* was found to have TTX and its analogs 4-epi-TTX and anhydro-TTX (Hwang et al., 2005). *Nassarius* spp. showed seasonal variations in TTX concentration, with two peaks which occurred during late May and June (Luo, Yu, Wang, & Zhou, 2012).

The family of Olividae inhabit warm and tropical waters and are carnivorous sand-burrowers (Hwang et al., 2007b). The approximate TTX concentrations of *O. miniacea*, *O. mustelina* and *O. hirasei* are 11, 8 and 14 MU/g, respectively (Hwang, Tsai, Lu, & Hwang, 2003).

The gastropods *Rapana rapiformis* and *R. venosa venosa* are found in temperate subtidal waters on sandy bottoms and burrow completely into the

sediment. Four out of 17 *R. rapiformis* specimens and 6 out of 28 *R. venosa venosa* specimens were toxic in the study by Hwang, et al. (1991) with the highest toxicity measured in the digestive gland. Tetrodotoxin-binding proteins have been isolated from five species of toxic gastropods *Polinices didyma, Natica lineata, Oliva miniacea, O. mustelina* and *O. hirasei* (Hwang et al., 2007a).

There is regional, individual and seasonal variation in the TTX concentration of gastropods. In *N. clathrata,* specimens that had been collected from Tungkang had higher TTX (digestive muscle 25 ± 3 MU/g in November 1990 and 19 ± 5 MU/g in July 1991, muscle 50 ± 15 MU/g in November 1990 and 46 ± 17 MU/g in July 1991) concentrations than those collected from Chiating (digestive muscle 8 ± 3 MU/g in November 1990 and 4 ± 2 MU/g in July 1991, muscle 4 ± 1 MU/g in November 1990 and 10 ± 6 MU/g in July 1991) and Putai (digestive muscle 10 ± 5 MU/g in July 1991, muscle 2 ± 1 MU/g in July 1991) (Cheng et al., 1995). In *N. semiplicatus*, they display two peaks of toxicity in late May and late July with TTX concentrations reaching a maximum of 846 MU/g on 24 May 2007, followed by a decrease in concentration to 160 MU/g in early July and then increasing again to 600 MU/g on 18 July with another decrease in early August to 130 MU/g (Luo et al., 2012).

Hwang et al. (2004) demonstrated that of the gastropods studied, the toxic ones were carnivorous and living on ocean sands whereas the non-toxic ones were omnivorous and living in freshwater. Silva et al. (2012) noted that TTX-bearing organisms were usually more typical of warmer waters, particularly in the Indian and Pacific, however they reported the potential expansion of TTX-bearing organisms into the temperate waters of the Atlantic Ocean during their study of two intertidal gastropod species *G. umbilicalis* and *M. lineata.* Although the TTX concentrations reported were low, the authors speculated that perhaps TTX synthesis was higher in warmer waters. Several species of carnivorous gastropods living in cold water have been discovered to possess tetramine in their salivary glands (Hwang et al., 2007b).

1.4 Origin of tetrodotoxin

Despite many decades of research on TTX, its origins are still controversial with researchers postulating either an endogenous or exogenous source (Cardall, Brodie, Brodie, & Hanifin, 2004; Honda et al., 2005; Lee et al., 2000).

1.4.1 Endogenous source

Most of the evidence for endogenous production comes from research on terrestrial organisms such as newts. Hanifin, Brodie, & Brodie, 2002 showed that TTX concentrations within captive newts (*Taricha granulosa*) remained stable and even increased over a time period of a year when maintained on non-toxic diets (Hanifin, Brodie, & Brodie, 2002). In other experiments with *T. granulosa*, it was observed that although TTX was released from the skin of *T. granulosa* following mild electrical stimulation, TTX concentrations increased again over a period of nine months despite being fed a TTX-free diet (Cardall et al., 2004). Although the above studies show that there is evidence for endogenous production of TTX, the question remains as to what the biosynthetic pathway is and thus far, biosynthesis studies on amphibians have not returned positive results (Daly, Myers, & Whittaker, 1987). No TTX-producing bacteria have been isolated from terrestrial organisms (Lehman, Brodie, & Brodie, 2004).

1.4.2 Exogenous source

Tetrodotoxin-producing bacteria have been cultured from marine organisms. Twenty (19 *Bacillus* strains and 1 *Actinomycete* strain) out of the 36 bacterial strains isolated from puffer fish *Fugu rubripes* produced TTX *in vitro* (Wu et al., 2005). TTX-producing bacterial strains were isolated from organs with high TTX concentrations. Toxic strains (mostly *Vibrio*) of bacteria have also been isolated from the gastropod *Nassarius semiplicatus* but the toxin concentrations were low (Wang et al., 2008). It has been suggested that in marine organisms, exogenous production of TTX occurs through marine bacteria entering the food chain via either dietary uptake or symbiosis with these bacteria (Noguchi & Arakawa, 2008). However, there is still doubt about bacterial production of TTX.

Chau, Kalaitzis, Wood, and Neilan (2013) attempted to isolate TTX-producing bacteria from *P. maculata,* however, this proved unsuccessful. It was suggested that there may be an overestimation of the number of bacterial TTX producers in the literature due to the fact that many studies that describe the finding of TTX-like activity use non-specific mouse bioassays or gas chromatography-mass spectrometry (GC-MS). In addition to this, the studies were not supported by the structure characterisation using nuclear magnetic resonance (NMR) or tandem mass spectrometry (MS/MS) of a purified compound (Chau et al., 2013). An example is the study by Wang et al. (2008) which had tested positive results for TTX using enzyme-linked immunosorbent assay (ELISA) but was not found to produce TTX when more sensitive spectrometric methods were used.

1.4.2.1 Dietary sources

Tetrodotoxin resistant organisms can accumulate the toxin from dietary sources. Feeding experiments with non-toxic cultured puffer fish have been undertaken with different TTX-containing diets (Honda et al., 2005). These demonstrated that puffer fish accumulated TTX differently depending on the dosage (low dosage was accumulated in the skin and liver, high dosage accumulated in the liver and ovary) and age (0 year olds accumulated 0-17%, 1 year olds accumulated over 30%). In a similar study, there was a difference in accumulation depending on what kind of TTX was fed to the puffer fish with the lowest accumulation occurring when puffer fish were fed crystalline TTX followed by methanol extract and toxic ovary (Matsui, Hamada, & Konosu, 1981).

The quantity and distribution of TTX within the common garter snakes (*Thamnophis sirtalis*) after they had eaten rough skinned newts (*Taricha granulosa*) varies (Williams et al., 2004). Toxin concentrations within the snake depended on the toxicity of *T. granulosa* consumed and remained in the snakes for a period of up to three weeks in the kidney and at least seven weeks in the liver. Collectively these studies demonstrate that *T. sirtalis* can accumulate TTX from a dietary source.

Another predator-prey relationship exists between the starfish *A. polyacanthus* and the trumpet shell *Charonia sauliae* (Noguchi, Narita, Maruyama, & Hashimoto, 1982). Parts of the starfish have been found in the contents of the trumpet shell's digestive tract. As previously mentioned, *A. polyacanthus* contains TTX and therefore it was concluded that the trumpet shell obtained its TTX at least partially from the starfish (Noguchi et al., 1982).

1.5 Discovery of tetrodotoxin in *Pleurobranchaea maculata*

Between July and September 2009, there were a series of dog poisonings on beaches in the Auckland region, New Zealand. Investigations identified TTX in the vomit of the dogs and also in beach-cast *P. maculata* (grey side-gilled sea slug). *Pleurobranchaea maculata* are opportunistic carnivores found in a variety of habitats from sandy sediment to rocky reefs. It was suggested that the dogs may have ingested TTX from *P. maculata* that washed up on the beaches or their egg sacs (McNabb et al., 2010). Since the initial identification of TTX in *P. maculata*, research has been carried out trying to elucidate its ultimate source.

Pleurobranchaea maculata populations in the Auckland, Whangarei and Tauranga region have been found with high concentrations of TTX while *P. maculata* populations in Wellington have low concentrations of TTX and South Island populations do not contain TTX (McNabb et al., 2010). Tetrodotoxin concentrations in *P. maculata* decreased from a peak in June-July, coinciding with egg-laying season, to only low concentrations in December (Wood et al., 2012). It was initially suggested that the difference in TTX concentrations between North and South Island populations were because they were two separate species but it has since been shown that *P. maculata* found in the North and South Islands are the same species with less than 1% sequence variability in the cytochrome *c* oxidase subunit 1 gene (Wood et al., 2012). There is no relationship between size and TTX concentrations as might be expected if TTX was endogenously produced (i.e. larger bodies should be capable of producing more TTX and therefore should have higher TTX concentrations) (Wood et al., 2012).

Pleurobranchaea maculata is an ideal study organism to explore the origins of TTX as they are found in relatively confined, easily accessible, shallow

sub-tidal areas, they can be reared in captivity under very controlled conditions, and contain high concentrations of TTX.

1.6 Research aims

The aim of this Master's research was to investigate whether the origin of TTX in *Pleurobranchaea maculata* (grey side-gilled sea slug) was dietary. This research is part of a larger project which aims to elucidate the origin of TTX in *P. maculata*. The hypothesis of this research was "*that TTX has a dietary origin in P. maculata and that they will preferentially feed on TTX-containing food*."

This Master's thesis had three distinct aims:

- To develop a non-lethal biopsy method and determine whether this is a feasible tool for future research concerning TTX in *P. maculata*
- To determine if non-toxic *P. maculata* can accumulate TTX from a dietary source and if there is uptake, to investigate how this is distributed throughout the organism
- To determine if *P. maculata* are attracted to TTX and whether this varies depending on the TTX content within the *P. maculata*

The methods in this thesis all involved the use of LC-MS analysis to determine TTX concentrations. I prepared the samples for analysis but the actual LC-MS procedure was run by the Cawthron Institute, Nelson.

Chapter Two details the development of a non-lethal biopsy method. Due to the high variability in TTX concentrations within *P. maculata* there have been challenges when performing controlled laboratory experiments. In addition to this, the current method of assessing TTX concentrations within *P. maculata* is lethal and requires a large number of individuals in order to obtain statistically significant results. The development of a non-lethal biopsy method would therefore aid considerably as each individual could act as its own control and changes in TTX concentrations could be plotted over time. I designed the biopsy experiment, undertook the experiments, analysed the data and wrote the manuscript with advice from my supervisors. This chapter has been published in Toxicon (Impact Factor: 2.924, Appendix 1) as:

Khor, S., Wood, S. A., Salvitti, L., Ragg, N. L. C., Taylor, D., McNabb, P., & Cary, S.C. (**2013**). Development of a non-lethal biopsy technique for estimating total tetrodotoxin concentrations in the grey side-gilled sea slug *Pleurobranchaea maculata*. Toxicon, 74(0), 27-33.

Chapter Three involves a feeding study and environmental survey. Although the ultimate source of TTX is a mystery, in certain species, there is little doubt that food chain transmission plays a role. This section deals with the question of whether non-toxic *P. maculata* are able to accumulate TTX through their diet, survive the intoxication and distribute it throughout their body. This chapter also details a benthic survey undertaken in the attempt to find a possible source of TTX for *P. maculata* in the wild. I designed the feeding experiment, undertook the majority of the experiment with help from staff at the Cawthron Institute, analysed the data and wrote the manuscript with advice from my supervisors but I was not directly involved in the benthic survey or analysis of the results. This chapter has been published in Marine Drugs (Impact Factor: 3.978, Appendix 2) as:

Khor, S., Wood, S., Salvitti, L., Taylor, D., Adamson, J., McNabb, P., & Cary, S.C. (**2014**). Investigating diet as the source of tetrodotoxin in *Pleurobranchaea maculata*. Marine Drugs, *12*(1), 1-16.

Chapter Four investigates whether *P. maculata* have a preference for TTX-containing food. If diet is a source of TTX, the premise is that organisms should be; attracted to it, able to detect it, and move towards it. Preference studies have often been run to elucidate inclinations in dietary sources and have been undertaken on several different TTX-containing organisms. In order to determine whether or not both toxic and non-toxic *P. maculata* have a preference for TTX-containing food, a series of three preference experiments were run; (1) a zonation experiment in which the time *P. maculata* spent in different zones of an aquarium containing either toxic or non-toxic agar blocks was monitored, (2) an agar trail experiment in which droplets of hot liquefied toxic or non-toxic agar were placed in parallel lines along the bottom of an aquarium and the behaviour of *P. maculata* monitored, and (3) a direct food preference experiment where *P. maculata* were presented with combinations of; toxic and toxic, non-toxic and

non-toxic, or toxic and non-toxic food and their choice was recorded. I designed the preference experiments, undertook these with the help of some volunteers, analysed and wrote the manuscript with advice from my supervisors. This chapter will be submitted as a manuscript to the Journal of Experimental Marine Biology and Ecology (Impact Factor: 2.263),

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Chapter Five is a closing chapter summarising the thesis results and findings and points to future directions pertaining to TTX and the study of *P. maculata*.

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Chapter 2 – Development of a non-lethal biopsy technique for estimating total tetrodotoxin concentrations in the grey side-gilled sea slug *Pleurobranchaea maculata*

2.1 Abstract

High concentrations of tetrodotoxin (TTX) have been detected in some New Zealand populations of *Pleurobranchaea maculata* (grey side-gilled sea slug). Within toxic populations there is significant variability in TTX concentrations among individuals, with up to 60-fold differences measured. This variability has led to challenges when conducting controlled laboratory experiments. The current method for assessing TTX concentrations within *P. maculata* is lethal, thus multiple individuals must be harvested at each sampling point to produce statistically meaningful data. In this study a method was developed for taking approximately 200 mg tissue biopsies using a TemnoEvolution® 18G × 11 cm Biopsy Needle inserted transversely into the foot. The correlations between the TTX concentrations in the biopsy sample and total TTX levels and in individual tissues were assessed. Six *P. maculata* were biopsied twice (nine days apart) and each individual was frozen immediately following the second sampling. Tetrodotoxin concentrations in biopsy samples and in the gonad, stomach, mantle and the remaining combined tissues and fluids were measured using liquid chromatography-mass spectrometry. Based on the proportional weight of the organs/tissues a total TTX concentration for each individual was calculated. There were strong correlations between biopsy TTX concentrations and the total (r^2 = 0.88), stomach (r^2 = 0.92) and gonad (r^2 = 0.83) TTX concentrations. This technique will enable more robust laboratory studies to be undertaken, thereby assisting in understanding TTX kinetics, ecological function and origin within *P. maculata*.

2.2 Introduction

Tetrodotoxin (TTX) is a potent neurotoxin that functions by binding and obstructing voltage-gated sodium channels in nerve cell membranes, preventing the propagation of action potentials (Lu et al., 2011). Tetrodotoxin derives its name from the puffer fish family *Tetraodontidae* where it was first discovered, but it is now known to occur in a wide variety of phylogenetically distinct marine and terrestrial organisms (Ito et al., 2006, Hanifin 2010, Williams 2010). Despite many decades of research on TTX, its origins are still controversial with researchers either postulating an endogenous or exogenous source. Evidence for endogenous production comes from research on terrestrial organisms such as newts. Studies on captive newts (*Taricha granulosa*) found that TTX concentrations remained stable or increased over one year when fed TTX-free diets (Hanifin et al., 2002). It has also been demonstrated that TTX was released from the skin of *T. granulosa* when stimulated by a mild electric current, but concentrations in the skin increased again over nine months despite only being fed TTX-free earthworms and crickets (Cardall et al., 2004). Although no TTX-producing bacteria have been detected from terrestrial organisms (Lehman et al., 2004), they have been cultured from multiple marine organisms. However, the low TTX concentrations produced by bacteria in concert with non-specific and cross-reactivity problems with techniques used to measure TTX-production in bacterial strains have resulted in uncertainty regarding the microbial origin of TTX (Matsumura, 1995, 2001).

Between July to September 2009, there were a series of dog poisonings on beaches in Auckland, New Zealand. Investigations identified TTX in the dogs' stomachs and vomit, and in beach-cast *Pleurobranchaea maculata* (grey side-gilled sea slug). It was suggested that the dogs had ingested TTX from *P. maculata* or their toxic egg masses that had washed up on the beaches (McNabb et al., 2010). *Pleurobranchaea maculata* are opportunistic scavengers that are commonly found in shallow sub-tidal areas around New Zealand, and have also been recorded in south-eastern Australia, China, Sri Lanka and Japan (Willan, 1983). Research on TTX in *P. maculata* has found that populations in Auckland, Whangarei and Tauranga (Upper North Island, New Zealand) contain high

concentrations of TTX (>1400 mg kg^{-1}) while populations in Wellington (Lower North Island, New Zealand) have low concentrations (*c.* 2.0 mg kg^{-1}) and South Island populations have no or very low concentrations (<0.01 mg kg^{-1}) of TTX (McNabb et al., 2010; Wood et al., 2012b). Wood et al. (2012b) showed that there was less than 1% sequence variability in the cytochrome *c* oxidase subunit 1 gene between the populations, providing evidence that they were the same species. In addition to between-population variability there are significant disparities in TTX concentrations within toxic *P. maculata* populations, with up to 60-fold differences occurring among individuals collected from one Auckland site (Wood et al., 2012b). Seasonal differences in TTX concentrations were also identified with a peak in June-July coinciding with egg-laying season (Wood et al., 2012b). A recent aquarium-based TTX-depuration study on *P. maculata* identified a similar trend when adults were maintained for 126 days on a TTX-free diet (Wood et al., 2012a). One of the limitations encountered during this study was the requirement to harvest multiple individuals at each sampling date due to the high natural variability in TTX concentrations and the need for lethal sampling to determine TTX concentrations (Wood et al., 2012a). For example, TTX concentrations measured in stomach tissue samples from Day 0 ranged from 14 to 1905 mg kg^{-1} among three individuals, making meaningful interpretation of changes in TTX concentrations challenging (Wood et al., 2012a). Development of a non-lethal technique for assessing TTX concentrations in *P. maculata*, allowing repeated assessment of one individual over time, would greatly assist future studies.

A non-lethal sampling method for assessing TTX concentrations in *T. granulosa* has been developed (Hanifin et al., 2004). They used a 5 mm diameter human skin biopsy punch to sample a region of the dorsal skin and developed a predictive model to estimate total TTX concentrations within the skin. Molluscs, however, present the further complication that their haemolymph ('blood') system lacks clotting factors; wounds may therefore haemorrhage uncontrollably, ultimately resulting in death (Armstrong et al., 1971; Hodgson, 1981; Taylor et al., 1994). Whilst maintaining *P. maculata* in aquaria we observed that when multiple individuals were kept together in one aquarium they often attacked each

other removing significant portions of foot tissue. This did not always result in death of the injured individual (Wood & McNabb, unpub. data). The foot region consists of muscle and connective tissue and therefore represents a promising site for biopsy, as sampling this area would minimise the chance of lethal damage to internal organs. In terrestrial gastropods, 'foot clipping' has been used previously to obtain tissue samples in order to prevent the need for lethal methods. For example, sampling of endangered Hawaiian tree snails (Achatinellinae) has been achieved using a sterile scalpel to obtain a 1–2 mm tissue slice from their foot region with no post-procedure mortality observed (Thacker & Hadfield, 2000).

The aims of this study were to develop a non-lethal biopsy method for sub-sampling *P. maculata* and to determine if the TTX concentrations in these sub-samples could be used to estimate total TTX concentrations in the entire organism, or among specific organs/tissues.

2.3 Methods

2.3.1 Field sampling and laboratory conditions

Nine *P. maculata* were collected by divers (18 July 2012) from Pilot Bay (Tauranga, New Zealand; 37°38'07"S, 176°10'29"E). Each was placed in a separate plastic bag containing seawater (300 mL) and transported back to the laboratory in an insulated container. *Pleurobranchaea maculata* were maintained in separate aquaria (19 L) filled with 14 L of aerated 0.22 µm-filtered seawater. At 2 to 3 day intervals, *P. maculata* were fed Greenshell™ mussel (*Perna canaliculus*) sourced from the Marlborough Sounds (South Island, New Zealand) and their water was exchanged. To confirm that the mussels contained no TTX, three from this batch were tested for TTX as described below.

2.3.2 Biopsies

Six of the nine *P. maculata* were biopsied (1 August 2012) while the remaining three were used as unmanipulated control samples. *Pleurobranchaea maculata* were removed from aquaria and transferred to beakers containing *c*. 300 mL seawater and allowed to acclimate for five minutes. Individuals were

transferred to a damp paper towel on a bench top and held firmly with one hand. Biopsies (*c*. 200 mg) were taken using a TemnoEvolution® 18G × 11 cm Biopsy Needle inserted transversely into the foot (Figure 2-1), dorsal to the pedal sinus and anterior to the pedal gland. Resulting tissue samples were placed into 1.7 mL tubes (Axygen). Control *P. maculata* were subject to the conditions above and were prodded with a metal rod to simulate the handling stress of the biopsy procedure, without breaking the epidermis. *Pleurobranchaea maculata* were returned to their separate aquaria immediately after biopsies were taken.

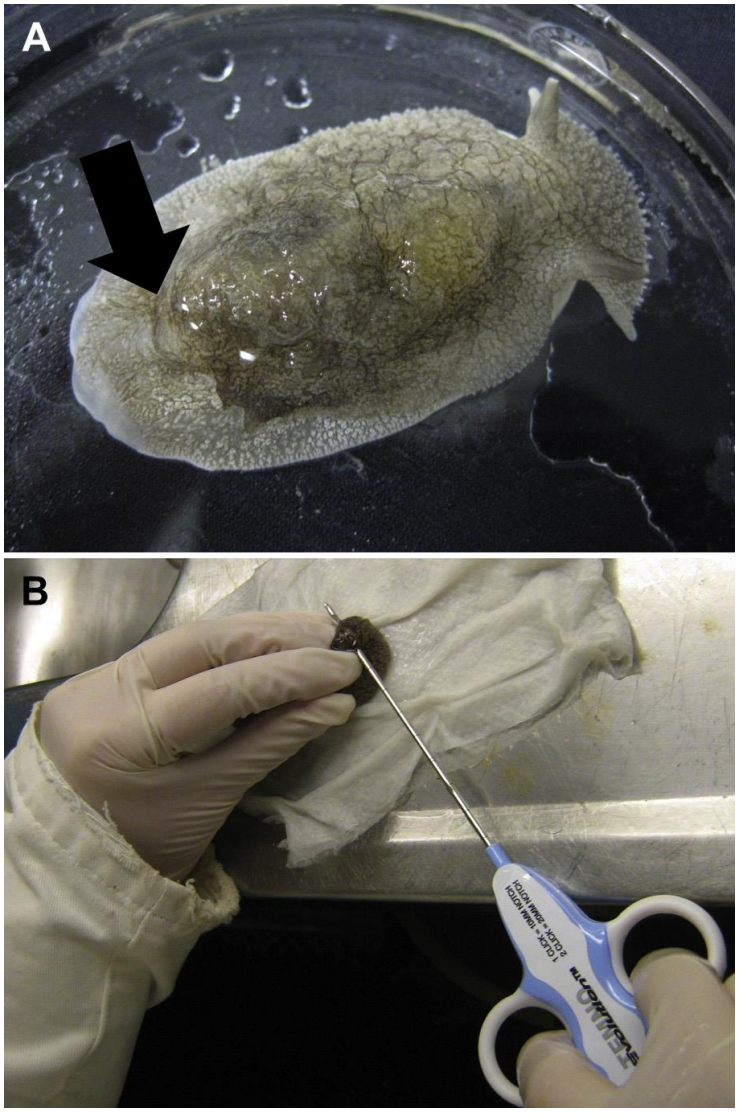

Figure 2-1. (A) *Pleurobranchaea maculata*, arrow shows approximate site of biopsy. (B) Biopsies were taken from the foot of *Pleurobranchaea maculata* using a TemnoEvolution® 18G ×11 cm Biopsy Needle.

Pleurobranchaea maculata were maintained in aquaria for nine days. During this time they were provided food on three occasions and monitored for signs of ill health (reduced activity and feeding; compromised adhesion). A second biopsy sample was taken (10 August 2012) using the method described above but with the exception that the beakers contained no seawater to allow a mucus sample to be collected. All *P. maculata* in the study were frozen (-20 ºC) whole immediately after biopsies were taken and a sample of mucus (*c.* 100 µL) collected from the beaker and frozen (-20 ºC).

2.3.3 Dissection, TTX extraction, and analysis

Pleurobranchaea maculata were partially defrosted and dissected using a sterile scalpel. The gonad, stomach including gut contents, and a section of the mantle were removed. The gonad is attached directly to the stomach and due to difficulties separating these organs, emphasis was placed on taking a clean sample rather than on trying to remove the entire gonad. All remaining fluids and tissue were combined, homogenized, and labelled as the 'rest'. All sub-samples were weighed and frozen (-20 °C) for later TTX analysis.

Sub-samples (*c.* 1 g) of each organ/tissue were extracted with 9 mL of Milli-Q water containing 0.1% v/v acetic acid, or a *pro rata* volume if the starting mass was less than 1 g. Each sample was homogenized (1 min; Heidolph Diax 600 Homogeniser, Heidolph, Germany). 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 with 100% methanol containing 0.1% v/v acetic acid. The mucus samples (100 µL) were defrosted and extracted with 900 µL Milli-Q water containing 0.1% v/v acetic acid. These were vortexed and sonicated (15 min) and then processed as described above. Samples were analysed for TTX using liquid chromatography-mass spectrometry (LC-MS) as described in McNabb et al. (2010). Total TTX concentrations for each *P. maculata* were calculated using the TTX concentration and the proportional weights of the gonad, stomach, mantle, and 'rest'.

Mann-Whitney U tests were undertaken in Statistica 8 (StatSoft Inc.) to compare mean TTX concentrations in the gonads, stomach, mantle and 'rest' among biopsied and control *P. maculata*. Sequential biopsy results were compared by paired *t*-test; as this procedure identified no significant difference, paired biopsies from each individual were subsequently treated as duplicates and an average calculated. Linear regression was used to test the relationship between average TTX concentrations in the biopsy, and total and individual organ/tissues.

2.4 Results

Immediately after the first biopsy two out of six *P. maculata* experienced some bleeding from the site of the needle insertion. One of these individuals displayed inhibited mobility for approximately two hours post-biopsy. No other signs of behavioural change or inhibited mobility were observed during the remaining nine days. The control and biopsied *P. maculata* showed no decline in their considerable appetite for Greenshell™ mussel flesh. All individuals ate two to three pieces (*c*. 0.5 g) of mussel flesh on the three occasions when they were fed post biopsy.

Tetrodotoxin was the only variant detected in the samples. The TTX concentrations in the biopsy samples varied from 8 to 167 mg kg wet tissue^{-1} (Table 2 1). In general there were only minor differences in TTX concentrations between the first and second biopsy samples (Table 2 1). The exceptions were the biopsies from the third individual in which the TTX concentration in the first biopsy was three times higher than the second, and the fifth individual where the second biopsy sample was 2.7 times higher than the first (Table 2 1). No consistent difference was detected between paired samples ($t_{0.025,\ 5\ df} = -0.126$), sequential biopsies were therefore treated as replicates.

Table 2-1. Weights of and tetrodotoxin concentrations in biopsy and mucus samples taken from six *Pleurobranchaea maculata*.

	TTX (mg kg wet tissue^{-1})					
No.	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**
***P. maculata* weight (g)**	16.4	7.9	7.2	19.8	13.3	20.0
Biopsy 1	13	19	81	144	62	61
Biopsy 2 (+9 days)	8	31	26	117	167	48
Difference ratio between biopsy samples	1.6	0.6	3.1	1.2	0.4	1.3
Mucus	Trace	Trace	Trace	1.2	Trace	1.1

There were strong correlations between total ($F_{1,5}$ = 30.44 p = 0.005, r^2 = 0.88), stomach ($F_{1,5}$ = 46.55, p = 0.002, r^2 = 0.92) and gonad ($F_{1,5}$ = 19.48, p = 0.012, r^2 = 0.83) TTX concentrations and TTX concentrations in the biopsy samples (Figure 2-2). The correlations were lower for the mantle ($F_{1,5}$ = 3.80, p = 0.12, r^2 = 0.49), and the 'rest' ($F_{1,5}$ = 7.88, p = 0.05, r^2 = 0.66). Only trace levels of TTX were detected in four of the six mucus samples. The mucus samples from the two remaining *P. maculata* (the fourth and sixth) contained 1.2 mg kg^{-1} and 1.1 mg kg^{-1} respectively.

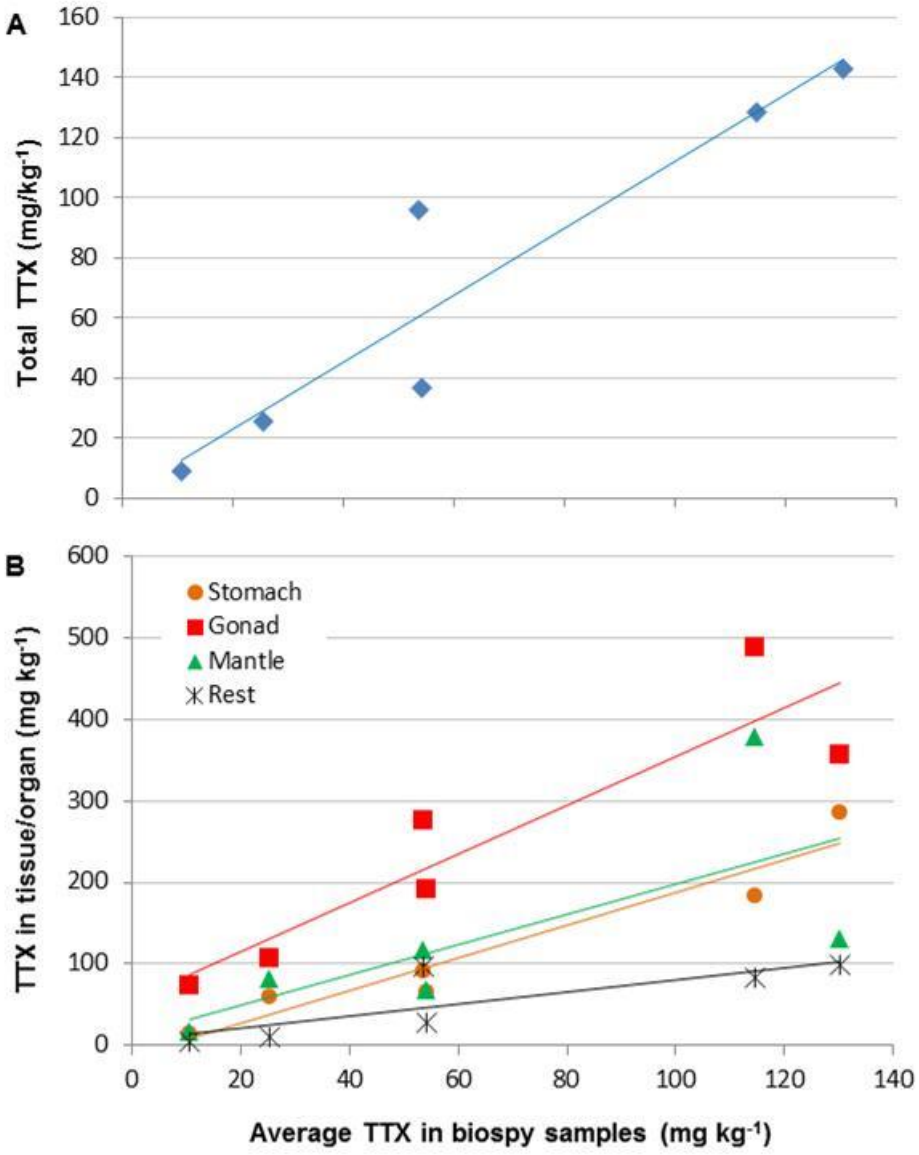

Figure 2-2. (A) Total tetrodotoxin (TTX) concentrations in six *Pleurobranchaea maculata* plotted against average TTX concentrations from two biopsy samples (*c.* 200 mg) taken nine days apart. (B) TTX concentrations in the stomach, gonad, mantle and 'rest' of six *Pleurobranchaea maculata* plotted against average TTX concentrations from two biopsy samples.

The average TTX concentrations in each organ/tissue were generally higher in the biopsied specimens than the control group (Figure 2-3). These differences, however, were not significant; stomach ($Z = 1.549$, $N = 6,3$, $p > 0.5$), gonad ($Z = 1.549$, $p > 0.05$), mantle ($Z = 1.29$, $p > 0.05$), and 'rest' ($Z = 1.29$, $p > 0.05$). There was greater variability in TTX concentrations in the biopsy group

(Figure 2-3). For example, stomach TTX concentrations ranged between 14 and 285 mg kg^{-1} in the biopsied *P. maculata*, whereas the range in concentrations was only 23 to 25 mg kg^{-1} in the control group.

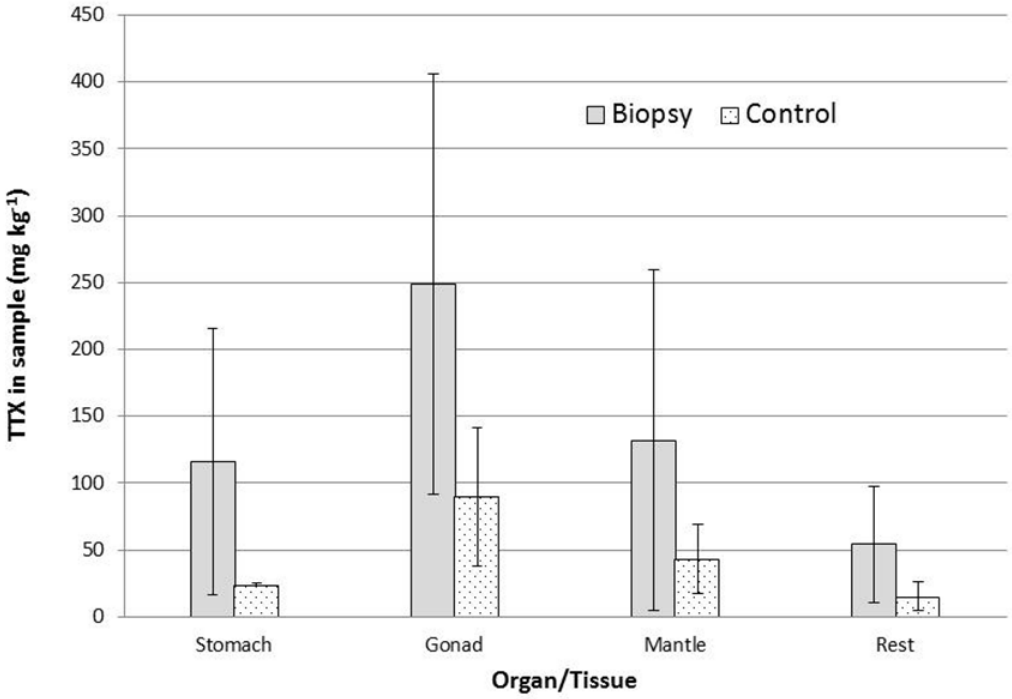

Figure 2-3. Tetrodotoxin concentrations in organs/tissue of biopsied (n = 6) and control (n = 3) *Pleurobranchaea maculata*. Error bars show one standard deviation.

2.5 Discussion

A range of non-lethal methods have been developed for sampling organisms in response to a need to investigate biological and physiological patterns, or to track contaminates within species and populations through time without causing long-term harm to individuals. Collecting representative samples from vertebrates is usually relatively straightforward and often involves collecting feathers, hairs, faeces, urine or taking skin samples; all of which can be undertaken with minimal disturbance to the individual (Beja-Pereira et al., 2009). Invasive sampling can also readily be undertaken on larger vertebrates, for example, biopsy techniques are routinely used in studies on marine mammals where non-lethal sampling techniques enable the acquisition of fresh skin and

blubber samples from free-ranging animals (Noren & Mocklin, 2012). Non-lethal sampling of invertebrates, especially small organisms, is more challenging. A variety of techniques have been developed and the type of method used is largely dependent on the downstream application of the sample. For example, a simple swab of foot tissue mucus has been shown to provide enough material for genetic analysis (Palmer et al., 2008), but this technique is unlikely to be sensitive enough to track concentrations of contaminates in individuals. This was illustrated in the current study where although trace concentrations of TTX were detected in the mucus samples, they did not correspond to the actual TTX concentrations in individuals. If tissue is sampled in molluscs, particular care is required to avoid damaging the main vascular spaces which, in the absence of clotting mechanisms, is likely to cause uncontrolled bleeding (Armstrong et al., 1971; Hodgson, 1981; Taylor et al., 1994). In bivalves several research groups have developed non-destructive biopsy methods which either involve removing small sections of the mantle (Berg et al., 1995; Buhay et al., 2002; Grobler et al., 2006; Kochzius & Nuryanto, 2008) or ligament (Doherty et al., 2007). There are far fewer studies exploring non-lethal sampling in gastropod molluscs. The effects of foot clipping on mortality and behaviour of terrestrial Cumberland Tigersnail (*Anguispira cumberlandiana*) was investigated in Tennessee, USA, with no mortality reported after a 2-week period, although short-term behavioural changes were observed (Haskell & Pan, 2010).

In this study, a non-lethal biopsy technique for sampling tissue from the marine opisthobranch *Pleurobranchaea maculata* was developed. Short-term behavioural changes were observed in one *P. maculata*, but these lasted less than two hours and no mortality was observed in any of the study organisms during the study period. It is likely that this technique could be applied to other Heterobranchia and the samples could be used for a range of applications. Measuring the TTX concentration in the biopsy sample also proved to be a robust method for estimating the total TTX concentrations within various tissues of adult *P. maculata.* The TTX concentrations in the small (*c.* 200 mg) tissue biopsy samples had a strong correlation (r^2 = 0.88) with the total TTX concentrations. We had anticipated that we would need to develop a predictive model, similar to

that of Hanifin et al. (2004) that took into account the difference in TTX concentration within various organs and their proportional weights; however, because of the strong correlation between the biopsy samples and total concentrations this was not necessary. Surprisingly, the lowest correlation was obtained when the biopsy TTX concentrations were compared to the mantle samples, which were taken in close proximity to each other. The biopsy sample was taken from within the foot rather than from the surface and this may explain why the biopsy sample is more predictive of total TTX concentrations rather than the surface concentrations. There were no significant differences in TTX concentrations between the biopsied and control *P. maculata,* indicating that the procedure had no effect on TTX concentrations within individuals.

The goal of the present study was to assess how much variability was present between biopsy samples taken within a short period and how these values related to TTX concentrations within specific organs and *P. maculata* as a whole. Wood et al. (2012a) showed marked decreases in TTX concentrations over three week sampling periods when *P. maculata* were maintained in captivity. To ensure that TTX concentrations did not decline relative to the biopsy samples, individuals in our current study were frozen immediately after conducting the second biopsy. This has limited our knowledge on potential long-term effects of the biopsies and how frequently biopsies can be taken. Wound closure in gastropods relies upon localised muscular contraction to occlude the lesion while a temporary plug of haemocytes forms; subsequent tissue regeneration typically requires 2 – 3 months (Armstrong et al., 1971; Taylor et al., 1994). In addition to its other vascular functions, haemolymph in the foot region serves a hydraulic function, antagonising muscle contraction, allowing extension and locomotion (Voltzow, 1986). A biopsy wound in the foot therefore incurs the additional risks, beyond haemorrhage and infection, that locomotion and adhesion could be impaired. However, given that there were no observed effects during the study and previous observation of long-term survival after conspecific attack (Wood, pers. obs.), we suggest that long-term effects would be minimal. A current feeding study is underway to assess the uptake of toxins into *P. maculata* and to date taking

biopsies at a frequency of one every three weeks has not resulted in mortalities (Wood & McNabb, unpub. data).

Hanifin et al. (2004) used a 5 mm diameter human skin biopsy punch to sample several regions of skin from newts (*T. granulosa*) and determined TTX concentrations. The predictive model they developed has been utilized for multiple studies including: monitoring change in TTX concentrations during experimentation (Cardall et al., 2004), feeding studies (Williams et al., 2004; Williams et al., 2010) and geographic distribution surveys (Hanifin et al., 2008). It is anticipated that the biopsy technique developed in this study will be used for similar studies on *P. maculata.*

The high variability in TTX concentrations between individual *P. maculata* measured in this study was similar to those observed previously (Wood et al., 2012a; 2012b). Marked spatial and temporal variability in TTX concentrations has been recorded for many TTX-containing organisms including; *T. granulosa* (Hanifin et al., 1999), the horseshoe crab *Carcinoscorpius rotundicauda* (Dao et al., 2009), and gastropods (*Rapana rapiformis* and *R. venosa venosa*) (Hwang et al., 1991). Variables that lead to the striking differences in TTX content in these organisms are unknown, but the results from the present study and Wood et al. (2012b) demonstrate that there is no correlation with the size or weight of an individual. In this study, variability amongst organs within individual *P. maculata* was shown with the gonads always containing the highest TTX concentrations. Wood et al. (2012a) undertook at 126-day captive study in which TTX-containing *P. maculata* were fed a TTX-free diet. Most organs depurated TTX, whereas there was only weak evidence for depuration of the gonad, indicating active transport of TTX to this organ. The ability to actively transport TTX may partially explain the variability observed within *P. maculata.* We speculate the individuals used in this study were close to egg laying, which would account for the high TTX-concentrations measured in the gonad.

In summary, the biopsy method developed by the present study provides a non-lethal method for sampling *P. maculata.* The aim of this study was to use biopsied samples to estimate TTX concentrations within individuals, but these samples could equally be used to monitor other physiological responses. For

example, changes in catecholamines, or to obtain samples for genetic analysis in *P. maculata*, and potentially other Heterobranchia. There was a strong correlation between the TTX concentration measured in the biopsy samples and the total TTX in each *P. maculata.* This method will minimise the number of *P. maculata* that need to be sacrificed during population studies and assist in future captivity experiments. This technique will enable TTX concentrations within individuals to be tracked over many months and will ultimately assist in understanding TTX kinetics, function and possibly the origin in *P. maculata.*

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Chapter 3 – Investigating diet as the source of tetrodotoxin in *Pleurobranchaea maculata*

3.1 Abstract

The origin of tetrodotoxin (TTX) is highly debated; researchers have postulated either an endogenous or exogenous source with the host accumulating TTX symbiotically or via food chain transmission. The aim of this study was to determine whether the grey side-gilled sea slug (*Pleurobranchaea maculata*) could obtain TTX from a dietary source, and to attempt to identify this source through environmental surveys. Eighteen non-toxic *P. maculata* were maintained in aquariums and twelve were fed a TTX-containing diet. Three *P. maculata* were harvested after 1 hr, 24 hrs, 17 days and 39 days and TTX concentrations in their stomach, gonad, mantle and remaining tissue/fluids determined using liquid chromatography-mass spectrometry. Tetrodotoxin was detected in all organs/tissue after 1 hr with an average uptake of 32%. This decreased throughout the experiment (21%, 15% and 9%, respectively). Benthic surveys at two sites with dense populations of toxic *P. maculata* detected very low or no TTX. This study demonstrates that *P. maculata* can accumulate TTX through their diet. However, based on the absence of an identifiable TTX source in the environment, in concert with the extremely high TTX concentrations and short life spans of *P. maculata*, it is unlikely to be the sole source for this species.

3.2 Introduction

Tetrodotoxin (TTX) is a low molecular weight neurotoxin which causes death in humans upon ingestion of only 1-2 mg. It is most famous for causing human fatalities associated with eating puffer fish or fugu (Noguchi & Arakawa, 2008). Tetrodotoxin was first isolated from and is named after the puffer fish family, Tetraodontidae (Ito et al., 2006; Yokoo, 1950). It has now been identified in a range of phylogenetically diverse marine (e.g., blue ringed octopus, horseshoe crabs, trumpet shells) and terrestrial (e.g., rough-skinned newt, common garter snake, atelopid frogs) organisms (Dao, Takata, Sato, Fukuyo, & Kodama, 2009; Mosher, Fuhrman, Buchwald, & Fischer, 1964; Noguchi & Arakawa, 2008). Among these organisms striking commonalities exist including; TTX and non-TTX-containing populations within species (Dao et al., 2009; Hwang, Lu, & Jeng, 1991), significant within-population variability in TTX concentrations (Hanifin, Yotsu-Yamashita, Yasumoto, Brodie, & Brodie, 1999; Noguchi, Arakawa, Daigo, & Hashimoto, 1986), marked seasonal differences in TTX concentrations within populations (Ji, Liu, Gong, Zhou, & Wang, 2011; Wood et al., 2012b), no correlation between weight and TTX-concentrations (Wood et al., 2012b), differential concentration of TTX among the organs/tissues of TTX-containing organisms (Matsui, Hamada, & Yamamori, 1982; Saito, Kohama, Ui, & Watabe, 2006; Williams & Caldwell, 2009) and the ability for adults to invest TTX in their progeny (Hanifin, Brodie, & Brodie, 2003; Williams, Lovenburg, Huffard, & Caldwell, 2011b). Elucidating the origin of TTX would greatly assist in explaining these observations. However, the ultimate source of TTX in marine and terrestrial ecosystems is still debated with three hypotheses postulated: endogenous (Cardall, Brodie, Brodie, & Hanifin, 2004; Hanifin, Brodie, & Brodie, 2002), through food-chain transmission (Gall, Stokes, French, Brodie, & Brodie, 2012; Honda et al., 2005; Noguchi, Narita, Maruyama, & Hashimoto, 1982; Williams, Brodie, & Brodie, 2004) or through microbial symbionts (Lee et al., 2000; Lu & Yi, 2009; Noguchi, Jeon et al., 1986; Wang, Yu, Luo, Zhou, & Lin, 2008; Wu et al., 2005; Yang et al., 2010; Yasumoto et al., 1986).

Feeding studies have been performed on a wide range of TTX-containing organisms including puffer fish, rough-skinned newts, garter snakes, comb sea-

stars, trumpet shells and caddisflies. These have; provided evidence for a dietary source of TTX (Gall et al., 2012; Noguchi, Arakawa, & Takatani, 2006; Noguchi et al., 1982; Williams, Hanifin, Brodie, & Brodie, 2012; Williams et al., 2004), showed differential uptake and conversion of TTX congeners (Kono, Matsui, Furukawa, Yotsu-Yamashita, & Yamamori, 2008; Matsui, Hamada, & Konosu, 1981), explored whether intoxication of non-toxic strains can occur (Honda et al., 2005; Matsui et al., 1990; Yamamori, Kono, Furukawa, & Matsui, 2004) and investigated the defensive function of TTX (Williams et al., 2012; Williams & Caldwell, 2009; Williams, Hanifin, Brodie, & Caldwell, 2011a; Williams et al., 2011b). Local variation in puffer fish TTX concentrations (Matsui et al., 1982) in concert with a study by Noguchi et al. (2006) in which over 5000 cultured puffer fish were reared in net and land based aquarium with TTX-free diets for 1 to 3 years and became "non-toxic", indicate a dietary source of TTX in this organism. Additionally, when non-toxic cultured puffer fish were fed with either a TTX-containing diet (toxic puffer fish tissue or liver) (Honda et al., 2005; Kono et al., 2008) or TTX-containing bacteria (Matsui et al., 1990), they accumulated the toxin in various parts of their bodies including the skin, liver and ovary. The form of TTX fed to the puffer fish affected accumulation. Puffer fish fed TTX-containing ovary accumulated TTX the fastest and it was detected in all their organs/tissues. Puffer fish fed TTX that was prepared by methanol extraction from toxic organs took longer to accumulate TTX, and no toxins were detected in puffer fish fed crystalline TTX (Matsui et al., 1981).

While the ultimate source of TTX remains a mystery there is little doubt that some species obtain TTX from their diet and use it to increase their fitness. For example, the garter snake (*Thamnophilis sirtalis*) consumes the TTX-containing rough-skinned newt (*Taricha granulosa*) (Williams et al., 2012; Williams et al., 2004), the trumpet shell (*Charonia sauliae*) preys on the toxic comb sea-star (*Astropecten polyacanthus*) (Noguchi et al., 1982) and caddisfly larvae (*Limnophilus* spp.) feed on the eggs of TTX-containing *T. granulosa* (Gall et al., 2012). Although diet is almost certainly the main source of TTX for these and other animals in higher trophic levels, there is far less certainty in organisms such as flat and ribbon worms, frogs and newts. Kim, Brown, Mosher, and

Fuhrman (1975) speculated on whether there is a common food source that is widely distributed enough whereby all the different TTX-containing organisms could access it. Under this scenario the most likely producers would be simple single cell organisms such as bacteria and algae, both of which have been implicated in TTX production previously (Lee et al., 2000; Lu & Yi, 2009; Wu et al., 2005; Yang et al., 2010).

Tetrodotoxin was first identified in New Zealand in the grey side-gilled sea slug (*Pleurobranchaea maculata*) in 2009 (McNabb et al., 2010). Populations in Auckland, Whangarei and Tauranga (Upper North Island, New Zealand) were found to contain high concentrations of TTX (up to 1414 mg kg^{-1}), while populations in Wellington (Lower North Island, New Zealand) had low concentrations (*c*. 2.2 mg kg^{-1}) and South Island populations had no or very low concentrations of TTX (McNabb et al., 2010; Wood et al., 2012b). In this study, non-toxic *P. maculata* sourced from the South Island were maintained in aquariums and fed TTX-containing food for 39 days to investigate whether; they would survive or be negatively affected by the toxin, and whether they could accumulate TTX, and if they did, how quickly and to where in the organisms this would be transported. One of the features that makes *P. maculata* a very amendable species to study the source of TTX is that it is found in relatively confined, easily accessible, shallow sub-tidal areas and populations can reach extremely high densities (*c.* 0.8 individual's m^2; Taylor, Wood & McNabb, 2011). To investigate whether *P. maculata* might obtain TTX from a dietary source in the wild, two extensive benthic surveys were undertaken at sites where dense populations of highly toxic *P. maculata* occurred.

3.3 Methods

3.3.1 Field sampling and laboratory conditions

Eighteen *P. maculata* were collected by divers (11 July 2012) from Tasman Bay (41°3'29"S, 173°5'28"E), Nelson, New Zealand. Each was placed in a separate plastic bag containing seawater (300 mL) and transported to the laboratory in an insulated container. *Pleurobranchaea maculata* were maintained in aquariums (19 L), filled with 14 L of filtered seawater (0.22 µm) and aerated using fish tank pumps. Initially due to limited aquariums in some cases two individuals were placed in aquariums (separated by a polystyrene block) (Appendix 3). After the first sampling, each was maintained in a separate aquarium. Aquarium water was exchanged weekly.

3.3.2 Preparation of TTX-containing food

A solution containing TTX was prepared using a homogenous mix of approximately ten *P. maculata* collected in September 2009 from Narrow Neck Beach, Auckland, New Zealand. These *P. maculata* were known to contain high concentrations of TTX (McNabb et al., 2010). A sub-sample (20 g) of the homogenate was extracted using 90 mL of Milli-Q water containing 0.1% v/v acetic acid. An aliquot (100 mL) of this was mixed with 5.51 g marine agar powder (DifcoTM) and 0.5 mL red food colouring (Queen Pillar Box, Australia). The red food colouring was added to assist in visually assessing whether each piece of agar was consumed during the feeding experiment. The mixture was microwaved (1100 W, 1 min 50 s) and poured into petri dishes (*c*. 15 mL). Once the agar had set, it was sectioned into small pieces (*c*. 0.24 g) using a sterile scalpel, weighed and placed into 1.7 mL tubes (Axygen). These were stored frozen (-20 ºC) and the required amount defrosted immediately prior to each feeding. Ten pieces of agar were kept and extracted to determine the concentration of TTX in the agar. Sub-samples (*c*. 0.3 g) were extracted using Milli-Q water (*c*. 2.7 mL) containing 0.1% v/v acetic acid. Each sample was homogenized using an ultrasonic probe (30 s, Heat Systems – Ultrasonics, Inc., Model W – 220F). Samples were centrifuged (3000 × *g*, 10 min) and an aliquot (100 uL) of the

supernatant added to 900 μL of 100% methanol containing 0.1% v/v acetic acid and frozen (-20 °C) for 1 hr. Samples were centrifuged (3000 × *g*, 10 min) and diluted 1:4 with 100% methanol containing 0.1% v/v acetic acid and analysed for TTX using liquid chromatography-mass spectrometry (LC-MS) as previously described (McNabb et al., 2010). The detection limit of TTX using the LC-MS method was 0.1 ng mL^{-1}.

3.3.3 Spiked recovery experiment

To determine if the extracted agar matrix resulted in any suppression or enhancement of the TTX signal during LC-MS analysis a spiked recovery experiment was undertaken. A sub-sample (1 g) of marine agar (made as per manufacturer's instructions) was homogenised in 9 mL of Milli-Q water containing 0.1% v/v acetic acid using an ultrasonic probe (30 s). A 2.5 mL aliquot was then added to 22.5 mL of methanol containing 0.1% v/v acetic acid. The solution was frozen (1 hr) and centrifuged (3000 × *g*, 10 min). An aliquot (5 mL) was added to 45 mL of methanol containing 0.1% v/v acetic acid. Ten aliquots (1 mL) of supernatant were taken and placed in LC-MS vials. Eight were spiked in duplicate with pure TTX (Tocris Bioscience, Cat. No: 1078) to give final concentrations of 1 ng mL^{-1}, 2 ng mL^{-1}, 10 ng mL^{-1} and 100 ng mL^{-1}. The final two vials were not spiked. The samples were analysed for TTX using LC-MS as previously described (McNabb et al., 2010).

3.3.4 Feeding experiment

Prior to the experiment all *P. maculata* were removed from their aquariums, patted dry using paper towels, weighed and returned immediately to the aquarium. To determine if feeding the TTX-containing food had any negative side-effects, each *P. maculata* was gently placed on its back on the floor of the aquarium and the time taken to turn over was measured prior to the experiment commencing and thereafter once weekly. Individuals were assessed sequentially and in triplicate.

Three *P. maculata* were harvested as controls prior to commencing the feeding experiment. These were frozen immediately (-20 °C) for later dissection

and TTX analysis. Twelve of the remaining *P. maculata* were fed three times a week with TTX-containing food and once a week this was supplemented with *c.* 0.3 g Greenshell™ mussel (*Perna canaliculus*) sourced from the Marlborough Sounds (South Island, New Zealand). To ensure these mussels were free of TTX, three individuals were tested for TTX as described below. The weight of TTX-containing food ingested was recorded for each individual. Three individuals were harvested at each of the following sampling points after the initial feeding; 1 hr, 24 hrs, 17 days and 39 days. These were frozen immediately (-20 °C) for later dissection and TTX analysis. The remaining three *P. maculata* were fed three times a week with Greenshell™ mussel and harvested at the end of the experiment (39 days). Eggs masses laid during the experiment were collected by gently scraping them off the aquarium wall using a spatula. These were washed (Milli-Q water), weighed and frozen (-20 °C) for later TTX analysis.

3.3.5 Dissection, TTX extraction and analysis

Pleurobranchaea maculata were partially defrosted and dissected using a sterile scalpel. The gonad, stomach, and a section of the mantle were removed. Emphasis was placed on taking a clean sample rather than on trying to dissect the entire gonad due to difficulties separating the gonad from the stomach. The remaining fluids and tissue were combined, homogenized, and labelled as 'rest'. All samples were weighed, frozen (-80 °C) and then lyophilized (FreeZone6, Labconco, USA) and reweighed.

The freeze dried organ/tissue or egg masses were then ground using a glass pestle. Sub-samples (0.16 g) were extracted with 15.58 mL of Milli-Q water containing 0.1% v/v acetic acid or a pro rata volume if the starting mass was greater than 0.16 g. The samples were ultrasonicated for 15 min (T-14, L and R Ultrasonics, NJ, USA), centrifuged (3000 × *g*, 10 min) and an aliquot (100 µL) of the supernatant 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 (100% methanol containing 0.1% v/v acetic acid) and then analysed for TTX using LC-MS as previously described (McNabb et al., 2010).

3.3.6 Statistical Analysis

The total TTX concentration for each *P. maculata* was calculated using the TTX concentration and the proportional weights of the gonad, stomach, mantle, and 'rest'. This was then compared with the amount of TTX ingested by each *P. maculata* to estimate percentage uptake.

The combined averages for the behavioural measure for the control *P. maculata* and TTX-fed *P. maculata* were calculated at each time point. Control and TTX-fed *P. maculata* averages at each time point were then compared using a *t*-test to determine if there was a statistical significance between the two groups using Statistica 8 (StatSoft Inc.).

3.3.7 Environmental Surveys

On 13 July 2010, a survey of benthic organisms on rocky reefs and *Musculista senhousia* (Asian date mussel) beds was undertaken by Scuba divers at Narrow Neck Beach (Auckland, New Zealand). During these surveys, all visible benthic flora and fauna were collected and bagged before freezing; where possible, five specimens of each species were collected (Table 3-1). The Narrow Neck Beach site had very high densities of *P. maculata* (up to 0.8 m^2; Taylor, Wood & McNabb, 2011) and these contained high levels of TTX (McNabb et al., 2010; Wood et al., 2012b). In August 2011, a second survey was undertaken by Scuba divers at Illiomama Rock (Auckland, New Zealand) and 51 samples were collected (Table 3-2). This site also contained high densities of toxic *P. maculata* (up to 0.3 individuals m^2; Taylor, Wood & McNabb 2011; Wood et al., 2012b). The specimens from both surveys commonly consisted of a consortium of species and most were covered with biofilms. The samples were homogenized in their entirety to enable all of these species to be assessed. Sub-samples (2 g) of each were prepared and tested for TTX using LC-MS as described above. All samples from Illiomama Rock were also screened for C9 using the methods described in McNabb et al. (2013).

3.4 Results

The average weight per individual at the start of the experiment was 8.4 g (± 6.0 g) and by day 39 this had increased to 19.8 g (± 5.4 g). A behavioral measure (time to turn over when placed on back) was used to investigate the possible negative effects of TTX on *P. maculata* during the experiment. This was run once a week throughout the course of the experiment. No significant differences were observed between the TTX-fed and control *P. maculata* during the study (time 0 - df = 16, $P = 0.36$, day 7 - df = 7, $P = 2.37$, day 14 - df = 7, $P = 2.4$, day 21 - df = 4, $P = 2.78$, day 28 - df = 4, $P = 2.78$ and day 35 - df = 4, $P = 2.78$).

Spiked recovery experiments demonstrated an average enhancement of the TTX peaks during liquid chromatography-mass spectrometry (LC-MS) analysis of 15.8% and all data were adjusted accordingly. Throughout the experiment only TTX was detected; it was not transformed into other variants. The average TTX concentration in ten pieces of TTX-containing agar was 373 mg kg^{-1} (± 8 mg kg^{-1}). This value was used to estimate the amount of TTX consumed by each individual. All *P. maculata* samples were subjected to lyophilization prior to TTX analysis. A comparison of weights pre- and post-lyophilization demonstrated that approximately 85% of the *P. maculata* was liquid. For comparative purposes (as all other research on *P. maculata* to date has presented TTX concentrations in wet weight (ww)) approximate ww are shown in brackets.

Four egg masses were laid during the experiment. The first (5.39 g) was laid on day one and had a TTX concentration of 0.9 mg kg^{-1} freeze dried weight (FDW) (ww *c.* 0.14 mg kg^{-1}); the individual *P. maculata* that laid this egg mass was harvested on the same day and had a total TTX concentration of 22 mg kg^{-1}FDW (ww *c.* 3.3 mg kg^{-1}). The remaining three egg masses were laid by one individual on days one, nine and twenty-five (12.46 g, 3.82 g and 7.91 g, respectively). The TTX concentrations in these egg masses progressively increased during the experiment with TTX concentrations of 11.6 mg kg^{-1} FDW (ww *c.* 1.7 mg kg^{-1}), 129 mg kg^{-1} FDW (ww *c.* 19.4 mg kg^{-1}) and 292 mg kg^{-1} FDW (ww *c.* 43.9 mg kg^{-1}), respectively. The individual *P. maculata* was

harvested on day thirty-nine and had a total TTX concentration of 361 mg kg^{-1} FDW (ww *c*. 54.3 mg kg^{-1}).

No TTX was detected in any of the organs/tissues from the three controls harvested prior to the experiment or in the three controls (fed non-TTX containing food) harvested on day 39. Tetrodotoxin was detected in the three *P. maculata* harvested at 1 hr. As expected TTX was detected in the stomach (ave. 120 mg kg^{-1} FDW, ww *c*. 18 mg kg^{-1}), but it was also present in reasonably high concentrations in the mantle (ave. 111 mg kg^{-1} FDW, ww *c*. 16.6 mg kg^{-1}), gonad (ave. 67 mg kg^{-1} FDW, ww *c*. 10.1 mg kg^{-1}) and 'rest' (ave. 46.0 mg kg^{-1} FDW, ww *c*. 6.9 mg kg^{-1}; Figure 3-1). The organ/tissue with the highest TTX concentration varied among individuals harvested at this time point. For example, the highest TTX concentration in individual "D" was in the stomach (185 mg kg^{-1} FDW, ww *c*. 27.7 mg kg^{-1}), whereas individual "E" had the highest TTX concentration in the gonad (182 mg kg^{-1} FDW, ww *c*. 27.3 mg kg^{-1}) and individual "F" in the mantle (216 mg kg^{-1} FDW, ww *c*. 32.5 mg kg^{-1}, Figure 3-1). After 1 hr, the average percentage uptake of TTX was 32% (range 21% - 43%). After 1 day, the average percentage uptake had decreased slightly (21% (range 16% - 27%)) and a similar pattern in variability in TTX concentrations among organs/tissues was observed (Figure 3-1). After 17 days, the average percentage uptake was 15% (range 15% - 16%). Concentrations in individual organs/tissue had increased markedly. In "J" the highest TTX concentration was the mantle (573 mg kg^{-1} FDW, ww *c*. 86 mg kg^{-1}) whereas "K" and "L" had the highest TTX concentrations in the stomach (1438 mg kg^{-1} FDW, ww *c*. 215 mg kg^{-1} and 733 mg kg^{-1} FDW, ww *c*. 110 mg kg^{-1} respectively; Figure 3-1). After 39 days the average percentage uptake decreased to 9% (range 6% - 13%). Individual "M" had the highest TTX concentrations in the stomach (888 mg kg^{-1} FDW, ww *c*. 133 mg kg^{-1}) whereas "N" and "O" had the greatest TTX concentrations in the mantle (1282 mg kg^{-1} FDW, ww *c*. 192 mg kg^{-1} and 1890 mg kg^{-1} FDW, ww *c*. 284 mg kg^{-1}), respectively.

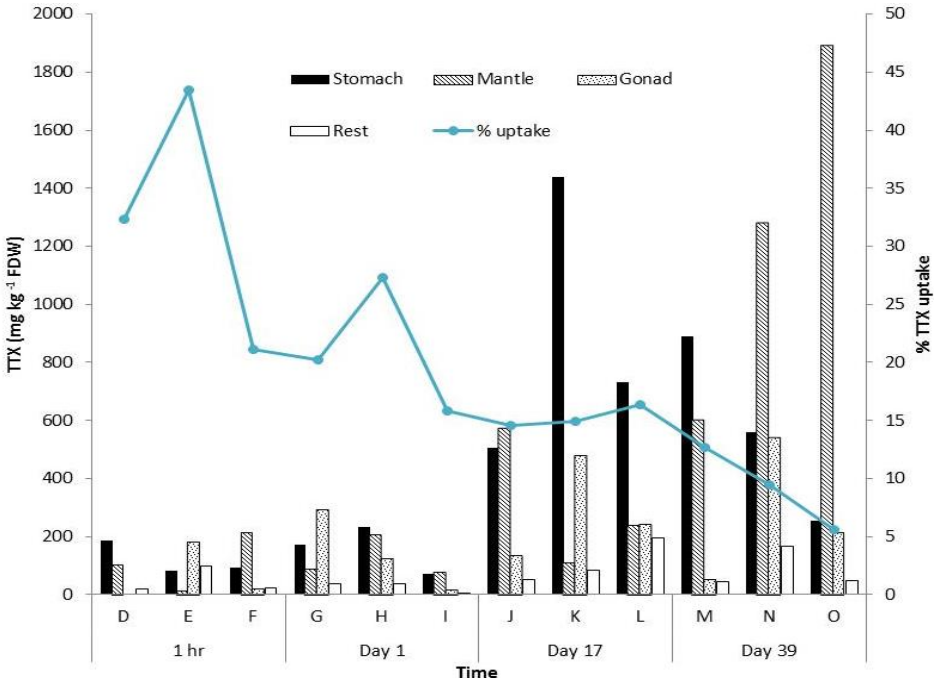

Figure 3-1. Tetrodotoxin (TTX) concentrations in tissue/organs of *Pleurobranchaea maculata* at various time points after being fed TTX-containing food. Data is presented as amount detected in lyophilized weight (FDW). Each letter represents a single individual.

Low concentrations of TTX (0.25 mg kg^{-1}) were found in sand dollars (*Arachnoides zelandiae*; Table 3-1, Figure 3-2) from Narrow Neck Beach. Trace amounts of TTX (<0. 2 mg kg^{-1}) were found the cats' eye snail (*Turbo smaragdus*), a crab (*Macrothalamus hirtipes*) and coralline algae (*Corallina officinalis*; Table 3-1) from Narrow Neck Beach. No TTX or C9 were detected in any of the samples from Illiomama Rock (Table 3-2).

Table 3-1. Benthic organisms/samples collected for tetrodotoxin (TTX) testing from rocky reed and *Musculista senhousia* (Asian Date Mussel) beds at Narrow Neck Beach and TTX concentrations detected. MS = *M. senhousia* beds.

Scientific Name	Common name/Description	Habitat	TTX Level (mg kg^{-1})
Echinodermata			
Australostichopus mollis	Sea cucumber	Reef	0
Stegnaster inflatus	Orange elevated cushion star	Reef	0
Patiriella regularis	Common cushion star	Reef and MS	0

Scientific Name	Common name/Description	Habitat	TTX Level (mg kg^{-1})
Coscinasterias calamaria	11 Arm star	Reef and MS	0
Evechinus chloroticus	Sea urchin (Kina)	Reef	0
Echinocardium australe	Heart urchin	MS	0
Arachnoides zelandiae	Sand Dollar	Reef	0.25
Mollusca			
Cominella virgata	Whelk	Reef	0
Musculista senhousia	Asian date mussel	MS	0
Saccostrea glomerata	Rock oysters	Reef	0
Cymatium spengleri	Whelk	Reef	0
Penion sulcatus	Whelk	Reef	0
Cominella adspersa	Whelk	Reef and MS	0
Turbo smaragdus	Cats eye	Reef	Trace
Haminoea zealandiae	Bubble shell slugs	Reef	0
Cellana radians	Limpets	Reef	0
Acanthochitona zelandica	Chiton	Reef	0
Cryptoconchus porosus	Chiton	Reef	0
Polychaeta			
Perinereis amblyodonta	Polychaete	Reef	0
Crustacea			
Plagusia chabrus	Red rock crab	Reef	0
Ovalipes catharus	Paddle Crab	MS	0
Petrolisthes elongatus	Porcelain crab	Reef	0
Chamaesipho columna	Barnacles	Reef	0
Macrophthalmus hirtipes	Crab	MS	Trace
Pagurus sp.	Hermit crabs	Reef	0
Callianassa filholi	Burrowing shrimp	MS	0
Crustacea			
Plagusia chabrus	Red rock crab	Reef	0
Corallina officinalis	Coralline turf algae	Reef	Trace
Other			
Sediment from *M. senhousia* beds		MS	0

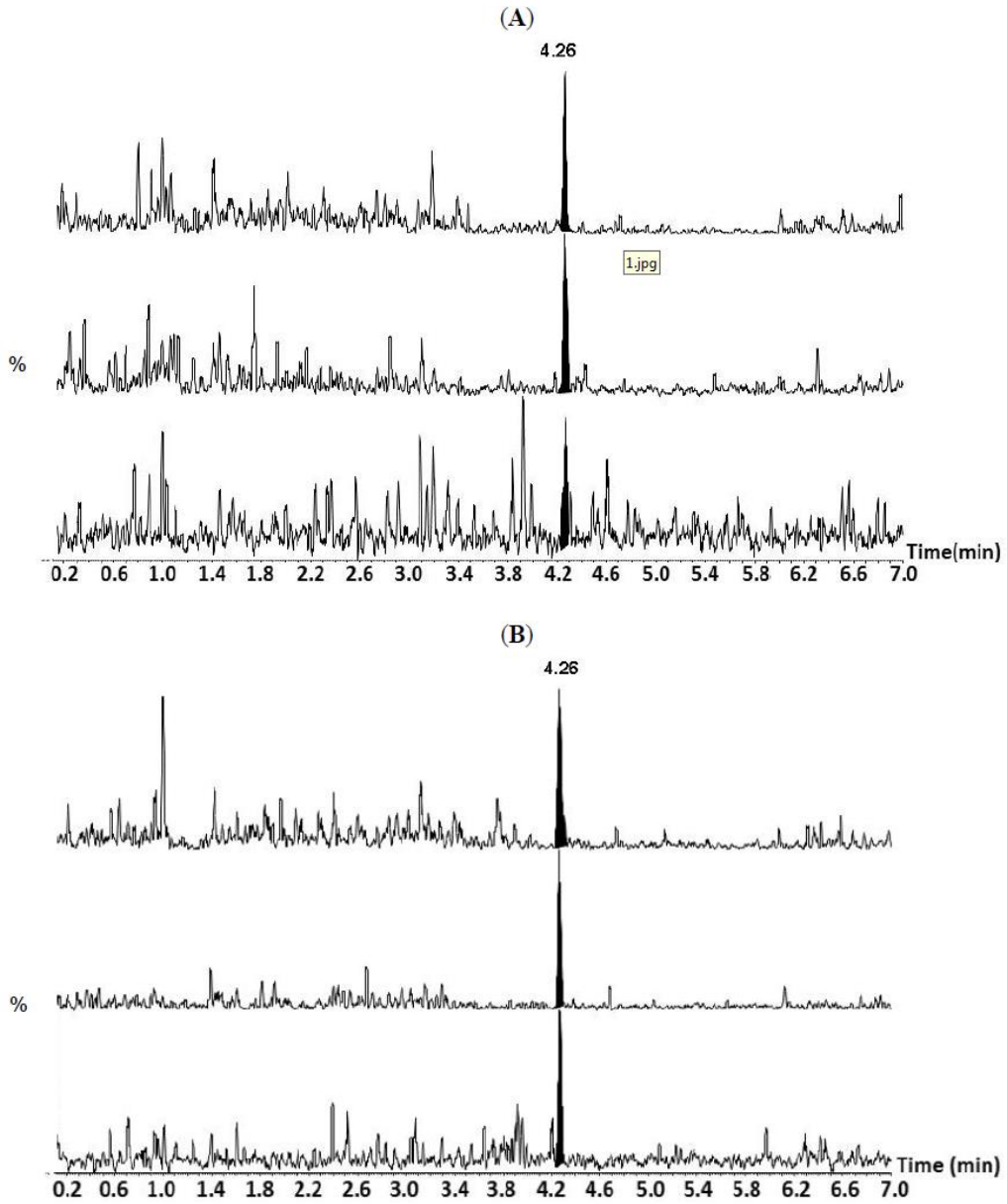

Figure 3-2. LC-MS/MS chromatograms showing three multiple reaction monitoring (MRM) traces *m/z* 320 > 302, 320 > 162 and 320 > 60 from top to bottomw (A) *Arachnoides zelandiae* with area ratios for each channel of 1.00:0.66:0.16 and (B) Authentic TTX at 0.5 ng/mL with area ratios for each channel of 1.00:0.63:0.21. The *x*-axis is time (min) and the *y*-axis is LC-MS/MS response normalised to the largest peak in each MRM channel.

Table 3-2. Benthic organisms collected for tetrodotoxin (TTX) testing from Iliomama Rock and TTX concentrations detected.

Scientific or Record Name	Common Name/Description	TTX level (mg kg $^{-1}$)
Echinodermata		
Patiriella regularis	Regular seastar	0
Coscinasterias calamaria	Spiny star	0
Echinocardium australe	Heart urchin	0
Arachnoides zelandiae	Snapper biscuit	0
Botryllus schlosseri	Star ascidian	0
Stichopus mollis	Sea cucumber	0
Porifera		
Polymastia sp.	Common sponge	0
Unknown orange sponge	Sponge	0
Mollusca		
Perna canaliculus	Greenshell^{TM} mussel	0
Bursatella leachii	Ragged Sea Hare	0
Buccinulum lineum	Lined whelk	0
Cockle shell	Cockle shell	0
Zelithophaga truncata	Bivalve	0
Atrina zelandica	Horse mussel	0
Cleidothaerus albidus	Bivalve	0
Mytilus galloprovincialis	Blue mussel	0
Crassostrea gigas	Pacific oyster	0
Sigapatella novazelandiae	Circular slipper limpet	0
Polychaeta		
Thelepus spectabilis	Polychaete	0
Annelida		
Chaetopterus sp.	Parchment worms	0
Crustacea		
Crab inside B39	Crab	0
Pagurus novazelandiae	New Zealand hermit crab	0
Algae		
Codium fragile	Green alga	0
Colpomenia sinuosa	Brown alga	0
Laurencia thyrsifera	Red alga	0
Lithothamnion sp.	Encrusting red alga	0
Lithothamnion sp.	Encrusting red alga	0

Scientific or Record Name	Common Name/Description	TTX level (mg kg $^{-1}$)
Plocamium sp.	Red alga	0
Sargassum sinclairii	Brown alga	0
Unknown brown algae	Alga	0
Unknown red algae	Alga	0
Other		
Beania discodermiae	Bryozoan	0
Biofilm from B39		0
Biofilm from rock		0
Biofilm from Scallop shell		0
Biofilm from shell		0
Ciona intestinalis	Sea squirt	0
Cnemidocarpa bicornuta	Sea squirt	0
Mussel biofilm		0
Pecten novazelandiae (scallop, biofilm)		0
Plumularia setacea	Hydroid	0
Rock with biofilm		0
Sargassum epifauna		0
Sediment -top of the tube (1cm)		0
Sediment- top of the tube (3 mm)		0
Styela plicata	Tunicate	0
Unknown ascidian		0

3.5 Discussion

Using a behavioural response measure (time taken to turn to upright position) we observed no negative influence of the TTX-containing diet on non-toxic *P. maculata*. Studies on puffer fish and newts have demonstrated that TTX-resistance comes from substitution of amino acids in the p-loop regions of skeletal muscle and neuronal Na_v channels (Soong & Venkatesh, 2006). Garter snakes and clams have a similar sodium channel mutation-based on TTX/STX resistance induced through ingestion of toxic prey (Bricelj et al., 2005; Geffeney, Fujimoto, Brodie, & Ruben, 2005). In contrast, the shore crab *Hemigrapsus sanguineus* possesses TTX-binding proteins in its hemolymph which enables this non-toxic

crab to be resistant to TTX. The mechanism via which *P. maculata* confers its TTX-resistance is unknown, however, results of this study suggest that all populations, regardless of whether they contain TTX or not, have evolved this adaptation. The presence of *P. maculata* populations that are resistant to TTX, yet do not contain it, is intriguing and may indicate that the source of TTX is not available at these locations, or that the ecological advantage that containing TTX confers is no longer necessary.

The results of this study demonstrate that non-toxic *P. maculata,* when fed with TTX-containing food, can rapidly sequester and transport the toxin around their bodies. After 1 hour TTX was detected in all organs/tissues tested. Excluding the stomach, the highest values were detected in the mantle. Wood et al. (2012a) suggested that the main function of TTX in *P. maculata* was as a chemical defense. The rapid transport to the mantle and the high concentration in this tissue on day 17 and 39 support this hypothesis. Four egg masses were laid during the experimental period. It was surprising that TTX was detected in the egg mass laid only 1 day after the first feeding of TTX-containing food. This demonstrates how rapidly *P. maculata* can transport this compound and provides further evidence to support the suggestion that adult *P. maculata* invest TTX into their offspring, presumably to function as a chemical defense (Wood et al., 2012a). In this study, there was not a pronounced transport of TTX to the gonads as might be expected based on this assumption. Many of the individuals were juvenile (only two laid egg masses during the experiment) and often during dissection we observed that the gonads were not well developed.

After 1 hour the *P. maculata* in this study had an average TTX uptake of between 21– 43 % uptake. Tetrodotoxin uptake decreased throughout the experiment (*c*. 9% by day 39) suggesting that *P. maculata* may have a maximum TTX concentration that they can retain. It is plausible that TTX concentrations may correlate with their level of TTX resistance, for example, Hwang et al. (1992) showed that puffer fish that were generally non-toxic could become weakly toxic in some habitats. They suggested that these species can accumulate a limited amount of TTX but because they only have a medium level of resistance to TTX they could not accumulate TTX to the same concentration as toxic puffer fish.

Despite the low uptake rates, the concentrations in the *P. maculata* at day 39 (ww *c*. 2-300 mg kg^{-1} in the highest organs/tissues) were within the ranges of wild populations (Wood et al., 2012b). Feeding studies on other marine TTX-containing organisms have shown comparable percentage uptakes. Yamamori et al. (2004) fed non-toxic cultured juvenile puffer fish (*Takifugu niphobles*) crystalline TTX for 30 days, followed by 170 days of a TTX-free diet. Initially the puffer fish accumulated *c*. 50% of the total TTX administered and this gradually decreased to *c*. 30% by day 80. Honda et al. (2005) fed non-toxic cultured puffer fish (*Takifugu rubripes*) TTX-containing diets for 60 days at low doses (less than 3 mouse units (MU) g^{-1}) and found that TTX accumulated in the skin and liver but at high doses of TTX (up to 57 MU g^{-1}), TTX was sequestered in the liver and ovary. Accumulation rates were age-dependent with <1 year old puffer fish having percentage uptakes of 0 – 17% whereas 1 year old puffer fish had accumulation rates of more than 30%. The *P. maculata* in the present study were late juvenile-young adult age. This age-group was selected to avoid egg-laying individuals as previous studies have shown that *P. maculata* depurate TTX through their egg masses (Wood et al., 2012a). Uptake rates in terrestrial organisms appear to be much lower. Caddisflies (*Limnophilus* spp.) fed TTX-containing rough-skinned newt eggs had an uptake of only 0.08 – 0.47%, although they were able to retain this for up to 134 days (Gall et al., 2012). Likewise, garter snakes sequestered only 0.68 – 3.4% of TTX seven days after consuming rough-skinned newts (Williams et al., 2004). Williams et al. (2004) and Gall et al. (2012) hypothesise that a binding protein may be responsible for TTX sequestration in these species, and that the lower uptake rate may be due to a functional limit in the amount of TTX that can be sequestered.

In this experiment the TTX concentrations measured among *P. maculata* harvested on the same day had less variability than TTX concentrations in wild (Wood et al., 2012a; 2012b). Variable consumption of a common food source high in TTX could explain this observation, with individuals in the wild that consume more TTX-containing food sequestering higher levels of TTX. Similar observations have been reported for garter snakes with individuals that consumed seven TTX-containing newts having higher TTX concentrations in their liver than

those that consumed only one (Williams et al., 2004). Despite this, and the demonstration in this study that *P. maculata* can accumulate TTX from their diet, during our environment surveys we were unable to detect any organisms or environmental material (i.e., sediment) that contained TTX concentrations high enough to account for the concentrations of toxin detected in *P. maculata*. Low concentrations were found in the detritivore *A. zelandiae*. The samples of this species were collected from sub-tidal areas at Narrow Neck Beach where *P. maculata* were abundant, and they may have accumulated TTX from *P. maculata* mucus trails. Previous studies have shown that *P. maculata* mucus contains TTX (Khor et al., 2013). The concentrations of TTX in *A. zelandiae* are too low to account for the concentrations in *P. maculata* and this species is unlikely to be a significant food source because of its extremely rigid skeleton. Attempts were made to sample a wide variety of habitats that were covered with diverse biofilms, however, we cannot rule out the possibility that we missed sampling the organism/s that provide the source of TTX to *P. maculata*. However, given how abundant *P. maculata* are at these sites, the high concentration of TTX (up to 1414 mg kg^{-1}) (Wood et al., 2012b), and their short life span (<1 year), we suggest that a dietary source is unlikely.

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Chapter 4 – Investigating the attraction of toxic and non-toxic *Pleurobranchaea maculata* to tetrodotoxin; a preference study.

4.1 Abstract

Tetrodotoxin (TTX) is a potent neurotoxin that is present in a wide range of phylogenetically distinct organisms. The ultimate source of TTX is still highly debated with endogenous and exogenous origins postulated. Among the possible exogenous sources, diet is highly cited. If the source of TTX for an organism is diet they should; be attracted to it, be able to detect it, and move towards it. Previous studies on the TTX-containing grey side-gilled sea slug *Pleurobranchaea maculata* have shown TTX concentrations decrease when fed a TTX-free diet and that non-toxic individuals can accumulate TTX via dietary sources. In this study eight non-toxic and seventeen toxic *P. maculata* were subjected to a series of three preference studies; (1) a zonation experiment in which the time spent at varying distances from a TTX food source was monitored, (2) a toxic/non-toxic agar trail experiment and, (3) a direct choice experiment utilizing various combinations of toxic and non-toxic agar blocks. A statistically significant preference for TTX was found for toxic *P. maculata* in the agar trails ($P < 0.001$) and direct choice experiments ($P < 0.001$). For the non-toxic *P. maculata*, a statistically significant preference was only found for the direct choice experiment ($P = 0.002$). These data and previous studies on *P. maculata* demonstrate that diet is a possible source. However, given the absence of identifiable TTX sources in environments where *P. maculata* are prevalent, in concert with their extremely high TTX concentrations and short life spans, it is unlikely to be the sole source for this species.

4.2 Introduction

Tetrodotoxin (TTX) is a potent neurotoxin that selectively binds to voltage-gated sodium channels. It derives its name from, and is infamously associated with the puffer fish family (*Tetraodontidae*) (Ito et al., 2006; Yokoo, 1950). Tetrodotoxin is found in a wide range of phylogenetically distinct organisms including those from terrestrial (e.g., rough-skinned newt, common garter snake, atelopid frogs) and marine (e.g., starfish, horseshoe crabs, trumpet shells) environments (Dao et al., 2009; Mosher et al., 1964; Noguchi & Arakawa, 2008). There is considerable speculation about the origin of TTX in these organisms with both endogenous and exogenous sources proposed (Honda et al., 2005; Lee et al., 2000; Lu & Yi, 2009; Noguchi & Arakawa, 2008).

Tetrodotoxin was recently discovered in New Zealand in the grey side-gilled sea slug (*Pleurobranchaea maculata;* McNabb et al., 2010). There is distinct geographic variability in concentration of TTX among populations, with those in the upper North Island of New Zealand containing high TTX concentrations (max. 1414 mg kg^{-1}), whilst those in the lower North Island have low toxicity (*c.* 2.2 mg kg^{-1}) and South Island populations are non-toxic (McNabb et al., 2010; Wood et al., 2012). There are several attributes of *P. maculata* that make them a very amenable marine organism for investigating the origin of TTX. They are found in relatively confined, easily accessible, shallow sub-tidal areas, populations can reach extremely high densities (*c.* 0.8 individual's m^{-2}; Taylor, Wood & McNabb, 2011), they can be reared in captivity under controlled conditions, and they can contain very high concentrations of TTX (McNabb et al., 2010; Wood et al., 2012). Wood et al. (2012) showed that toxic *P. maculata* depurate TTX while maintained in captivity and Khor et al. (2014) demonstrated that non-toxic *P. maculata* can accumulate the toxin when fed a TTX-containing diet. Thus a dietary source of TTX is one of the favoured hypotheses for this organism.

A dietary source has also been proposed for other marine TTX-containing organisms. For example, accumulation of TTX was demonstrated in non-toxic puffer fish fed a TTX-containing diet (Honda et al., 2005; Kono et al., 2008; Matsui et al., 1990). The results from Noguchi et al. (2006) also support a dietary

source. In their study, they cultured over 5000 specimens of the puffer fish *Takifugu rubripes* in net-cages and land-based aquaria. The puffer fish were fed TTX-free diets for between 1–3 years and all tissues tested negative for TTX.

If diet is a source of TTX for an organism, the premise is that they should; be attracted to it, be able to detect it, and move towards it (i.e., they should have chemosensory abilities). Chemoreception is well known in gastropods. For example, Murphy and Hadfield (1997) showed that the nudibranch *Phestilla sibogae* detects its prey via chemosensory endings on its rhinophores. Likewise, Uchida et al. (2010) demonstrated that a feeding response (shell lifting, pedal sole expansion and lifting and food seizing with pedal sole) could be elicited in abalone (*Haliotis discus hannai*) when brown algae (*Laminaria japonica*) was placed in contact with any of its three tentacles (cephalic, epipodial or mantle).

Preference studies are commonly used to elucidate inclinations in dietary sources and have been undertaken on several TTX-containing organisms. Hwang, Noguchi, & Hwang (2004) investigated eight TTX-containing and two non-TTX-containing aquatic snail species (Hwang, et al., 2004). Tetrodotoxin-containing food and control food were placed in separate partitions of a circular tank for 24 hrs and the locations of the snail species at set time points were recorded. Among toxic snails, it was found that higher toxicity snails were more attracted to TTX-containing food and that different species had varying preferences and abilities to accumulate TTX. The non-toxic species were not attracted to TTX. Saito et al. (2000) state that if TTX is an essential substance for the puffer fish *T. rubripes* they should be able to recognise and seek out TTX sources. They undertook a study in which ten puffer fish were placed into aquarium with removable partitions. After *c.* 30 min the partitions were removed and the movement of fish toward food with or without TTX observed. All fish appeared to preferentially choose TTX-containing food; however, differences in concentration of TTX did not appear to affect the puffer fish's behaviour.

The aim of this study was to determine whether *P. maculata* have a preference for TTX-containing food. A series of three preference studies were undertaken: (1) a zonation experiment in which the amount of time *P. maculata* spent in different zones of an aquarium containing either toxic or non-toxic agar

blocks was monitored, (2) an agar trail experiment in which droplets of hot liquefied toxic or non-toxic agar were placed in parallel lines along the bottom of an aquarium and the behaviour of *P. maculata* was monitored, and (3) a direct choice experiment where *P. maculata* were presented with combinations of toxic and non-toxic agar blocks and their response was recorded. The experiments were undertaken using toxic and non-toxic *P. maculata.*

4.3 Methods

4.3.1 Field sampling and laboratory conditions

Eight non-toxic *P. maculata* were collected by divers (31 October 2012) from Tasman Bay (41°3' S, 173°5' E; Nelson, New Zealand) and seventeen toxic *P. maculata* (19 June 2013) from Matakana Island (37°35′ S, 176°05′ E; Tauranga, New Zealand). *Pleurobranchaea maculata* were placed in individual plastic bags or jars containing seawater (300 mL) and transported to the laboratory in insulated containers. Non-toxic *P. maculata* were held in captivity until 28 January 2013 and toxic *P. maculata* until 24 June 2013. Between collection and experimentation, *P. maculata* were fed tuna or shrimp.

Non-toxic *P. maculata* were maintained in aquariums (19 L), filled with 14 L of filtered seawater (0.22 µm) and aerated using fish tank pumps. Toxic *P. maculata* were maintained in aquariums (14 L) with a flow through seawater system (Appendix 4). Water flowed out of the tanks into a sump with a mesh to trap larger debris and this was then recycled back through the system. The sump was cleaned or replaced twice weekly.

4.3.2 Preparation of agar blocks

Tetrodotoxin-containing agar blocks were prepared using a homogenous mix of approximately ten *P. maculata* collected in 2009 from Narrow Neck Beach (Auckland, New Zealand). Individuals from this population in 2009 contained high concentrations of TTX (McNabb et al., 2010). Non-TTX-containing agar blocks were prepared using non-toxic *P. maculata* collected from Tasman Bay (Wood et al., 2012).

Sub-samples (20 g) of each homogenate were extracted using 90 mL of Milli-Q water containing 0.1% v/v acetic acid. An aliquot (100 mL) was mixed with 5.51 g marine agar powder (Difco™). The mixture was microwaved (1100 W, 1 min 50 s) and poured into petri dishes (*c.* 15 mL). TTX concentrations were determined in each batch using liquid chromatography–mass spectrometry (LC-MS) as described in Khor et al. (2013). Stainless steel weights (*c.* 5 g) were embedded in the agar to prevent these floating during experimentation. Agar blocks were removed from the petri dishes prior to commencing each preference experiment.

The diffusion of TTX from the agar blocks was investigated. Eighteen TTX-containing agar blocks were placed in an aquarium (42 L) containing 14 L of filtered seawater. Three blocks were removed immediately and then at 10 min, 30 min, 1 hrs, 2 hrs and 3 hrs. Agar blocks were frozen immediately (-20 ºC). Sub-samples (*c.* 0.6 g) from each block were extracted as described in Khor et al. (2013) and their TTX concentrations determined using LC-MS (McNabb et al., 2010). LC-MS data were decreased by 15.8% to account for enhancement of the signal caused by the agar matrix (Khor et al., 2013).

4.3.3 Preference experiments

Three preference experiments were undertaken (Figure 4-1). The first two experiments took place in glass (non-toxic *P. maculata*) or plastic (toxic *P. maculata*) aquariums (42 L) filled with 14 L of filtered (0.22 µm) seawater.

All *P. maculata* were weighed prior to experimentation (ave. 17.7 g (non-toxic), 10.4 g (toxic)). *Pleurobranchaea maculata* were randomly selected for each trial and each treatment using a random number generator (randomizer.org/form.htm) where the numbers tallied with numbers given to each aquarium. Experimentation ran over the course of 5 days for non-toxic *P. maculata* and 10 days for toxic *P. maculata*. All *P. maculata* were starved for two days prior to experimentation and thereafter fed with a piece of tuna or shrimp every 1-2 days.

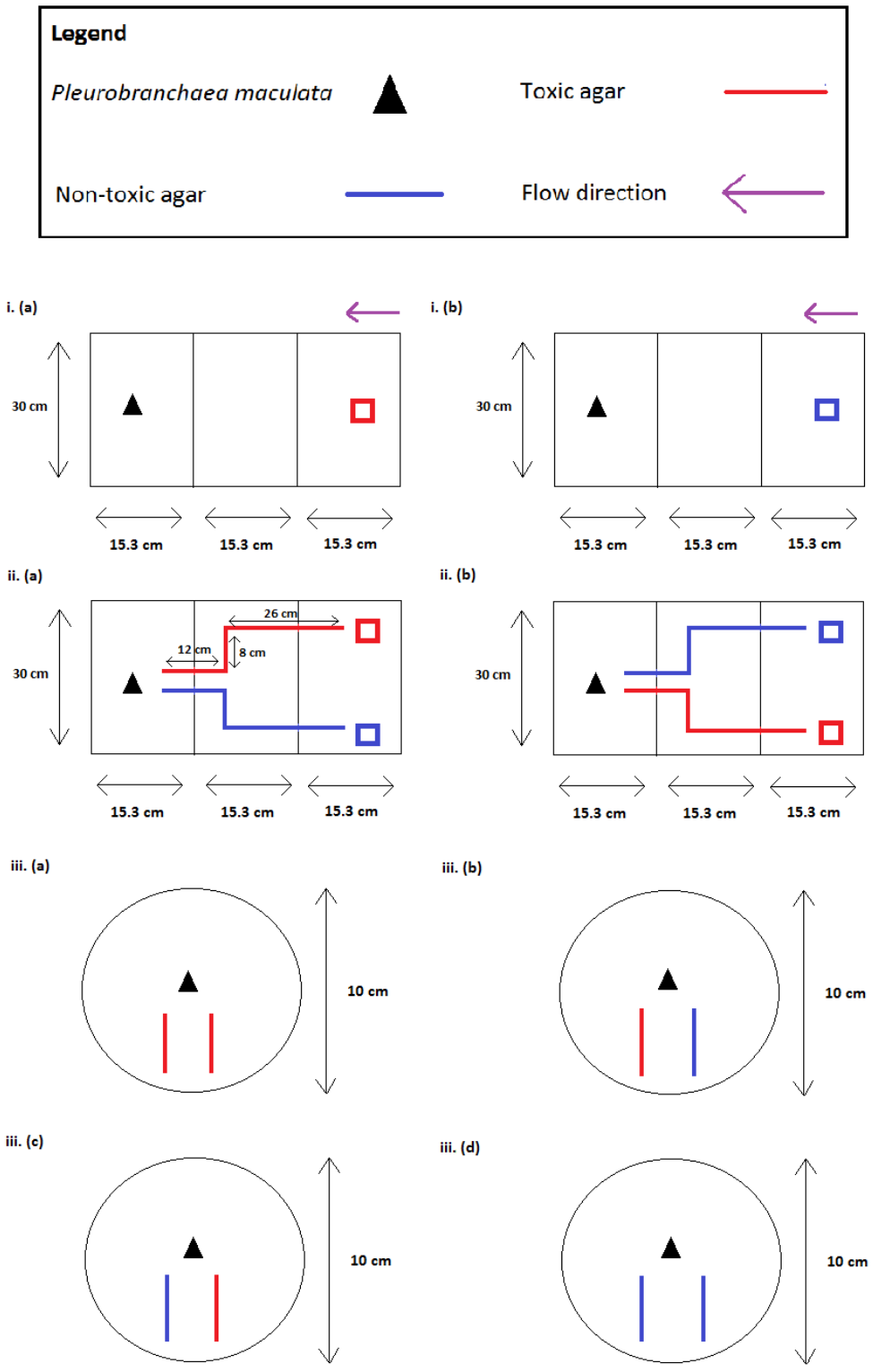

Figure 4-1. Schematic of the three preference tests carried out on *Pleurobranchaea maculata*. (i- a,b) Aquarium zonation, (ii- a,b) agar trails, (iii- a,b) direct choice experiments.

4.3.3.1 Aquarium zonation

A peristaltic pump (501Z, Watson-Marlow, MA, USA) was connected to 4-way distributor splitter lever control valve tap to ensure relatively unidirectional flow (Figure 4-1, Appendix 5). The flow rate was set at 1 mL per second and checked periodically throughout the experiments. Agar blocks were replaced every 3-4 trials and all pump tubing was flushed with fresh seawater (*c.* 5 min) and aquariums rinsed and cleaned between each trial.

A marker board positioned underneath the aquarium was used to divide it into three 15.3 cm zones and to mark the starting point for each *P. maculata* (Figure 4-1 (i)). An agar block was placed 1-2 cm in front of the valve taps 5 min before addition of a single *P. maculata*. The amount of time spent in each zone was assessed for 10 min. *Pleurobranchaea maculata* were defined as entering a new zone when 50% or more of their body crossed the dividing line. Both TTX-containing and non-TTX containing agar blocks were assessed and the experiment was repeated three times for each non-toxic *P. maculata* and four times for each toxic *P. maculata*. During these experiments some individuals consumed portions of the agar block and this was recorded.

4.3.3.2 Agar trails

Droplets (*c.* 30 µL) of hot liquefied (1 min microwave or melted on a hot plate) toxic or non-toxic agar were pipetted at regular intervals (1 cm) and three droplets was placed at the end of the trail (Figure 4-1 (ii), Appendix 6).

The trail taken by each *P. maculata* was recorded (i.e. the "decision" made at the "fork") as well as the time taken and the distance travelled along the trail. The experiment was repeated three times with each non-toxic *P. maculata* and twelve times with each toxic *P. maculata*. To control for side biases the toxic and non-toxic agar trails were switched between experiments (Figure 4-1 (ii)).

4.3.3.3 Direct choice experiment

Pleurobranchaea maculata were individually removed from aquariums and placed into glass containers (10 cm dia., 20 mL volume) containing 15 mL filtered (22 µm) seawater. Using tweezers, paired sub-samples (*c.* 0.1 g) from the

agar bocks were presented to them using one of the following combinations; toxic and toxic, non-toxic and non-toxic, or toxic and non-toxic (Figure 4-1 (iii)). The agar blocks were held *c.* 1 cm in front of the *P. maculata* and a positive response was recorded when the *P. maculata* extended its mouth piece out towards the agar block and oriented its body towards it. The toxic and non-toxic combination was repeated 21 times with each non-toxic *P. maculata,* and 16 times with each toxic *P. maculata*. The toxic/toxic and non-toxic/non-toxic combinations were repeated six times with each non-toxic and toxic *P. maculata*. The test container was washed and filled with fresh seawater between each experiment. One non-toxic *P. maculata* died during this experiment and its data was removed (i.e. this experiment was run with only seven non-toxic *P. maculata*).

4.3.4 Statistical Analysis

Paired student *t*-tests (Microsoft Excel) were used to determine statistically significant differences among data.

4.4 Results

4.4.1 Tetrodotoxin diffusion from agar blocks

The average TTX concentration in ten pieces of TTX-containing agar was 373 mg kg^{-1} (± 8 mg kg^{-1}). The TTX diffused rapidly out of the agar blocks. Within 10 min the concentrations had decreased by 19.4% (Table 4-1). The diffusion continued over the 180 min period, although the rate decreased after 60 min (Table 4-1).

Table 4-1. Average tetrodotoxin (TTX) concentrations in agar blocks during diffusion experiment. n = 18.

Time (min)	Average Decrease (%)	Std. Dev. (%)
0	N/A	N/A
10	19.4	25.7
30	33.3	3.8
60	54.9	11.4
120	53.8	6.8
180	63.7	9.7

4.4.2 Preference experiments

4.4.2.1 Aquarium zonation

When toxic agar blocks were present, toxic *P. maculata* spent the majority of their time in Zone 1 (furthest from the agar block, ave. 313 sec) followed by Zone 3 (closest to the agar block, ave. 202 sec) and Zone 2 (ave. 85 sec, Figure 4-2). Paired student *t*-tests showed that the average amount of time spent in Zone 1 was significantly greater than Zone 2 ($P < 0.001$) and Zone 3 ($P = 0.02$) and the time in Zone 3 was significantly greater than in Zone 2 ($P < 0.001$).

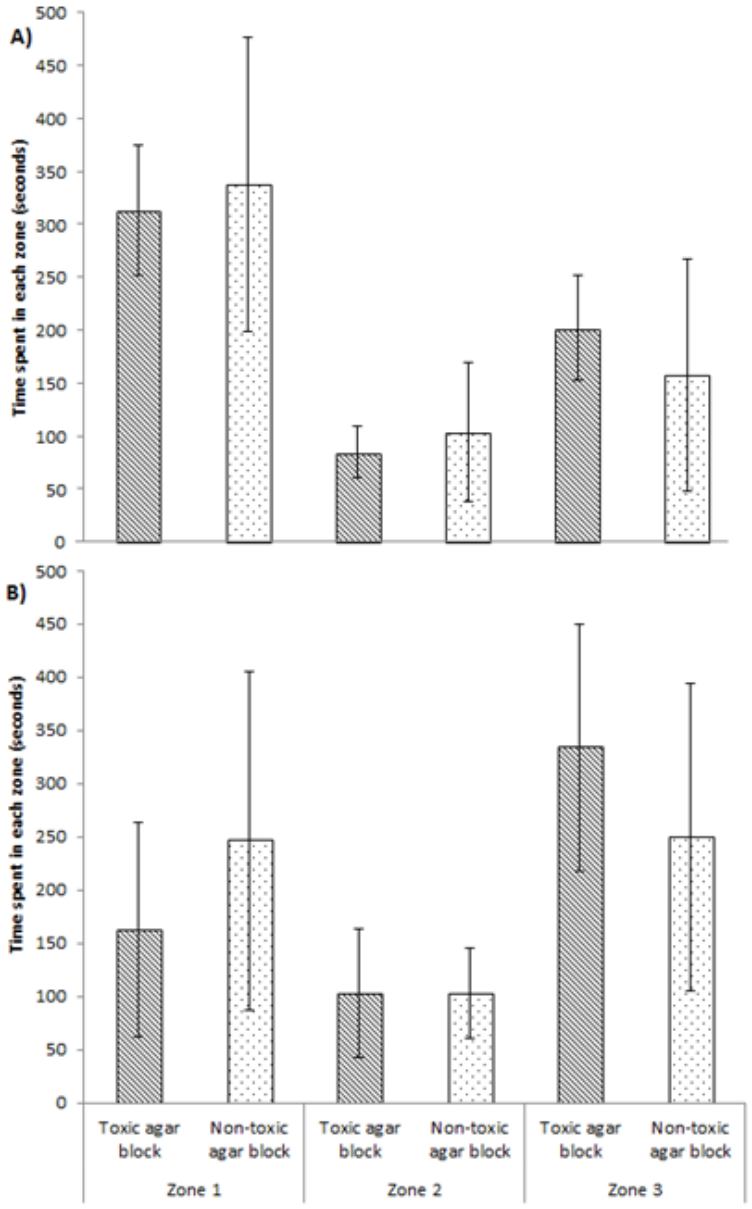

Figure 4-2. Average time spent by (A) toxic *Pleurobranchaea maculata* (n = 136) and (B) non-toxic *P. maculata* (n = 48) in each of the zones of the aquarium. Zone 1 is furthest from the agar block and Zone 3 is closest to the agar block. No choice was made in 5% (toxic *P. maculata*) and 0.7% (non-toxic *P. maculata*) of the experiments. Error bars show one standard deviation.

When non-toxic agar blocks were present, toxic *P. maculata* spent the majority of their time in Zone 1 (ave. 338 sec) followed by Zone 3 (ave. 158 sec) and Zone 2 (ave. 104 sec, Figure 4-2). Paired student *t*-tests showed that the average amount of time spent in Zone 1 was significantly greater than Zone 2 (P <

0.001) and Zone 3 (P = 0.001) but there was not a significant difference between time spent in Zone 2 and Zone 3 (P = 0.04). Paired student *t*-tests also showed that toxic *P. maculata* did not spend significantly more time in Zone 3 when there was toxic agar blocks compared to non-toxic agar blocks (P = 0.17) available. Toxic *P. maculata* ate toxic agar blocks in 18% and non-toxic agar blocks in 10% of the trials (P = 0.21).

When toxic agar blocks were present, the non-toxic *P. maculata* spent most of their time in Zone 3 (ave. 335 sec) followed by Zone 1 (ave. 162 sec) and Zone 2 (ave. 103 sec, Figure 4-2). Paired student *t*-test showed that there was not a significant difference between the average time spent in Zone 1 compared to Zone 2 (P = 0.2) or Zone 3 (P = 0.08). There was, however, a significant difference between the average time spent in Zone 2 compared to Zone 3 (P = 0.002).

When non-toxic agar blocks were present, non-toxic *P. maculata* spent approximately the same amount of time in Zone 1 (ave. 247 sec) and Zone 3 (ave. 250 sec) and spent the least amount of time in Zone 2 (ave. 103 sec, Figure 4-2). Paired student *t*-tests showed that there was a significant difference between the average amount of time spent in Zone 1 compared to Zone 2 (P = 0.01) and between Zone 2 compared to Zone 3 (P < 0.001). There was not a significant difference between the average amount of time spent in Zone 1 compared to Zone 3 (P = 0.9). Paired student *t*-tests also showed that non-toxic *P. maculata* did spend, on average, significantly more time in Zone 3 when there was toxic agar blocks compared to non-toxic agar blocks (P = 0.02). Non-toxic *P. maculata* ate toxic agar blocks in 79% and non-toxic agar blocks in 25% of the trials (P = 0.002).

4.4.2.2 Agar trails

Toxic *P. maculata* chose the toxic agar trail significantly more often than the non-toxic trail (30% vs. 9%; P < 0.001, Table 4-2). Non-toxic *P. maculata* did not choose the toxic trail significantly more often than the toxic trail (29% vs 25%, P = 0.8; Table 4-2). If an individual did not progress pass the first 12 cm of the trail to the fork and make a clear "choice", it was recorded as "no choice".

Toxic and non-toxic *P. maculata* did not make a choice in 61% and 46% of the experiment, respectively.

Table 4-2. Average number of times tetrodotoxin (TTX) versus non-TTX trails were followed by toxic (n = 204) and non-toxic (n = 24) *Pleurobranchaea maculata* and the average percentage of the trail followed before the *P. maculata* deviated off the trail.

	Ave. % of times chosen			**Ave. % of trail followed**	
	TTX	**Non TTX**	**No choice**	**TTX**	**Non-TTX**
Toxic *P. maculata*	30.4	8.8	60.8	57	51
Non-toxic *P. maculata*	29.2	25	45.8	47	59

4.4.2.3 Direct choice experiment

During the rounds where the toxic and non-toxic combination was presented, *P. maculata* chose the toxic agar blocks significantly more often (68% ($P < 0.001$) for toxic and 73% ($P = 0.002$) for non-toxic *P. maculata*; Figure 4-3). During all the rounds there was a difference between the number of times toxic *P. maculata* chose agar from the left compared to the right tweezer ($P = 0.05$). This was not observed for the non-toxic *P. maculata* ($P = 0.16$).

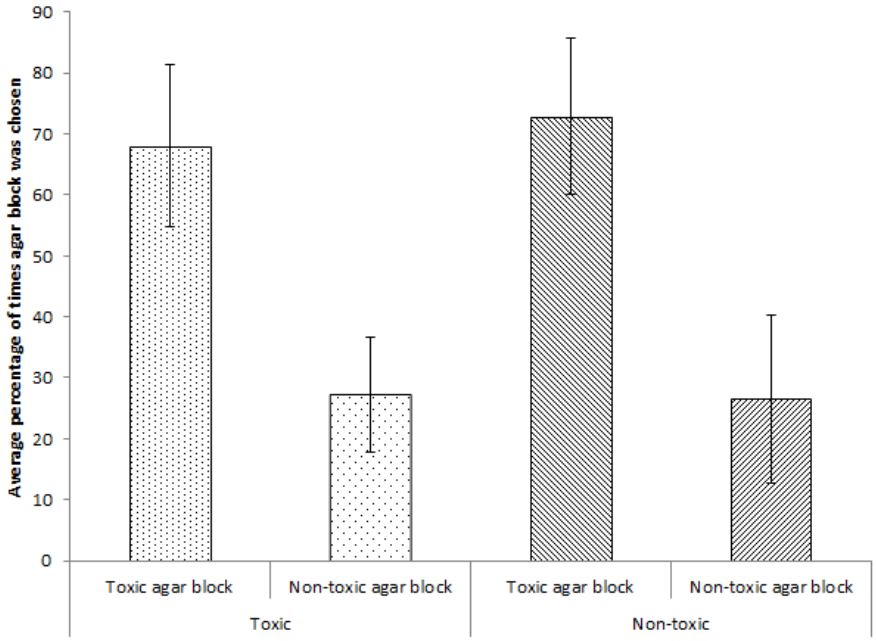

Figure 4-3. Average number of times the toxic versus non-toxic agar blocks were chosen by toxic (n = 272) and non-toxic (n = 147) *Pleurobranchaea maculata*. No choice was made in 5% (toxic *P. maculata*) and 0.7% (non-toxic *P.maculata*) of the experiments. Error bars show one standard deviation.

4.5 Discussion

The diffusion experiments demonstrated that TTX rapidly leached out of the agar blocks confirming that this was a suitable matrix to use for these experiments. It was essential to find a matrix from which TTX diffused to enable *P. maculata* to sense and locate the source of the TTX using chemosensory or other mechanisms. The use of agar also enabled the size of blocks and TTX concentrations within them to be standardized across experiments. Additionally, agar was also shown to be palatable for *P. maculata*, and in some experiments consumption was observed.

Food preference is dependent on many factors and typically does not remain constant over time or space (Emlen, 1966). Animals are generally less selective when food is scarce but become more selective if food is abundant (Emlen, 1966). To account for this in the present study, *P. maculata* were fed three days prior to the experiment and any uneaten food was removed from the aquarium. In the agar choice experiment there were very few cases (5%, toxic and

0.7%, non-toxic) when the *P. maculata* made no 'choice', indicating that they were suitably interested in seeking out food. This value was much higher (46-61%) in the agar trail experiment, but it is likely this was due to other reasons including the complexity of the set-up.

Animals must be able to differentiate food via sensory means otherwise no choice is possible (Forbes & Kyriazakis, 1995). This is particularly important for gastropods such as *P. maculata* that rely on odor release patterns to find the source (Voigt & Atema, 1997). Voigt and Atema (1997) suggest that the best methods for such studies should include 'first choice', 'time spent' or 'final preference'. In this study, the zonation experiment assessed 'time spent' while the agar trails and direct choice experiments investigated 'first choice' responses. The results of the three preference experiments were variable between the methods and among toxic and non-toxic *P. maculata* (Table 4-3).

Table 4-3. Summary of preference experiments showing where statistically significant preferences were observed. × = no preference. PT = preference observed for toxic agar blocks. No statistically significant preferences were observed for non-toxic agar blocks.

	Toxic *P. maculata*	Non-Toxic *P. maculata*
Zonation	×	×[#]
Agar Trails	PT	×
Direct choice	PT	PT

During the aquarium zonation experiment, the difference between zones 2 and 3 was significant, but between 1 and 3 it was not.

The zonation experiment relies on *P. maculata* being able to sense the source of the food offered via sensory means. This type of orientation behaviour is best studied in a flow-through tank to prevent the build-up of the stimulus. However, we used a single aquarium with unidirectional flow and it is possible that because TTX rapidly diffused from the agar that it was present in all zones of the experiment at similar concentrations. This may have confused the *P. maculata*

and hindered them finding the actual TTX source and may explain the confounding results of this experiment.

The results of the agar trail experiments were significant for the toxic *P. maculata*. It is plausible that *P. maculata* need to have direct contact with the TTX rather than just rely on sense. In a study on the gastropod *Navanax*, Paine (1963) observed that when encountering a mucus trail, it would deviate from its former course to follow it. The inner fold of the head shield was observed to remain in contact with the mucus trail. It was presumed that these were chemoreceptors and if one of them lost contact with the mucus trail, the movement of the *Navanax* was adjusted (Paine, 1963). The author concluded that prey is located by direct contact rather than through distance chemoreception. In the zonation experiment in this study, *P. maculata* would have had to rely on concentration gradients and distance chemoreception, however, the agar trails experiment would have allowed direct contact with TTX and this may explain why the results were significant for this experiment.

The differences in the results found among methodologies in our study may also be attributed to demand elasticity (Hursh, 1980). In behavioural economics, it is accepted that not all "commodities" are equally important to the consumer (Hursh, 1980). Under this scenario there are differences in the demand elasticity. If an organism continues to "work" hard despite an increased cost to obtaining the commodity, this is known as an "inelastic demand" but if the organism does not continue to "work" hard, this is known as an "elastic demand" (Hursh, 1980). In this study when *P. maculata* did not have to "work" for their food (i.e. in the direct choice experiment), there was a clear preference shown, however, when they did have to "work" for their food (i.e. the aquarium zonation experiment), their preference was weaker.

Mpitsos and Collins (1975) observed that *Pleurobranchaea* can exhibit strong associative learning. During the conditioning phase prior to the start of each experiment non-toxic individuals were hand fed from tweezers, whilst food was dropped into the tanks of toxic individuals where it was left for them to scavenge. This may explain the differences observed between toxic and non-toxic specimens in the agar trail experiments. This experiment relies on the individuals

seeking the food source. It is possible that the non-toxic individuals had become too acclimatised to being directly provided food.

In the direct choice experiment, the non-toxic *P. maculata* showed a preference for TTX. To the best of our knowledge, this study is the first to investigate TTX preference among toxic and non-toxic individuals within a species. Previous studies have only investigated preferences among different species. For example, Hwang et al. (2004) showed that non-toxic snail species only had a very limited attraction to TTX-containing food. They suggested that non-toxic snails lack TTX-binding proteins and therefore reject a TTX diet. By comparison, Khor et al. (2014) showed that non-toxic *P. maculata* can accumulate TTX indicating the presence of appropriate mechanisms to sequester this toxin (i.e. TTX-binding proteins or modified sodium channels).

If TTX confers ecological advantages for *P. maculata*, as suggested previously (Wood et al., 2012), and when given a simple choice of TTX or non-TTX containing food, they will preferentially select the TTX-containing food, then the question remains as to why large populations of non-toxic *P. maculata* exist. The most likely explanation is that the ultimate source of TTX is absent from these sites. Wood et al. (2012) suggested that latitudinal and current-induced gradients may result in differences in microbial diversity at *P. maculata* populations around New Zealand, although evidence to support this hypothesis is lacking. Additionally, extensive environmental surveys at sites with dense *P. maculata* populations have failed to identify organism(s) with TTX in them at concentrations that could account for the levels measured in *P. maculata* (Khor et al., 2014).

In conclusion, both toxic and non-toxic *P. maculata* showed a varying degree of preference for TTX among the three experiments. It seems most likely that this is due to the differences in the methodologies. We suggest that in the zonation experiment, the presence of the highly water soluble TTX in all zones of the experiment in the 'closed' system may have limited the ability of *P. maculata* to find the TTX source and thus confounded these results. During the agar trail experiment, the toxic *P. maculata* showed a statistically significant preference for the TTX-trails, whilst the non-toxic did not. This may be due to 'learnt' feeding

behaviours during the acclimation phase of the experiment. However, in both cases there was a high percentage of 'non-choices' suggesting that the experimental design was not optimal. In the simplest experiment, the direct choice experiment, which was also the one which required the least "work" from the test subjects, there was a statistically significant preference for the TTX-containing agar by both the toxic and non-toxic individuals. These data in concert with our previous studies (Wood et al. 2012; Khor et al. 2014) suggest that diet could be a source of TTX for *P. maculata*. Additionally, it is possible that *P. maculata* only seek out TTX at a certain life stage e.g., as juveniles or just prior to egg laying, and therefore response may vary and further studies tracking preferences across all life stages would be valuable. Given the high abundance of very toxic *P. maculata* at some sites (Wood et al. 2012), the inability to detect other organism with high TTX concentrations at these sites (Khor et al. 2014), and their short life span (<1 year, which only allows a short period for accumulation of TTX), we suggest that a dietary source is unlikely to be the only source of TTX for this species.

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Chapter 5 – Conclusions and Future Directions

The origin of TTX has been highly debated with endogenous and exogenous sources postulated (Honda et al., 2005; Lee et al., 2000; Lu & Yi, 2009; Noguchi & Arakawa, 2008). Additionally, within a species both toxic and non-toxic strains may exist.

In this Master's project, a successful non-lethal biopsy method was developed which will allow future studies with *P. maculata* to be undertaken where each individual can act as its own control and thus, will reduce variability and minimise the number of individuals required per study. It will also allow the tracking of TTX concentrations over time rather than only at specific time points. A key finding of this Masters project was that non-toxic *P. maculata* can accumulate TTX from their diet and that both toxic and non-toxic strains have a preference for TTX-containing food. The results of this thesis indicate that diet is a possible source in *P. maculata*. However, given the absence of identifiable TTX sources in environments where *P. maculata* are prevalent (Khor et al., 2014), in concert with their extremely high TTX concentrations and short life spans (Wood et al., 2012b), diet is unlikely to be the sole source of TTX for this species.

In terms of future directions, I think it would be interesting to expand on the current studies. For instance, previous work has demonstrated that toxic *P. maculata* depurate TTX when held in captivity (Wood et al., 2012a), however, it would be interesting to determine whether after feeding them with TTX-containing food, non-toxic *P. maculata* also depurate and how long this takes. The preference study could also be extended by undertaking further experiments where toxic and non-toxic individuals are held under similar conditions and with similar number of trials. In addition to this, the use of a flow-through aquarium for the aquarium zonation experiment would be an improvement on the current methodological design.

Comparisons of diet using new molecular tools such as Next Generation Sequencing would also be useful. These could be undertaken on toxic and non-toxic *P. maculata* populations from a variety of locations around New Zealand.

This may help identify commonalities in diet which may help guide future surveys.

In terms of the overarching question of where TTX originates from within *P. maculata*, it might be useful to radioactively label TTX in a food source and feed this to non-toxic *P. maculata*. If this was combined with a method that enabled all the TTX within the body of an individual to be visualised, it might be possible to determine how much TTX came from the food. If there is TTX present that did not come from food, this TTX would have been either produced by *P. maculata* and would potentially indicate that *P. maculata* requires the ingestion of TTX to "trigger" TTX production internally or through a bacterial symbiont.

Since the first isolation of TTX by Yokoo (1950) over 60 years ago, the origins of TTX have remained a mystery. Although the results in this thesis have shed light on a possible source of TTX in *P. maculata*, a definitive answer is still lacking. As technology progresses and more studies emerge on a range of species, the true source on TTX may eventually be revealed.

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Appendices

Appendix 1. Copy of first page of journal article published in Toxicon.

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Development of a non-lethal biopsy technique for estimating total tetrodotoxin concentrations in the grey side-gilled sea slug *Pleurobranchaea maculata*

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ABSTRACT

High concentrations of tetrodotoxin (TTX) have been detected in some New Zealand populations of *Pleurobranchaea maculata* (grey side-gilled sea slug). Within toxic populations there is significant variability in TTX concentrations among individuals, with up to 60-fold differences measured. This variability has led to challenges when conducting controlled laboratory experiments. The current method for assessing TTX concentrations within *P. maculata* is lethal, thus multiple individuals must be harvested at each sampling point to produce statistically meaningful data. In this study a method was developed for taking approximately 200 mg tissue biopsies using a TerumoEvolution® 18G × 11 cm Biopsy Needle inserted transversely into the foot. Correlation between the TTX concentrations in the biopsy sample and total TTX levels and in individual tissues were assessed. Six *P. maculata* were biopsied twice (nine days apart) and each individual was frozen immediately following the second sampling. Tetrodotoxin concentrations in biopsy samples and in the gonad, stomach, mantle and the remaining combined tissues and fluids were measured using liquid chromatography-mass spectrometry. Based on the proportional weight of the organs/tissues a total TTX concentration for each individual was calculated. There were strong correlations between biopsy TTX concentrations and the total ($r^2 = 0.88$), stomach ($r^2 = 0.92$) and gonad ($r^2 = 0.83$) TTX concentrations. This technique will enable more robust laboratory studies to be undertaken, thereby assisting in understanding TTX kinetics, ecological function and origin within *P. maculata*

1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin that functions by binding and obstructing voltage-gated sodium channels in nerve cell membranes, preventing the propagation of action potentials (Lu et al., 2011). Tetrodotoxin derives its name from the pufferfish family *Tetraodontidae* where it was first discovered, but it is now known to occur in a wide variety of phylogenetically distinct marine and terrestrial organisms (Ito et al., 2006; Hanifin, 2010; Williams, 2010). Despite many decades of research on TTX, its origins are still controversial with researchers either postulating an endogenous or exogenous source. Evidence for endogenous production comes from research on terrestrial organisms such as newts. Studies on captive newts (*Taricha granulosa*) found that TTX concentrations remained stable or increased over one year when fed TTX-free diets (Hanifin

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Article

Investigating Diet as the Source of Tetrodotoxin in *Pleurobranchaea maculata*

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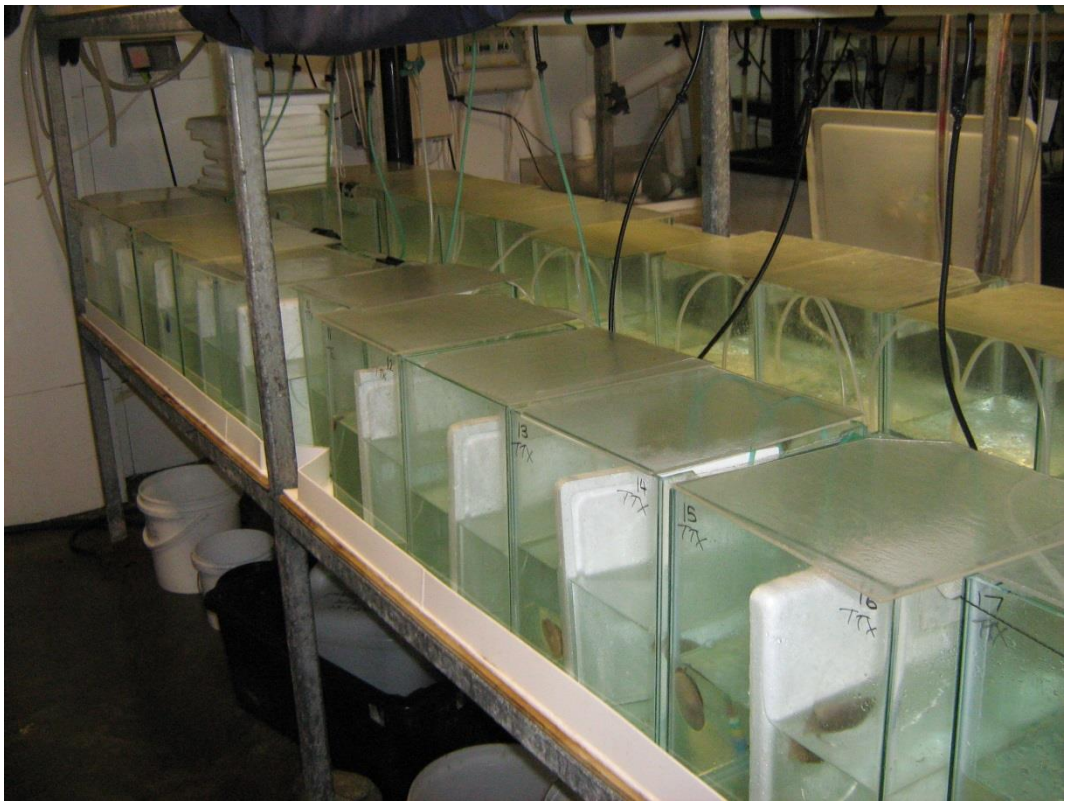
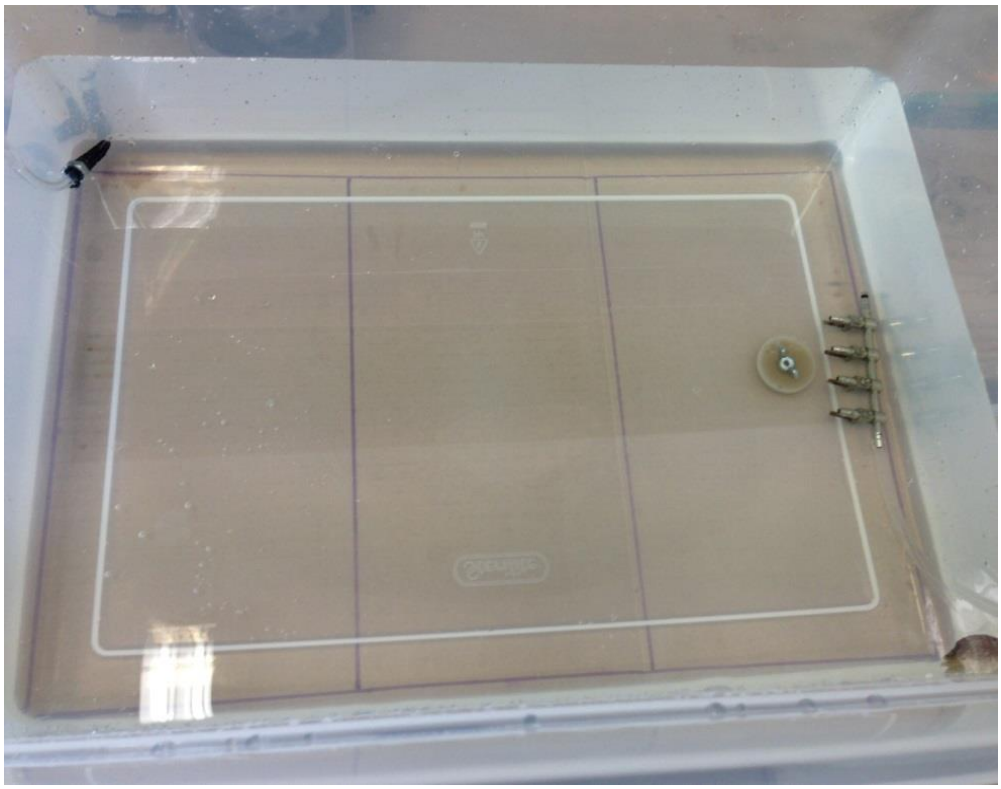
Abstract: The origin of tetrodotoxin (TTX) is highly debated; researchers have postulated either an endogenous or exogenous source with the host accumulating TTX symbiotically or via food chain transmission. The aim of this study was to determine whether the grey side-gilled sea slug (*Pleurobranchaea maculata*) could obtain TTX from a dietary source, and to attempt to identify this source through environmental surveys. Eighteen non-toxic *P. maculata* were maintained in aquariums and twelve were fed a TTX-containing diet. Three *P. maculata* were harvested after 1 h, 24 h, 17 days and 39 days and TTX concentrations in their stomach, gonad, mantle and remaining tissue/fluids determined using liquid chromatography-mass spectrometry. Tetrodotoxin was detected in all organs/tissue after 1 h with an average uptake of 32%. This decreased throughout the experiment (21%, 15% and 9%, respectively). Benthic surveys at sites with dense populations of toxic *P. maculata* detected very low or no TTX in other organisms. This study demonstrates that *P. maculata* can accumulate TTX through their diet. However, based on the absence of an identifiable TTX source in the environment, in concert with the extremely high TTX concentrations and short life spans of *P. maculata*, it is unlikely to be the sole TTX source for this species.

Appendix 3. Initial set-up of laboratory conditions for non-toxic *Pleurobranchaea maculata* during feeding experiment.



Appendix 4. Laboratory set-up for toxic *Pleurobranchaea maculata* during preference studies.

Appendix 5. Photograph of aquarium zonation study.



Appendix 6. Photograph of agar trails study.