ELUCIDATING THE ORIGIN AND TRANSMISSION OF TETRODOTOXIN IN NEW ZEALAND BIVALVES

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Abstract

Tetrodotoxin (TTX) is a potent neurotoxin that acts by selectively targeting sodium channels, blocking propagation of action potentials and causing paralysis. It has been responsible for countless human intoxications and deaths around the world, particularly in Japan, from consumption of pufferfish. The distribution of TTX and its analogues is remarkably diverse, and the toxin has been detected in organisms from marine, freshwater and terrestrial environments. Increasing detections of TTX in aquaculture species not typically associated with TTX, such as edible bivalves and gastropods, has drawn considerable attention to the toxin, reinvigorating scientific interest and regulatory concerns. There have been reports of TTX in 18 species of edible shellfish species from ten countries since the 1980's, with some reports above the safe threshold established by the European Food Safety Authority (EFSA) of 44 µg of TTX per kg of shellfish.

Despite decades of research, the exact origin of TTX remains a mystery. Current literature supports three hypotheses: endogenous production, symbiotic bacteria, or direct bioaccumulation through a dietary source. In 2009, the sea slug *Pleurobranchaea maculata* was found to contain high concentrations of TTX in New Zealand and an extensive research programme to explore the origin of TTX in *P. maculata* in New Zealand was launched. During this research, *Paphies australis*, an endemic clam, was found to accumulate high concentrations of TTX (800 µg kg^{-1}). The aim of this thesis was to elucidate the source of TTX in these clams that are a common food source and are culturally important in New Zealand. *Paphies australis* are largely sessile and found in subtidal habitats making them a highly amedable organisms to investigate the source of TTX.

In this thesis, I used a multiple line of evidence approach to investigate potential TTX producers. This included histological and analytical techniques to explore the micro-distribution of TTX within the organs of *P. australis*, aquaria studies to investigate the depuration and uptake of TTX, field studies to explore the variations in TTX concentrations from different *P. australis* populations and other bivalve species in New Zealand, and molecular analyses. Immunohistological analysis of

P. australis tissues employing a TTX monoclonal antibody demonstrated that TTX was present in the outer cells of the siphons, but also in their digestive system, foot and gill tissues. These results were also supported by chemical analysis using liquid chromatography with tandem mass spectrometry. Observing TTX in organs involved in feeding provided initial evidence to support the hypotheses of an exogenous source in *P. australis* and presence in siphon tips supports the hypothesis that TTX is used as a chemical defence against predators.

The TTX depuration rate in *P. australis* was then assessed by maintaining TTX-bearing individuals in captivity and feeding them a non-toxic diet for 150 days. The bivalves significantly depurated the toxin (0.4% of total toxin content per day) and there was a rapid decline of the toxin in their digestive glands with only traces amounts remaining after 21 days. This result provides evidence to support the hypothesis that *P. australis* do not endogenously produce TTX.

During field surveys, *P. australis* from 10 different sites around New Zealand were harvested and tested for TTX. There were significant differences in TTX concentrations from the different sites. All *P. australis* contained TTX but bivalves from the warmer North Island sites contained significantly higher concentrations than those from the South Island. These results provided further evidence to support the proposition that the source of TTX in *P. australis* is likely exogenous, varying in abundance by location. I hypothesised that the source is a warm-water-adapted TTX producer that is mostly present or more prevalent in warmer climates, or that TTX production is triggered by warmer temperatures.

To explore this possibility further, I used metabarcoding to investigate the bacteria present in the digestive glands and siphons of *P. australis* from the 10 sites with different TTX concentrations and collected monthly over a year from one site with high TTX concentrations. Marine cyanobacteria, in particular picocyanobacteria (e.g., *Cyanobium*, *Synechococcus*, *Pleurocapsa*, and *Prochlorococcus*), were found in all samples collected from sites containing the highest amount of TTX and were present in the core microbiome of TTX-bearing *P. australis*. Cyanobacteria are well known for producing a wide range of marine and freshwater toxins and this finding warrants further investigation.

Lastly, I assessed if wild *P. australis* could sequester and accumulate TTX from a dietary source. I developed a novel method to artificially feed a controlled amount of TTX to bivalves. The micro-encapsulation method incorporated humic acid so that the water-soluble TTX bound to a solid substance. The main finding from this study was that *P. australis* can rapidly accumulate of TTX from an external dietary source and concentrations were much higher (> 100 μg kg^{-1}) than the safe threshold in bivalves established by the EFSA (44 μg kg^{-1}) in under two weeks. This further strengthens the hypothesis that bivalves most likely accumulate TTX from their diet.

This thesis has addressed several critical knowledge gaps in our understanding of the source of TTX in bivalves, particularly in the clam *P. australis*. A detailed account of each study is presented in this thesis and future research to elucidate the origin of TTX in bivalves are suggested.

Acknowledgements

My PhD has been an incredible journey and there are many people that I would like to acknowledge and thank.

Firstly, I would like to thank my supervisors Dr's Susie Wood, Kirsty Smith and Professor Ian Hawes, I could never have dreamt of a better supervisory team. Susie and Kirsty, thank you so much for allowing me the opportunity to start my PhD while working with you and for trusting me, it means so much to know that I can always count on you. Ian, thank you so much for all the sound advice, for allowing me to do my PhD outside of the university and for always making sure 'reviewer #2' did not have much to complain about. Susie, Kirsty and Ian, your constant support, time (and the amazingly quick reviews!) and help has made completing this PhD a possibility. Thank you for all the encouragement, ideas, and critiques along the way, I would not have done it with anyone else by my side.

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with my studies, even though you still joke that I never seem to do any work. I loved our weekly skype chats when you asked me if the PhD was finished already. I miss you every day. To my sister Karine and my niece Iris, thanks for been so supportive even when it meant I had to be far away for a few years. I want to dedicate this thesis to my late Mamie Vonne who was my biggest inspiration and who always told me to follow my dream when I made the decision to move to New Zealand. I also want to say thank you to Janine, Tony, Helen and Rodney Bowater, Gail and David Collingwood for their support and for being a second family in New Zealand for me. You have all been so supportive and interested, even though I'm not sure you had any idea what I was doing. I am so glad to call New Zealand home.

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Statement of Authorship

This thesis has been written as a series of manuscripts, already published and there is, therefore, inevitable repetition between introductions and discussions of these chapters. This thesis is the intellectual and analytical work of the author and Prof. Ian Hawes, and Drs Susie Wood and Kirsty Smith provided advice and support throughout. The contributions of all co-authors and the publications for each chapter are outlined in the Appendices A1 to A5 and below:

Chapter 2 is a review compiling reports on the occurrence of TTX accumulation in edible bivalves and gastropods around the world. This thesis chapter was published in the journal *Chemosphere* in July 2019 and a modified version is presented, with some sections modified to avoid repetition with Chapters 1 and 6. This paper is presented in its published format in Appendix 6. The full reference for the original publication is as follows:
Biessy L, Boundy MJ, Smith KF, Harwood DT, Hawes I, Wood SA (2019) Tetrodotoxin in marine bivalves and edible gastropods: a mini-review. Chemosphere, 124404. https://doi.org/10.1016/j.chemosphere.2019.124404.

Chapter 3 was published in the journal *Toxins* in July 2018 and is presented in near identical form. The citation for the original publication is:
Biessy L, Smith, KF, Boundy MJ, Webb S, Hawes I, Wood SA (2018). Distribution of tetrodotoxin in the New Zealand clam, Paphies australis*, established using immunohistochemistry and liquid chromatography-tandem quadrupole mass spectrometry. Toxins: 10, 282. https://doi.org/10.3390/toxins10070282.*

Chapter 4 was published in *Toxicon:X* in February 2019 and is presented in near identical form. The full reference for this paper is:
Biessy L, Smith KF, Harwood DT, Boundy MJ, Hawes I, Wood SA (2019). Spatial variability and depuration of tetrodotoxin in the bivalve Paphies australis *from New Zealand. Toxicon: X: 100008. https://doi.org/10.1016/j.toxcx.2019.100008.*

Chapter 5 was published in the journal *Frontiers in Microbiology* in July 2020 and is presented in near identical form. The full reference for this publication is:
Biessy L, Pearman JK, Smith KF, Hawes I and Wood SA (2020). Seasonal and spatial variations in bacterial communities from tetrodotoxin-bearing and non-tetrodotoxin-bearing clams. Frontiers in Microbiology: 11, 1860.
https://doi.org/10.3389/fmicb.2020.01860.

Chapter 6 was published in the journal *Marine Drugs* in January 2021 and is presented in near identical form. The citation for the original publication is:
Biessy L, Smith KF, Wood SA, Tidy A, van Ginkel R, Bowater JRD, Hawes I (2021). A microencapsulation method for delivering tetrodotoxin in bivalves to investigate uptake and accumulation. Marine Drugs: 19, 33.
https://doi.org/10.3390/md19010033.

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CHAPTER 1

General introduction, overview and thesis structure

1.1 Tetrodotoxin

1.1.1 Overview

Tetrodotoxin (TTX) is a potent neurotoxin (Campbell, Barnes et al. 2013) named after the order of fish Tetraodontidae (Tetraodon pufferfish; Hwang and Noguchi 2007) and has long been known as the causative agent in pufferfish poisoning events (Kodama, Noguchi et al. 1983, Noguchi 2006). Records of pufferfish poisoning are documented in ancient literature particularly in Japan (from 200 AD) and China (at least 200 years ago; Miyazawa and Noguchi 2001). The poisoning was first described in European literature in 1774 by Captain James Cook who detailed the consumption of a local tropical fish from New Caledonia, now thought to be a species of pufferfish, and the resulting symptoms of weakness, numbness, and vomiting (Isbister, Son et al. 2002). TTX was first isolated and named in 1910 (Tahara 1910). It is a low-molecular weight (319.3 g mol^{-1}), non-protein molecule with a highly unusual structure (Miyazawa and Noguchi 2001). Its formula is $C_{11}H_{17}N_3O_8$, it is a zwitterion, with a positively charged guanidinium group, and a negatively charged hemiacetal alcohol group (Figure 1-1.A; Fuhrman 1967). It was isolated as a crystal in the 1950s (Yokoo 1950) and its structure was confirmed more than a decade later by several groups (Tsuda, Ikuma et al. 1964, Woodward 1964, Goto, Kishi et al. 1965). Although the structure of TTX has been confirmed, its biosynthetic pathway remains unknown. Several proposed biosynthetic pathways have been published but are yet to be verified (Kotaki and Shimizu 1993, Yotsu-Yamashita, Abe et al. 2013, Ueyama, Sugimoto et al. 2018).

Tetrodotoxin is one of the most toxic natural substances known with a median lethal dose of 1-2 mg for a 50 kg human (Noguchi and Ebesu 2001). The neurotoxin selectively binds and blocks voltage-gated sodium channels, hence inhibiting the propagation of action potentials in muscle and nerve cells often with fatal effects at extremely low doses. Following TTX ingestion, intoxication symptoms start

rapidly with numbness and nausea, and can be followed by vomiting, breathing difficulties, paralysis, and at high enough concentrations, death by respiratory failure (Noguchi and Ebesu 2001, Knutsen, Alexander et al. 2017). The symptoms of intoxication by TTX are the same as those observed with saxitoxin (STX; Figure 1-1.B) which can also found in organisms containing TTX (Bane, Lehane et al. 2014). Although the chemical structures of TTX and STX are considerably different (Figure 1-1), both toxins exert their effects through an interaction with voltage-gated sodium channels resulting in inhibition of neuromuscular transmission (Narahashi 1988). Both toxins are active on the α-subunit of the sodium channels although there are some differences in the affinities of TTX and STX for different sodium channel isoforms (Walker, Novick et al. 2012).

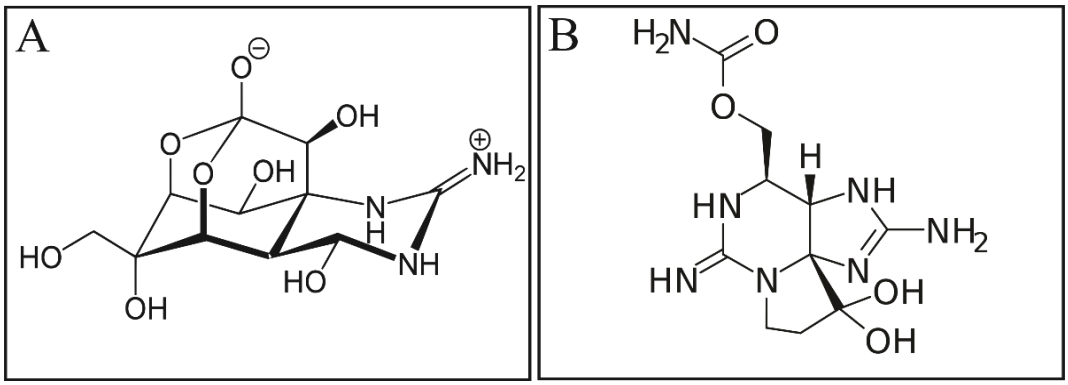


Figure 1-1. The structure of (A) tetrodotoxin, and (B) saxitoxin.

There is no known antidote for TTX (Soong and Venkatesh 2006) and it has been responsible for numerous human fatalities, partly because the molecule is tasteless and heat-stable (Turner, Powell et al. 2015). Most documented poisoning cases have occurred in countries where pufferfish is regarded as a delicacy. Toda (2012) reported 651 incidents of TTX poisoning including 56 fatalities in Japan due to pufferfish ingestion between 1989 and 2010. In 2008 in Bangladesh, three poisoning events resulted in 17 deaths (Islam, Razzak et al. 2011) and Azanza et al. (2019) reported 93 cases of TTX poisoning with 21.6% leading to fatalities between 2005 and 2018 in the Philippines. In the last decade, TTX has been newly reported in a range of organisms from temperate environments, particularly in marine shellfish (Silva, Azevedo et al. 2012, Lago, Rodríguez et al. 2015, Turner, Powell et al. 2015, Turner, Higgins et al. 2015). Tetrodotoxin concentrations in shellfish are considerably lower than in pufferfish species (approximately 1,000 times lower).

Despite the amount of data available being limited to date, the risk to humans cannot however be ignored due to: 1) the high amounts of shellfish consumed worldwide, and 2) the variability of TTX concentrations measured in shellfish.

1.1.2 Tetrodotoxin methods of detection

The mouse bioassay was the first method used for the detection of TTX in seafood. This technique involves aliquots of sample extract being injected into mice and the median death times used to calculate the toxicity (in mouse units; MU; Hungerford 2006). It has been used for decades but aside from the ethical concerns, the MBA is not specific to TTX and positive results could also be caused by the presence of STX-group toxins. Both classes of neurotoxin exhibit the same symptomology in mice and can co-occur in the marine environment (Dell'Aversano et al. 2019, Jen, Lin et al. 2014), therefore some historical intoxications may have been incorrectly assigned to TTX or STX.

Another method used is the mouse neuro-2A (N2a) neuroblastoma cells assay (Kogure, Tamplin et al. 1988). In the presence of ouabain, there is an increase in sodium influx in the N2a cells, causing cellular swelling and death. When TTX is present, the sodium channels of the cells are blocked, enabling cell growth to continue. The N2a assay is a slow process with weeks required to culture the cells and cannot differentiate TTX from STX or other compounds inhibiting sodium influx, but the assay does not require multiple expensive reference standards and is very sensitive. This technique has been reported to be used for monitoring below the European Food Safety Authority (EFSA) limit and is able to detect the toxin concentrations around 20 µg kg^{-1} (Gerssen, Bovee et al. 2018). Immunological assays also exist for the detection of TTX, with enzyme-linked immunosorbent assays (ELISA), using an alkaline phosphatase-labelled monoclonal antibody being the most common (Raybould, Bignami et al. 1992). ELISA kits for TTX are now commercially available although variability of performance and availability could hinder monitoring programmes relying on the assay for regular high-throughput testing.

Early chemical detection methods for TTX were based on chemical conversion with alkali treatment of TTX and its analogues to fluorescent 2-amino-quinazoline

derivatives and were able to be analysed in a fluorescence spectrophotometer (Nunez, Fischer et al. 1976). This technique was utilised and further developed on a high-performance liquid chromatography (HPLC) system, by first separating the TTX analogues using ion exchange or ion pairing chromatography then performing the derivatisation to this fluorescent C_9-base post-chromatographic separation continuously in line to the detector (Yasumoto, Nakamura et al. 1982, Yasumoto and Michishita 1985). The analysis of TTX can also be undertaken by gas chromatography-mass spectrometry (Suenaga and Kotoku 1980) using a similar derivatisation procedure to the HPLC but analysis requires an additional derivatisation process in order to get a GC-suitable product for analysis. The analysis of the C_9-base is, however, not specific to TTX, and therefore toxicity may be overestimated as less toxic analogues of TTX can be converted to the C_9-base and lead to false positives (Matsumura 1995). To mitigate the issues of specificity, additional techniques such as thin layer chromatography, electrophoresis and nuclear magnetic resonance have been used to confirm the presence of TTX (Noguchi, Maruyama et al. 1981).

Liquid chromatography-mass spectrometry (LC-MS) is now a well-established tool for the analysis of many different classes of marine toxins and has been used for over a decade for regulatory monitoring of shellfish for human consumption (McNabb, Selwood et al. 2005, Stobo, Lacaze et al. 2005). This technique has been demonstrated to be very robust for the analysis of TTX and its analogues (Leung, Fong et al. 2011, Rodríguez, Alfonso et al. 2012, McNabb, Taylor et al. 2014, Boundy, Selwood et al. 2015). Tetrodotoxin and its analogues can be monitored directly with an excellent level of specificity, or alternatively, may be derivatised and monitored as the C_9-base. The EFSA recommended that liquid chromatography with tandem mass spectroscopy (LC–MS/MS) methods were the most suitable for identification and quantification of TTX and its analogues (Knutsen, Alexander et al. 2017).

1.1.3 Tetrodotoxin in New Zealand

Tetrodotoxin was historically detected only in tropical regions, but has been reported in sub-tropical and temperate regions in recent years, including Europe (Turner, Powell et al. 2015) and New Zealand (McNabb, Selwood et al. 2010). Tetrodotoxin was first detected in New Zealand in 2009 when dogs in Auckland became unwell after eating native grey side-gilled sea slugs, *Pleurobranchaea maculata,* that had washed up on the beach (McNabb, Selwood et al. 2010). Dogs had symptoms similar to neurotoxin poisoning and an investigation was launched to find the source of these poisonings. The investigation resulted in the identification of high concentrations TTX in *P. maculata* individuals (up to 360 mg kg^{-1}; McNabb, Selwood et al. 2010). This raised concerns about the potential presence of TTX in seafood from regions were *P. maculata* occurred and a research project was initiated with a total of 383 samples (from 53 species) analysed over 14 months (Ogilvie, Taylor et al. 2012). The neurotoxin was present in five non-seafood species: at low levels in *Arachnoides zelandiae* (echinoderm; 0.25 mg kg^{-1}), *Astrostole scabra* (echinoderm; 0.17 mg kg^{-1}), and *Turbo smaragdus* (mollusc; 0.11 mg kg^{-1}), and at trace levels in the crab *Macrophthalmus hirtipes* and the macroalga *Corallina officinalis*. Tetrodotoxin was also found at low levels in two seafood species: the New Zealand rock oyster *Saccostrea glomerata* (0.14 mg kg^{-1}*)* and the Pacific oyster *Crassostrea gigas* (0.08 mg kg^{-1}), a significant finding because TTX was rarely found in shellfish at that time (Ogilvie, Taylor et al. 2012). While sampling in Tauranga (North Island, New Zealand), the flatworm *Stylochoplana* sp. was observed in high abundance in the same shallow subtidal area as *P. maculata*. The flatworms were found to contain high concentrations of TTX (38 -115 mg kg^{-1}; Salvitti, Wood et al. 2015).

Since 2012, TTX has been actively monitored in New Zealand, especially in seafood, and low concentrations have been found in other species. Tetrodotoxin was first reported at a significant concentration in the bivalve *Paphies australis* (0.8 mg kg^{-1}; McNabb, Taylor et al. 2014) and TTX was including in New Zealand's non-commercial shellfish marine biotoxin monitoring programme (Boundy and Harwood 2017). Between 2015-2016, 697 samples were analysed and 61.1% contained traces of TTX and 27% of samples had detectable TTX but at levels below the recommended safe guidance level (0.002–0.044 mg/kg). The highest

sample was a single *Perna canaliculus* (green-lipped mussel) with a concentration of 1.6 mg kg^{-1} collected from the Hauraki Gulf in Northland. During that monitoring period, *P. australis* from the Hokianga Harbour (Northland) were found to contain TTX over the entire year at higher concentrations and more frequently than the other sites across New Zealand (average of 0.15 mg kg^{-1}; Boundy, Biessy et al. 2020), suggesting that the Hokianga Harbour could be a TTX hot-spot and a central study site for this thesis.

1.1.4 *Paphies australis*

The study species of this thesis is *Paphies australis* (Gmelin 1791), a marine bivalve belonging to the Mesodesmatidae family. They are known as pipi in te reo Māori and are burrowing clams endemic to New Zealand. The term 'clam' is a common name applied to some bivalves having commercial significance (Hooker 1995). Most clam species belong to the Heterodonta subclass and have been described as having two equally-sized smooth valves joined by adductor muscles (Powell 1979). *Paphies australis* have two white, symmetrical shells (average size: 80 mm in length, 50 mm in width; Figure 1-2A) and are abundant in coastal regions throughout New Zealand and its outlying islands (Powell 1979). They are restricted to flat sandy beaches and in muddy estuaries and harbours with considerable water flow, where they form beds in the top few centimetres of sediments at mid-tide or below the tide level (Morton and Miller 1973). They are found in high densities, sometimes up to 1,000 per m^2 (Ministry of Fisheries, New Zealand), and use their foot to orient themselves in the substratum (Figure 1-2B; Forrest, Keeley et al. 2009). Although they are usually sedentary, they can produce a mucus thread which enables them to float in the water column and move using coastal currents (Hooker 1995). *Paphies australis* are filter feeders, consuming phytoplankton and other particulate material from the water column through the inhalant siphon (Figure 1-2B). The gills trap particles in mucus, which is then transported to the mouth, then sorted and either consumed or rejected (i.e., expelled from the bivalve; Mamat 2010). The siphons are the only organ protruding from the sand in the water column and available to predators under normal conditions (Figure 1-2B).

Paphies australis are edible, easily collected from the intertidal zone and have long been a valuable species for food and bait (Hooker 1995). For Māori, *P. australis*

are a traditional food resource, and were gathered in specific flax baskets that would let the smaller specimens fall back into the beds. They are an important recreational and customary harvest species with a small commercial fishery of around 120 tons annually (Haddon 1989).

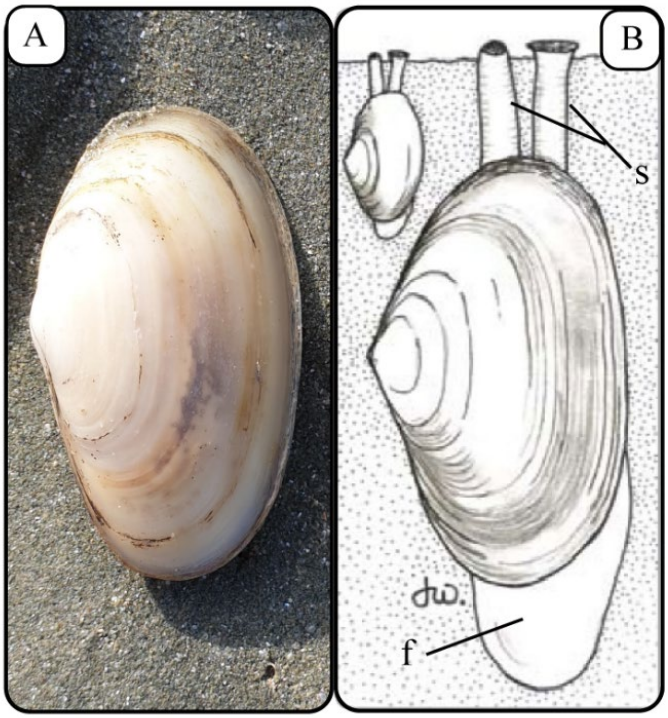


Figure 1-2. *Paphies australis* A) collected from Delaware Bay (Nelson, New Zealand), and B) a schematic showing the pair of siphons (s) and foot (f) from Morton and Miller (1973).

1.2 Aims, objectives and thesis structure

Paphies australis have been shown to accumulate high concentrations of TTX, are commonly harvested for human consumption, are prevalent across New Zealand and are mostly sedentary, making them an ideal study organism to investigate the source of TTX in bivalves. The overarching aim of this project was to elucidate the source of TTX in *P. australis* by combining molecular techniques, such as high-throughput sequencing, immunohistochemistry and chemical detection methods with environmental surveys and manipulative experiments. This thesis includes a literature review and five data chapters covering the following objectives:

1. To identify where TTX is located within tissues of *P. australis*.
2. To establish the TTX concentration variations in *P. australis* populations across New Zealand.
3. To determine if TTX-bearing *P. australis* depurate the toxin when kept in captivity and whether the toxin migrates among organs or tissues over time.
4. To determine if there is any relationship between bacterial communities and TTX concentrations in *P. australis* organs and identify organism/s which could be responsible for TTX production in *P. australis*.
5. To assess whether *P. australis* accumulate TTX as part of their diet when fed the toxin.

Chapter 1 introduces the topic and the main aims of this thesis. **Chapter 2** is a published literature review and compiles reports on the occurrence of TTX accumulation in edible bivalves and gastropods around the world, reviews the knowledge on the possible sources of TTX in shellfish, and evaluates the risk it poses to humans and assesses whether it should be regulated in shellfish. Key questions identified in Chapter 2 were addressed in Chapters 3, 4, 5 and 6. **Chapter 3** describes the micro-distribution of TTX in the tissues of *P. australis* and the potential ecological roles of the toxin. **Chapter 4** investigates the spatial variability of tetrodotoxin in populations of *P. australis* across New Zealand and the depuration rate of the toxin in the bivalves when maintained in a TTX-free environment. In **Chapter 5**, microbial communities between sites with the lowest and highest TTX concentrations (determined in Chapter 4) were investigated using metabarcoding. The core microbiome from TTX-bearing individuals was also

analysed over a one-year period. In **Chapter 6**, non-TTX-containing *P. australis* were fed a controlled amount of TTX. To do so, I developed a microencapsulation method were TTX was bound to a solid and encapsulated in agar-gelatine before being fed over-time to *P. australis*. **Chapter 7** is the general discussion, which summarise the results from the data chapters, identify limitations of this study, and discusses avenues for future research.

CHAPTER 2

Literature review

2.1 Introduction

Mollusca is one of the largest phyla in the animal kingdom with more than 100,000 existing species living in marine, freshwater and terrestrial habitats (Barnes 1980). It is subdivided into seven classes with Bivalvia (more than 20,000 living species described) and Gastropoda constituting 95% of known mollusc species (Kantha 1989). Bivalvia are a highly successful class of invertebrates found in aquatic habitats worldwide (Dame 2011) and are an important food source for humans globally. The Food and Agriculture Organization estimated that 15 million tonnes of bivalves are traded each year (FAO 2012), representing around 14% of the total marine aquaculture production in the world (Wijsman, Troost et al. 2019). Bivalves are filter feeders, passing large quantities of water through their gills to capture particulate food such as phytoplankton (Arapov, Ezgeta–Balić et al. 2010). The consumption of raw or insufficiently cooked shellfish can be associated with infectious diseases (Rippey 1994). These are caused either by bacteria naturally present in the sea such as *Vibrio* spp. or by human pathogenic viruses and/or bacteria sourced from contaminated waters (Huss 1997). In addition, organic and inorganic particles retained by bivalves can readily accumulate substances such as heavy metals (Landsberg 2002, Deeds, Landsberg et al. 2008). A third possible risk for human consumers occurs when bivalves accumulate marine biotoxins as a result of ingesting toxic microalgae. For example, dinoflagellates from the genus *Alexandrium* (MacKenzie 2014) can produce saxitoxin (STX) group toxins and, when concentrated in filter-feeding bivalves, these toxins are responsible for paralytic shellfish poisoning (PSP) events. Human and animal intoxications resulting from the ingestion of seafood contaminated with toxic microalgae are recorded worldwide every year (Hallegraeff, Anderson et al. 2003).

Gastropoda is the most diverse Molluscan class with over 75,000 existing species (30,000 are marine) and include both snail and slug species. They are highly abundant in marine ecosystems, playing important ecological roles as grazers,

predators and major food sources for higher trophic levels (Holan, King et al. 2017). Marine gastropods are also an important source of animal protein to humans, especially in Asia (Hamli, Idris et al. 2013). Similar to bivalves, they are very susceptible to environmental contaminants and have been shown to accumulate metals (e.g., copper; Grosell, Blanchard et al. 2007) and marine biotoxins (e.g., ciguatoxins and STXs; Hwang, Lin et al. 1992, Hwang, Tsai et al. 2007, Luo, Yu et al. 2012).

A marine biotoxin of increasing concern worldwide is tetrodotoxin (TTX). Tetrodotoxin is a potent toxin and its poisoning is described as one of the most violent forms of marine toxin intoxication (Bagnis, Berglund et al. 1970). This toxin used to be reported as a threat only in Asian countries, mostly from pufferfish (Lago, Rodríguez et al. 2015), but has now been reported in seafood in the Pacific and Mediterranean (Biessy, Smith et al. 2019, Katikou 2019). Although there is increasing evidence that TTX is produced by microorganisms such as bacteria or marine microalgae (Chau, Kalaitzis et al. 2011), there is still considerable uncertainty regarding its source and biosynthetic pathway. The evidence regarding the production of TTX by microalgae is limited to one study that suggested the dinoflagellate *Alexandrium tamarense* produced this toxin (Kodama, Sato et al. 1996). However, the experimental conditions of this study are questionable as TTX was isolated from a large culture extract by Bio-Gel P-2 column, which could also be used for isolation of other materials. In recent years, there has been an increase in reports of TTX in marine organisms, especially in commonly eaten bivalves like blue mussels (*Mytilus* spp.), but concentrations are not routinely monitored (Knutsen, Alexander et al. 2017). Additionally, it has recently been demonstrated that TTX and STX toxicities are additive (Finch, Boundy et al. 2018), making it appropriate to calculate TTX analogues as STX equivalents to determine a combined total toxicity of these toxin groups during monitoring. Most studies investigating TTX in edible shellfish report on the presence and concentrations of TTX in specific species, but very few explore toxin variability, location within organisms, or possible sources.

The aims of this review are: (1) to compile reports on the occurrence of TTX accumulation in edible bivalves and gastropods, and (2) evaluate the risk that TTX

in edible shellfish poses to humans and assess whether it should be regulated in shellfish.

2.1.1 Tetrodotoxin in edible shellfish

In this review, to enable a comparison between studies and to keep all units consistent, mouse unit (MU, the amount of toxin required to kill a 20 g female mouse in 30 minutes via intraperitoneal injection; Yu, Yu et al. 2004)) was converted to μg kg^{-1}; the unit currently most commonly used to report biotoxins concentrations. The MU conversion calculation used in this review is approximate as some of the manuscripts did not specify the calibrated conversion factor in their study. In these cases, a conservative estimate was used: 1 MU = 0.2 μg TTX, unless otherwise specified in the paper (for example, Luo et al. (2012) reported 1 MU = 0.220 μg TTX and Jen et al. (2007) reported 1 MU = 0.178 μg TTX). Some manuscripts only reported the amount of toxin in whole organisms (i.e., μg/organism) rather than a concentration (i.e., μg kg^{-1}) which makes it difficult to compare the toxicity across different studies.

2.1.1.1 *Tetrodotoxin in marine bivalves*

Tetrodotoxin has been identified in ten bivalve species from seven countries (Table 2-1; Figure 2-1). The first report of TTX in marine bivalves was in Japan in 1993 when the toxin was found at high concentrations in the digestive gland of the scallop *Patinopecten yessoensis* following a bloom of the dinoflagellate *Alexandrium tamarense* (Kodama, Sato et al. 1993). The digestive glands of these scallops reached 8,000 μg kg^{-1}, a concentration 10 times higher than concentrations found in other bivalve species. Concentrations as high as those have not been reported since, questioning the method of detection used in this study. Two decades later, TTX was detected in the endemic clam *Paphies australis* from New Zealand (max. 800 μg kg^{-1}; McNabb, Taylor et al. 2014). The detection of TTX in edible bivalves like *P. australis* led to concerns about health risks for human consumers and prompted further research globally. Tetrodotoxin has now been detected in mussel species from five countries (at the date of publication): England (*Mytilus edulis*), the Netherlands (*M. edulis*), China (*M. edulis* and *M. coruscus*), New Zealand *(Perna canaliculus)*, and Greece where the highest concentrations were detected

(up to 203 µg kg^{-1} in *M. edulis*; Table 2-1). The neurotoxin has also been detected in oysters from Europe, New Zealand, and Asia with maximum concentrations of 140 µg kg^{-1} in the New Zealand rock oysters *Saccostrea commercialis*. Four species of clams have also been shown to contain TTX in five countries with the highest concentrations measured in the clams *Venus verrucosa* in Greece (176 µg kg^{-1}; Table 2-1).

Table 2-1. Reports of tetrodotoxin in marine bivalves to date DG = digestive gland; WF = whole flesh; HPLC = High performance liquid chromatography; MBA = Mouse Bioassay; LC-MS/MS = LC tandem mass spectrometry; LC-HRMS = Liquid chromatography high resolution mass spectrometry; N2a = mouse neuro-2A neuroblastoma cell assay; TLC = Thin layer chromatography; * Recalculated from MU/g assuming 1MU = 0.2 µg TTX.

Sampling dates	Country detected	Species	Maximum TTX concentration (µg kg^{-1})	Organs analyzed	Detection methods	References
1993	Japan	*Patinopecten yessoensis*	8,000*	DG	HPLC, MBA, TLC	Kodama et al. (1993)
2011	New Zealand	*Saccostrea commercialis*	140	WF	LC-MS/MS	Ogilvie et al. (2012)
2011	New Zealand	*Crassostrea gigas*	80	WF	LC-MS/MS	Ogilvie et al. (2012)
2012	Greece	*Venus verrucosa*	176.5	DG	MBA, LC-MS/MS	Vlamis et al. (2015)
2012	Greece	*Mytilus edulis*	179.1 202.9	WF DG	MBA, LC-MS/MS	Vlamis et al. (2015)
2013-2014	China	*Ruditapes philippinarum* *Sinonovacula constricta*	2.2 16	WF WF	LC-MS/MS	Han et al. (2018)
2013-2014	China	*Mytilus edulis* *Mytilus coruscus*	2.7 4.4	WF WF	LC-MS/MS	Han et al. (2018)
2014	New Zealand	*Paphies australis*	800	WF	LC-MS/MS	McNabb et al. (2014)
2013-2015	England	*Mytilus edulis*	120	WF	LC-MS/MS	Turner et al. (2015)
2013-2015	England	*Crassostrea gigas*	5	WF	LCMS/MS	Turner et al. (2015)
2015-2017	Netherlands	*Mytilus edulis*	33.3	WF	LC-MS/MS, N2a	Knutsen et al. (2017)
2015-2017	Netherlands	*Ostrea edulis*	124.1	DG	LC-MS/MS, N2a	Knutsen et al. (2017)
2015-2017	Italy	*Mytilus edulis*	64	WF	LC-HRMS	Dell'Aversano et al. (2019)
2017	New Zealand	*Perna canaliculus*	160	WF	LC-MS/MS	Boundy and Harwood (2017)

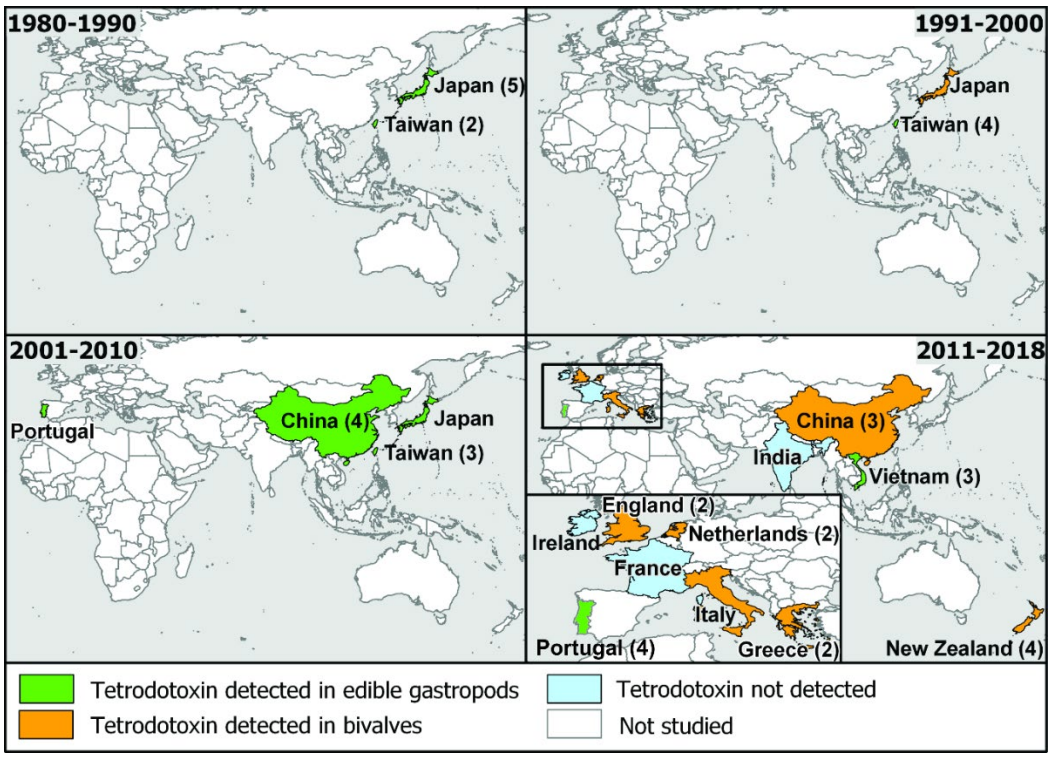


Figure 2-1. Global detection of tetrodotoxin in edible gastropods and bivalves in the last four decades. When more than one species has been found to contain TTX in the same country, the number of species is given in brackets.

2.1.1.2 *Tetrodotoxin in edible gastropods*

Food poisoning cases caused by gastropod consumption have severe consequences to human health and the toxin responsible in most poisoning cases is TTX (Hwang, Tsai et al. 2007). Tetrodotoxin has been reported in eleven marine gastropod genera from five countries, with the first detection occurring in 1980 (Table 2-2, Figure 2-1; Shiomi, Tanaka et al. 1984).

Tetrodotoxin is generally found at the highest concentrations in the digestive glands (Table 2-2), possibly suggesting that accumulation is via dietary sources. For example, the trumpet shell *Charonia lampas* (previously *C. sauliae*) was reported to accumulate TTX from starfish, a TTX-bearing food source of the gastropod (Noguchi, Maruyama et al. 1982). Rodriguez et al. (2008) found very high TTX concentrations (315,000 µg kg^{-1}) in the digestive glands of the predatory sea snail *C. lampas lampas* harvested from the south coast of Portugal after reports of food poisoning. However, in some species, TTX was exclusively detected in the flesh

and not in the digestive gland which may indicate a different binding mechanism or alternative source in those species (Table 2-2).

Human poisonings from gastropods mostly occur in Asian countries such as Taiwan, Japan and China, where the highly nutritious molluscs are a popular food (Hwang, Tsai et al. 2007). TTX-poisoning from gastropod ingestions has occurred in every decade since the 1980s (Table 2-2), and from 1994 to 2006, nine food poisoning incidents occurred in Taiwan and three people died, mostly from eating gastropods of the Nassariidae (mud snails) family. Taiwanese studies have found nine species from this family contain TTX in their tissues and eight of these species have been associated with poisoning incidents (Hwang, Tsai et al. 2007). There is currently no regulation or monitoring of TTX in edible marine gastropods.

Table 2-2. Reports of tetrodotoxin (TTX) in edible marine gastropods. † Reports linked to human intoxications; * Recalculated from MU/g assuming 1 MU = 0.2 µg TTX unless otherwise specified in the paper; # Average concentrations of tetrodotoxin reported in each paper ; DG = digestive gland, WF = whole flesh; MBA = Mouse bioassay; GC-MS = Gas chromatography-mass spectrometry; HPLC = High performance liquid chromatography; LC-MS/MS = Liquid chromatography tandem mass spectrometry; NMR = Nuclear magnetic resonance; TLC = Thin layer chromatography.

Sampling dates	Country detected	Species	Maximum TTX concentration (µg kg^{-1})	Organs analyzed	Detection methods	References
1980	Japan	*Babylonia japonica*	11,000*	DG	MBA, GC-MS, TLC	Noguchi et al. (1981)
1981	Japan	*Nassarius siquijorensis*	680*	Edible parts	MBA, GC-MS, TLC	Narita et al. (1984)
1979-1980 †	Japan	*Charonia lampas*	390,000*	DG	MBA, GC-MS, TLC, NMR	Noguchi et al. (1982)
1984	Japan	*Tutufa lissostoma*	140,000*	DG	MBA, GC-MS, TLC, NMR	Noguchi et al. (1984)
1988-1989	Taiwan	*Rapana rapiformis* *Rapana venosa venosa*	28,000* 2,600*	DG DG	MBA, HPLC, TLC	Hwang et al. (1991)
1988	Taiwan	*Natica lineata*	144,000* 2,400* 5,600*	Muscle DG Other parts	MBA, HPLC, TLC	Hwang et al. (1990)
1988-1989	Taiwan	*Natica vitellus* *Polinices didyma* *Polinices tumidus*	4,500*# 1,300*# 14,000*# 3,600*# 800*	Muscle DG Muscle DG DG	MBA, HPLC	Hwang et al. (1991)
1989-1990	Taiwan	*Nassarius conoidalis*	220,000*# 45,000*#	DG Other parts	MBA, HPLC	Hwang et al. (1992)
1989-1990	Taiwan	*Nassarius scalaris* *Zeuxis castus-like*	28,000 µg/specimen*	WF	MBA, HPLC, TLC	Hwang et al. (1992)

			2,600 µg/specimen*	WF		
2002	China	*Nassaris sinarum*	37,000 µg/specimen*	WF	MBA, HPLC, TLC	Sui et al. (2002)
2002 †	Taiwan	*Oliva miniacea* *Oliva mustelina* *Oliva nirasei*	3,600* 3,200* 5,600*	Edible parts (no DG) Edible parts (no DG) Edible parts (no DG)	MBA, HPLC, GC-MS, LC-MS	Hwang et al. (2003)
2004 †	China	*Nassarius glans*	2,100,000* 400,000* 550,000*	WF DG Muscle	MBA, GC-MS	Yin et al. (2005)
2005 †	Taiwan	*Nassarius papillosus*	110,000* 96,000*	Muscle DG	MBA, HPLC, LC-MS/MS	Jen et al. (2007)
2007-2008	Japan	*Nassarius glans*	2,000,000* 470,000*	DG Muscle	MBA, LC-MS	Taniyama et al. (2009)
2007	China	*Nassarius sinarum* *Nassarius variciferus*	190,000* 3,800*	WF WF	MBA, LC-MS	Luo et al. (2012)
2007 †	Portugal	*Charonia lampas lampas*	315,000	DG	MBA, LC-MS	Rodriguez et al. (2008)
2007	China	*Nassarius nitidus*	1,350	Edible parts	MBA, LC-MS	Huang et al. (2008)
2010	Portugal	*Charonia lampas lampas*	66.6 22.4	Muscle DG	LC-MS/MS	Nzoughet et al. (2013)
2011	Portugal	*Phorcus lineatus* *Charonia lampas* *Nucella lapillus* *Gibbula umbilicalis*	< 5.46 < 5.46 < 5.46 < 5.46	WF WF WF WF	HPLC	(Silva, Rodríguez et al. 2019)
2014	Vietnam	*Oliva hirasei* *Oliva ornata* *Oliva annulata*	4,800* (~5-6% TTX) 4,600* (~5-6% TTX) 3,400* (~5-6% TTX)	Muscle Muscle Muscle	MBA, LC-MS/MS	Jen et al. (2014)

Tetrodotoxin has also been reported at very high concentrations (up to 500 mg kg^{-1}, over 60 times higher than in bivalves) in marine worms and flatworms. These species are known to live inside or on the shells of other organisms, including shellfish (Carroll, McEvoy et al. 2003, Stokes, Ducey et al. 2014, Salvitti, Wood et al. 2015, Turner, Fenwick et al. 2018). Although there are no confirmed poisoning cases from TTX-bearing worms, the presence of one toxic marine worm or flatworm in an edible shellfish could result in an adverse health effect to the consumer, and this risk should be investigated further.

2.1.1.3 *Tetrodotoxin regulations in marine gastropods and bivalves*

Japan, where pufferfish is a delicacy, is one of the only countries to have policies, guidance values and regulations for TTX to manage the risk of intoxication (Davis 2000). This includes certification of chefs trained in handling the fish and identification of species and organs known to contain high levels of TTX. In Japan, a guidance value of 2 mg TTX eq kg^{-1} is used to classify pufferfish as high or low toxicity (Tani 1945, Endo 1984). In other areas such as Europe, fish belonging to families Tetraodontidae, Molidae, Diodontidae and Canthigasteridae (the main species containing TTX) are prohibited to enter the food market (Regulation 2012). In New Zealand, the regulation allows the importation of Korean pufferfish as long as it is accompanied with certification that identifies the species, a guarantee that the product has been gutted and prepared by a certified person, and is therefore deemed fit for human consumption (Dansted 2019). In the United States, illegal importations of fish containing TTX have also been reported and often results in human poisoning (Cohen, Deeds et al. 2009).

With the detection of TTX in edible seafood, the need to regulate the toxin in shellfish is currently under debate by regulatory agencies in affected countries. The EFSA published a scientific opinion on the evaluation of the toxicity of TTX and analogues in bivalve molluscs and marine gastropods, and determined that concentrations above 44 µg kg^{-1} would indicate a concern for consumers of a large portion of shellfish (400 g or larger) (Knutsen, Alexander et al. 2017). Following the EFSA recommendation, the Netherlands adopted the concentration of 44 µg TTX per kg as the action limit after detecting TTX in mussels and oysters from their

shellfish production areas (Gerssen, Bovee et al. 2018). Some gastropods and bivalves analysed in different parts of the world have been shown to contain TTX at levels greater than the EFSA recommended level (Tables 2-1 and 2-2). A recent study demonstrated that TTX has similar potency to STX and that the toxicities of both toxins are additive when administered together (Finch, Boundy et al. 2018). This indicates that TTX should potentially be considered together with the STX-group toxins for regulatory monitoring. Further studies are required to better understand the need for regulation and the effects of TTX on humans. This is further highlighted by results from Boente-Juncal et al. (2019) who demonstrated that chronic low oral doses of TTX might have deleterious effects on renal and cardiac tissues.

2.1.2 Possible sources of tetrodotoxin and its dynamics within edible shellfish

To manage the risk associated for TTX in edible seafood, it is important to understand its origin, the mechanism in which the toxin enters the food supply and how the concentrations observed are accumulated. For TTX, this is difficult as controversy remains regarding its origin. The two most common hypotheses are that it is produced by symbiotic bacteria (endogenous) or that it is accumulated through the diet (exogenous).

The evidence for bacteria producing TTX is contradictory. According to some literature, there are a number of bacterial species that can produce TTX and its analogues (Pratheepa and Vasconcelos 2013). The most commonly reported bacterial genera associated with TTX production are: *Vibrio*, followed by *Bacillus*, *Pseudomonas*, *Actinomyces* and *Micrococcus* (Turner, Fenwick et al. 2018). However, in a review of bacteria reported to produce TTX, no evidence of production of TTX was found when using modern highly-specific analytical methods (Chau 2013). This indicates that there may have been an overestimation of the number of bacteria that produce TTX due to analytical constraints. The two methods of analysis that have commonly been used to investigate the production of TTX in bacterial cultures are: 1) HPLC followed by fluorescence detection, which has low specificity and can give rise to false positives due to matrix interferences (Matsumura 1995); and 2) GC-MS, which has also been shown to generate false

positives from extraction of culture medias (Matsumura 1995). The TTX concentrations reported in bacterial cultures are also usually very low, making it difficult to explain the extremely high levels found in higher trophic species and bioaccumulation in the food chain from TTX-producing bacteria. Furthermore, most of the publications on bacterial production do not provide controls to eliminate the possibility of contamination from TTX source material, and some demonstrate loss of production with further inoculations, which could also indicate contamination from the starting material.

Knowledge of TTX production and accumulation in higher organisms is also hindered by an incomplete understanding of the biosynthetic pathways for TTX production (Chau, Kalaitzis et al. 2011). Katikou (2019) recently summarised the knowledge to date on the biosynthetic pathway of TTX and outlined that it is likely that TTX biosynthetic pathways were different between terrestrial and marine animals. This hypothesis was further strengthened by the recent work of Ueyama et al. (2018), reporting the discovery of seven novel spiro bicyclic guanidino compounds isolated from the pufferfish *Tetraodon biocellatus* that share the same carbon skeleton as TTX. These new compounds have not been detected in terrestrial species. It was further suggested that marine TTX and analogues are produced by marine microorganisms and then accumulate in marine animals in higher trophic levels. Further studies are required to investigate the presence of the new compounds in marine bivalves and gastropods, which would help confirm the proposed TTX biosynthetic pathway.

Limited research has been performed on the source of TTX in marine bivalves or edible gastropods, but available evidence indicates an exogenous source. Gammaproteobacteria, particularly *Vibrio* and *Pseudomonas* species, have been linked to the accumulation of TTX in bivalves. Two recent studies (Turner, Dhanji-Rapkova et al. 2017, Leão, Lozano-Leon et al. 2018) found a correlation between the presence of *Vibrio* and *Pseudomonas* and shellfish that contained TTX, but they were unable to culture any TTX-producing bacteria from these samples. The hypothesis that bacteria or microalgae are the source of TTX is fuelled by reports of toxic episodes in bivalves during warmer months, in particularly late spring in Europe (Gerssen, Bovee et al. 2018, Leão, Lozano-Leon et al. 2018) and New

Zealand (Biessy, Smith et al. 2019). This may indicate the presence of a warm-water adapted TTX-producing microorganism.

No data are available regarding the toxin depuration in gastropods, but bivalves have been shown to depurate TTX. In the field, Turner et al. (2017) reported that blue mussels (*M. edulis*) depurated TTX over a period of several weeks. Tetrodotoxin-containing clams (*P. australis*) from New Zealand depurated the toxin over 150 days when kept in captivity and fed a non-toxic diet (Biessy, Smith et al. 2019). These authors also show significant variations in TTX concentrations between different sites across the country. Clams from warmer waters were significantly more toxic than the ones from colder waters and when collected in winter, adding further evidence for the producer being associated with warmer waters. Using LC-MS/MS combined with immunohistochemistry Biessy et al. (2018) demostrated that toxic *P. australis* mostly accumulate TTX in their siphons and organs used for feeding, supporting the exogenous toxin source hypothesis.

In New Zealand, *Pleurobranchaea maculata*, the grey side-gilled sea slug has been shown to lay eggs that contain relatively high TTX levels compared to other parts of the slug (Wood, Casas et al. 2012). Small *P. maculata* larvae have also been shown to contain high concentrations of TTX. High densities of this sea slug have been found in aquaculture areas in New Zealand (Taylor, Wood et al. 2015). In England, a new invasive nemertean species *Cephalothrix simula* has been shown to contain high levels of TTX (Turner, Fenwick et al. 2018). In Japan, highly toxic Nemertans of the genus *Cephalothrix* were also found on the shells of cultured oysters in Japan (Asakawa, Ito et al. 2013). *Cephalothrix simula* and the eggs and larvae of *P. maculata* are small enough to be filtered by shellfish and the presence of these organisms in shellfish aquaculture areas could serve as a possible vector or source of the toxin (Wood, Taylor et al. 2012).

2.2 Conclusions

This review compiled reports on the occurrence of TTX in bivalves and edible gastropods and showed that there has been an increase in TTX and/or in the frequency of TTX reports in these organisms. Increased monitoring would help understand if this is due to more sensitive detection techniques or if the toxin

prevalence is increasing with global environmental changes. Tetrodotoxin concentrations reported in some bivalves and edible gastropods are exceed the guidance level of 44 µg TTX kg^{-1} suggested by the European Food Safety Authority. However, this conservative concentration determined by EFSA is significantly lower than the current regulatory limit applied for STXs (800 µg kg^{-1}). Cases where human intoxication from gastropods was reported, the concentration of TTX was significantly higher than the regulatory limit for STX. This supports that the existing regulatory limit established for STX continues to be appropriate for food safety and inclusion of TTX within the existing regulation of the STX-group toxins would also be appropriate.

CHAPTER 3

Distribution of tetrodotoxin in the New Zealand clam, *Paphies australis*, established using immuno-histochemistry and liquid chromatography-tandem quadrupole mass spectrometry

3.1 Abstract

Tetrodotoxin is one of the most potent neurotoxins known. It was originally thought to only occur in pufferfish but has now been identified in twelve different classes of freshwater and marine organisms, including bivalves. Despite being one of the world's most studied biotoxins, its origin remains uncertain. There is contradictory evidence regarding the source of TTX and its pathway through food webs. To date, the distribution of TTX has not been examined in bivalves. In the present study 48 *Paphies australis*, a TTX-containing clam species endemic to New Zealand, were collected. Thirty clams were dissected, and organs and tissues pooled into five categories (siphons, digestive gland, adductor muscles, foot and the 'rest') and analyzed for TTX using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The micro-distribution of TTX was visualized in the remaining 18 individuals using an immunohistological technique incorporating a TTX-specific monoclonal antibody. The LC-MS/MS analysis revealed that siphons contained the highest concentrations of TTX (mean 403.8 μg kg^{-1}). Immunohistochemistry analysis showed TTX in the outer cells of the siphons, but also in the digestive system, foot and gill tissue. Observing TTX in organs involved in feeding provides initial evidence to support the hypothesis of an exogenous source in *P. australis*.

3.2 Introduction

Tetrodotoxin is a lethal neurotoxin that selectively binds and blocks voltage-gated sodium channels (Cestèle and Catterall 2000). It is one of the most potent natural substances known, with ingestion of 1 to 2 mg enough to kill a 50-kg human (Noguchi and Ebesu 2001). Tetrodotoxin was named after the pufferfish family Tetraodontidae and was first isolated in 1910 (Tahara 1910), although characterization of the chemical structure was not achieved until 1950 (Noguchi and Arakawa 2008).

Tetrodotoxin has long been known as the causative agent in pufferfish poisoning (Kodama, Noguchi et al. 1983, Noguchi 2006) and was first recorded in 1774 by Captain James Cook who detailed the effects on his crew from the consumption of a fish from New Caledonia, now thought to be a pufferfish (Isbister, Son et al. 2002). It was originally thought that TTX only occurred in pufferfish, but has since been identified in 12 different classes of marine, freshwater and terrestrial vertebrate and invertebrate organisms (Chau, Kalaitzis et al. 2011).

Despite TTX being one of the most studied biotoxins in the world, its origin remains uncertain. There is contradictory evidence regarding whether the source of TTX is exogenous or endogenous, and the pathways and mechanisms through which TTX is incorporated in the food web are unknown (Bane, Lehane et al. 2014). The wide distribution of TTX in many genetically unrelated species and its high spatiotemporal variation among TTX-containing species suggest that the toxin comes from an exogenous source such as accumulation through diet or symbiotic bacteria (Yasumoto, Yasumura et al. 1986, Wu, Yang et al. 2005, Noguchi and Arakawa 2008). However, there is also evidence for an endogenous source in terrestrial species. Research suggesting this began in the late 1980s when TTX-containing tree frogs *Atelopus oxyrhynchus* were shown to retain high levels of toxicity in controlled environment for three years (Yotsu-Yamashita, Mebs et al. 1992). Hatched frogs *A. varius* raised in captivity were also found to contain TTX (Daly, Padgett et al. 1997). Similar experiments undertaken in newts *Taricha granulosa* have shown that individuals regenerated their levels of TTX in their skin when kept in captivity and fed a TTX-free diet (Cardall, Brodie et al. 2004).

Tetrodotoxin has been identified in nine different bivalve species from six countries. The first report of TTX in marine bivalves was in Japan in 1993 (Kodama, Sato et al. 1993) when the toxin was found in the digestive gland of scallop *Patinopecten yessoensis* after a bloom of the dinoflagellate *Alexandrium tamarense*. Two decades after the first detection in marine bivalves, McNabb et al. (2014) reported high levels (800 μg kg^{-1}) of TTX in the surf clam *Paphies australis* in New Zealand. That research triggered further investigations on TTX in bivalves globally. Turner et al. (2015) detected TTX in Pacific oysters *Crassostrea gigas* and common blue mussels *Mytilus edulis* in England. Shortly after, Vlamis et al. (2015) reported moderate levels (up to 223 μg kg^{-1}) of TTX in *M. edulis* harvested in 2012 from the Greek islands. Oysters and mussels from production areas in the Netherlands also tested positive for TTX (Knutsen, Alexander et al. 2017) and trace detections (2.22 μg kg^{-1}) have also been reported in a clam species *Ruditapes philippinarum* in China (Zhang, Yan et al. 2015).

Recent advances in chemical detection and quantification methods (i.e., Liquid Chromatography-Tandem Quadrupole Mass Spectrometry; LC-MS/MS) have allowed the development of very specific, sensitive and accurate methods to measure TTX concentrations (McNabb, Selwood et al. 2005, Boundy, Selwood et al. 2015). The use of these methods makes it possible to investigate and quantify the distribution of TTX in specific organs within bivalves. To date, the micro-distribution of TTX has been studied in a wide range of organisms including: three species of pufferfish (Tanu, Mahmud et al. 2002, Mahmud, Okada et al. 2003); a sea slug and a flatworm (Salvitti, Wood et al. 2015); a nemertean and a turbellarian (Tanu, Mahmud et al. 2004); and a ribbon worm (Magarlamov, Shokur et al. 2016) but has never been investigated in bivalves. Improving knowledge on the location of TTX in marine bivalves may provide new insights on the ecological functions and sources of the neurotoxin in these organisms. For example, finding the toxin in the outside epithelium or in the reproductive system could indicate a defense mechanism, whereas the identification of TTX in the digestive tract could indicate a possible exogenous source.

In the present study, LC-MS/MS was used to determine which *P. australis* organs contained TTX. These data were then used to guide immunohistological experiments using a TTX-specific monoclonal antibody (Rivera, Poli et al. 1995,

Kawatsu, Hamano et al. 1997) to determine the micro-distribution of TTX within these organs. Based on similar studies on the neurotoxin saxitoxin (produced by planktonic marine dinoflagellates and freshwater cyanobacterial species) in clams *Saxidomus gigantea* (Smolowitz and Doucette 1995) and in other *Paphies* species (MacKenzie, White et al. 1996), we hypothesized that TTX would be located in the siphons and reproductive system of *P. australis*, where it provides protection for the adult during feeding and would be transferred to larvae to aid in their protection post-spawning.

3.3 Materials and methods

3.3.1 *Paphies australis* collection

Paphies australis (n = 48) were collected from the Hokianga Harbour (Northland, New Zealand; 35°28'S, 173°24'E) on 28 September 2017 and placed in a metal shellfish collection basket. *Paphies australis* from this location had previously been shown to contain TTX (Boundy, Biessy et al. 2020). Individuals were rinsed in seawater and placed in a plastic bag inside an insulated container (9-12 °C). Within two hours of collection, twelve large *P. australis* were sectioned and six small individuals (<20 mm) were kept whole and processed for immunohistochemistry as described below. Once in the laboratory (<24 h), five individuals for TTX analysis were frozen whole (-20 °C) immediately and the remaining *P. australis* (n = 30) were kept in an aerated aquarium (30 L), maintained at 18±1 °C with a 14:10 h light:dark cycle with recirculating water for gut voidance. Bivalves were fed the microalga *Isochrysis galbana* (2 L; 12 x 10^6 cells.mL^{-1}), known to be free of TTX, every second day for nine days. The aquariums were cleaned and the water changed after seven days to maintain dissolved oxygen concentrations (7-8 mg L^{-1}) and salinity (34-35‰).

3.3.2 Sample processing

3.3.2.1 *Sectioning for immunohistochemistry*

The twelve *P. australis* were measured (side-to-side axis) and manually shucked with a sterile blade. A longitudinal cross-sectional slice (3-5 mm) was prepared that included the stomach, adductor muscle, digestive gland, mantle, foot, gills and

gonads as described in Howard et al. (2004). Sections of siphon tissues were also prepared for histology. The six *P. australis* were left whole because of their small sizes (less than 20 mm long). Cross-sections, siphons and the small whole *P. australis* were then placed in histological cassettes (ABC Scientific, CA, USA) and fixed in 10% formalin in seawater for 48 h. The cross-sections and siphons from the same individual were combined in one cassette. After fixation, the cassettes were transferred to 70% ethanol until further processing.

3.3.2.2 *Dissection for tetrodotoxin analysis*

Paphies australis (n = 10) fed a TTX-free diet were collected from the aquarium after day 3, 6 and 9 and were rinsed with Milli-Q water. These, and the five that had been frozen immediately, were aseptically dissected using a sterile scalpel. The tissues were pooled into five groups (keeping each time point separate): siphon, foot, digestive gland, adductor muscles and the 'rest' which included the mantle and the gonads. The pooled samples were frozen (-20 °C) for later TTX analysis using LC-MS/MS as described below.

3.3.2.3 *Immunohistochemistry*

Dissected tissue samples in cassettes were then sent to Medlab (Taranaki, New Zealand) to be dehydrated, embedded in paraffin and sectioned at 5 µm thickness. Three histological sections on individual slides were received per sample, two unstained and one 'control' slide stained with hematoxylin and eosin.

The following protocol was adapted from Salvitti et al. (2015). Immunohistological sections were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol (100-70%) before treatment with 3% H_2O_2/10% methanol to remove endogenous peroxidase activity followed by incubation with normal goat serum (VectorLabs, Burlingame, CA, USA) to prevent non-specific binding. Both the H_2O_2/methanol mixture and normal goat serum were diluted with 1× Phosphate Buffered Saline (1× PBS; pH 7.2). Slides were then incubated with a TTX-specific monoclonal antibody (mAb) T20G10 diluted to 1 µg mL^{-1} (Rivera 1995) in concert with VECTASTAIN® ABC kit (VectorLabs, Burlingame, CA, USA) according to the manufacturer's instructions (Table 3-1). Visualization of the antigen-antibody complex was conducted using 3,3′-diaminobenzidine (DAB)-Nickel substrate

solution. Sections were counterstained with Gill's II Hematoxylin (Sigma-Aldrich, Darmstadt, Germany) and were dehydrated using ascending grades of ethanol (60-100%), followed by two rinses in xylene for 10 min each. The slides were mounted with DPX mountant (Sigma-Aldrich), and observed using an inverted microscope (CKX41, Olympus) and photographs were taken using a slide scanner (VENTANA iScan Coreo, Tucson, AZ, USA).

Table 3-1. Immunohistological incubation scheme. Steps were undertaken at room temperature unless otherwise specified. PBS = phosphate buffered saline, mAB = monoclonal antibody, DAB = 3,3′-diaminobenzidine. * Reagents were diluted in 1x PBS, pH 7.2.

Step	Solution	Time (min)
1.	3% H2O2/10% methanol	10
2.	1× PBS	10 × 3
3.	Normal Goat Serum	20
4.	1× PBS	10 × 3
5.	mAB T20G10 *	Overnight at 4 °C
6.	1× PBS	10 × 3
7.	Biotinylated secondary antibody (anti-rabbit IgG) *	60
8.	1× PBS	10 × 3
9.	VECTASTAIN® ABC reagent *	60
10.	1× PBS	10 × 3
11.	DAB *	5 - 10
12.	Deionized H_2O	5
13.	Counterstain (Gill's II Hematoxylin)	5

Antigen-antibody complexes were visualized as brown colour deposit in positive sections and the same protocol was followed for the negative controls with the slides being incubated overnight with 1× PBS instead of the TTX-specific monoclonal antibody.

3.3.2.4 *Tetrodotoxin analysis*

Each sample (ca. 0.3-3.0 g) was weighed, cut into small pieces with a sterile blade and placed in a sterile tube (50 mL) with a corresponding volume (ca. 300-3,000 µL) of Milli-Q water containing 1% acetic acid. Samples were homogenized (Ultra-Turrax®, IKA®, NC, USA) for 45 s to ensure complete disruption of tissues. The

tubes were placed in boiling water (5 min) and then cooled in an ice bath (5 min) before briefly vortexing. Samples were centrifuged (3,200 × *g*, 10 min) and 0.3-1 mL of the supernatant transferred to a centrifuge tube (1.7 mL) containing 2.5-5 µL of 25% ammonia (Honeywell). Samples were then centrifuged (17,000 × *g*, 1 min) and the supernatant (400 µL) cleaned with the graphitized carbon Solid Phase Extraction (SPE) method as described by Boundy et al. (2015) using Supelclean™ ENVI-Carb 250 mg/3 mL SPE cartridges (Sigma-Aldrich). Tetrodotoxin was analysed and quantified by liquid chromatography tandem-mass spectrometry analysis as described by Turner et al. (Turner, Boundy et al. 2017).

3.3.3 Statistical analysis

Tetrodotoxin data from the LC-MS/MS were analysed using R (RCoreTeam 2020). Data from the three time points (3, 6 and 9 days) were combined to enhance replicate number (after showing no significant difference) and an ANOVA with a Tukey's post-hoc test were undertaken to determine if there are statistically significant differences in TTX concentrations among tissues and organs.

3.4 Results and discussion

3.4.1 Siphons

Quantification of TTX in *P. australis* organs by LC-MS/MS identified the highest concentration of toxin in the siphons (mean 403.8 ± 32.8 µg kg^{-1}, n = 5; Figure 3-1). One-way ANOVA showed a significant difference between the mean concentrations in organ groups ($p < 0.001$), with Tukey's post-hoc test identifying the difference between the siphons and all other groups ($p < 0.001$; Figure 3-1). The identification of high concentrations in the siphon is similar to studies which showed that butter clams *S. gigantea* and the surf clam *Paphies subtriangulata* sequester saxitoxin in their siphons (Kvitek 1991, Smolowitz and Doucette 1995, MacKenzie, White et al. 1996). The immunostaining provided further evidence for the location of accumulation of TTX in the siphon sections. It was visualized as small brown deposits in the outside and inside layers of cells (Figure 3-2a-f). Slides stained with eosin and hematoxylin were used to identify organs and tissues but were also used to identify brown cells or rhogocytes (Hine 1999; Figure 3-2g), to demonstrate that they had not been misinterpreted as TTX-specific staining. No

TTX-like staining was observed in control slides not stained with anti-TTX, confirming that there was no confusion with brown cells (Figure 3-2h).

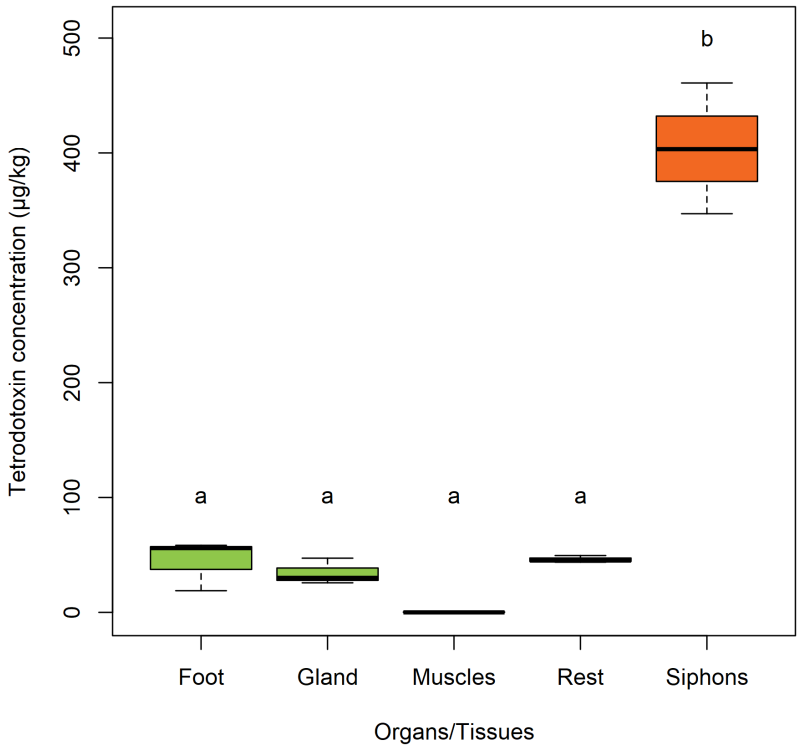


Figure 3-1. Tetrodotoxin concentrations in the organs and tissues of *Paphies australis* determined using liquid chromatography-mass spectrometry. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the inter-quartile range if there are data that far from it. Letters indicate where significant differences (one-way ANOVA with Tukey's post-hoc test, $p < 0.001$) occur between organs/tissues.

Bivalve siphons were an evolutionary development which enabled them to diversify into many new ecological niches after the Palaeozoic Era (Stanley 1968). In bivalves, the two siphons are paired: the inhalant siphon carries the water into the mantle cavity, and the exhalant siphon ejects the water from the internal cavity (Gosling 2008). The water flow from the siphons is variously used for feeding, respiration, reproduction and in locomotion for some bivalves (Gosling 2008). Siphons also play a critical role in survival by allowing water flow while buried (Zwarts and Wanink 1989). *Paphies australis* and many other bivalves, bury themselves in the sand and keep their siphons above the sediment surface to filter feed. It is well known that siphons are a targeted food for predators including siphon-nipping fish such as the flatfish *Pleuronectes platessa* (de Goeij, Luttikhuizen et al. 2001) or shrimp (Kamermans and Huitema 1994). Bivalves are negatively impacted by siphon-nipping predators because they require extra energy

for siphon regeneration and it reduces feeding opportunities (Kamermans and Huitema 1994). Researchers have suggested previously that biotoxins are accumulated as a chemical defense against predation. For example, Kvitek (1991) suggested that the clam *S. gigantea* retains saxitoxin in their siphons as a defense mechanism against siphon-nipping predators and Kvitek & Bretz (2004) demonstrated that sea otters stop grazing on shellfish with high saxitoxin concentrations. We speculate that TTX accumulation in siphon tissues could provide a similar chemical defense in *P. australis*. An elevated level of TTX in *P. australis* siphons could also suggest that TTX is present in the water or within organisms in the water that they are filtering while feeding, possibly indicating an exogenous source.

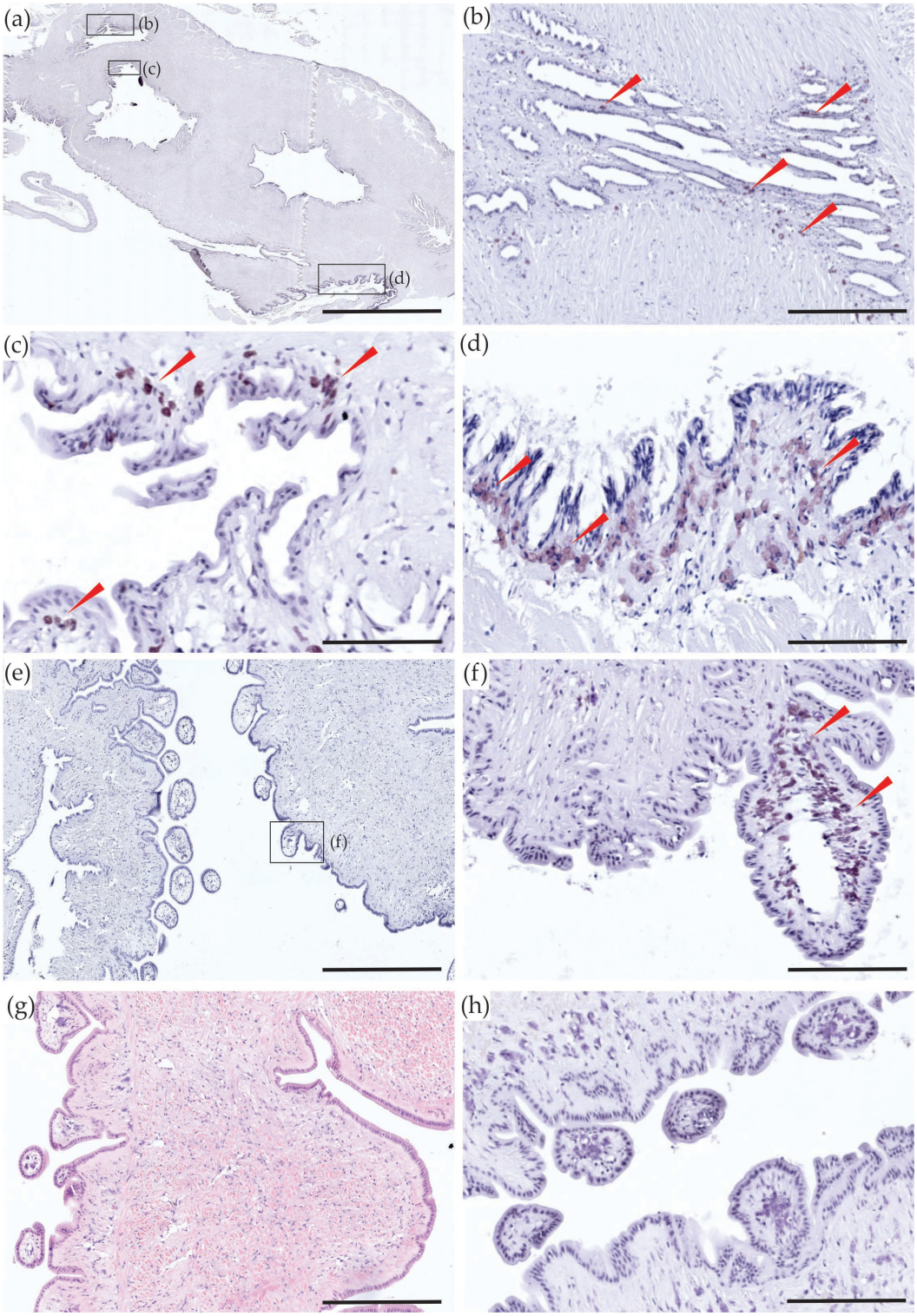


Figure 3-2. Longitudinal sections of the inhalant and exhalant siphons of *Paphies australis*. (a) Tetrodotoxin (TTX)-specific monoclonal antibody (mAB) immunohistological staining in longitudinal section of siphons; (b, c and d) enlargements of different boxes on (a) to show micro-location of TTX staining, identified by the brown colour deposits shown by red arrows; (e) another view of TTX-specific mAB immunohistological staining in the outer layer of the siphon; (f) enlargement of the box on (e) to show detailed view of the staining in the siphons loops; (g) Hematoxylin and Eosin staining; (h) mAB negative control. Scale bars = 200 µm (a), 50 µm (b-d, f, h), 150 µm (e) and 100 µm (g).

After the Palaeozoic era, certain bivalve species developed an extensive foot, opening up new life history strategies (Stanley 1968). The development of a foot gave bivalves a distinct competitive advantage by allowing them to rapidly dig down into the sand (Kondo and Stace 1995). Some are also able to swim short distances using their foot (e.g. *Mytilus edulis*; Gosling 2008). The LC-MS/MS analysis identified lower concentrations of TTX in foot tissue (mean 44.4 ± 12.8 µg kg^{-1}, Figure 3-1) but no TTX was visualized after immunostaining. In *P. australis* the foot is directly attached to the gonads. The toxic *P. australis* collected for this experiment were relatively small (<1.5 grams) and not mature, hence the difficulty in separating the small gonads from the foot during the dissection. The LC-MS/MS detection is most likely 'contamination' from other organs. However, previous studies have detected low levels of saxitoxin in the foot of other bivalves including different clam species (Hwang, Tsai et al. 1992, Bricelj and Shumway 1998). Pereira et al. (2004), also detected small amounts of saxitoxin in the foot of freshwater mussels *Anodonta cygnea* after two days of exposure to the toxin.

3.4.2 Adductor muscles

Adductor muscles are a significant part of the muscular system in bivalves, they are strong and connect the two valves (Gosling 2008). Swimming movements of bivalves (in swimming species such as *Pecten* spp.) are produced by a series of sudden shell valve closures powered by rapid cycles of contraction and relaxation of the adductor muscles (Baldwin and Opie 1978). In this study, the muscle group included the posterior and anterior adductors muscles. No TTX was detected in the muscle using either immunostaining or LC-MS/MS quantification. Previous studies have shown that accumulation of marine biotoxins in the muscles of bivalves is very low or non-existent (Shumway and Cembella 1993). To take advantge of the low toxicity of this tissue in regions of the world where saxitoxin is present, sushi is now being made with bivalve adductor muscles (Bricelj and Shumway 1998).

3.4.3 Digestive system

In bivalves, a short oesophagus leads from the mouth to the stomach. The stomach is surrounded by the digestive gland and an opening from the stomach leads to the intestine that extends into the foot, ending in the rectum and eventually the anus (Helm, Bourne et al. 2004). In this study, the digestive system consisted of the

digestive gland, the oesophagus and the gut when it was large enough to identify and dissect.

Tetrodotoxin was detected in the digestive system of the *P. australis* using LC-MS/MS (mean 34.3 ± 6.5 μg kg^{-1}; Figure 3-1) and was also observed in the inside cells of the epithelium of the intestine and the rectum in the immunostained sections (Figure 3-3). A number of studies have identified biotoxins in the digestive tracts of bivalve species, for example, the highest concentration of saxitoxin was found to be stored in the viscera of *M. edulis* (Harada, Oshima et al. 1982); domoic acid was mostly contained (>80%) in mussel's and oyster's digestive systems (Mafra Jr, Bricelj et al. 2010) and greater than 50% of microcystins were present in the digestive glands of freshwater mussels (Chen and Xie 2005). This result could indicate that TTX is sourced from the diet of *P. australis*. Tetrodotoxin may be initially stored in the organs of the digestive system and could be transported to other tissues and organs, to use as a chemical defense.

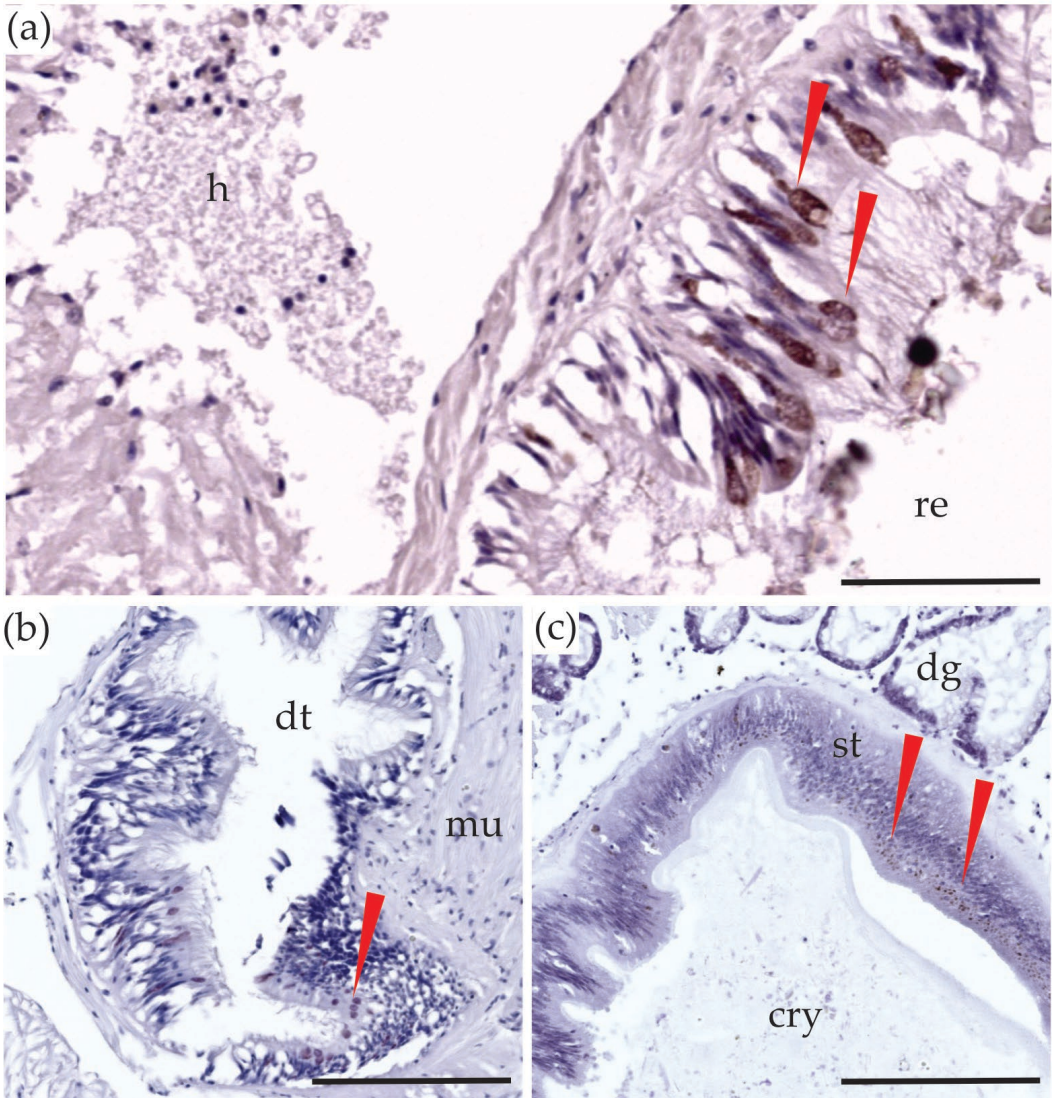


Figure 3-3. Sections of the digestive system of *Paphies australis*. Tetrodotoxin-specific monoclonal antibody immunohistological staining in: (a) the rectum; (b) the intestinal epithelium; (c) the inside layer of the stomach wall. cry = crystalline style, dg = tubules in the digestive gland, dt = digestive tube, mu = muscle tissue, re = rectum. Scale bars =50 µm (a) and 100 µm (b,c).

3.4.4 The 'rest'

The 'rest' group contained all the organs and tissues that were not described above. It mainly included the gonads, the mantle, the labial palps and the gills. The separation of these tissues was not possible due to the fragility and small size of the *P. australis* collected. The 'rest' contained low concentrations of TTX (mean 46.1 ± 1.8 µg kg^{-1}; Figure 3-1). Although these sections could not be dissected for LC-MS/MS analysis, they could be visualised using the immunohistological staining and key findings are described below.

3.4.5 Gonads

Like most bivalves, *P. australis* are dioecious and fertilization usually occurs in the water column after spawning (Gosling 2008). In immunostained sections of *P. australis*, the gonads did not contain TTX. After hematoxylin staining, the eggs, which contain high quantities of lipids were dark purple (Figure 3-4), making the visualisation of TTX challenging. Based on the immunostained sections, we do not believe TTX was present in the eggs. Additionally, the majority of the *P. australis* collected for this study were not sexually mature (<40 mm shell length) and this may have affected our results if TTX migrates to the gonads only at sexual maturity.

Studies on other marine organisms have shown the presence of TTX in reproductive organs (e.g., in the gonads of the sea-slug *Pleurobranchaea maculata* and the flatworm *Stylochoplana* sp.; Salvitti, Wood et al. 2015), and on the body surface of larvae from the pufferfish *Takifugu alboplumbeus* (Itoi, Suzuki et al. 2018). Further fine-scale studies are required to determine if *P. australis* eggs contain TTX using sexually mature individuals, and rearing studies could be used as the presence in mature eggs has significance to whether TTX is passed onto successive generations.

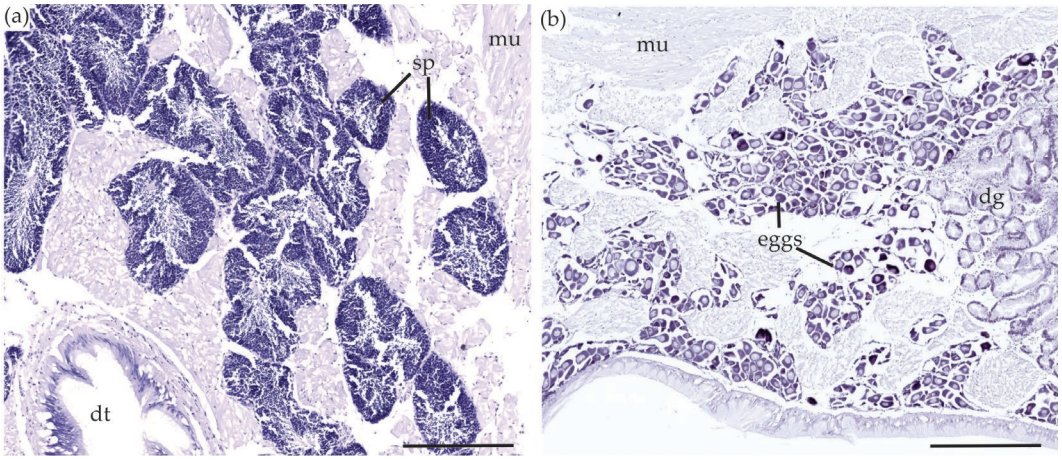


Figure 3-4. Sections of the gonads of Paphies australis. Gonads contain high concentrations of lipids and turned dark purple after hematoxylin staining, but no evidence of TTX. (a) Male gonads; (b) Female gonads. dg = tubules in the digestive gland, dt = digestive tube, mu = muscle tissue, sp = sperm. Scare bars = 100 µm.

3.4.6 Mantle

The mantle of the bivalves has a sensory function and can initiate closure of the valves in response to unfavourable conditions. It also has a respiratory function by

controlling the inflow of water (Helm, Bourne et al. 2004). No TTX was visualized in the mantle after the immunostaining experiment. These results correlate with other studies investigating biotoxin distribution in bivalves. Studies by Pereira et al. (2004) and Harada et al. (1982) did not detect any saxitoxins in the mantle of several bivalve species.

3.4.7 Gills and labial palps

Gills in bivalves are primarily used for respiration and feeding (Helm, Bourne et al. 2004). The pair of labial palps, situtated in the mantle cavity near the mouth, are involved in feeding. Each of the pair has two components: the inner palp is used for sorting food and transferring the selected particles to the mouth while the function of the outer palp is the production of pseudo-faeces (Purchon 1968). Tetrodotoxin from an external source (e.g. food) would be expected to be present in these tissues of *P. australis*. It was visualized in the labial palps (Figure 3-5a) and in the gills (Figures 3-5c and d) of *P. australis*, explaining why TTX was detected in low concentrations in the 'rest' tissue group with LC-MS/MS. Other biotoxins have previously been detected in the gills of bivalves: low levels of microcystins (Chen and Xie 2005) and saxitoxins (Harada, Oshima et al. 1982) have been detected in gills of mussels and three species of bivalves in Palau. This result provides further evidence to support the hypothesis of a dietary source of TTX in *P. australis*.

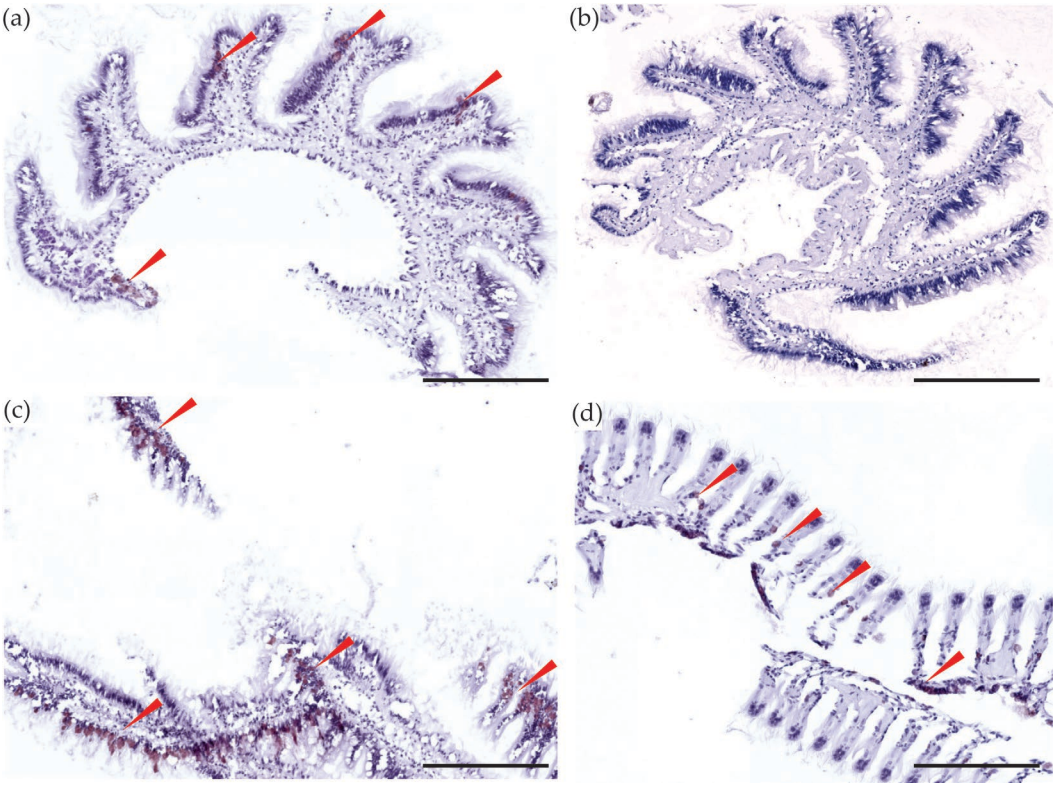


Figure 3-5. Sections of *Paphies australis* gills and labial palps tissues sectioned at 7 µm. Tetrodotoxin (TTX)-specific monoclonal antibody (mAB) immunohistological staining of: (a) the labial palps with the TTX-mAB; (b) the labial palps with mAB negative control; (c) and (d) the gills with the TTX-mAB. TTX was identified by the brown colour deposits shown by red arrows. Scale bars = 100 µm (a, b) and 50 µm (c, d).

3.4.8 Parasites

Digenean trematode metacercariae (sub-adult stages) were seen (Figure 3-6) encysted in *Paphies australis* mantle tissues (55% of *P. australis* observed were infected). Further identification of these gymnophallid-like worms was prevented by their poor internal condition. Most of their internal organs were missing and the body cavity of each contained many grey/purple spheres resembling colonies of rickettsia hyperparasites. No TTX was observed in the parasites in immunostained sections.

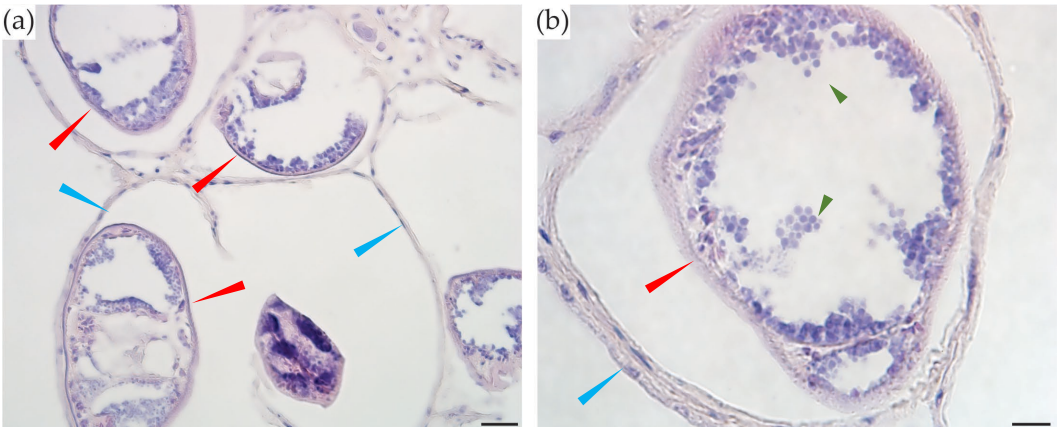


Figure 3-6. Metacercariae encysted in mantle tissues of *Paphies australis*. (a) View of a group of encysted metacercariae (red arrows) and cyst walls (blue arrows); (b) one metacercaria (red arrow) inside cyst wall (blue arrow); rickettsial hyperparasite colonies inside metacercaria (green arrows). Scale bars = 20 µm (a) and 10 µm (b).

3.5 Conclusions

Tetrodotoxin analysis of *P. australis* organs and tissues using LC-MS/MS showed that the siphons contained the highest concentrations of the biotoxin. Lower concentrations of TTX were found in the foot, digestive system, mantle and the combined group of organs/tissues ('rest') which could not be individually dissected. The immunohistochemistry experiment demonstrated the micro-localization of TTX inside these organs/tissues. The toxin was primarily present in the outer layers of the siphons, rectum, digestive tubes, gills and labial palps. Observing TTX in organs involved in feeding provides initial evidence to support the hypothesis that the source of the neurotoxin is exogenous in *P. australis*. The presence of TTX in the siphon suggests that one of its ecological roles in this species may be to reduce predation. Further studies are needed to determine if TTX migrates to other organs over time, such as those involved in reproduction, and whether the toxin is transferred to subsequent generations.

CHAPTER 4

Spatial variability and depuration of tetrodotoxin in the bivalve *Paphies australis* from New Zealand

4.1 Abstract

Tetrodotoxin is a potent neurotoxin responsible for many human intoxications globally. Despite its potency and widespread occurrence in taxonomically diverse species, the primary source of TTX remains uncertain. *Paphies australis*, an endemic clam found in New Zealand, has been found to contain TTX in several locations. However, it is unknown if this represents endogenous production or accumulation from an external source. To address this question, the concentrations of TTX in whole *P. australis* and dissected organs (siphons, foot, digestive gland and the 'rest') from thirteen sites around New Zealand were determined using liquid chromatography-tandem quadrupole mass spectrometry analysis (LC-MS/MS). Depuration rate of TTX was also investigated by harvesting and measuring concentrations in *P. australis* maintained in captivity on a toxin-free diet every three to 15 days for 150 days. The LC-MS/MS analyses of the spatial samples showed that TTX was present in *P. australis* from all regions tested, with significantly ($p < 0.001$) higher concentrations (15-50 µg kg^{-1}) observed at lower latitudes of the North Island compared with trace levels (0.5-3 µg kg^{-1}) in the South Island of New Zealand. Tetrodotoxin was detected in all the dissected organs but the siphons contained the highest concentrations of TTX at all sites analysed. A linear model of the depuration data identified a significant ($p < 0.001$) decline in total TTX concentrations in *P. australis* over the study period. The siphons maintained the highest amount of TTX across the entire depuration study. The digestive glands contained low concentrations at the start of the experiment, but this depurated rapidly and only traces remained after 21 days. These results provide evidence to suggest that *P. australis* does not produce TTX endogenously but obtains the neurotoxin from an exogenous source (e.g., diet) with the source more prevalent in warmer northern waters. The association of higher TTX concentrations in shellfish with warmer environments raises concerns that this toxin's distribution and abundance could become an increasing human health issue with global warming.

4.2 Introduction

Tetrodotoxin is a potent neurotoxin that blocks voltage-gated sodium channels (Noguchi and Ebesu 2001). Globally, it has been responsible for up to 100 human intoxications per annum (Tsuda, Ikuma et al. 1964, Isbister and Kiernan 2005). It is named after the pufferfish family, Tetraodontiformes, from which it was first identified and isolated (Chau, Kalaitzis et al. 2011). Tetrodotoxin was initially thought to only occur in pufferfish but has now been identified in a wide range of marine, freshwater and terrestrial vertebrates and invertebrates (Bane, Lehane et al. 2014). The source of TTX remains unknown and controversial: there are studies which provide evidence to support an exogenous source such as bacteria and algae (Yasumoto, Yasumura et al. 1986, Wu, Yang et al. 2005, Chau, Kalaitzis et al. 2011, Vlamis, Katikou et al. 2015), and conversely, compelling experiments indicating TTX could be produced endogenously by some organisms (Daly, Padgett et al. 1997, Cardall, Brodie et al. 2004). Tetrodotoxin was predominantly found in tropical regions (Lange 1990) but in recent years, it has been detected in a greater number of species from temperate regions including: bivalves from the Mediterranean Sea (Vlamis, Katikou et al. 2015) and England (Turner, Powell et al. 2015); and bivalves, gastropods and platyhelminths in New Zealand (McNabb, Selwood et al. 2010, Salvitti, Wood et al. 2015). Increasing reports of TTX in farmed aquaculture species, such as bivalves, has drawn attention to the toxin, reinvigorating scientific interest and regulatory concerns (Knutsen, Alexander et al. 2017).

The first report of TTX in bivalves was in the early 1990s when it was detected in the digestive glands of the Japanese scallops (*Patinopecten yessoensis;* 8 μg kg^{-1}; Kodama, Sato et al. 1993). In 2011, McNabb et al. (2014) recorded high concentrations (800 μg kg^{-1}) in an endemic clam *Paphies australis* in New Zealand. The identification of high concentrations in edible shellfish triggered further research on TTX in bivalves globally. The neurotoxin has now been detected in mussels *Mytilus edulis* from England (Turner, Powell et al. 2015) and Greece (Vlamis, Katikou et al. 2015); in oysters *Crassostrea gigas* from the UK (Turner, Powell et al. 2015, Turner, Dhanji-Rapkova et al. 2017); and in oysters, mussels and clams *Mercenaria mercenaria* from the Netherlands (Knutsen, Alexander et al. 2017, Turner, Dhanji-Rapkova et al. 2017, Gerssen, Bovee et al. 2018). In 2015,

researchers in China detected trace concentrations in clams *Ruditapes philippinarum* purchased from markets (Zhang, Yan et al. 2015). There are multiple attributes of bivalves that make them an ideal organism to investigate the source and dynamics of TTX, including: they are stationary and occur in relatively confined shallow sub-tidal areas making obtaining high number of individuals plausible and allowing systematic sampling of benthic communities; they can be maintained and in many instances reared in captivity, allowing long-term or manipulative experiments; and as filter feeders they occur low in the trophic pyramid (2nd order).

The detection of TTX in *P. australis* in New Zealand has led to concerns about health risks for human consumers and prompted further research as they are a common non-commercial shellfish species consumed in the country. Biessy et al. (2018) showed that TTX is primarily located in the siphons of *P. australis*, although low concentrations were also detected in the foot, digestive system, gills and labial palps. McNabb et al. (2014) detected the neurotoxin in individuals from several locations around New Zealand, however, there is uncertainty as to whether all *P. australis* populations in New Zealand contain TTX, and how variable the concentrations are within and between locations. In a study on the TTX-containing sea slug *Pleurobranchaea maculata*, Wood et al. (2012) showed marked variability in toxin concentrations within populations, with a general pattern of decreasing toxin content between populations along a north to south latitudinal gradient. This led the authors to speculate that TTX may be sourced exogenously in this species, with ocean currents, such as those that flow through Cook Strait (the water body that separates the North and South Islands of New Zealand) acting as a barrier for the producer. Studies in the northern hemisphere also indicate a potential latitudinal gradient or link to warmer water temperatures, with higher TTX concentrations in bivalves during summer months (Turner, Dhanji-Rapkova et al. 2017) and in places with warmer waters (Vlamis, Katikou et al. 2015).

A further important knowledge gap, which would enhance TTX management and risk assessment, is information on depuration rates of the neurotoxin in harvested shellfish. These data would assist in providing guidance on when it may be safe to recommence shellfish harvesting if a contamination event occurred. To our knowledge, no studies have investigated TTX depuration in shellfish or bivalves in

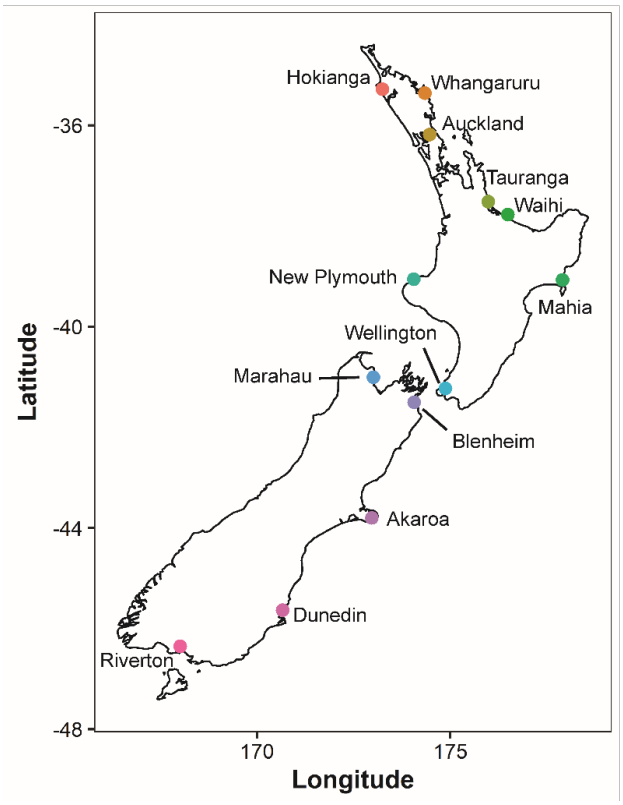
a controlled environment. However, research on other marine biotoxins has shown that depuration rates vary among toxins and bivalve species, and range from days to several months (Lee, Lovatelli et al. 2008). Depuration of TTX has been investigated in *Pl. maculata*, and a significant decrease in concentrations was observed over 126 days with the toxin migrating to the gonads, and largely depurating through laying of egg masses (Wood, Casas et al. 2012).

The overarching aims of the present study were: (1) to investigate TTX concentrations within and between *P. australis* populations around New Zealand; (2) to determine whether depuration of TTX occurs in *P. australis* kept in captivity for 150 days and fed a TTX-free diet, and (3) to explore if TTX migrates between *P. australis* organs over a 150-day period. We hypothesised: (1) that a north to south latitudinal gradient of decreasing TTX exists, as observed in *Pl. maculata* (Wood, Taylor et al. 2012), and (2) that in *P. australis,* the neurotoxin migrates over time from organs involved in filtering or digestion to the siphon, where it functions as a chemical defence mechanism. Increasing knowledge on the distribution and within organism transport of TTX in the sessile *P. australis* may provide new insights to the toxin's origin and function in this species.

4.3 Materials and methods

4.3.1 Distribution of TTX in *Paphies australis* around New Zealand

Paphies australis (n = 5 or 15) were collected from thirteen sites around New Zealand between September 2017 and September 2018 (Figure 4-1; Appendix 7, Table A7.1). The *P. australis* were rinsed in seawater, chilled (ca. 8 °C) and sent overnight to the laboratory (Cawthron Institute, Nelson, New Zealand). Once in the laboratory, five individuals were rinsed with Milli-Q water, and stored frozen (-20 °C) until later TTX analysis. Toxin extractions were not possible on individual organs because of their small sizes (a minimum of 300 mg of material is required). When possible, ten of the fifteen individuals were aseptically dissected in a laminar flow cabinet using sterile blades disinfected between each organ and individual, and the tissues pooled into four groups: the pair of siphons, foot, digestive gland, and the 'rest' which mostly included the mantle, gills, adductor muscles and gonads. The pooled samples were frozen (-20 °C) for later TTX analysis.



Collection dates

Hokianga	September 2017	Spring
Whangaruru	January 2018	Summer
Auckland	August 2018	Winter
Tauranga	February 2018	Summer
Waihi	August 2018	Winter
Mahia	September 2018	Spring
New Plymouth	March 2018	Autumn
Wellington	March 2018	Autumn
Marahau	January 2018	Summer
Blenheim	January 2018	Summer
Akaroa	March 2018	Autumn
Dunedin	September 2018	Spring
Riverton	July 2018	Winter

Figure 4-1. *Paphies australis* collection locations, dates and seasons from around New Zealand.

4.3.2 Depuration study

4.3.2.1 *Paphies australis collection and maintenance*

Paphies australis (n = 435) were collected from the Hokianga Harbour (Northland, New Zealand, 35°28'S, 173°24'E; Figure 4-1) on 28 September 2017 and placed in a metal shellfish collection basket. *Paphies australis* from this location had previously been shown to contain TTX (Biessy, Smith et al. 2018). Individuals were rinsed in seawater and placed inside an insulated container (9-12 °C) for 24 h while being transported to the laboratory. Once in the laboratory, *P. australis* were rinsed with sterile seawater and 15 individuals were dried and stored frozen (-20 °C) until later TTX analysis. The remainder were kept in two separate aerated aquariums (60 L), each containing 210 individuals to avoid an anoxic environment. The aquariums were maintained at 18 ± 1 °C with a 14:10 h light:dark cycle and the water was recirculated through the two aquariums. The bivalves were fed *Isochrysis galbana* (2 L; ca. 12 x 10^6 cells mL^{-1}) every second day for 150 days. The aquariums were cleaned, and the water changed weekly to maintain the dissolved oxygen levels (7-8 mg/L) and salinity (34-35‰).

4.3.2.2 *Tetrodotoxin depuration study*

Individuals (n = 15) were harvested from the tanks every third day for 15 days, every sixth day for 57 days, and then every twelfth day until the conclusion of experiment at day 150. Due to budgetary constraints samples collected on days 81, 93 and 141 were not analysed. These were selected as there were no notable changes in TTX concentrations between the adjacent time periods. After collection, *P. australis* were rinsed with Milli-Q water and 10 of the 15 individuals were aseptically dissected and the tissues pooled into four groups as above: the pair of siphons, foot, digestive tract and the 'rest'. The pooled samples and the remaining five 'whole' *P. australis* were frozen (-20 °C) for later TTX analysis. Subsamples of *I. galbana* prior to feeding (50 mL) and faeces from the bottom of the aquariums were collected weekly. These samples were centrifuged (3,000 × *g*, 5 min), the seawater decanted and the pellet frozen (-20 °C) for later analysis. Negative extraction controls (Milli-Q water) were also extracted, following the extraction protocol described below, and analysed for TTX.

4.3.3 Tetrodotoxin extraction and analysis

The following protocol was adapted from Biessy et al. (2018). Each sample (whole organism or organs of *P. australis*, algae, faeces and controls) was weighed (ca. 0.3-3.0 g), cut into small pieces (*P. australis* only) with a sterile blade and placed in a sterile tube (50 mL) with a corresponding volume (ca. 300-3,000 µL) of Milli-Q water containing 1% acetic acid. Samples were homogenized (Ultra-Turrax®, IKA®, NC, USA) for 45 s to ensure complete homogenization. The tubes were boiled (5 min) and cooled in an ice bath (5 min) before briefly vortexing. Samples were centrifuged (3,200 × *g*, 10 min) and 0.5-1 mL of the supernatant transferred to a centrifuge tube (1.7 mL) containing 25% ammonia (2.5-5 µL; Honeywell, Seelze, Germany). Samples were then centrifuged (17,000 × *g*, 1 min) and the supernatant subjected to the graphitised carbon solid phase extraction (SPE) method as described in Boundy *et al*. (2015) using Supelclean™ ENVI-Carb 250 mg/3 mL cartridges (Sigma-Aldrich, MO, USA). Briefly, cartridges were conditioned with 20% acetonitrile and 1 % acetic acid (3 mL) and 0.025% ammonia (3 mL). Sample extracts (400 µL) were added to the cartridges and bound TTX was then eluted with 20% acetonitrile and 1 % acetic acid (2 mL). Tetrodotoxin was analysed and quantified by liquid chromatography tandem-mass spectrometry analysis using a Waters™ Xevo TQ-S (Waters Corporation, Milford, MA, USA) with Waters™ Acquity i-Class UPLC (Waters Corporation, Milford, MA, USA) as described by Turner et al. (2017).

4.3.4 Statistical analysis

Statistical analyses were performed using the R statistical package (RCoreTeam 2020). Normality was checked through inspection of Quantile-Quantile plots and conducting a Shapiro-Wilk test. Normality was improved by log transformation of the spatial data. Statistical differences in TTX concentrations between sites was assessed using a one-way analysis of variance (ANOVA). A Tukey's honestly significant difference (HSD) post-hoc test was used to identify which sites were responsible for the significant differences. Levene's test was used to assess the equality of variance in TTX concentrations between sites (Carroll and Schneider 1985).

A linear regression model was constructed to investigate the relationship between log transformed total TTX concentrations in *P. australis* and time (day) of the experiment. Generalized additive models (GAMs; Hastie and Tibshirani 1990) were used to model nonlinear trends in organ TTX concentrations in relation to time (days). Models were selected with a stepwise procedure based on the general Akaike information criterion (AIC) and were validated by inspecting the deviance residuals. The GAMs models were run with the *mgcv* package in R (Wood and Wood 2015).

4.4 Results

4.4.1 Distribution of tetrodotoxin in *Paphies australis* around New Zealand

Tetrodotoxin was detected in *P. australis* from all sites around New Zealand (Figure 4-2). The samples from Akaroa were under the limit of quantification of the method (< 2.0 µg kg^{-1}) but the chromatograms clearly indicated that trace levels of TTX were present in these samples. Tetrodotoxin was the main congener (> 99%) detected in all bivalve samples analysed (Boundy, Biessy et al. 2020).

One-way ANOVA showed a significant difference between TTX concentrations among all sites ($F = 39.2$, $p < 0.001$), with a Tukey's HSD post-hoc test identifying a complex overlap between sites (Figure 4-2; Appendix 7, Table A7.2). The highest concentrations were measured in *P. australis* from the northernmost site, the Hokianga Harbour (median concentration 47.6 µg kg^{-1}). Median toxin concentrations in *Paphies australis* from the South Island sites were significantly lower (Marahau, 3.2 µg kg^{-1}; Blenheim, 3.8 µg kg^{-1}; Dunedin, 8.5 µg kg^{-1}; Riverton, 2.4 µg kg^{-1}) than the North Island sites (Hokianga Harbour; Whangaruru Harbour, 24.3 µg kg^{-1}; Auckland, 20.9 µg kg^{-1}; Tauranga, 35.9 µg kg^{-1}; Waihi 13.5 µg kg^{-1}; Mahia, 17.3 µg kg^{-1}; New Plymouth, 12.3 µg kg^{-1}; and Wellington, 15.0 µg kg^{-1}). A Levene's test showed that the degree of variance did not differ among sites ($F = 0.62$).

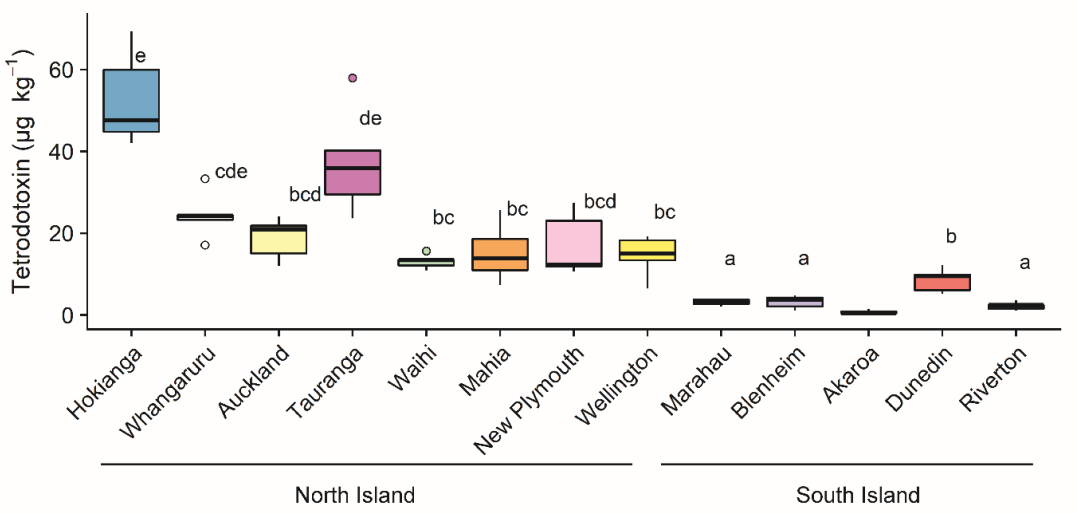


Figure 4-2. Tetrodotoxin concentrations in populations of *Paphies australis* collected around the New Zealand coastline, determined using liquid chromatography-mass spectrometry (n = 5). Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the inter-quartile range. Dots outside the whiskers are considered as outliers. Different letters indicate where significant differences occur between sites (one-way ANOVA with Tukey's HSD post-hoc test, $p < 0.001$). Sites are ordered by increasing latitude for each Island (i.e., North and South Island). Akaroa was not included in the statistical as the concentrations of toxin were under the limit of quantification of the method (< 2.0 µg kg^{-1}).

At all sites, and regardless of TTX concentrations in whole *P. australis*, the siphons contained the highest amount of toxin compared to the other tissue types (Figure 4-3). *Paphies australis* from the North Island populations also contained TTX in their foot, digestive gland and mantle (except in the Mahia samples). Tetrodotoxin was detected in low concentrations (ca. 6 µg kg^{-1}) in the digestive glands and of *P. australis* from two of the South Island sites (Marahau and Blenheim) and in the mantle from the Dunedin samples (Figure 4-3).

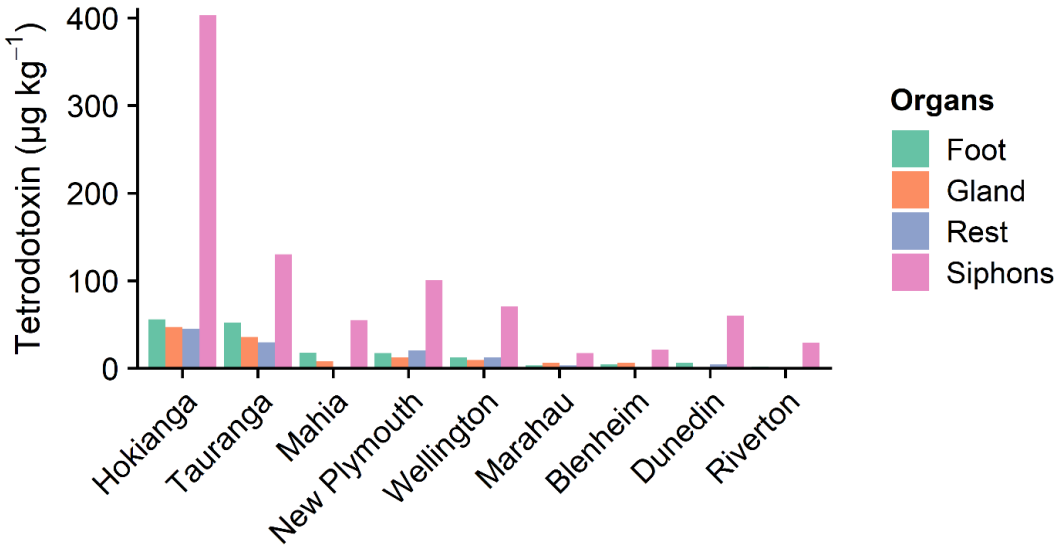


Figure 4-3. Tetrodotoxin concentrations in the organs and tissues of *Paphies australis* from different sites around New Zealand. Data are a composite of organs from 10 different individuals pooled together to enable sufficient mass for the toxin extraction. Organ dissections and extractions were not possible from sites where less than 15 *P. australis* were collected (i.e., Whangaruru, Auckland, Waihi and Akaroa sites). Sites are ordered by increasing latitude for each island (i.e., North and South Islands).

4.4.2 Tetrodotoxin depuration

4.4.2.1 *Depuration of tetrodotoxin in Paphies australis maintained in captivity*

Paphies australis stayed healthy for the duration of the experiment, except for six individuals that died and were removed from the aquariums. The feed was consumed within four hours when placed in the aquariums, showing that the bivalves were healthy and feeding well. All negative extraction controls (Milli-Q water), *I. galbana* and faeces samples were negative for TTX. Tetrodotoxin was the main congener (> 99%) detected in all samples analysed over the entire study.

The average TTX concentration in whole *P. australis* at day 0 was 56.5 ± 5.7 µg kg^{-1} and declined to 21.7 ± 6.3 µg kg^{-1} after 150 days in captivity (Figure 4-4). A linear model was fitted to the data ($R^2 = 0.24$, $F = 28.05$, $p < 0.001$) which showed an average depuration rate of TTX over the entire experiment in *P. australis* of 0.23 µg kg^{-1} per day.

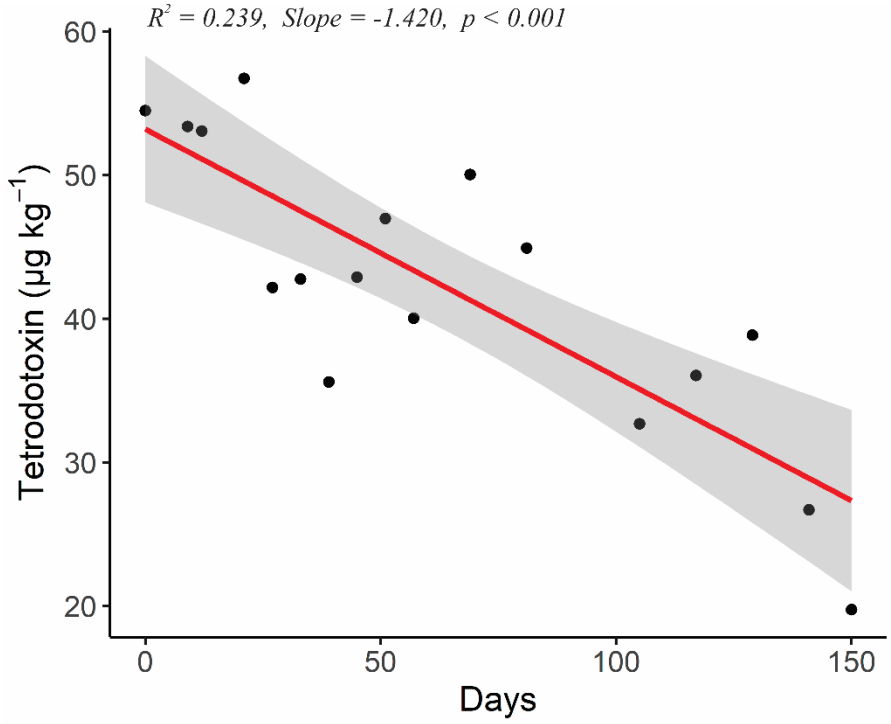


Figure 4-4. Tetrodotoxin (TTX) concentrations in whole *Paphies australis* maintained in captivity and fed a TTX-free diet for 150 days. Data are averages from five individuals. The linear model indicated a significant ($p < 0.001$) decrease in TTX concentrations after 150 days.

4.4.2.2 *Changes in TTX concentrations in the organs of Paphies australis*

Overall, the depuration pattern was relatively consistent across all organs with a decline in TTX concentrations over 150 days. At day 0, the siphons contained the highest concentrations of TTX (404 µg kg^{-1}, Figure 4-5). Low concentrations (35-45 µg kg^{-1}) were also detected in the foot, digestive gland and mantle. In general, the TTX concentrations increased from day 0 until day 28 in the siphons, foot and mantle. Concentrations in these organs then remained relative stable until day 45 for the siphons and in the foot until day 28 after which concentrations decreased (Figure 4-5A and B). The TTX concentrations remained relatively constant in mantle, with notably lower concentrations recorded on the final day of the experiment (150; Figure 4-5C). In contrast, after 10 days, the digestive gland contained only traces of TTX (< 5.1 µg kg^{-1}; Figure 4-5D). The GAMs showed that there was a significant ($p < 0.001$) decrease in TTX concentrations in all the organs over the 150-day experiment. Tukey HSD pairwise comparisons demonstrated that the average TTX concentrations over the experiment was significantly different between all four organs ($p < 0.001$), except between the mantle and foot ($p = 0.76$).

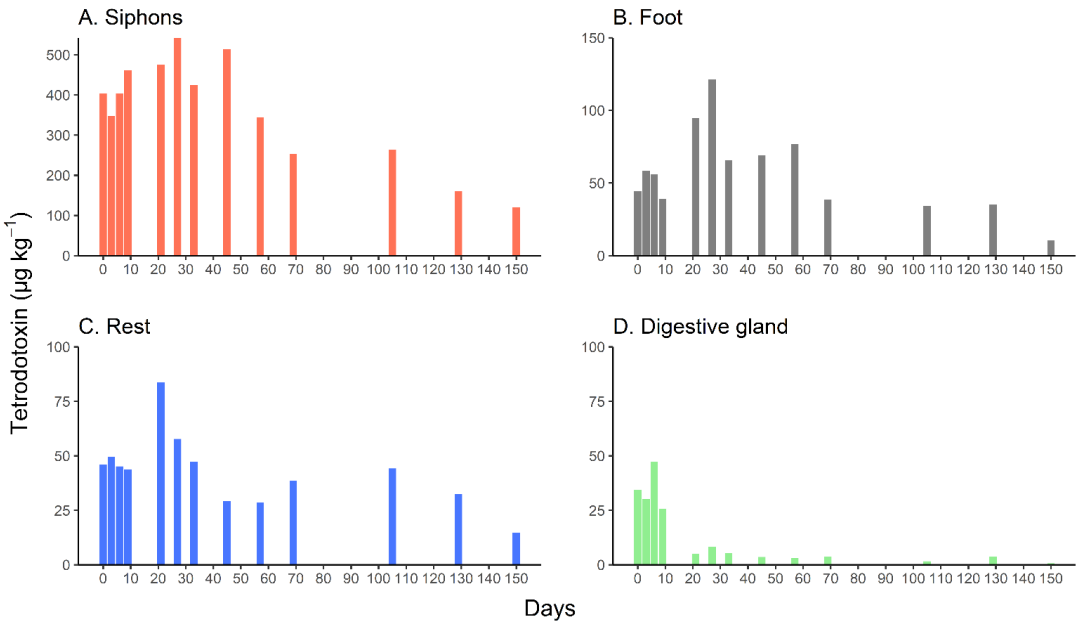


Figure 4-5. Tetrodotoxin (TTX) concentrations, in the organs of *Paphies australis* maintained in captivity and fed a TTX-free diet for 150 days, measured using liquid chromatography-tandem mass spectrometry. Results at each time point are a total of 10 pooled *P. australis* individuals (n = 1).

4.5 Discussion

4.5.1 TTX concentrations in *Paphies australis* populations around New Zealand

All *P. australis* populations sampled around New Zealand contained TTX, albeit only trace amounts were detected in the Akaroa samples. However, there was marked variability in toxin concentrations between regions, with sites from the North Island containing significantly higher amounts than those from the South Island. Wood et al. (2012) found a similar north-south pattern during their study on TTX in the sea slug *Pl. maculata*. Two plausible explanations for the north-south gradient in toxin concentrations are the difference in the abundance of the TTX source, should it be exogenous; or genetically disconnected populations, should the source be endogenous.

The Cook Strait is a 70-km wide body of water separating the North and South Islands of New Zealand (Bowman, Kibblewhite et al. 1983). The winds are highly variable, often leading to sharp accelerations and gale conditions (Brodie 1960), resulting in Cook Strait having some of the world's strongest tidal and ocean

currents, which may limit the exchange of planktonic organisms resulting in different diets between North and South Island populations (Walters, Gillibrand et al. 2010).

The strong currents of Cook Strait also significantly alter the structure of the sea shelf, which can have significant biological consequences (Bowman, Kibblewhite et al. 1983). For example, natural populations of bivalves such as *Paphies subtriangulata* and *Perna canaliculus* from the North and South islands show distinct genetic differentiations (Ross, Hogg et al. 2009, Gardner, Bell et al. 2010). Studies on the population genetics of *P. australis* suggest three distinct genetic groups (Northern, South Eastern and South Western groups; (Hannan 2014). Thus, a further possible reason for varying TTX-concentrations among geographic regions is that the genetically different groups may have varying propensities to uptake and accumulate TTX.

The trend for increasing concentrations of TTX with decreasing latitudes (i.e., moving closer to the equator) and warmer water has been observed previously with higher TTX concentrations recorded in the sunniest and warmest parts of southern England (Turner, Dhanji-Rapkova et al. 2017). In Europe, high TTX has also been found in warm waters, including Greece (Vlamis, Katikou et al. 2015) and Portugal (Rodriguez, Alfonso et al. 2008, Silva, Azevedo et al. 2012). The latitudinal/temperature associated patterns identified in the present study, in concert with the relationship with warmer climates in the Northern Hemisphere, could indicate the presence of a warm-water-adapted TTX-producer, or that the biosynthesis of TTX or the synergistic relationships between the TTX-producer and host organism is enhanced under these conditions (Pratheepa, Alex et al. 2016). The study from Gerssen et al. (2018) also identified a peak in TTX in the bivalves just prior to summer in The Netherlands. This led them to suggest that TTX is produced exogenously. In this study, the Waihi estuary and the Tauranga sampling sites had significantly (p = 0.008) different concentrations of TTX despite being less than 100 km apart. In this study, samples were collected at different times of the year: Tauranga in summer (February) and Waihi in winter (August). These results correlate with the hypothesis that a TTX producer might be most prolific in warmer waters, or that TTX synthesis is upregulated at hotter temperatures. In the present study, water temperatures at sampling locations were not measured, hence we were

unable to determine if there were relationships between TTX concentrations and waters temperatures.

As global sea temperature rises, there may be a pressing need to enhance monitoring of TTX in edible marine species in locations where the toxin has traditionally been absent. The European Food Safety Authority recently released a scientific opinion (Knutsen, Alexander et al. 2017) on the risk to public health related to the presence of TTX in marine bivalves and gastropods. They suggest that TTX levels above 44 µg kg^{-1} in bivalves would be a concern for consumers when a large portion size (> 400 g) is consumed. In the present study, the *P. australis* from the Hokianga Harbour (median concentration 47.6 µg kg^{-1}) are above this threshold but there have not been any official reports of intoxication from this site to date.

Statistical analyses of the organ data were not possible because the organisms collected were usually small (< 25 mm long) and once dissected there was not enough biomass for individual analysis, thus the five samples were pooled. However, the results from all sites consistently demonstrated that the siphons contained the highest amounts of TTX. These results concur with the study from Biessy et al. (2018), where immunohistochemistry was used to show the presence of TTX within cells in the siphons, and LC-MS/MS to demonstrate that toxin concentrations (on a per mass basis) were significantly higher in the siphon than other organs. Other clam species like *Paphies subtriangulata* and *Saxidomus gigantea* have been shown to sequester saxitoxin, another neurotoxin with similar mode of action and toxicity to TTX, in their siphons (Smolowitz and Doucette 1995, MacKenzie, White et al. 1996). As noted by Biessy et al. (2018), the accumulation of TTX in siphon tissue could suggest that TTX is present in the seawater or within planktonic organisms that are filtered while feeding, possibly indicating that the toxin comes from an exogenous source.

4.5.2 TTX concentrations in *Paphies australis* maintained in captivity

This study demonstrated that when *P. australis* containing TTX are kept in a controlled environment and fed a TTX-free diet, the toxin significantly depurated over 150 days. Bivalve species can be classified as rapid (weeks to detoxify; up to 15% toxin loss day^{-1}) or slow detoxifiers (months to years to detoxify; ≤ 3% loss day^{-1}). When averaged out of over the 5 months of the experiment, the depuration

rate of TTX in *P. australis* was 0.23 μg kg^{-1} per day (0.41% loss day^{-1}), classifying them as a slow detoxifier for TTX (Bricelj and Shumway 1998). Toxin biotransformation, which may lead to changes in net toxicity, varies greatly among species and between biotoxins. Some species exhibit rapid enzymatic decarbamoylation (e.g., the clam *Protothaca staminea* in presence of saxitoxin), whereas other bivalves (e.g., *M. edulis*) show limited toxin metabolism and thus are useful indicators of the toxigenic source (Bricelj, Haubois et al. 2012).

Similar studies on TTX persistence and depuration in marine and terrestrial organisms fed non-toxic diets concur with the findings of the present study. Wood et al. (2012) demonstrated that the TTX concentrations in the sea slug *P. maculata* significantly decreased overtime when kept in aquariums for 126 days with the stomach depurating the fastest (6.7 μg kg^{-1} day^{-1}). Yotsu-Yamashita et al., (2012) showed that newts *Notophthalmus viridescens* lost their toxin after being kept in captivity and fed a TTX-free diet over several years. Recently, Turner et al. (2017) investigated the depuration of TTX in wild populations of marine bivalves in the field and found that mussels (*M. edulis*) and oysters (*C. gigas*) rapidly depurated the toxin with 75% of the total lost in four weeks (from 80 to 20 μg kg^{-1}), though this in uncontrolled conditions. The authors also showed that uptake and depuration patterns were species-specific. One important consideration with the Turner et al. (2017b) study is that it was a non-controlled field study and the difference in TTX concentrations might be due to the natural variability between individuals, and the source of TTX may still be present in the environment and being accumulated, making accurately measuring depuration rates difficult. In a recent study, Biessy et al. (2018) demonstrated that TTX was mostly stored in the siphons of *P. australis*, potentially a defensive strategy (Kvitek, 1991), but it is important to note that not all bivalves have functioning siphons. Those that live on or above the substrate (e.g., *M. edulis* or *C. gigas*) do not need protruding siphons (Gibson, Atkinson et al. 2008), which could explain why some species might hold onto TTX more than others.

Tetrodotoxin was detected in all organs of *P. australis* investigated in the present study, and in the test animals the siphons again contained the highest amount of toxins, which remained the case for the entire study period. As noted above, because of the small size of the organs and the need to pool them to obtain a sufficient sample for TTX analysis. This also makes it challenging to determine if patterns

such as the increase in TTX concentrations in the digestive glands and the mantle (only one point in time) were a real shift, analytical or natural variability. The increase in TTX concentrations in the siphons and the foot was more gradual (over six time points) and suggests a migration of the toxin between organs, possibly from the digestive tract and mantle to the siphons and/or the foot. Only one other study has investigated TTX depuration from different organs: Wood et al. (2012) showed that the slugs *P. maculata* contained TTX in all their organs with the highest concentrations initially occurring in the stomach and migrating to the gonads, which is then transferred to the eggs, where TTX may act as a chemical defence deterring predators from eating the egg masses. Other studies on marine organisms have also proposed that the ecological function of TTX is a chemical defence (reviewed in Williams 2010). For instance, the snails *Natica lineata* released TTX from their 'muscle cavity' upon disturbance (Hwang, Chueh et al. 1990); some species of pufferfish secrete TTX in their skin after electrical stimulation (Kodama, Ogata et al. 1985); and gonads (i.e., ovaries, oocytes, eggs) of many toxic pufferfish and larvae of flatworms often harbour the highest concentrations of TTX (Williams 2010).

Tetrodotoxin has previously been detected in the intestinal epithelium, the rectum and the stomach wall of *P. australis* (Biessy, Smith et al. 2018), leading us to hypothesize that the bivalves might obtain TTX from their diet and that over a few days or weeks, the toxin might migrate to other organs such as the foot and siphons. Over the experimental period, TTX did also depurate from the foot and siphons, potentially indicating that *P. australis* are not endogenously producing TTX, rather they accumulate the toxin from an exogenous source. Previous studies have shown that TTX can accumulate through the food chain: for example, Lin and Hwang (2001) demonstrated the starfish *Astropecten scoparius* accumulates TTX from consuming the TTX-containing gastropod *Umbonium suturale*. Kono et al. (2008) demonstrated that the non-toxic pufferfish *Fugu niphobles* accumulated TTX when fed a highly toxic diet and recent study (Itoi, Ueda et al. 2018) confirmed that the pufferfish *Takifugu niphobles* becomes toxic after feeding on toxic flatworms. The mechanism of depuration in marine bivalves remains unknown but it is possible that the molecule is broken down and/or metabolised inside the organisms at a slow rate.

We cannot completely rule out the possibility that *P. australis* endogenously produces TTX and that this is enhanced when exposed to certain abiotic or biotic triggers (e.g., pH, temperature, predators), a pattern which has been shown for toxin production in other bacteria and microalgae. For instance, hepatotoxin production in cyanobacteria is thought to be influenced by parameters such as pH, light or temperature (Neilan, Pearson et al. 2013); and brevetoxin production can be triggered by a decrease in salinity in the dinoflagellate *Karenia brevis* (Errera and Campbell 2011). However, the results from the spatial and the depuration studies undertaken to date provide compelling evidence to support the hypothesis of an exogenous source of TTX in *P. australis*. When collated with results from similar depuration studies in other marine organisms such as sea slugs (Wood, Casas et al. 2012) or pufferfish (Noguchi, Arakawa et al. 2006), which span a range of trophic levels, live in varying habitats, in distinct parts of the globe, and whose diets would be markedly different from *P. australis,* the evidence suggests an ubiquitous microbial source as the likely producer; a suggestion that has been touted for many decades but not conclusively proven (Khor, Wood et al. 2013, Bane, Lehane et al. 2014, Magarlamov, Melnikova et al. 2017). Because *P. australis* are stationary filter feeders, they may prove a more amenable organism to study TTX-containing dietary organisms. Although the source of TTX may be relatively prevalent, we suggest not all organisms can accumulate this toxin, for example, when other New Zealand clam species including *Austrovenus stutchburyi* and *Paphies subtriangulata*, were collected at the same sites as toxic *P. australis* individuals as part of this study, no TTX was detected (Biessy, unpublished data). We hypothesise that *P. australis* contains unique TTX-binding proteins similar to those found in the pufferfish *Fugu pardalis* (Yotsu-Yamashita, Sugimoto et al. 2001) or the crab *Hemigrapsus sanguineus* (Nagashima, Yamamoto et al. 2002), that allow them to store the toxin in different organs.

CHAPTER 5

Seasonal and spatial variations in bacterial communities from tetrodotoxin- and non-tetrodotoxin-bearing clams

5.1 Abstract

Tetrodotoxin (TTX) is one of the most potent naturally occurring compounds and is responsible for many human intoxications worldwide. *Paphies australis* are endemic clams to New Zealand containing varying concentrations of TTX. Research suggests that they accumulate the toxin exogenously, but the source remains uncertain. The aim of this study was to identify potential bacterial TTX-producers by exploring differences in bacterial communities in two organs of *P. australis*: the siphon and digestive gland. Samples from the digestive glands of a non-toxic bivalve *Austrovenus stutchburyi* that lives amongst toxic *P. australis* populations were also analysed. Bacterial communities were characterized using 16S ribosomal RNA gene metabarcoding in *P. australis* sourced monthly from the Hokianga Harbour, a site known to have TTX-bearing clams, for one year, and from ten sites with varying TTX concentrations around New Zealand, and in *A. stutchburyi* from the Hokianga Harbour. TTX was detected in *P. australis* from sites all around New Zealand and in all *P. australis* collected monthly from the Hokianga Harbour with an average of 150 μg kg^{-1} over the year of sampling in the but no TTX was detected in the *A. stutchburyi* samples from the same site. Bacterial species diversity differed amongst sites ($p < 0.001$) and the diversity in siphon samples was significantly higher than in digestive glands ($p < 0.001$). Spirochaetaceae (4-60%) and Mycoplasmataceae (16-78%) were the most abundant families in the siphons and the digestive glands, respectively. The bacterial communities were compared between sites with the lowest TTX concentrations and the site with the highest TTX concentrations, and the core bacterial communities from TTX-bearing individuals were analysed. The results from both spatial and temporal studies corroborate with previous hypotheses that *Vibrio* and *Bacillus* could be responsible for the source of TTX in bivalves but also indicate that marine cyanobacteria, in particular picocyanobacteria (e.g., *Cyanobium*, *Synechococcus*, *Pleurocapsa* and *Prochlorococcus*), should be investigated further as potential TTX producers.

5.2 Introduction

Tetrodotoxin (TTX) is one of the most potent naturally occurring neurotoxins in the world (Noguchi et al., 2011). This toxin was thought to occur only in tropical to sub-tropical climates but has now been reported from more temperate climates in the Pacific and Mediterranean (Lago, Rodríguez et al. 2015, Biessy, Boundy et al. 2019). Tetrodotoxin is found in a wide variety of phylogenetically unrelated terrestrial, marine and freshwater organisms (Bane, Lehane et al. 2014) and the consumption of seafood, primarily pufferfish and gastropods, contaminated with TTX has resulted in many human intoxications (Noguchi et al., 2011). It was first identified in marine bivalve Japanese scallops, *Pactinopecten yessoensis*, in 1993 (Kodama, Sato et al. 1993), but recently there has been an increase in detection and published reports with TTX now identified in ten bivalve species from seven countries (Biessy, Boundy et al. 2019). The detection of TTX in harvested edible bivalves has led to concerns about health risks for human consumers and prompted further studies worldwide.

Despite substantial research over three decades, the origin of TTX remains uncertain (Chau, Kalaitzis et al. 2011). The two most common hypotheses regarding its origin are: (1) it is endogenously produced by symbiotic bacteria, or (2) it is exogenously accumulated through dietary sources (e.g., bacteria, microalgae). Exogenous or symbiotic bacteria are commonly touted as the most likely TTX-producers, with a wide diversity of species and strains implicated. There are reports in the literature of at least 150 TTX-producing bacterial strains, with *Vibrio* species comprising more than 30% of records, followed by *Bacillus*, *Pseudomonas*, *Actinomyces* and *Micrococcus* (Turner, Fenwick et al. 2018, Katikou 2019). However, it is estimated that less than 1% of all bacteria are culturable, making isolation, culturing and confirmation of toxin production in bacteria a significant challenge (Chau, Kalaitzis et al. 2011). Studies have claimed to isolate bacteria that potentially produce TTX, but the concentration of toxin identified in laboratory cultures is usually low compared to amounts in the host animals. For example, 184 ng g^{-1} of TTX was reported from an isolated *Vibrio* sp. in comparison to 36 µg g^{-1} in the tissue of its host, the sea snail *Nassarius semiplicatus* (Wang, Yu et al. 2008). Additionally, there are indications that some of the methods (e.g., high performance-liquid chromatography and gas chromatography-mass spectrometry)

used to analyse the TTX-producing bacteria in culture generate false positives (Matsumura 1995).

Limited research has been undertaken on the source of TTX in marine bivalves but, as in other organisms, available evidence indicates an exogenous source (Biessy, Smith et al. 2018, Biessy, Boundy et al. 2019). Gammaproteobacteria, particularly *Vibrio* and *Pseudomonas* species, have been linked to the accumulation of TTX in bivalves, with two recent studies finding correlations between the presence of *Vibrio*, *Pseudomonas* and TTX in shellfish (Turner, Dhanji-Rapkova et al. 2017, Leão, Lozano-Leon et al. 2018). However, these studies were unable to culture any TTX-producing bacteria from the shellfish samples. The hypothesis that bacteria or microalgae are the source of TTX in these organisms is also fuelled by reports of toxic episodes in bivalves being more prevalent during warmer months, particularly in late spring in Europe (Gerssen, Bovee et al. 2018, Leão, Lozano-Leon et al. 2018) and New Zealand (Biessy, Smith et al. 2019). This may indicate seasonal changes in bacterial communities and an increase in warm-water adapted TTX-producing microorganisms.

The application of molecular genetics-based techniques to characterize entire communities (e.g., metabarcoding) is escalating rapidly (Taberlet, Coissac et al. 2012, Deiner, Bik et al. 2017). Metabarcoding is a culture-independent technique that enables taxa in an environmental sample or organ to be identified simultaneously and has previously been used as a tool to investigate the source of TTX by characterizing the core bacterial communities of toxin-bearing organisms. Salvitti et al. (2017) used metabarcoding to investigate the foregut content of the highly toxic, carnivorous sea slug *Pleurobranchaea maculata* from New Zealand. The authors found a high abundance of sequences taxonomically related to Cnidaria and Annelida, taxa known to contain neurotoxins that could have been ingested by *P. maculata*. Turner et al. (2018) studied the bacterial communities of the TTX-bearing nemertean worm *Cephalothrix simula* and showed the prevalence of a large number of bacterial genera previously associated with TTX production including *Alteromonas*, *Vibrio* and *Pseudomonas*. In a recent study, Melnikova and Magarlamov (2020) investigated the core bacterial communities of TTX-bearing and non-bearing nemertean worms from Japan and demonstrated that toxic individuals tend to have more potential TTX-producing bacterial strains (e.g.,

Pseudomonas, *Vibrio* and *Bacillus*) in their microflora but they were unable to identify a possible TTX producer. One issue with microbiome analyses of motile organisms is that it only provides a 'snapshot' of what the organisms have ingested at one point in time and location. These organisms may move from the site of TTX-ingestion, making it challenging to identify TTX sources in the environment. Analysis of bacterial communities of sedentary animals, such as marine bivalves, would provide novel insights into the source of TTX, especially if investigated over spatial and temporal scales.

In 2011, McNabb et al. (2014) recorded high concentrations (800 µg kg^{-1}) of TTX in an endemic marine clam *Paphies australis* in Whangapoua (Coromandel, New Zealand). Immunohistochemistry and chemical analyses of *P. australis* individuals from Hokianga Harbour (Northland, New Zealand) revealed that the siphons and organs involved in feeding contained the highest concentrations of TTX (means of 403 and 34 µg kg^{-1}, respectively), providing evidence to support the hypothesis of an exogenous source (Biessy, Smith et al. 2018). This hypothesis was further strengthened during an experiment where *P. australis* individuals were maintained in captivity for 150 days and showed significant depuration of TTX. Tetrodotoxin present in the digestive glands at the start of the experiment depurated rapidly and only traces remained after 21 days (Biessy, Smith et al. 2019). Furthermore, *P. australis* sourced from different locations around New Zealand have significant variations in TTX concentrations (Biessy, Smith et al. 2019). The bivalves collected from warmer sites (at lower latitudes) contained higher TTX concentrations than the ones from the colder regions.

The overarching goal of the present study was to further investigate the source of TTX in *P. australis.* We used metabarcoding to characterize bacterial communities in the digestive glands and siphons sourced monthly from one site with high concentrations, for one year, and from single time points at ten sites with *P. australis* that contained varying TTX concentrations. The aims of the study were: 1) to explore differences in bacterial communities between *P. australis* populations with high and low TTX concentrations and a non-toxic bivalve species, the cockle *Austrovenus stutchburyi*, found in the same habitat at one site; 2) to evaluate the core bacterial communities of *P. australis* at one site where TTX concentrations were relatively stable over time; and 3) use the genetic information from bacterial

amplicon sequence variants and corresponding toxin data to identify potential TTX-producing bacterial species. Intra- and inter-species variation in toxin concentration provides a unique opportunity to examine the differences in bacterial communities of these populations and identify any specific bacterial communities correlated with toxin concentrations in the host.

5.3 Materials and methods

5.3.1 Sample collection

5.3.1.1 *Spatial study*

Paphies australis (n = 8 per site) were collected from ten sites spanning the length of New Zealand between September 2017 and February 2019 (Appendix 8, Figure A8.1). The *P. australis* were chilled (ca. 8 °C) and sent overnight to the laboratory (Cawthron Institute, Nelson, New Zealand). Once in the laboratory, three of the eight individuals were aseptically dissected for genetic analysis: the siphons and digestive glands were placed in individual tubes and stored frozen (-20 °C) until further analysis. The remaining five *P. australis* from each site were used for toxin extraction. *Austrovenus stutchburyi* were identified amongst the *P. australis* beds in the Hokianga Harbour and were also collected (n = 8) in September 2017 for further analysis. Five *A. stutchburyi* were used whole for toxin extraction and the remaining (n = 3) were aseptically dissected and the digestive glands were placed in individual tubes and stored frozen (-20 °C) until DNA was extracted.

5.3.1.2 *Temporal study*

Paphies australis were collected from the Hokianga Harbour (Northland, New Zealand, 35°28’S, 173°24’E) monthly from April 2017 to March 2018 (except May 2017, n = 11 sampling events), chilled and sent to the laboratory overnight. *Paphies australis* from this location had previously been shown to contain TTX (Biessy, Smith et al. 2018). *Paphies australis* used for the temporal study were collected as part of a larger survey investigating TTX concentrations in recreationally harvested bivalves in New Zealand (Boundy, Biessy et al. 2020). The *P. australis* received each month were pooled together to follow the standard operating procedure which is used in the accredited laboratory that undertook the analysis of these samples.

Three *P. australis* were put aside each month and were then dissected and stored for further genetic analysis as described above.

5.3.2 Toxin extractions

Tetrodotoxin extraction was performed as previously described in Biessy et al. (2018). Briefly, for the spatial samples, *P. australis* and *A. stutchburyi* were shucked and individually weighed (ca. 0.5-3.0 g) and placed in a sterile tube (50 mL) with a corresponding volume (ca. 500-3,000 µL) of Milli-Q water containing 1% acetic acid. Organisms were homogenized (Ultra-Turrax®, IKA®, NC, USA) for 45 s. The temporal samples were freshly opened, left to drain (5 min), pooled together for homogenisation (Ultra-Turrax®) for 5 min and a sub-sample was weighed (5 g) and placed in a sterile tube containing Milli-Q water and 1% acetic acid (5 mL). Subsequently extraction for both the spatial and temporal samples followed the same procedure. The tubes were boiled (5 min) and cooled in an ice bath (5 min) before briefly vortexing. Samples were centrifuged (3,200 × *g*, 10 min) and 0.5-1 mL of the supernatant transferred to a centrifuge tube (1.7 mL) containing 25% ammonia (2.5-5 µL; Honeywell, Seelze, Germany). Samples were then centrifuged (17,000 × *g*, 1 min) and the supernatant subjected to the graphitised carbon solid phase extraction (SPE) method as described in Boundy et al. (2015) using Supelclean™ ENVI-Carb 250 mg/3 mL cartridges (Sigma-Aldrich, MO, USA). Tetrodotoxin was analysed and quantified by liquid chromatography tandem-mass spectrometry analysis as described by Turner et al. (2017). The toxin results for samples collected in 2017 and 2018 were previously published by Biessy et al., (2019) but are integrated with the molecular dataset in this study.

5.3.3 Statistical analysis

Statistical analyses for toxin concentrations were performed using the R statistical package (RCoreTeam 2020). Normality was checked through inspection of Quantile-Quantile plots and conducting a Shapiro-Wilk test. As the data deviated from a normal distribution the spatial data were log transformed. Levene's test was used to assess the equality of variance in TTX concentrations between sites (Carroll and Schneider 1985). Statistical differences in TTX concentrations between sites was assessed using a one-way analysis of variance (ANOVA; Factor: Site; 10

levels). A Tukey's honestly significant difference (HSD) post-hoc test was used to identify which sites were responsible for the significant differences.

5.3.4 Metabarcoding

5.3.4.1 *DNA extractions and PCR*

Dissected organs from both studies were individually placed in the first tube containing bashing beads of the DNA extraction kit (DNeasy Powersoil Pro kit, Qiagen, MD, USA). The DNA was then extracted following manufacturer's instructions using an automated homogenizer (1600 MiniG Automated Tissue Homogenizer and Cell Lyser, SPEX SamplePrep, NJ, USA) and a robotic workstation for DNA extraction (QIAcube, Qiagen). Negative extraction controls were performed every 23 samples. Amplification and sequencing PCR amplicons were generated covering the V3 and V4 regions of the 16S ribosomal RNA (rRNA) gene using the primer sets as described in Klindworth et al. (2013) and were modified to include Illumina™ overhang adaptors following the dual-indexing method from Kozich et al. (2013): Bact341F- 5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG –3' and Bact785R- 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C –3'. PCR reactions were undertaken in triplicate with 450 nM of each primer, 25 µL of 2X MyFi™ Mix (Bioline, UK), ca. 5 ng of DNA, and sterile water for a total reaction volume of 50 µL. Cycling conditions were: 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 45 s, and a final extension of 72 °C for 7 min. Triplicates of PCR products were pooled and visualized on 1.5% agarose gel with Red Safe™ DNA Loading Dye (Herogen Biotech, USA) and UV illumination. PCR negatives were run to assess for contamination during the PCR steps. The PCR products were purified, cleaned of primer dimers and normalized using SequalPrep™ Normalisation plate (ThermoFisher, MA, USA), and submitted to Auckland Genomics (University of Auckland, New Zealand) for library preparation. Sequencing adapters and sample-specific indices were added to each amplicon via a second round of PCR using the Nextera™ Index kit (Illumina Inc., USA). Amplicons were pooled into a single library and paired-end sequences (2 × 250 bp) generated on a MiSeq® instrument. The sequencing libraries were prepared following the Illumina 16S Metagenomics

Library Prep manual with the exception that after the indexing PCR 5 μL of each sample (including three water samples acting as sequencing blank) was pooled and a single clean-up was undertaken on the pool instead of samples being individually cleaned. Quality control was undertaken using a bioanalyzer before the library was diluted to 4 nM and denatured. A 15% PhiX spike was used and the final loading concentration was 7 ρM. Sequence data were automatically demultiplexed using MiSeq® Reporter (version 2, Illumina Inc.), and forward and reverse reads assigned to samples.

5.3.4.2 *Bioinformatics*

Raw reads were processed, subsequent to primers being removed with *cutadapt* (Martin 2011), using the *DADA2* package (Callahan, McMurdie et al. 2016) within R. Reads were truncated to 228 and 230 bp and filtered with a maxEE (maximum number of "expected errors") of 2 and 4 for forward and reverse reads respectively (reads not reaching this threshold were discarded). *DADA2* constructs a parametric error matrix (based on the first 10^8 bps in the dataset), the samples are dereplicated and sequence variants for the forward and reverse reads are inferred based on the derived error profiles from the samples. Singletons observed in the inference step are discarded. Subsequently, paired-end reads were merged with a maximum mismatch of 1 bp and a required minimum overlap of 10 bp. Forward and reverse reads, which did not merge were not included in further analysis. Chimeras were removed using the function removeBimeraDenovo. The resulting chimera-checked, merged Amplicon Sequence Variants (ASV) were used for taxonomic classification using the SILVA v132 database (Pruesse, Quast et al. 2007) within the *DADA2* package, which is based on the rdp classifier (Wang, Garrity et al. 2007) with a bootstrap of 50. The results were parsed into a table using the *phyloseq* package (McMurdie and Holmes 2013), and reads assigned as eukaryotes, chloroplasts and mitochondria were removed. Negative controls were assessed and the sum of reads from contaminating ASVs was subtracted from the samples. Samples were subsampled to an even depth of 9,500 sequences for comparison.

5.3.4.3 *Spatial study*

Phylogenetically annotated 16S rRNA sequences were used to characterize bacterial community composition of each tissue type at the family level. Donut

charts were generated using the package *ggplot2* (Wickham 2016) in R based on the average relative abundance of sequence reads attributed to a given bacterial family within each tissue type and for each site. A two-way permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) was used to examine the potential differences in community composition as a function of tissue types (digestive gland, siphon or cockle) and sampling sites, assessed at the ASV level. PERMANOVA was run on triangular similarity matrices derived from the fourth root transformed data calculating the Bray-Curtis. Visualization of the observed patterns was obtained by means of a principal coordinates analysis (PCoA) using a Bray-Curtis similarity matrix. Alpha diversity numbers were calculated using the estimate_richness script in R. Differences in alpha diversity between tissues were assessed by an analysis of variance (ANOVA) for the 'Observed' diversity index. Comparative analysis of the bacterial communities from sites with low *P. australis* TTX concentrations (Blenheim, Marahau and Riverton) with those from Hokianga Harbour which has *P. australis* with high TTX was undertaken to identify potential members of the bacterial community correlated with changes in TTX concentrations. The second replicate for the Hokianga siphons did not contain enough reads and was lost after rarefaction. The bacterial communities from the digestive glands of both *P. australis* and *A. stutchburyi* were compared using *phyloseq*. Relationships between TTX concentrations and the 20 most abundant ASVs in the 20 most abundant phyla were determined using linear regressions.

5.3.4.4 *Temporal study*

The core bacterial communities of digestive glands and siphons from *P. australis* over a one-year period was identified using the *microbiome* package in R (Lahti, Shetty et al. 2017-2019). ASVs present in at least 70% of samples were chosen as members of the core bacterial communities. A two-way PERMANOVA was used to identify potential differences in community composition as a function of organs and sampling months, assessed at the ASV level. PERMANOVA was run on triangular similarity matrices derived from the fourth root transformed data calculating the Bray-Curtis. Alpha diversity numbers were calculated using the estimate_richness script in R. Differences in alpha diversity between tissues were assessed by an analysis of variance (ANOVA) for the 'Observed' diversity index Visualization of the observed patterns was plotted with PCoA using the Bray-Curtis

similarity matrix. The core bacterial communities of the digestive glands from *A. stutchburyi* were also identified using the *microbiome* package.

5.3.5 Functional characterization

Functional characterization was undertaken by performing a pathway analysis. To achieve this, the reference sequence of each ASV present in each organ and at each site was obtained and replicated to match the relative abundance of the ASV. Metabolic inferences were made for the community by phylogenetic placement of the ASVs sequences using the *Paprica* pipeline (Bowman and Ducklow 2015). *Paprica* contains reference phylogenetic trees based on the 16S rRNA gene of sequenced genomes and query ASVs are placed within this tree. The metabolic functions of published genomes deposited in MetaCyc (Caspi, Foerster et al. 2008), a highly curated database containing metabolic pathways that have been experimentally validated and reported in the scientific literature, were used as a basis for the inference of metabolic functions. Each node within the phylogenetic tree has a consensus genome, derived from the reference genomes comprising the node. ASVs are assigned the inferred functions based on the closest node in the phylogenetic tree. Enzyme codes were obtained from the *Paprica* outputs and a sample × enzyme table imported into *phyloseq.* To assess differences in the enzyme abundance of the bacterial community in high TTX *P. australis* (Hokianga Harbour) and low TTX *P. australis* (Blenheim, Riverton and Marahau) samples from these sites were imported into *DESeq2* (Love, Huber et al. 2014) using the variable TTX concentration. Log2 fold changes were calculated with the function *DESeq* using a parametric fitType and filtered for significance at a 0.01 level.

5.4 Results

5.4.1 Tetrodotoxin analysis

5.4.1.1 *Spatial study*

Tetrodotoxin was detected in *P. australis* from sites all around New Zealand (Figure 5-1A) with TTX as the main congener (> 99%) in all samples. A Levene's test showed that the degree of variance did not differ among sites ($F = 0.59$). One-way ANOVA showed a significant difference between TTX concentrations among all sites ($F = 51.6$, $p < 0.001$), with a Tukey's HSD post-hoc test identifying a complex

overlap between sites (Figure 5-1A). The highest concentrations were measured in *P. australis* from the northernmost site, the Hokianga Harbour from both years tested. Median toxin concentrations in *P. australis* from the South Island sites were significantly lower than the North Island sites. No TTX (< 3 µg kg^{-1}) was detected in the *A. stutchburyi* samples from the Hokianga Harbour site.

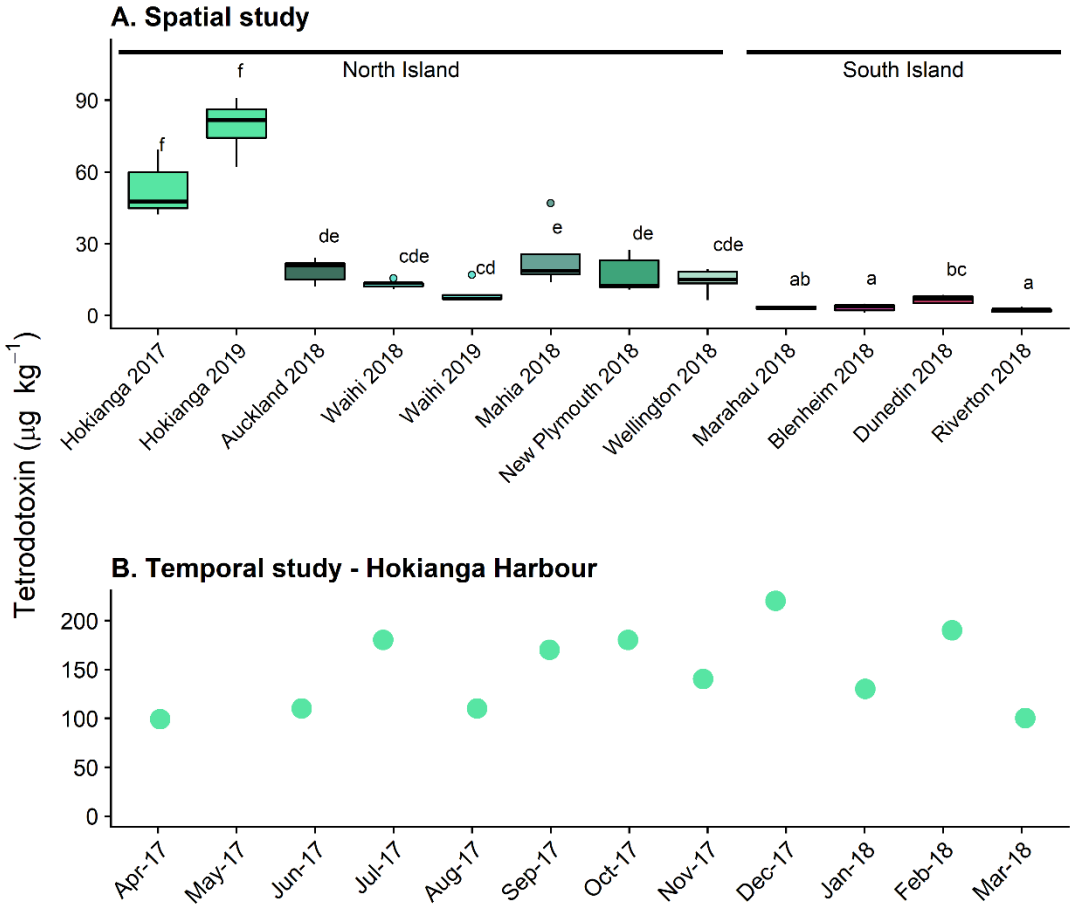


Figure 5-1. Tetrodotoxin concentrations in populations of *Paphies australis,* A) collected around the New Zealand coastline, determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS; n = 5). Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the inter-quartile range. Dots outside the whiskers are considered as outliers, defined as beyond 1.5× interquartile range. Different letters indicate where significant differences occur between sites (one-way ANOVA with Tukey's HSD post-hoc test, $p < 0.001$). Sites are ordered by increasing latitude for each island. Data from 2018 and earlier was previously reported in Biessy et al. (2019). B) Tetrodotoxin concentrations in populations of *P. australis* collected from the Hokianga Harbour site over a one-year period, determined using LC-MS/MS; (n = 1 pooled sample).

5.4.2 Temporal study

Tetrodotoxin was detected in all *P. australis* collected monthly from the Hokianga Harbour between April 2017 and March 2018 (Figure 5-1B). The lowest concentration was 99 μg kg^{-1} in April 2017 and the highest was 220 μg kg^{-1} in December 2017, and the toxin averaged 150 μg kg^{-1} over the year of sampling. Only one sample was extracted for toxin analysis per time point and statistical analyses were not possible. The differences for the Hokianga Harbour toxin concentration between the spatial and temporal study are likely due to the minor methodological differences in toxin extraction.

5.4.3 Metabarcoding

5.4.3.1 *Spatial study*

Across the 64 samples, encompassing the 10 sites and two tissues, the 16S rRNA gene sequencing yielded 6,865,560 read sequences that resulted in a total of 17,188 distinct ASVs after processing and rarefaction.

Species diversity significantly differed amongst the ten sites around New Zealand ($p < 0.001$, $F = 5.9$) but no differences between the two islands were observed (Appendix 8, Figure A8.2). The siphons had bacterial communities with significantly higher alpha diversity than the digestive glands ($p < 0.001$, $F = 65.8$). When examining the most abundant (top ten) families in term of read numbers (Figure 5-2), Spirochaetaceae (ranging from 4 to 60% across all samples) and Mycoplasmataceae (16 – 78%) accounted for the highest proportions of reads in the siphons and digestive glands respectively for both islands. Proteobacteria (purple and pink hues; Figure 5-2) were present at all sites and in both organs. Bacteroidetes (green hues, Figure 5-2) were present at all sites, except Waihi, and in higher abundances in the siphons. Cyanobiaceae were detected in two sites, Hokianga and Mahia (Figure 5-2).

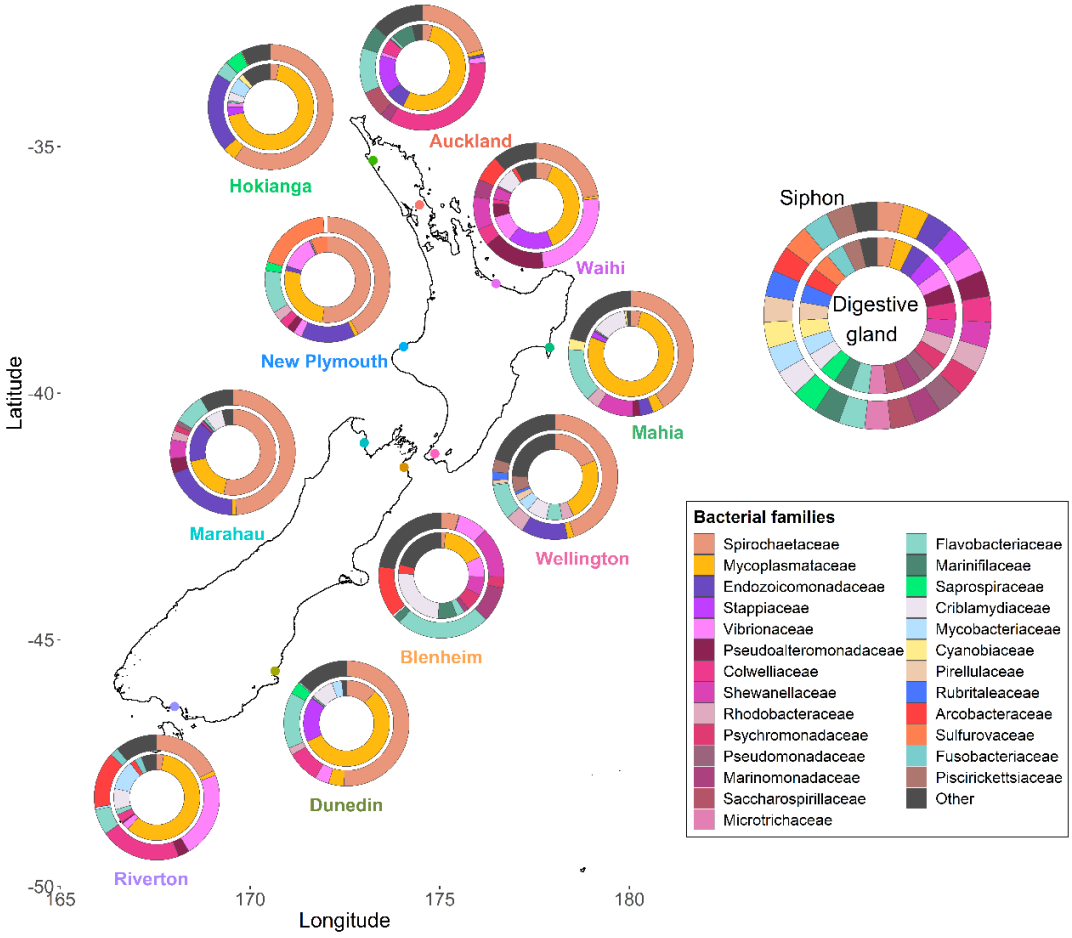


Figure 5-2. Bacterial community composition at the family level from the digestive glands and siphons of the New Zealand endemic clam *Paphies australis* collected from ten sites across the north and south islands. The relative abundance of the ten most abundant families in terms of sequence number for each site and organ are shown. Family are represented by different colours but the phylum Proteobacteria is represented by the purple-pink hues and the Bacteroidetes are represented by the green hues.

Multidimensional scaling based on Bray-Curtis similarities between bacterial communities (using ASVs), showed a clear separation between the two organs from *P. australis* and *A. stutchburyi* digestive glands (Figure 5-3). Comparison of bacterial communities across tissues and sites showed that the communities structure had a significant interaction term (PERMANOVA, $p < 0.001$, $F = 1.61$), suggesting inconsistent differences amongst the sites between organs.

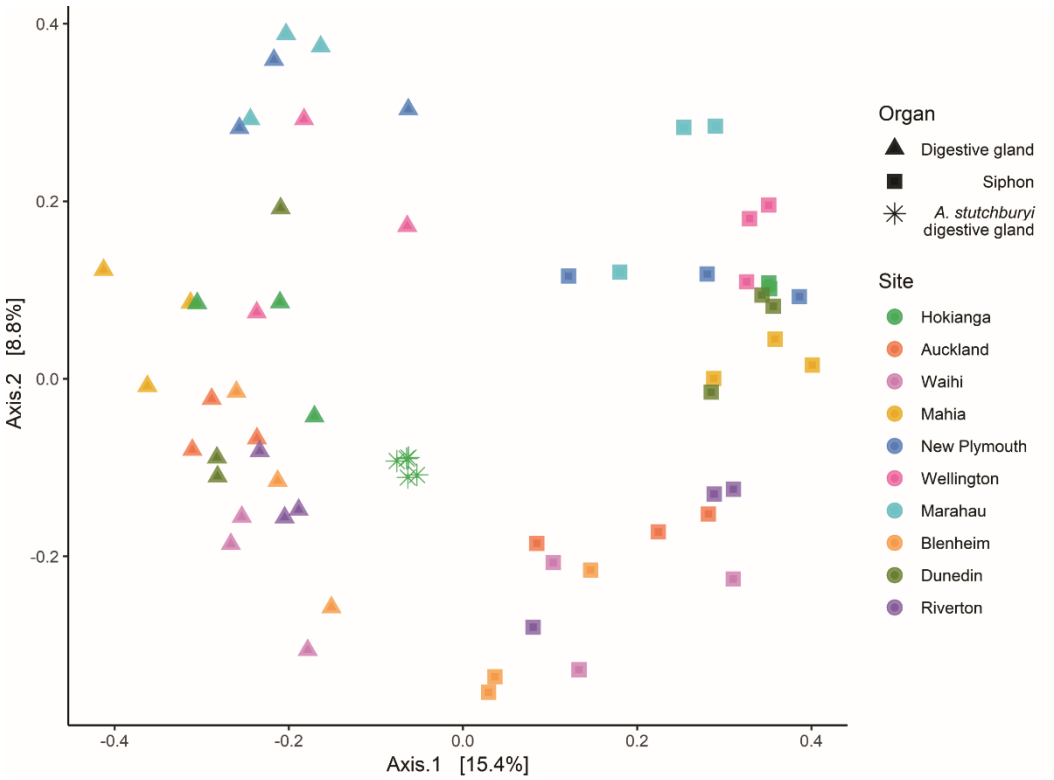


Figure 5-3. Principal Coordinates Analysis based on Bray-Curtis dissimilarities of bacterial community composition of *Paphies australis* organs (unless indicated otherwise) at the Amplicon Sequence Variant level from ten different sites around New Zealand (999 permutations). For each axis, in square brackets, the percent of variation explained was reported. Sites are ordered by increasing latitude for New Zealand. *A. stutchburyi* = *Austrovenus stutchburyi*.

The bacterial community was compared between the pooled sites with the lowest TTX concentrations (Blenheim, Marahau and Riverton) and the ones from the Hokianga Harbour containing the highest toxin concentration. The most abundant bacterial genera in *P. australis* siphons from the Hokianga Harbour (Figure 5-4A) that were not present in low TTX sites were *Kistimonas* and *Spirochaeta* followed by *Pleurocapsa* and *Roseibacillus*. For the digestive glands, *Mycoplasma* was the main genus remaining in the *P. australis* from the Hokianga Harbour after ASVs from the low TTX sites had been subtracted (Figure 5-4B). Other genera present included *Bacillus*, *Clostridium*, *Prochlorococcus* and *Vibrio*. Finally, when comparing the bacterial communities present in the digestive glands of the TTX-bearing *P. australis* to those in the non-toxic *A. stutchburyi*, *Mycoplasma* was also the most abundant genus remaining. *Bacillus*, *Mycobacterium*, *Spirochaeta* and *Vibrio* were also present (Figure 5-4C).

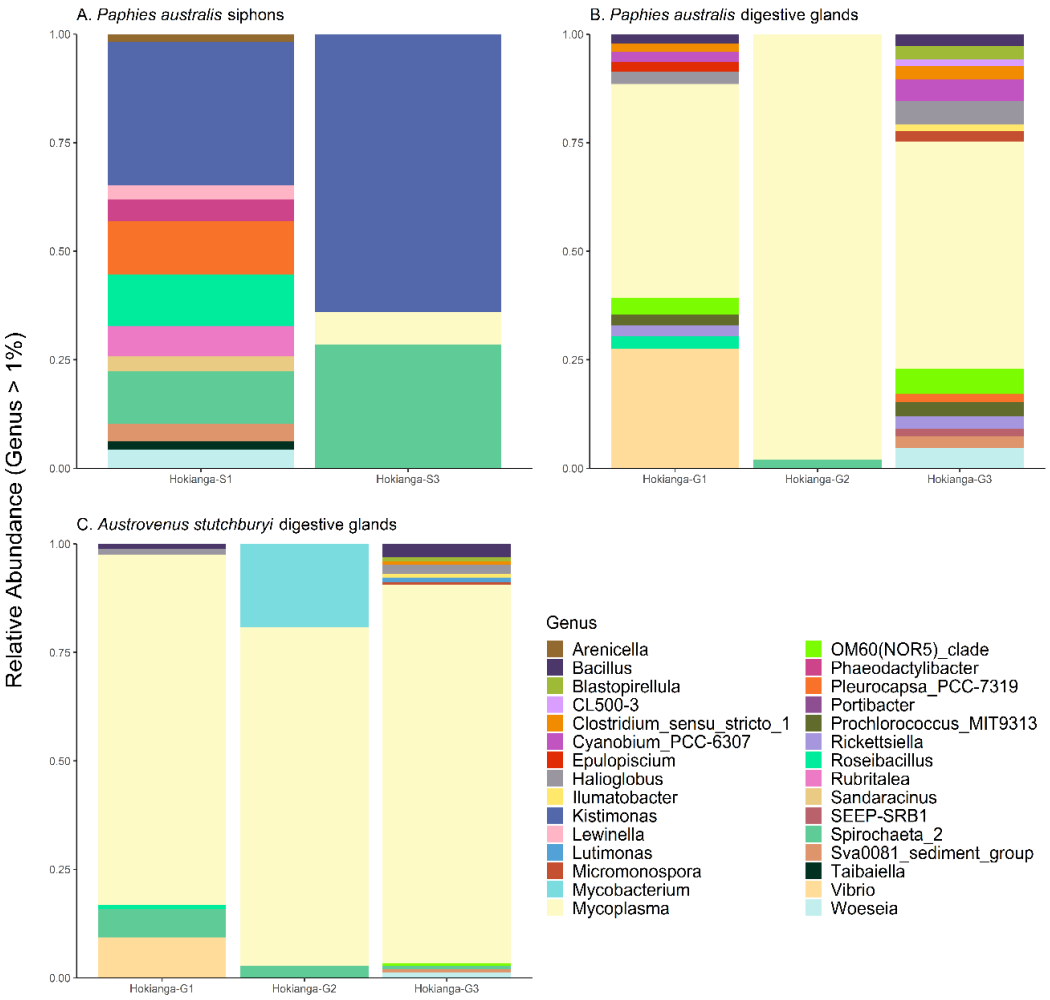


Figure 5-4. Stacked barplots showing the relative abundance of bacterial 16S ribosomal RNA sequences, at the genus level (> 1% of total abundance), that are present in tetrodotoxin (TTX)-bearing *Paphies australis* from the Hokianga Harbour samples but not in tissue samples from sites with low TTX. A) and B) show communities present in *P. australis* siphons and digestive glands, respectively, at the Hokianga site but not in similar tissues from the three lowest TTX sites. C) shows relative abundance of bacteria communities present in TTX-bearing *P. australis digestive glands* and the non-toxic *Austrovenus stutchburyi* digestive gland from the same bed in the Hokianga Harbour. S1: Siphon replicate 1, S3: Siphon replicate 3, G1: digestive gland replicate 1, G2: digestive gland replicate 2, G3: digestive gland replicate 3.

The results from the linear regression analysis identified 17 ASV that were significantly and positively correlated to TTX concentrations; however all relationships were weak ($R^2 < 0.35$; Appendix 8, Table A8.1). The phyla present were: Verrucomicrobia (4 ASVs from the *Rubritaleaceae* family), Tenericutes (4 ASVs from the *Mycoplasmataceae* family), Acidobacteria (2 ASVs), Cyanobacteria (2 ASVs including the genus *Synechococcus*_CC9902), Marinimicrobia (2 ASVs), and one ASVs from each Firmicutes (*Romboutsia* genus), Planctomycetes and Proteobacteria phyla.

5.4.4 Temporal study

5.4.4.1 *Bacterial composition*

Bacterial DNA was isolated from *P. australis* siphons and digestive glands every month for 11 months (n = 3 per month), totalling 62 samples after rarefaction and contamination removal. The 16S rRNA gene sequencing yielded 589,000 reads that resulted in 14,782 distinct ASVs.

Taxonomic community composition of the dominant families (abundance > 5%) by tissue type revealed that similar numbers were present in both *P. australis* tissues (Figure 5-5). There were high relative abundances of Mycoplasmataceae (65-95%) and Spirochaetaceae (60-90%) in the digestive glands and siphons, respectively. In the siphons, Flavobacteriaceae (0-14%) and Mycoplasmataceae (0-12%) were also substantial components of the community occurring in samples from most months. Endozoicomonadaceae (0-18%) were also present half of the year, mostly in winter (July-September). In the digestive glands, Spirochaetaceae (4-37%) and Criblamydiaceae (0-25%) were present in samples from the majority of the sampling period. Stappiaceae (0-8%), Endozoicomonadaceae (0-4%) and Vibrionaceae (0-2%) were less frequently observed in the digestive gland communities (Figure 5-5).

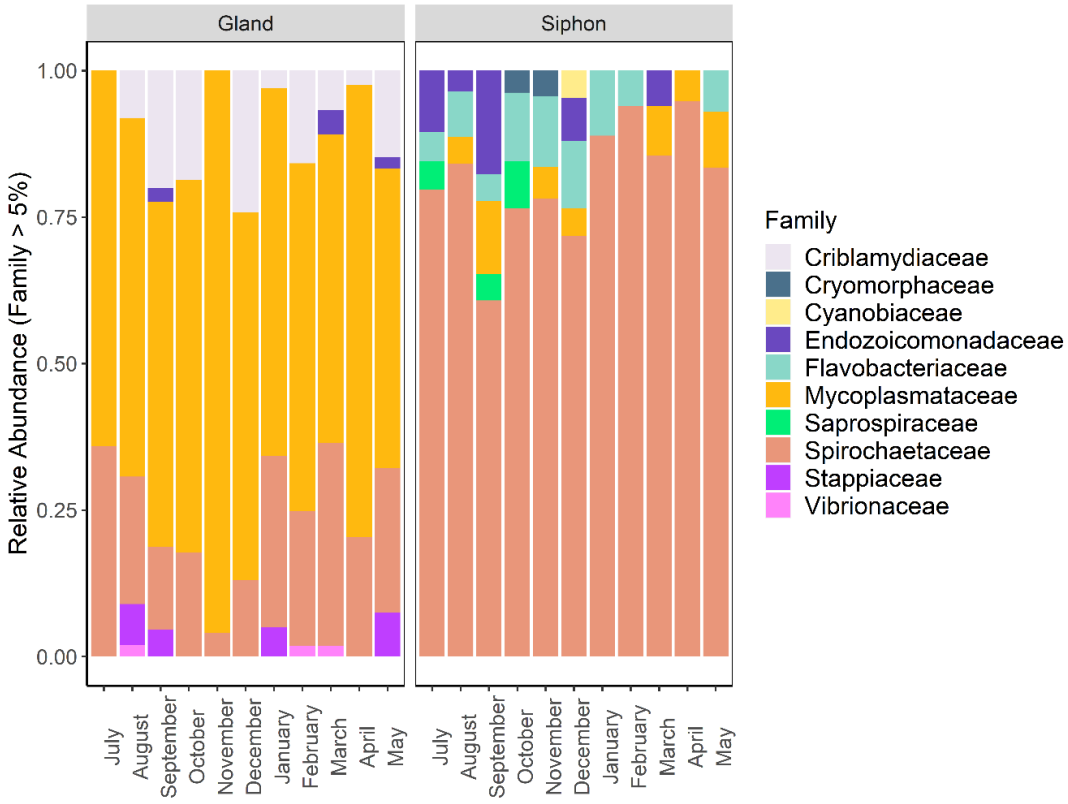


Figure 5-5. Stacked bar plot showing the relative abundance of bacterial 16S ribosomal RNA sequences at the family level (contributing to > 5% of total abundance) in *Paphies australis* digestive glands and siphons from the Hokianga Harbour (Northland, New Zealand), sampled every month from July 2017 to May 2018.

Multidimensional scaling based on Bray-Curtis similarities between bacterial communities (using ASVs), showed a clear separation between the two organs (Figure 5-6). Analysis of overall bacterial community structure revealed a significant difference between siphons and digestive glands (PERMANOVA: $p < 0.0001$, F = 11.5). No significant interaction was found between sampling months and tissue type (PERMANOVA, $p = 0.12$, $F = 1.26$). The digestive glands did not display a seasonal pattern in species diversity but had a significantly lower diversity than the siphons ($p < 0.001$, $F = 181.85$; Figure 5-6). In contrast, the siphons had a higher diversity in warmer months (October to January; Appendix 8, Figure A8.3).

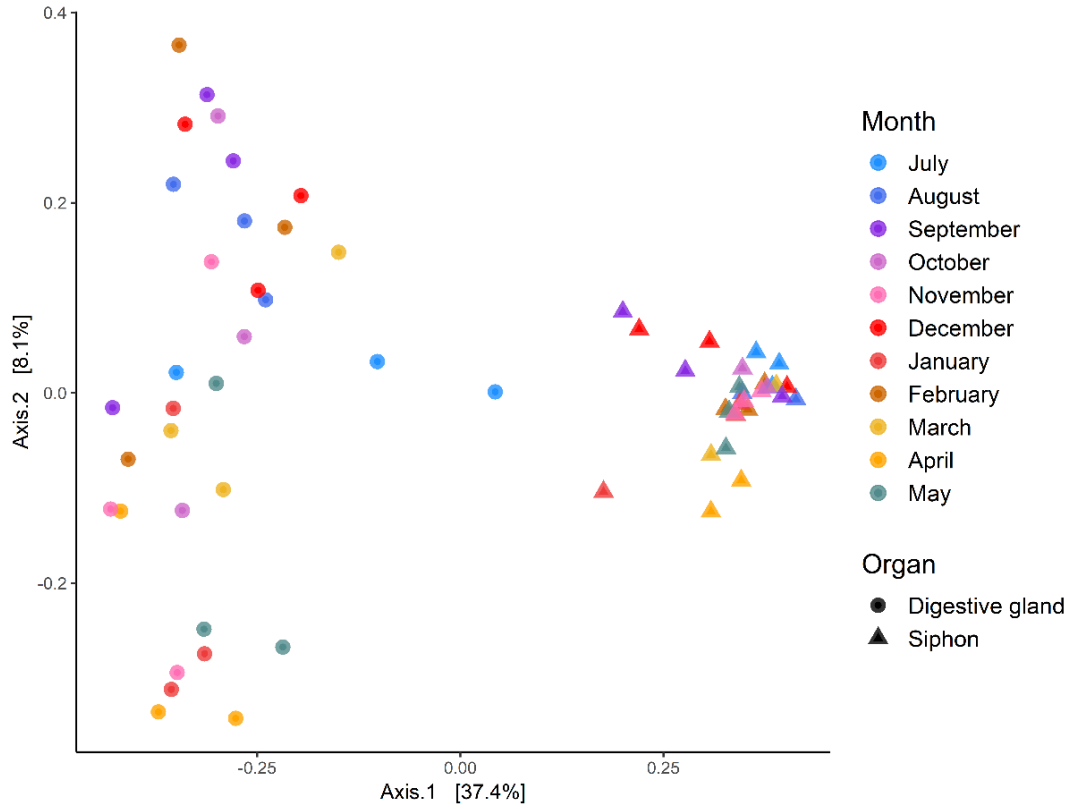


Figure 5-6. Principal Coordinates Analysis based on Bray-Curtis dissimilarities of bacterial community composition of *Paphies australis* organs at the Amplicon Sequence Variant level(999 permutations), sampled every month from July 2017 to May 2018 from the Hokianga Harbour (Northland, New Zealand). For each axis, in square brackets, the percent of variation explained is reported.

5.4.4.2 *Core bacterial communities*

A total of 38 and 74 bacterial ASVs present in at least 70% of all samples were identified as members of the core bacterial communities of the *P. australis* digestive glands and siphons, respectively (Tables 5-1 and 5-2). Mycoplasmatales (15 ASVs total) was the most dominant order in the digestive gland bacterial communities, followed by Spirochaetales (9 ASVs total). Many more orders were present in the core bacterial communities of the siphons from *P. australis* with the Flavobacteriales the most abundant (12 ASVs total), followed closely by Chitinophagales (7 ASVs), Microtrichales (7 ASVs) and Steroidobacterales (5 ASVs; Table 5-2). Only 12 ASVs were present in the core bacterial communities of *A. stutchburyi* digestive glands (Appendix 8, Table A2.2): Clostridiales was the most dominant order (4 ASVs), followed by Mycoplasmatales (3 ASVs).

Table 5-1. Summary of bacterial Amplicon Sequence Variants (ASV) corresponding to the core bacterial communities of *Paphies australis* digestive glands, defined as present in at least 70% of all samples. In bold are the genera detected in more than 90% of all samples. Unclass. = unclassified.

Phylum	Class	Order	Family	Genus	ASVs
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	***Mycoplasma***	14
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	***Spirochaeta_2***	6
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Unclass.	3
Actinobacteria	Actinobacteria	Corynebacteriales	Mycobacteriaceae	*Mycobacterium*	1
Bacteroidetes	Bacteroidia	Chitinophagales	Saprospiraceae	Unclass.	1
Chlamydiae	Chlamydiae	Chlamydiales	Criblamydiaceae	Unclass.	1
Cyanobacteria	Oxyphotobacteria	Synechococcales	Cyanobiaceae	*Synechococcus_CC9902*	1
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	1
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Epulopiscium*	1
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	*Romboutsia*	1
Proteobacteria	Alphaproteobacteria	Rhizobiales	Stappiaceae	Unclass.	1
Proteobacteria	Unclass.	Unclass.	Unclass.	Unclass.	1
Proteobacteria	Alphaproteobacteria	Rhizobiales	Unclass.	Unclass.	1
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Unclass.	1

Table 5-2. Summary of bacterial Amplicon Sequence Variants (ASVs) corresponding to the core bacterial communities of the clam *Paphies australis* siphons, present in at least 70% of all samples. In bold are the genera detected in more than 90% of all samples. Unclass. = unclassified.

Phylum	Class	Order	Family	Genus	ASVs
Bacteroidetes	Bacteroidia	Chitinophagales	Saprospiraceae	Unclass.	7
Proteobacteria	Gammaproteobacteria	Steroidobacterales	Woeseiaceae	*Woeseia*	5
Actinobacteria	Acidimicrobiia	Microtrichales	Microtrichaceae	*Sva0996_marine_group*	4
Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Aquibacter*	4
Actinobacteria	Acidimicrobiia	Microtrichales	Ilumatobacteraceae	*Ilumatobacter*	3
Proteobacteria	Gammaproteobacteria	Unclass.	Unclass.	Unclass.	3
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	***Spirochaeta_2***	3
Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Muriicola*	2
Actinobacteria	Acidimicrobiia	Actinomarinales	Unclass.	Unclass.	2
Cyanobacteria	Oxyphotobacteria	Synechococcales	Cyanobiaceae	***Cyanobium_PCC-6307***	2
Cyanobacteria	Oxyphotobacteria	Synechococcales	Cyanobiaceae	***Synechococcus_CC9902***	2
Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae	Unclass.	2
Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	*Halioglobus*	2
Proteobacteria	Gammaproteobacteria	Gammaproteobacteria	Unknown_Family	Unclass.	2
Proteobacteria	Gammaproteobacteria	UBA10353_marine_group	Unclass.	Unclass.	2
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Unclass.	2
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	*Mycoplasma*	2

Phylum	Class	Order	Family	Genus	ASVs
Acidobacteria	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	*Subgroup_10*	1
Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Eudoraea*	1
Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Lutimonas*	1
Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Polaribacter_4*	1
Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Robiginitalea*	1
Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Winogradskyella*	1
Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Zeaxanthinibacter*	1
Cyanobacteria	Oxyphotobacteria	Leptolyngbyales	Leptolyngbyaceae	Unclass.	1
Cyanobacteria	Oxyphotobacteria	Synechococcales	Cyanobiaceae	*Prochlorococcus_MIT9313*	1
Proteobacteria	Alphaproteobacteria	Rhizobiales	Unclass.	Unclass.	1
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_Incertae_Sedis	*Anderseniella*	1
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Boseongicola*	1
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclass.	1
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Silicimonas*	1
Proteobacteria	Gammaproteobacteria	Arenicellales	Arenicellaceae	Unclass.	1
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Endozoicomonadaceae	*Kistimonas*	1

5.4.5 Functional characterization

Paprica analysis of samples from the Hokianga Harbour, Blenheim, Marahau and Riverton sites, was used to identify metabolic pathways in bacteria related to functional properties of the communities in *P. australis* digestive glands and siphons. Sixteen metabolic pathways were significantly represented in the digestive gland samples and 28 metabolic pathways in the siphon samples from the Hokianga Harbour (Appendix 8, Table A8.3). Many of these metabolic pathways related to bacterial internal cellular processes (e.g., phospholipid or fatty acids biosynthesis), growth and development (e.g., L-Ascorbate, sugar or β-Alanine biosynthesis or degradation), or carbon fixation pathways (i.e., the photosynthetic ability of cyanobacteria present). No pathways known to be directly linked to marine toxin or secondary metabolite production were identified, but several pathways could not be classified.

5.5 Discussion

The endemic New Zealand clam *Paphies australis* is an excellent study organism to investigate the source of TTX. Individual clams are mostly stationary, and samples can be repeatedly collected from the same location over time, reducing the effects of seasons, tides and weather conditions. This study explored seasonal and spatial differences in bacterial communities present in the siphons and digestive glands of *P. australis* and aimed to identify any relationships with TTX-production. To achieve this, the bacterial communities were characterized using metabarcoding in *P. australis* sourced monthly from the Hokianga Harbour for one year and the bacterial communities of *P. australis* from ten sites with varying TTX concentrations spanning the length of New Zealand were compared. Previous studies have shown that *P. australis* from the Hokianga Harbour contained relatively high levels of TTX and that toxin concentrations in clam populations were variable around the country (Biessy, Smith et al. 2019).

The bacterial community present in the siphons was more diverse than the digestive glands, potentially due to being in direct contact with the external environment (Pierce and Ward 2018). Previous studies on other bivalves have shown similar patterns with the gills of oysters *Crassostrea gigas* containing a higher diversity of

bacteria than digestive glands (Hernández‐Zárate and Olmos‐Soto 2006). Kueh and Chan (1985) also found that total bacterial counts were comparable between seawater and organs in contact with the external environment like the gills, mantle and adductor muscles of the oyster *C. gigas*. The most abundant phylum in the siphon was Spirochetes. Species from this phylum have previously been found associated with the crystalline style or stomach of bivalves (Gunnar, Gunnar et al. 2010) but are also free-living bacteria that are abundant in marine environments, explaining their abundance in the siphons of shellfish (Paster and Dewhirst 2000). The bacterial communities of the digestive glands were dominated by ASVs from the genus *Mycoplasma*, a genus that is also consistently associated with bivalves, often in high abundances (Lokmer, Goedknegt et al. 2016, Aceves, Johnson et al. 2018, Pierce and Ward 2018). Proteobacteria such as Vibrionaceae, Stappiaceae or Endozoicomonadaceae were also abundant in both organs, which corroborates previous studies of TTX-bearing bivalves, for example in the clams *Ruditapes philippinarum* (Milan, Carraro et al. 2018).

Many bacterial genera have been reported as potential producers of TTX, the most common being *Bacillus*, *Pseudomonas*, *Aeromonas*, *Vibrio*, *Actinomyces* and *Shewanella* (Pratheepa and Vasconcelos 2013), and over 150 TTX-producing strains are described in the literature (Magarlamov, Melnikova et al. 2017, Katikou 2019). With less than 1% of bacteria being culturable there are challenges with isolating, culturing and thus characterizing TTX producers and it is likely that many other strains may be TTX producers (Chau, Kalaitzis et al. 2011). Many marine bacteria are capable of producing toxic secondary metabolites including cyanobacteria (Williamson, Singh et al. 2004, Mejean, Peyraud-Thomas et al. 2010), *Actinomycetes* (Fiedler, Bruntner et al. 2005) and *Bacilli* (Teasdale, Liu et al. 2009). The development of molecular techniques, such as metabarcoding, have revolutionized environmental surveys and are increasingly used worldwide for biodiversity monitoring (Taberlet, Coissac et al. 2012, Valentini, Taberlet et al. 2016, Stat, Huggett et al. 2017). Metabarcoding has previously been used for detecting rare, toxin-producing dinoflagellates (Smith, Kohli et al. 2017), to first report harmful algal species in new territories (Grzebyk, Audic et al. 2017) and to monitor toxic cyanobacterial blooms in freshwater reservoirs (Casero, Velázquez et al. 2019). Salvitti et al. (2017) used metabarcoding to investigate the diet and gut

content of the TTX-containing sea slug *P. maculata* and observed that most TTX-bearing individuals contained cnidarian and annelid sequences, two phyla known to contain toxic species (Miyazawa and Noguchi 2001, Salvitti, Wood et al. 2017). However, the bacterial content of the slug's foregut was not analysed and blocking primers to remove the background sequences of the sea slugs themselves were not used, reducing the number of taxa that could be identified (Vestheim and Jarman 2008). Another study investigating the microbiome of TTX-containing marine nemertean *Cephalothrix simula* showed the prevalence of a large number of bacterial genera previously associated with TTX production including *Alteromonas*, *Vibrio* and *Pseudomonas* but could not conclusively identify a potential producer (Turner, Fenwick et al. 2018).

The present study comprised of a spatial and temporal component. When analyzing the spatial study data, we subtracted the bacterial ASVs from sites containing very low TTX concentrations (approx. limit of detection; 2-3 ug kg^{-1}) from the Hokianga Harbour bacterial ASVs. In the siphons, the first genus of interest was *Phaeodactylibacter,* a gram-negative bacteria associated with the diatoms *Phaeodactylum* spp. (Chen, Lei et al. 2014) that has been shown to produce the neurotoxin non-protein amino acid β-methylamino-L-alanine (BMAA; Réveillon, Séchet et al. 2016). *Bacillus* (phylum: Firmicutes) was detected in the digestive glands from *P. australis* from high TTX sites and has previously been described as a potential TTX producer (Chau 2013) and TTX was recently detected in the strain *Bacillus* sp. 1839 tested after five years of cultivation in the laboratory (Melnikova, Vlasenko et al. 2019). However, the concentrations detected in the culture were low and would not likely account for the extremely high concentrations in higher trophic organisms. The last genus of interest in the differential analysis was *Vibrio,* which comprise more than 30% of all the suggested 150 TTX-producing strains reported in the literature (Turner, Fenwick et al. 2018). When the bacteria present in the digestive glands from the non-toxic *A. stutchburyi* were subtracted from those in the glands of toxic *P. australis*, the genera *Bacillus* and *Vibrio* were also present indicating that these taxa remain candidates for further investigation as potential TTX producers.

In the temporal study, the focus was on the core bacterial communities of TTX-bearing *P. australis* over an entire year. The core microbiome of a species is defined

as the group of microbes present in all individuals regardless of the environment (Turnbaugh et al., 2007). Given that TTX was present in all samples in the temporal study at Hokianga Harbour, we hypothesized that the TTX producer would be present in the core bacterial communities of *P. australis.* The siphons and the digestive glands had some similarities: two genera, *Mycoplasma* and *Spirochaeta,* were present in both organs' bacterial communities. *Spirochaeta* and *Mycoplasma* are common in the core bacterial communities of bivalves (Pierce and Ward 2018) and were also present from all sites in the spatial study but have never been previously associated with toxin production. In the digestive glands, *Bacillus* was present in 70% of all samples.

When analysing the spatial study data, three cyanobacterial genera remained when subtracting the bacterial ASVs from sites containing very low TTX concentrations from the Hokianga Harbour bacterial ASVs. The first was *Pleurocapsa* strain PCC-7319, a cyanobacterium that was found to be abundant in a toxic bacterial mat, although the toxin was not identified (Aboal, Puig et al. 2002). Two other cyanobacterial genera, *Cyanobium* strain PCC-6307 and *Prochlorococcus* strain MIT-9313, were also detected. *Cyanobium* have been shown to produce toxins similar to microcystins (Jakubowska and Szeląg-Wasielewska 2015) and one strain (in addition to other cyanobacteria) was toxic to brine shrimp, but the analysis did not reveal the production of known toxins (Frazão, Martins et al. 2010). *Prochlorococcus* is one of the smallest and most abundant photosynthetic organisms in the ocean (Partensky, Hess et al. 1999) and at least one strain has been shown to produce BMAA (Jakubowska and Szeląg-Wasielewska 2015). *Prochlorococcus* and *Cyanobium* are picocyanobacteria (i.e., have a cell size between 0.2 and 2 μm) and are common in freshwater and marine ecosystems but remain poorly studied due to their small size and difficulty to isolate (Jakubowska and Szeląg-Wasielewska 2015). Cyanobacterial ecotoxicology is particularly well documented in freshwater habitats where they have been found to produce a wide range of toxins (Jakubowska and Szeląg-Wasielewska 2015, Rastogi, Madamwar et al. 2015, Cirés, Casero et al. 2017) including saxitoxin. Saxitoxin is a potent neurotoxin produced by cyanobacteria and marine dinoflagellates and is known to exert the same toxic effect as TTX through an interaction with voltage gated sodium channels resulting in inhibition of neuromuscular transmission (Narahashi 1988).

Both toxins are active on the α-subunit of the sodium channels (Walker, Novick et al. 2012) and have been detected simultaneously in several aquatic species (Bane, Lehane et al. 2014). However, toxicological studies on cyanobacteria have not been performed to the same extent in marine environments (Frazão, Martins et al. 2010), and while no specific links have previously been reported between cyanobacteria and TTX, they are an interesting new lead for future studies with the aim to identify TTX-producing bacterial species.

The unicellular cyanobacterial genus *Synechococcus* was significantly correlated to higher TTX concentrations in the spatial study and was also present in the bacterial communities of both *P. australis* organs in the temporal study. Marine *Synechococcus* are abundant throughout the world's oceans and are significant primary producers in coastal environments but very little is known about their toxic effects on other marine organisms (Hamilton, Paz-Yepes et al. 2014). Some strains produce substances with neurotoxic effects in mice and negative effects on invertebrates (Martins, Pereira et al. 2005, Martins, Fernandez et al. 2007). Although no toxin production has been associated with the strain CC9902 (the strain whose 16S rRNA sequences were most closely related to the ASV in this study; Genbank accession number CP000097.1), it has been shown to alter fish behaviour after a 3-day exposure (Hamilton, Paz-Yepes et al. 2014). More toxicological studies should be undertaken on *Synechococcus* using analytical methods. When investigating the core bacterial communities of the siphons, *Cyanobium* PCC-6307 and *Synechococcus* CC9902 were again present in 90% of all samples and *Prochlorococcus* MIT9313 in 70% of all samples, strengthening the hypothesis that cyanobacteria (especially picocyanobacteria) could be responsible for the presence of TTX in *P. australis*. This is further reinforced by the fact that no cyanobacteria were present in the core bacterial communities of the non-TTX bearing *A. stutchburyi*.

In this study, metabolic pathways and their differential abundances were investigated (via inference from 16S rRNA gene metabarcoding data) in TTX-bearing and non-bearing organisms. The *paprica* pipeline identifies the metabolic processes most relevant to the functional properties of microbial communities (Bowman and Ducklow 2015). Our aim was to identify toxin or secondary metabolite production pathways that could be responsible for the increased TTX

concentrations observed in *P. australis* from the Hokianga Harbour. The metabolic pathways observed here were generally linked to the organisms' habitat and some of their active cellular processes. No metabolic pathways could be definitively linked to toxin or secondary metabolite production but there was a high abundance of photosynthetic enzymes in the digestive glands, possible indicating the presence of cyanobacteria. There are many limitations of using *paprica* for this purpose, in particular the genomic databases do not contain many environmental taxa and can only identify known pathways or enzymes. Investigating differences in bacteria community function between toxic and non-toxic strains of the same host species warrants further investigation and the use of metatranscriptomic techniques (the study of gene transcripts (RNA-sequencing) from environmental samples) is recommended.

5.6 Conclusion and future research

This is the first study to evaluate the bacterial communities of TTX-bearing and non-TTX bearing bivalves using high-throughput sequencing. Our results from both spatial and temporal studies correlate with some previous hypotheses that *Vibrio* and *Bacillus* could be responsible for the source of TTX in the New Zealand endemic clams *P. australis*. This study highlights that further investigation of marine cyanobacteria as potential TTX producers is warranted, especially *Synechococcus, Cyanobium* and *Prochlorococcus,* which were present in TTX-containing clams in both spatial and temporal studies. Culturing, isolating and testing these bacterial species, in particular picocyanobacteria, for toxin production would be a logical next step in the search to identify the source of TTX in marine organisms. It has recently been shown that DNA from toxic flatworms were detected in the intestinal content of juvenile pufferfish (Okabe, Oyama et al. 2019). So alternatively, it is also feasible that *P. australis* could source TTX from ingesting larvae, eggs, or pieces of the highly toxic sea slug *P. maculata* and flatworm *Stylochoplana* sp., which are common throughout New Zealand, Japan and Europe (Miyazawa, Jeon et al. 1987, Salvitti, Wood et al. 2015, Turner, Fenwick et al. 2018). Other eukaryotes, such as dinoflagellates, should also be investigated as many of them produce other marine biotoxins. Molecular techniques such as qPCR, droplet digital PCR or metabarcoding with the use of blocking primers could be

used to assess whether these toxic species are present in the digestive system of *P. australis.*

CHAPTER 6

A microencapsulation method for delivering tetrodotoxin to bivalves to investigate its uptake and accumulation

6.1 Abstract

Most marine biotoxins are produced by microalgae. The neurotoxin tetrodotoxin (TTX) has been reported in many seafood species worldwide but its source is unknown, making accumulation and depuration studies in shellfish difficult. Tetrodotoxin is a water-soluble toxin and cannot be directly ingested by shellfish. In the present study, a method was developed which involved binding TTX to solid particles of humic acid and encapsulating them in agar-gelatine capsules. A controlled quantity of TTX-containing microcapsules (size range 20-280 μm) was fed to *Paphies australis*, a bivalve known to accumulate TTX in the wild. The TTX-containing microcapsules were fed to *P. australis* every second day for 13 days. Ten *P. australis* (including five controls fed non-toxic microalgae) were harvested after 7 days and ten after 13 days. *Paphies australis* accumulated TTX, reaching concentrations of up to 103 μg kg^{-1} by day 13, exceeding the European Food Safety Authority recommended concentration of 44 $\mu g.kg^{-1}$ in shellfish. This novel method will allow future studies to explore the effects, accumulation and depuration rates of TTX in different animals and document how it is transferred through food webs.

6.2 Introduction

Shellfish are a rich source of protein, essential minerals, vitamins and are an important food source worldwide (James, Carey et al. 2010). However, bivalves filter large volumes of water and can concentrate contaminants including bacterial pathogens and phycotoxins (Huss 1997). With over 66 million tonnes of shellfish now consumed by humans annually, the risk of poisoning through contaminated seafood is an increasing public health concern (Whittle and Gallacher 2000, Özogul and Hamed 2018). With regards to phycotoxins (biotoxins produced by microalgae), the risk of poisoning increases exponentially during harmful algal blooms (HABs), when microalgal populations form dense concentrations of cells and sometimes visible water discoloration (Hallegraeff 2003). HABs have negative environmental impacts and can cause mass mortalities of fish, birds and marine mammals, and human illness (James, Carey et al. 2010), when they produce biotoxins that contaminate seafood through biomagnification up the food web (Visciano, Schirone et al. 2016). About 300 marine microalgal species are known to produce biotoxins and more than 100 of these can cause intoxication or even death in humans and animals (Visciano, Schirone et al. 2016).

The microalgal species responsible for producing most of the known biotoxins have been identified. For example, in the marine environment, saxitoxin (STX), a potent neurotoxin regularly present in edible shellfish and responsible for paralytic shellfish poisoning, is produced by dinoflagellates species from the genera *Alexandrium, Gymnodinium, Centrodinium* and *Pyrodinium* (Harada, Oshima et al. 1982, Negri, Stirling et al. 2003, Murray, Wiese et al. 2012, Shin, Li et al. 2020). Saxitoxin is also produced by freshwater cyanobacteria, the most common genera being *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, and *Planktothrix* (Moustafa, Loram et al. 2009, Pearson, Mihali et al. 2010, Quiblier, Wood et al. 2013). The fate of microalgal biotoxins in marine and freshwater organisms has been evaluated in a number of species. For example, green-lipped mussels *Perna viridis* were exposed to STX-producing dinoflagellate *Alexandrium fundyense* that were then fed to black sea bream *Acanthopagrus schlegeli and* the accumulation, distribution, transformation, and elimination of STX in specific organs were evaluated (Kwong, Wang et al. 2006). The hepatopancreas in the mussels and the viscera in the fish accumulated most of the STX. Differences in uptake, distribution,

and elimination of STX were observed between mussels and fish, and this may influence trophic transfer of microalgal toxins in marine organisms. Pereira et al. (2004) evaluated the accumulation and depuration of STX by a freshwater mussel *Anodonta cygnea* exposed to the STX-producing cyanobacterium *Cuspidothrix (Aphanizomenon) issatschenkoi,* and concluded that when removing/metabolizing 40% of the daily toxin consumed, the feeding behaviour of the mussels was affected (i.e., erratic feeding patterns and lower clearance rate of the toxic species).

While the sources and pathways of many marine biotoxins are well known, the origin of tetrodotoxin (TTX), a widespread and potent neurotoxin naturally occurring in organisms from marine, freshwater and terrestrial environments, remains uncertain (Chau, Kalaitzis et al. 2011). There is contradictory evidence regarding whether the source of TTX is exogenous or endogenous, and the pathways and mechanisms through which TTX is incorporated in the food web are unknown (Bane, Lehane et al. 2014). The wide distribution of TTX in many genetically unrelated species suggest that the toxin comes from an exogenous source such as accumulation through diet or symbiotic bacteria (Wu, Yang et al. 2005, Noguchi and Arakawa 2008) with reports in the literature of at least 150 TTX-producing bacterial strains (Katikou 2019). However, there is also evidence for an endogenous source in terrestrial species such as frogs or newts (Yotsu-Yamashita, Mebs et al. 1992, Cardall, Brodie et al. 2004). The neurotoxin is responsible for 30-50 global human intoxications every year and has the highest fatality rate of all marine biotoxins, mainly from pufferfish (e.g., fugu) consumption (Lago, Rodríguez et al. 2015). Tetrodotoxin also occurs in marine shellfish and has been reported in 21 species of bivalves and edible gastropods from ten countries since the 1980s (Biessy, Boundy et al. 2019). The risk of TTX intoxication to humans via shellfish cannot be ignored due to the high and increasing amounts consumed worldwide. Understanding the accumulation of TTX in the marine food web will help evaluate and reduce this risk. Without a known producer, studies which explore TTX accumulation in bivalves are very challenging. To date, there has been no feeding studies in bivalves, but feeding studies involving other organisms such as the sea slug *Pleurobranchaea maculata* (Salvitti, Wood et al. 2015) and the pufferfish *Takifugu niphobles* (Okabe, Oyama et al. 2019) suggested that TTX is accumulated via the diet.

A possible option for administrating toxins to bivalves is through microencapsulation. This involves the encapsulation of a solid within a thin protective coating, creating small particles (Espinosa, Barillé et al. 2007). This technology has been readily used within food and pharmaceutical industries as it allows a core material to be completely isolated from the external environment (Vidhyalakshmi, Bhakyaraj et al. 2009). The technique has recently been used to provide sustained and controlled release of bioactives in aquaculture (Masoomi Dezfooli, Gutierrez-Maddox et al. 2019). Microencapsulation has been used to feed live microalgae to the oyster *Crassostrea gigas* (Espinosa, Barillé et al. 2007) but the present study is the first which uses the encapsulation of a pure toxin which is then fed to shellfish. Tetrodotoxin is a relatively small, water-soluble molecule (Turner, Powell et al. 2015), making it impossible to directly encapsule TTX without it being bound to a solid first.

The aims of this study were to; 1) develop a method to encapsulate TTX into a food suitable for bivalves, and 2) feed known amounts of TTX to a bivalve species to determine accumulation. This technique would allow future studies to investigate the effects, accumulation and depuration rates of the neurotoxin in different animals, as well as its bioaccumulation in higher organisms via feeding of contaminated shellfish. This method could then be applied to other biotoxins where the source is unknown, isolation of the causative organisms is challenging or production of the biotoxin is not stable. In this experiment, TTX bound to humic acid was encapsulated in an agar-gelatine solution. A known quantity of the TTX microcapsules were fed to *Paphies australis*, an endemic New Zealand clam that has been shown to accumulate high TTX concentrations in the wild (McNabb, Taylor et al. 2014, Biessy, Smith et al. 2019).

6.3 Material and methods

6.3.1 *Paphies australis* collection and acclimation

Adult *Paphies australis* (n = 30) of similar size (ca. 40 mm long) were collected from Delaware Bay (Nelson, New Zealand; 41°09'S, 173°27'E) between March and August 2020, chilled (ca. 8 °C) and transported to the laboratory (Cawthron Institute, Nelson, New Zealand) within one hour. The shells were scrubbed and rinsed with sterile seawater to remove biofouling. Control samples (hereafter

environmental controls; $n = 5$) were stored frozen (-20 °C) until later TTX analysis to ensure that there was no or only very low TTX in the environmental population. The remaining individuals were placed in aquariums (15-L) which had been thoroughly cleaned and rinsed pre-experiment using detergent and bleach (10%). These were maintained with a recirculating flow of seawater and continuous aeration at 18±1 °C with a 14:10 h light:dark cycle. The bivalves were left to acclimatize for one week and fed the microalgal species *Isochrysis galbana* (2 L, ca. 12×10^6 cells mL^{-1}) every second day.

6.3.2 Microencapsulation method development

6.3.2.1 *Micro-algal encapsulation*

To develop the encapsulation method, the dinoflagellate *Alexandrium minutum* (CAWD12, maintained in the Cawthron Institute Culture Collection of Microalgae) was used to determine if microalgal cells could be encapsulated and if the capsules were impermeable. The preparation of agar–gelatine based microcapsules was modified after Lam et al. (2012). Agar (2% in Milli-Q water; Sigma-Aldrich, MA, USA) and gelatine (1% in Milli-Q water; Sigma-Aldrich), solutions were mixed in glass beakers using a magnetic stirrer (Labnet International, Inc AccuPlate™) at 80 °C until fully dissolved. In a separate beaker, 10 mL of each solution was added and stirred at 1,000 rpm (10 min, 60 °C). *Alexandrium minutum* culture (ca. 5,000 cells mL^{-1}; 10 mL) was added to the agar-gelatine mixture and this mixture was stirred for a further 5 min (1,000 rpm, RT). A solution of olive oil (400 mL; extra-virgin) and Span® 80 (2 mL; Sigma-Aldrich) was made up separately and the mixture slowly added to the agar-gelatine-algae solution with continuous stirring at 1,000 rpm. This was then homogenized (OMNI International, Inc®, 1 min) to create an emulsion. The emulsion was left to cool whilst stirring (1,000 rpm, 3 h). Following stirring, the solution was transferred into a separating funnel along with 500 mL of sterile seawater to promote separation from the oil causing the microcapsules to aggregate at the bottom layer of the separating funnel. The capsules were collected, washed three times with acetone, filtered through 100 μm and 20 μm filters until all olive oil was removed, rinsed with tap water and then dispersed in seawater.

6.3.2.2 *Tetrodotoxin encapsulation*

The encapsulation method was subsequently trialled using liquid TTX solution (0.7 mg kg^{-1}) in lieu of a biological producer. Unfortunately, due to the high solubility of TTX in water, the capsules did not contain any toxin after encapsulation (results not shown). Liquid TTX was then substituted for TTX bound to solid humic acid, a strong chelator, to create a solid substrate to encapsulate. To validate that TTX was binding to the humic acid before adding it to the capsules, a small experiment was undertaken where TTX (10 µL, 6 mg kg^{-1}) was diluted in MilliQ-water (1 mL), before adding humic acid (60 mg; Sigma-Aldrich) and vortexing (2 min, max. speed). Finally, formic acid (10 µL, >98%; Sigma-Aldrich) was added to the solution to investigate the release of TTX from the particles of humic acid and simulate what would happen in the stomach of *P. australis* (pH ~ 4, as measured in this study). Sub-samples of the solution were taken at each step and analysed for TTX concentration (see methods below).

After ensuring that TTX was binding to humic acid, the TTX solution (500 µL; 0.7 mg kg^{-1}) was diluted in Milli-Q water (9,500 µL) and added to humic acid (500 mg) which was then vortexed (5 min). The protocol described in 4.2.1 was then undertaken using TTX-humic acid solution instead of *A. minutum* cells. Three test individuals of *P. australis* were fed the capsule solution and sacrificed 24 h later. Their digestive glands were dissected before being observed under the microscope. The final concentration of TTX in the capsule solution and its accumulation rate in individual *P. australis* were calculated using the formula:

$$\%\, TTX\ accumulated = \frac{amount\ TTX(\mu g)\ in\ each\ organ - amount\ TTX\ (\mu g) in\ controls}{506\ \mu g\ TTX\ given \times 0.007\ L \times number\ of\ TTX\ feedings} \times 100$$

6.3.2.3 *Microscopic characterization of microcapsules*

For the entire method development, microcapsules were microscopically examined using an Olympus CKX41 microscope equipped with a digital camera. Digital images were captured and processed using the cellSens imaging acquisition software (Olympus Life Science).

6.3.3 Feeding experiment

Following the acclimation period, *P. australis* of similar size were transferred to individual 1-L glass jars (n = 1 per jar) containing 500 mL of filtered seawater with constant aeration. The bivalves (n = 20) were individually fed a mixture of concentrated *I. galbana* (93 mL; ca. 12×10^{12} cells mL^{-1}) and freshly-made TTX capsules (7 mL) five times at days 0, 3, 6, 9 and 12, or were fed *I. galbana* only on the same days for experimental controls. The mixture of TTX and algae instead of TTX capsules only was used to trigger the *P. australis* to open and start filtering their food. A subsample (50 mL) of *I. galbana* culture was collected and analysed for TTX. Five *P. australis* fed TTX microcapsules were harvested at day 7, along with five control individuals. The remaining *P. australis* (five fed the solution of *I. galbana* and TTX capsules and five fed *I. galbana* only) were harvested on day 13 for TTX analysis. Using the weight and TTX concentration from each tissue sample and the total weight of individual *P. australis*, total TTX concentrations in whole organisms were calculated. Faeces samples were harvested from each jar at days 7 and 13 but on closer inspection, we observed that these samples also contained humic acid particles that had aggregated at the bottom of the jar and it was not possible to separate these. No further analysis was undertaken on the faeces as it would not have provided meaningful data. The jars were cleaned and the water was replaced at day 7. At each sampling point, *P. australis* were rinsed with Milli-Q water, left to drain (5 min) and were frozen (-20 °C) until toxin extraction.

6.3.4 Tetrodotoxin extraction and analysis using LC-MS/MS

The TTX extraction protocol was adapted from Biessy et al. (2018). Frozen *P. australis* were shucked, then aseptically dissected into two tissue groups: (1) the siphons and digestive gland combined (DGS), and (2) the 'rest' mostly composed of the foot, gonads, gills and mantle. Each sample was weighed (ca. 0.3-3 g) and placed in a sterile tube (50 mL) containing a corresponding volume (ca. 300-3,000 µL) of 1% acetic acid in Milli-Q water. The samples were then homogenized (OMNI International, Inc®, 45 s or until complete homogenization). Samples were boiled (5 min) and cooled in an ice bath (5 min) followed by brief vortexing. The samples were centrifuged (3,200 × g, 10 min) and 1 mL of the supernatant was transferred to a centrifuge tube (1.7 mL) containing 5 µL of 25% ammonia (Honeywell, USA). The samples were centrifuged again (17,000 × g, 1 min) and

the supernatant was cleaned with the graphitized carbon Solid Phase Extraction (SPE) method (Boundy et al., 2015) using Supelclean™ ENVI-Carb 250 mg/3 mL SPE cartridges (Sigma-Aldrich). The extracted tetrodotoxin was analysed and quantified using liquid chromatography tandem-mass spectrometry as described in Turner et al. (2017).

6.3.5 Statistical Analysis

The effect of time, sample type, and their interactions on TTX accumulation was assessed using a mixed effect linear model (Zuur, Ieno et al. 2009). This modelling framework addresses the non-independence of the data, considering the individual *P. australis*. "Animal id" was incorporated into the model as a random effect. TTX concentration was log-transformed to linearize the relationship and reduce heteroscedasticity. The log-transformed TTX data were tested for normality with an Anderson-Darling normality test (Thode 2002). The marginal means for the combination of the day, sample type and TTX concentrations were estimated and the comparisons among them. Adjusted *p* values for the differences were calculated using the Tukey method. All statistical analyses were performed within the 'R' statistical and programming environment (RCoreTeam 2020). The package *lme4* (Bates, Mächler et al. 2015) was used for the mixed effect linear models and the package *emmeans* (Lenth, Singmann et al. 2020) to estimate the marginal means (least-squares means) for the factor combinations from the mixed effects linear models.

6.4 Results

6.4.1 Microscopic characterization of microcapsules

To develop the encapsulation method, microalgae that could be observed under microscope were first used to determine if cells could be encapsulated and if the capsules were impermeable.

The microscopic analysis showed the microcapsules were intact and approximately spherical (Figure 6-1). The encapsulation of the live *Alexandrium minutum* cells was successful (Figure 6-1A) and the microalgae stayed in the capsules until they disintegrated (42 h). The microscopic analysis also showed that the humic acid was encapsulated (Figure 6-1 B and C). The smallest capsules agglutinated to each other

but were easily disrupted with gentle agitation. The capsules stayed intact for a minimum of 34 h, their diameters ranged from 20 to 280 µm and >85% contained humic acid particles. Each fresh batch of capsules (made just prior to feeding) varied between 18,000 and 33,000 capsules per mL and ca. 75 mL of concentrated capsule solution was produced, containing ca. 506 µg TTX per L^{-1}. On average, each *P. australis* filtered the 100 mL of solution (93 mL of microalgae with 7 mL of TTX capsules) within 4 h. Capsules containing humic acid particles were found partially digested in the digestive glands of *P. australis* (Figure 6-1D), showing that the clams were capable of filtering and digesting the agar-gelatine capsules.

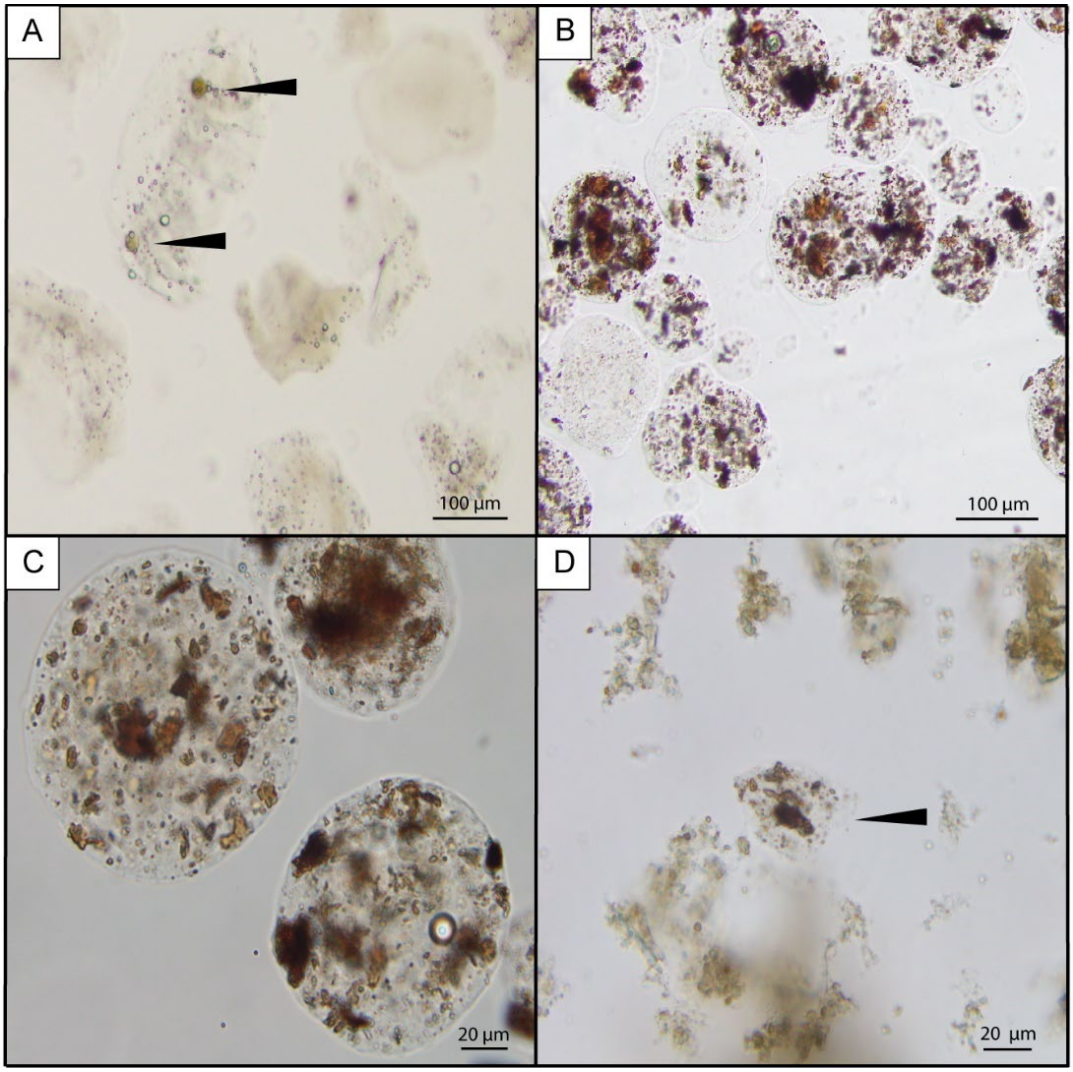


Figure 6-1. Agar-gelatine microcapsules containing *Alexandrium minutum* cells (A; black arrows) and humic acid (the brown solids; B, C and D). The black arrow in D shows a partially digested capsule containing humic acid that was found in the digestive gland of a *Paphies australis*.

6.4.2 Tetrodotoxin encapsulation

The next step was to validate that TTX was binding to humic acid before adding it to the capsules. The results showed that ca. 70% of the TTX was bound to the humic acid and that most (ca. 80%) of the bound TTX was released after addition of the formic acid to break the ionic bond between TTX and the humic acid, the same way as it would in the acidic digestive glands of *P. australis* (Table 6-1).

Table 6-1. Tetrodotoxin (TTX) concentrations in solution at various stages in the testing of adsorption and release (under acidic conditions) from humic acid.

Sample	**TTX concentration in solution (ng mL^{-1})**
TTX in water (n = 6)	102.3 ± 5.5
TTX in water + humic acid (n = 6)	29.7 ± 1.8
TTX in water + humic acid + formic acid (n = 6)	82.7 ± 2.1

6.4.3 Feeding experiment

The culture of the microalga *Isochrysis gabana* did not contain any TTX. The environmental controls (wild *P. australis* harvested from Delaware Bay, Nelson, New Zealand) tested positive for TTX with an average of 15 µg kg^{-1}. The TTX concentrations in the experimental controls remained stable during the feeding experiment (average of 17 and 15 µg kg^{-1} in whole *P. australis* after 7 and 13 days respectively). TTX concentrations in the different samples of the experimental controls also remained constant with the digestive glands and siphons (DGS) containing an average of 44 µg kg^{-1} after 7 days and 39 µg kg^{-1} after 13 days and the 'rest' of the organs containing 9.8 µg kg^{-1} after 7 days, and 7.5 µg kg^{-1} after 13 days. (Figure 6-2).

Tetrodotoxin concentrations were significantly higher in the DGS of *P. australis* fed TTX for 7 days (average of 108 µg kg^{-1}, $p = 0.001$) and 13 days (average of 238 µg kg^{-1}, $p < 0.0001$), compared to the experimental control organisms fed microalgae only (average of 44 µg kg^{-1}). The TTX concentration was also significantly higher in the 'rest' samples compared to the controls (average of 9 µg kg^{-1}) after 7 days of feeding (average of 18 µg kg^{-1}, $p = 0.026$) and after 13 days (average of 49 µg kg^{-1}, $p < 0.0001$). The *P. australis* accumulated more TTX over

time, the concentration was significantly higher in DGS and in the 'rest' samples at day 13 compared to day 7 (p = 0.0136 and p = 0.001, respectively; Figure 6-2). TTX concentrations in whole *P. australis* (calculated by summing results from DGS and rest samples on a pro rata weight) reached 39.2 µg kg^{-1} after 7 days and 105.9 µg kg^{-1} after 13 days.

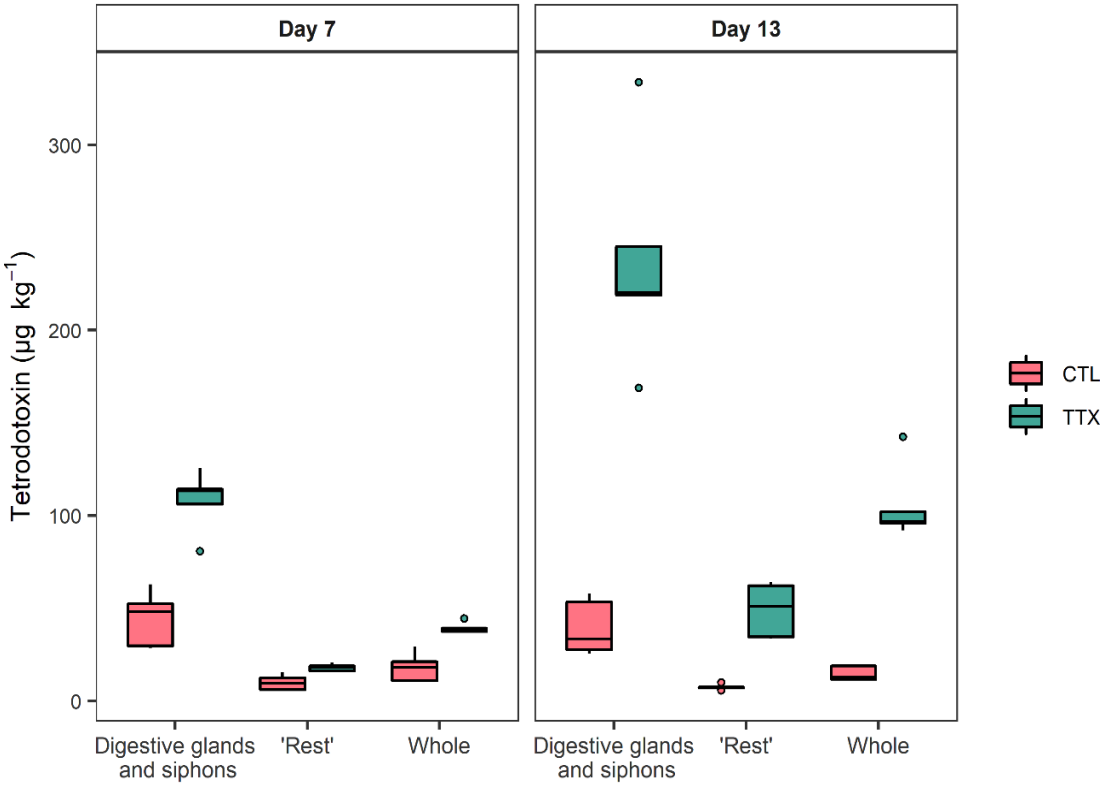


Figure 6-2. Tetrodotoxin (TTX) concentrations in experimental controls (CTL) and treatment samples (TTX) for the two tissue types (combined digestive gland and siphon, and everything else 'rest') and 'whole' *Paphies australis* (n=5) individuals after being fed controlled amounts of TTX every three days for 7 and 13 days. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the inter-quartile range. Dots outside the whiskers are considered as outliers.

A mixed effect linear model showed that there was no significant difference in the control groups from the DGS and the 'rest' groups (p = 0.999 and p = 0.993, respectively) between days 7 and 13 (Table 6-2). The interactions between days, organs and treatment (TTX versus experimental controls) were not significant. There were significant differences in the control treatments between the DGS and 'rest' sample types from both sampling days (p < 0.0001 for days 7 and 13) and there were significant differences between the two sample types and controls (p < 0.0001) and at each day (p < 0.0001).

Based on the amount of TTX provided at each feeding (ca. 506 µg L^{-1} in the capsule solution), the *P. australis* accumulated on average 0.5% of the TTX provided for the first 7 days (0.6% in the DGS and 0.32% in the 'rest') and 0.98% on average after 13 days (0.88% in the DGS and 0.42% in the 'rest').

Table 6-2. Pairwise comparisons (Tukey HSD test) of mean tetrodotoxin concentrations in *Paphies australis* during the feeding experiment, among days, sample type and treatments (CTL = controls fed microalgae only, TTX = organisms fed tetrodotoxin capsules and microalgae, DGS = Digestive gland and siphons pooled together. Bolded values represent statistically significant differences ($p < 0.05$).

			Day 7						**Day 13**					
			DGS		**Rest**		**Whole**		**DGS**		**Rest**		**Whole**	
			CTL	**TTX**	**CTL**	**TTX**	**CTL**	**TTX**	**CTL**	**TTX**	**CTL**	**TTX**	**CTL**	**TTX**
Day 7	**DGS**	**CTL**		**0.022**	**<0.0001**	**0.0052**								
		TTX			**<0.0001**	**<0.0001**								
	Rests	**CTL**				**0.0444**								
		TTX												
	Whole	**CTL**						0.0055						
		TTX												
Day 13	**DGS**	**CTL**	0.9999	**0.0005**	**<0.0001**	**0.0234**				**<0.0001**	**<0.0001**	**<0.0001**		
		TTX	**<0.0001**	**0.0131**	**<0.0001**	**<0.0001**					0.9607	**<0.0001**		
	Rests	**CTL**	**<0.0001**	**<0.0001**	0.9927	**0.0038**						**<0.0001**		
		TTX	0.9998	**0.0136**	**<0.0001**	**0.001**								
	Whole	**CTL**					0.9998	**0.001**						**<0.0001**
		TTX					**<0.0001**	**0.0011**						

6.5 Discussion

The overarching aim of this study was to develop a method to encapsulate TTX in a way that made it possible to feed it in a controlled experiment to bivalves. Achieving this would allow studies on accumulation rates and add to evidence that TTX might be obtained through food web. In this study, we used the clams *P. australis*, which are endemic to New Zealand and have been shown to accumulate TTX to reasonably high levels in the environment as our test organisms (McNabb, Taylor et al. 2014).

An initial concern when generating the microcapsules was that the wild *P. australis* had never been exposed to artificial food. The microcapsules containing TTX needed to be within the size range of particles that the organisms were able to ingest. Most bivalves filter particles greater than 5 µm in diameter with nearly 100% efficiency (Riisgård 1988) and several studies reported an efficient uptake of particles with diameters of 200 to 300 µm in bivalves (Cognie, Barillé et al. 2003, Espinosa, Barillé et al. 2007). The microcapsules produced in the present study were within this size range (20 to 280 µm). Our observations supported the posit that the microcapsules were of a digestible size as the capsule-microalgae solution was filtered and the water was clear within 4 h and microscopic analysis showed capsules present in the digestive glands of *P. australis*.

The encapsulation process used in in our study did not destroy or degrade the TTX as evidenced by the high concentration remaining in the final capsule solution. Additionally, the agar, gelatine and humic acid were not detrimental to *P. australis* survival or feeding, as all the individuals stayed healthy for the duration of the experiment. The advantage of using an agar-gelatine capsule is that it is stable (the capsules stayed intact for at least 34 h) and impermeable, but easily dissolves when placed in a slightly acidic medium (i.e., the digestive system of bivalves). The use of humic acid as a solid binding agent was an important adaptation which allowed the water-soluble TTX to be contained inside a microcapsule instead of leaking out into the water.

Once the encapsulation method was developed and the ingestion of TTX-filled capsules by *P. australis* was confirmed, the second aim of this study was to feed them a controlled amount of TTX and investigate its accumulation rate. TTX has

been detected in all *P. australis* tested in previous studies (McNabb, Taylor et al. 2014, Biessy, Smith et al. 2019, Boundy, Biessy et al. 2020) and we anticipated to detect the low concentrations of TTX (average of 15 µg kg^{-1}) found in the 'environmental control' *P. australis*. The amounts detected after both seven (3 feedings) and 13 days (5 feedings) were significantly higher than this and were *on par* with concentrations recently detected in wild *P. australis* in New Zealand (Biessy, Pearman et al. 2020, Boundy, Biessy et al. 2020). An exception to this, was the detection of very high concentrations (800 µg kg^{-1}, i.e. 80-fold higher than detected in other studies) of TTX in 2014 (2014). One hypothesis is that the *P. australis* in that study had consumed a highly toxic source such as eggs or larvae from the sea slug *Pleurobranchaea maculata* or flatworm *Stylochoplana* sp. that have been shown to contain extremely high levels of TTX (Wood, Casas et al. 2012).

The concentration measured in whole *P. australis* after only 13 days of feeding (103 µg kg^{-1}) were well above the European Food Safety Authority recommendation concentration of 44 µg kg^{-1} for TTX in shellfish without seeing adverse effects in human health (Knutsen, Alexander et al. 2017). This result shows that monitoring TTX concentrations in edible bivalves is important and should be established in areas or countries where shellfish were shown to contain high TTX concentrations and are regularly harvested for consumption. Food-borne TTX is a realistic scenario as previous studies have shown that its source in bivalves is likely exogenous, with reports of bacterial species such as *Vibrio* and *Pseudomonas* (Chau, Kalaitzis et al. 2011, Katikou 2019) or cyanobacteria, especially picocyanobacteria (Biessy, Pearman et al. 2020, Li, Tian et al. 2020) suggested as potential producers. If one of these species is confirmed as the producer, the risks of TTX in edible seafood are likely to increase with climate change as both cyanobacteria and bacteria thrive at warmer temperatures (Paerl and Paul 2012, Baker-Austin, Trinanes et al. 2013). This study shows that *P. australis* can accumulate TTX from an external source and strengthens the hypothesis that bivalves accumulate TTX from their diet.

The method developed in this study can now be used to feed a range of bivalve species to determine if they can also accumulate TTX to concentrations above the recommended threshold. In addition to accumulation rates, depuration could also be studied, which will add valuable information for the management of commercial

species (i.e., blue mussel, oysters) that have been shown to accumulate TTX in several countries (Biessy, Boundy et al. 2019). Investigating TTX accumulation in a wide range of bivalves will also assist in understanding the mechanisms of TTX accumulation, such as the potential presence of a TTX-binding protein. Lastly, this method will also pave the way for further studies that explore TTX accumulation through the food web. For example, once the shellfish have accumulated the TTX, these could be fed to higher trophic organisms in the food web (e.g., fish). Similar studies have previously been undertaken, for example, Oikawa et al. (2005) investigated the accumulation and depuration rates of STX in crabs that were fed toxic mussels after being exposed to toxic cyanobacteria (2005).

We estimated that only 0.5-1% of the TTX administered was accumulated by *P. australis*. The low accumulation relative to the amount filtered could be due to some TTX molecules breaking down with the acid from the *P. australis* digestive system. Unfortunately, we were unable to test the amount of TTX in the faeces, so the quantity of TTX directly excreted is unknown. It is also possible that *P. australis* are only capable of accumulating a certain amount of TTX. It has been hypothesized that these bivalves contain unique TTX-binding proteins similar to those found in pufferfish and crabs (Yotsu-Yamashita, Sugimoto et al. 2001, Nagashima, Yamamoto et al. 2002, Biessy, Smith et al. 2019) that allow them to store the toxin in certain organs. It is possible that the TTX-binding protein could become saturated when exposed to a high amount of the toxin in a short amount of time. The low accumulation could also be due to the 20% of TTX remaining bound to the humic acid and not being absorbed by the bivalves once ingested. Despite the low accumulation rate of TTX observed in this study, the fact that *P. australis* have been shown to be slow detoxifiers/depurators for TTX (Biessy, Smith et al. 2019), suggests that for this species to reach similar concentrations in the wild, the producer would either be present most of the time but in low quantities, or only present occasionally but in higher concentrations. The amount of TTX filtered in the wild could thus be very high for a brief period and the *P. australis* retains the toxin for a very long time. Additionally, it is possible that the bivalve species accumulate a TTX precursor molecule and biosynthesize it to TTX, making the TTX producer very difficult to find.

In this study, the siphons and the digestive glands accumulated more TTX than the remaining tissue, corroborating with previous studies that reported these organs accumulated the highest amount of TTX in the wild (Biessy, Smith et al. 2018). The 'rest' group did not show an increase in TTX accumulation after 7 days of feeding but did increase after 13 days, suggesting that the toxin is migrating between the clam's organs as it was previously indicated (Biessy, Smith et al. 2019). Studies have found reduced predation on shellfish with high STX concentrations (Kvitek 1991, Kvitek and Bretz 2004), suggesting that the concentrated levels of TTX within *P. australis* siphons may be to protect the vulnerable organ which protrudes out of the sand in their natural environment. Previous studies on bivalves have shown greater toxin accumulation in the digestive gland-stomach complex and viscera which may contribute up to 98% of the total toxin, but in some bivalves a reversal of toxin content from the digestive gland and viscera has been observed, with toxins migrating to alternative tissues over time (Martin, White et al. 1990, Bricelj and Shumway 1998). To fully understand toxin micro-distribution within each species from these experiments, it would be necessary to undertake further fine scale dissections of each organ as well as feeding for a longer period to allow for toxin exchange between tissues to occur. However, our demonstration of initial toxin accumulation occurring within the digestive glands is useful for the analysis of wild populations, potentially inferring the recency of a contamination event, and the occurrence of ongoing toxin uptake.

6.6 Conclusions

A method which involved binding TTX to solid particles of humic acid and encapsulating them in agar-gelatine capsules was successfully developed. This is the first study binding TTX to a solid particle that was then fed to aquatic organisms. This experiment involved a known quantity of TTX-containing microcapsules being fed to *P. australis*. The bivalves only accumulated 0.5 to 1% of the TTX given at each feeding but their TTX concentrations reached up to 103 μg kg^{-1} after only 13 days, a concentration similar to those found in wild populations and well above the safe threshold recommended by EFSA of 44 μg kg^{-1} in bivalves. This result demonstrated that *P. australis* can accumulate TTX from an external source, thus strengthening the hypothesis that bivalves accumulate TTX from their diet. The method developed in this study will enable TTX dynamics to be explored

within different species of bivalves, and other aquatic species. Enhancing knowledge on accumulation and depuration rates in a range of aquatic species will help establish the time needed for safe consumption to occur following the discovery of contamination with TTX and may provide new insights into why some bivalves accumulate TTX and others do not.

CHAPTER 7
General discussion

The source of tetrodotoxin (TTX) remains one of the main mysteries in marine toxin research. Previous research strongly indicates an exogenous, bacterial origin but it has not yet been definitely proven. This chapter provides some background on the source of TTX in marine organisms globally and in New Zealand, a synthesis of the research undertaken in my thesis and suggests future directions I believe are necessary to gain insights into the origin of TTX and its ecological role(s) in marine bivalves.

7.1 Review of the possible sources of tetrodotoxin in marine organisms

In Chapter 2, I described how the concentrations of recent TTX detections in bivalves around the world are significantly lower than those observed historically in seafood species (e.g., gastropods and pufferfish) that have been linked with intoxication in humans. However, there has been an increase in the detection of TTX in bivalves worldwide. It is unclear if the increase is due to improved sensitivity of modern techniques, increased monitoring, or if the accumulation of TTX in bivalves in an increasing phenomenon, perhaps due to an environmental change. The only way to answer this question would be to analyse shellfish that would have been archived from previous studies over a decade ago. Regardless, there is an urgent need to evaluate the risk TTX in bivalve poses to human health.

There have been records of exceptionally high TTX concentrations in bivalves including the Japanese scallop *Patinopecten yessoensis* (8,000 µg kg^{-1}; Kodama, Sato et al. 1993), the clam *Paphies australis* (800 µg kg^{-1}; McNabb, Taylor et al. 2014) and more recently in the mussel *Mytilus galloprovincialis* (541 µg kg^{-1}; Bordin, Dall'Ara et al. 2021). None of these studies investigated the potential source of TTX in these edible shellfish.

Over the last few years, multiple researchers have explored the source of TTX in different organisms at higher trophic levels (Noguchi, Arakawa et al. 2006). Most

of the research has focused on pufferfish and it has been historically hypothesised that different species accumulate TTX through the food chain (Matsumura 1998).

Additional evidence was provided recently in an experiment where non-toxic *Takifugu obscurus* (pufferfish species) were fed wild TTX-containing gastropods and subsequently accumulated TTX in their liver and skin (Zhang, Zong et al. 2020). Okabe et al. (2019) found DNA from the toxic flatworm *Planocera multitentaculata* was detected in the intestine of the pufferfish *T. niphobles*, Further investigation showed that the pufferfish were accumulating TTX from consuming the flatworms' highly toxic eggs.

Studies investigating the source of TTX in ribbon worms (Nemertea) have hypothesised that they also obtain TTX from dietary sources. Tanu et al. (2004) investigated TTX localization in the body of the nemertean *Cephalothrix* sp. by immunohistochemistry and demonstrated that TTX was mainly present in its digestive tract. Vlasenko and Magarlamov (2020) recently confirmed this result using more sensitive detection assays and found high concentrations of TTX in the intestine of *Cephalothrix* cf. *simula*.

In New Zealand, the source of TTX in the highly toxic sea slug *Pleurobranchaea maculata* was investigated in an extensive research programme (2012-2015). Experiments on slugs maintained in aquariums demonstrated that they could accumulate TTX through their diet within one hour of being fed the toxin (Khor, Wood et al. 2013). This research programme also identified high TTX concentrations in the marine flatworm *Stylochoplana* sp. This flatworm was later identified in the stomach of the slug *P. maculata* and hypothesised as the likely source of TTX (Salvitti, Wood et al. 2015). However, due to the lack of *Stylochoplana* sp. in areas with dense slug populations, it was deemed unlikely that the flatworms represented the sole source of TTX in *P. maculata*. The researchers suggested that the source of TTX in *P. maculata* was mostly likely from a dietary source through several possible pathways including; (1) the consumption of the flatworm *Stylochoplana* sp., (2) ingestion of microbial organisms from the benthos that produce a precursor compound which is converted to TTX with the organism, and (3) when abundances of *P. maculata* are dense, cannibalism (Salvitti 2015).

To date, studies on the source of TTX have been undertaken on highly motile organisms, and these species also had high variabilities in toxin concentrations, making it difficult to pinpoint the exact place or source of contamination and accumulation. The overarching aim of this thesis was to elucidate the origin of TTX in New Zealand bivalves, specifically in the endemic New Zealand clam *Paphies australis*, a species culturally important in New Zealand and a common food source. They are also largely sessile, indicating that if the source of TTX is indeed exogenous and comes from their diet, the toxin producer will be in the surrounding environment, making it easier to identify compared to other organisms studied to date.

7.2 Summary of studies

The ultimate goal of this thesis was to elucidate the origin of TTX in *P. australis*. A multi-lines-of-evidence approach was used including histological and analytical techniques to explore the micro-distribution of TTX within the organs of *P. australis*, aquaria studies to investigate the depuration and uptake of TTX, field studies to explore the variations in TTX concentrations from different *P. australis* populations and other bivalve species in New Zealand, and molecular analyses to investigate potential TTX producers. The following sections provide short summaries and key findings from the individual studies in this thesis.

7.2.1 Micro-distribution of TTX in *Paphies australis* organs (Chapter 3)

Immunohistochemistry analysis showed that TTX was present in the outer cells of the siphons, but also in the digestive system, foot and gill tissues. The finding was also supported by chemical analysis using LC-MS/MS. Observing TTX in organs involved in feeding provided initial evidence to support the hypothesis of an exogenous source in *P. australis.* I hypothesised that the clams sequester TTX in their siphons, the only organ protruding out of the sand, as a defence mechanism, for example against siphon-nipping predators such as birds or fish.

7.2.2 Depuration rate of tetrodotoxin in *Paphies australis* (Chapter 4a)

After being maintained in captivity and fed a non-toxic diet for 150 days, wild TTX-bearing *P. australis* significantly depurated the toxin (0.4% of total toxin content per day). Of note, was the rapid decline of TTX in their digestive glands compared

to other tissues, with only traces amounts remaining after 21 days. This result provided further evidence to support the hypothesis that they do not endogenously produce TTX but likely obtain it from an exogenous source.

7.2.3 Geographic variation of tetrodotoxin in *Paphies australis* populations (Chapter 4b)

There were significant differences in TTX concentrations among *P. australis* populations from 10 different sites across New Zealand. All *P. australis* contained TTX but the bivalves from the North Island of New Zealand contained significantly higher concentrations than the ones from the South Island. There also was a seasonal trend with organisms collected during the warmer months containing higher concentrations of TTX. I hypothesised that the source could be a warm-water-adapted TTX producer that is mostly present or more prevalent in warmer climates, or in which TTX production is triggered with warmer temperatures. These results provided further evidence to support the proposition that the source of TTX in *P. australis* is likely exogenous (microbial or eukaryotic).

7.2.4 Spatial and temporal changes in bacteria communities (Chapter 5)

Using metabarcoding, the bacteria present in the two organs of *P. australis* containing the highest amount of TTX, the digestive glands and siphons, from the Hokianga Harbour were investigated. Samples were also collected every month over a year and were shown to have stable TTX concentrations across seasons. The most significant finding was the presence of marine cyanobacteria, in particular picocyanobacteria, in sites containing the highest amount of TTX and their continuous presence in the core microbiome of the *P. australis* from the Hokianga Harbour. Cyanobacteria are well known for producing a wide range of marine and freshwater toxins and this finding warrants further investigation.

7.2.5 Sequestration of tetrodotoxin from an artificial diet (Chapter 6)

In this chapter, I developed a novel method to artificially feed a controlled amount of TTX to bivalves. The micro-encapsulation method incorporated humic acid so that the water-soluble TTX bound to a solid substance. The main finding from this study was that *P. australis* can accumulate TTX from a dietary source. This further

strengthens the hypothesis that they most likely accumulate from their diet in the wild.

7.2.6 Survey of tetrodotoxin in New Zealand shellfish (Co-authored peer-reviewed publication, presented in Appendix 9)

During my PhD, I contributed to a survey of TTX in New Zealand shellfish. In this study, over 700 samples from eight bivalve species were harvested from different sites around New Zealand over 16 months and were analysed for TTX using LC-MS/MS. All *P. australis* tested (n = 44, mostly from the Hokianga Harbour) contained detectable levels of TTX and 66% of them had TTX concentrations over the safe recommended level of 44 μg kg^{-1}. However, cockles *Austrovenus stutchburyi* also collected from the same shellfish bed in the Hokianga Harbour did not contain any TTX. Samples of *Paphies subtriangulata*, a clam species closely related to *P. australis*, had detectable TTX concentrations but always under the safe recommended level (10% of samples had concentrations between 2 – 44 μg kg^{-1}). Samples of green-lipped mussel *Perna canaliculus* and the oyster *Saccostrea glomerata* were also positive. This study demonstrates that not all bivalve species are able to accumulate or sequester TTX.

7.3 Synthesis of studies

The results from this thesis strongly suggest that the source of TTX in *P. australis* is exogenous, that they accumulate it through their diet and that the producer of TTX is more prevalent or produces higher amounts of the toxin in warmer water temperatures. As climatic conditions change and anthropogenic pressures on waterways intensify, it thus seems likely that the prevalence of TTX in shellfish will increase. Tetrodotoxin-bearing organisms are also migrating with climate change and have been detected in more geographically diverse locations worldwide than previously detected in recent decades (Kalogirou 2013, Turner, Fenwick et al. 2018). The presence, bioavailability and potency of marine toxins like TTX are predicted to intensify (Roggatz, Fletcher et al. 2019), making monitoring of toxic species important.

Based on the key results from my thesis and in concert with previous studies on the subject, I hypothesize that *P. australis* are obtaining TTX from a dietary source.

There are three main possible routes and hypotheses of accumulation which are summarised in Figure 7.1.

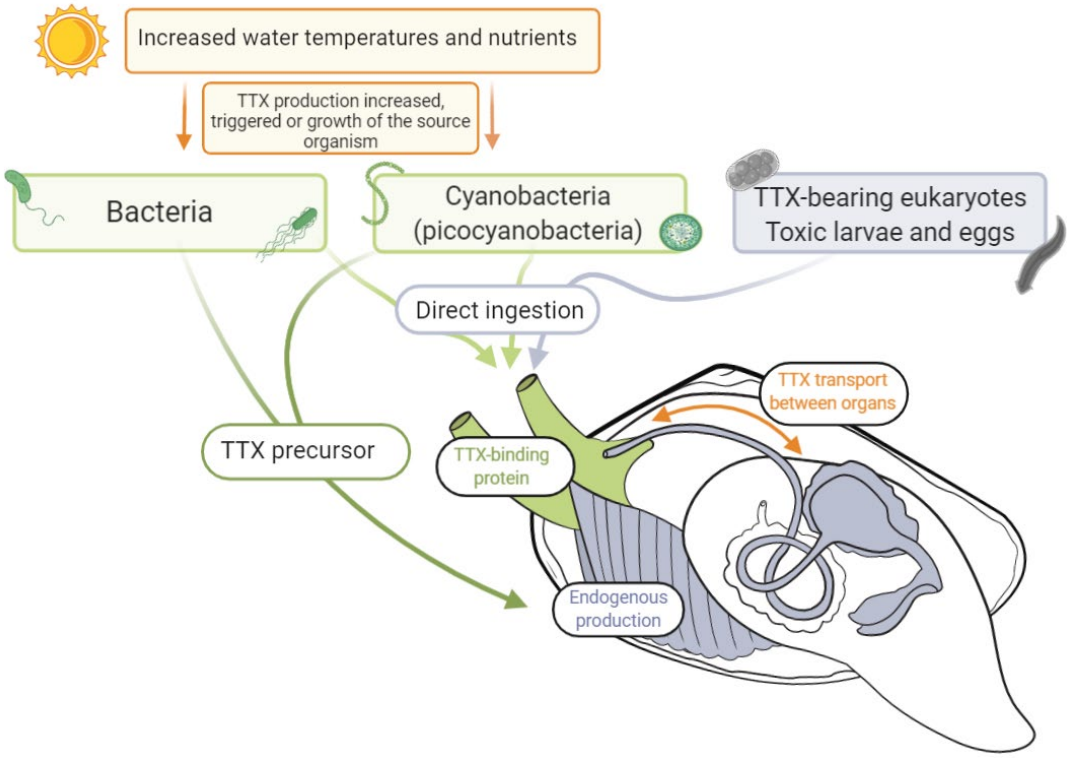


Figure 7-1. Diagram synthesising the main hypotheses regarding the accumulation of tetrodotoxin in the different organs of the New Zealand clam *Paphies australis*. Created with *BioRender.com*.

The first hypothesis, supported by the results of my thesis, is the direct consumption of a microorganism (such as cyanobacteria or bacteria) that is continually producing low amounts of TTX. After demonstrating that *P. australis* depurate the toxin, it is likely that constant uptake is needed. Finally, given that many different species of bivalves can accumulate TTX at various concentrations in countries with different climates, this scenario seems more likely than the suggestion that each of these species has evolved to endogenously produce TTX.

Some species such as the cockles *A. stutchburyi* do not accumulate TTX and more research is needed to understand why. This could be the result of different diets but given that they are also filter feeders and co-occur within *P. australis* beds, this seems improbable as it would require highly selective filtration. It is more likely that some species, including *P. australis,* contain TTX-binding proteins, similar to the one purified from the plasma of the pufferfish *Fugu pardalis* (Yotsu-Yamashita, Sugimoto et al. 2001) that facilitate uptake, retention and transfer of TTX. It has

been hypothesised that when a large amount of TTX circulates in the blood of pufferfish, the TTX-binding proteins get saturated, leading to a decrease in the TTX accumulation. The saturation of the TTX-binding protein would explain why *P. australis* accumulate TTX at a slow rate after exposure to high TTX concentrations. The accumulation of TTX in some bivalves seem to follow a seasonal pattern with higher concentrations during warmer seasons.

The second hypothesis is that *P. australis* ingests benthic or planktonic organisms that produce a precursor compound which is later converted to TTX within *P. australis* and is then transported to their siphons. A number of authors have speculated this (Shimizu and Kobayashi 1983, Chau, Kalaitzis et al. 2011) but to date there is no evidence to support it, especially after demonstrating that TTX can be directly accumulated from food in *P. australis*. New TTX analogues are regularly discovered (Pires Jr, Sebben et al. 2005, Kudo, Yamashita et al. 2014, Tonon, de Azevedo et al. 2020) but the full biosynthesis pathway remains a mystery.

Lastly, the direct accumulation of TTX in *P. australis* by the filtration of contaminated eggs from toxic organisms such as *P. maculata* or *Stylochoplana* sp. is another possibility and could explain the occasional high concentrations of TTX in some species of bivalves (e.g., 800 μg kg^{-1} in *P. australis*; McNabb, Taylor et al. 2014). It is unlikely that this is sole source of TTX in bivalves as spawning of the flatworm and slug is seasonal and would not explain the continuous detection of TTX *P. australis* from the Hokianga Harbour over a year.

7.4 Future directions

The results from this thesis and the novel technologies that are now available provide the basis for new directions of future research. Some of the techniques and experiments that I have used in this thesis could be used on other TTX-bearing organisms worldwide to test these hypotheses and compare the results between different species. To date, studies on the origin of TTX has mostly focused on single organisms in isolation but to solve the mystery of the source of TTX in the marine environment, the most significant advancements may occur when researchers from different fields share knowledge and compare multiple organisms to explore commonalities and differences.

Bivalves are easy to find, harvest and rear through life stages. As sessile filter feeders, they are an ideal model to assist with elucidating the origin of TTX in the marine environment. A novel method to feed TTX to filter feeding bivalves was developed in this thesis and it will now be possible to undertake controlled studies on accumulation and depuration of TTX in several species of bivalves. Another important aspect of accumulation studies will be exploring differences in toxin uptake between closely related species. More experimental studies (i.e., spatial and seasonal variations) on known TTX-accumulating species are needed rather than focusing on new species that accumulate the neurotoxin at low concentrations. These studies would provide new insights into the source of TTX. Further studies are also required to determine if the eggs of TTX-bearing bivalves contain the toxin, and rearing studies could be used to investigate whether TTX is passed onto successive generations

It would be valuable to isolate, culture and test marine cyanobacteria from environments where TTX accumulates in bivalves. Live shellfish should be collected, and their digestive glands and siphons should be lightly homogenised and resuspended in cyanobacteria specific media to allowing them to grow and have enough biomass to test for TTX. It would also be worthwhile to extend the molecular study I undertook on bacterial communities to microeukaryotes. Some microalgae have been associated with producing or containing TTX (Kodama, Sato et al. 1993, Vlamis, Katikou et al. 2015) and their presence in *P. australis* digestive tract should be investigated. I attempted to explore these eukaryotic communities, but the samples were swamped with DNA from *P. australis* tissues. This issue could be overcome by designing primers that specifically block *P. australis* DNA sequences (Vestheim and Jarman 2008) while amplifying all other eukaryotic organisms.

A further avenue for research is the investigation of known TTX-bearing organisms (e.g., *P. maculata* and *Stylochoplana* sp.) inside the bivalve digestive system using quantitative molecular methods (i.e., quantitative PCR or droplet digital PCR). This would determine if bivalves consuming toxic eggs or larvae from TTX-bearing organisms.

7.5 Conclusion

The research conducted in this thesis collectively supports the hypotheses that *P. australis* are filtering and ingesting an exogenous TTX-producer that is of microbial origin, and concentrating this toxin in select organs. The producer is likely more abundant in warmer water temperatures and its abundance may increase due to climate change. I was unable to definitively identify the source of TTX in *P. australis,* but this thesis highlighted a new possibility, picocyanobacteria, that should be further explored as these organisms are known to produce an array of toxins in marine and freshwater environments.

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APPENDIX 1

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Thesis Chapter 2. Published as: Biessy L, Boundy MJ, Smith KF, Harwood DT, Hawes I, Wood SA (2019) Tetrodotoxin in marine bivalves and edible gastropods: a mini-review. Chemosphere, 124404.

Nature of contribution by PhD candidate	I prepared and wrote the manuscript. The editing of the manuscript was conducted as detailed below.
Extent of contribution by PhD candidate (%)	90%

CO-AUTHORS

Name	Nature of Contribution
Michael J. Boundy	Helped with the chemistry sections, overall literature review and with the construction of the two tables.
Kirsty F. Smith	Advisor and helped with the writing and editing of the manuscript
D. Tim Harwood	Helped with the writing and editing of the manuscript
Ian Hawes	Advisor and main point of contact at Waikato University. Aided in overall writing and manuscript preparation.
Susanna A. Wood	Advisor and helped with manuscript development, writing and editing.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
Michael J. Boundy		09/12/20
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D. Tim Harwood		09/12/20
Ian Hawes		17/12/20
Susanna A. Wood		16/12/20

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Nature of contribution by PhD candidate	I was involved with sample collections, tissue preparation, immunohistological methodology development, and writing of the manuscript.
Extent of contribution by PhD candidate (%)	85%

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Name	Nature of Contribution
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Michael J. Boundy	Preformed all liquid chromatography-mass spectrometry analysis and was consulted regarding data analysis.
Steve Webb	Advised and helped on tissue preparation and histological techniques.
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Susanna A. Wood	Advisor, helped with method development, data analysis, manuscript writing and editing.

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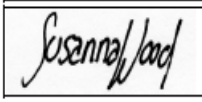
Ian Hawes		17/12/20
Susanna A. Wood		16/12/20

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Nature of contribution by PhD candidate	I was involved in sample collections, experimental design, bivalves maintenance, methodology development, results analysis and writing of the manuscript
Extent of contribution by PhD candidate (%)	95%

CO-AUTHORS

Name	Nature of Contribution
Kirsty F. Smith	Advisor, helped with sample collection, manuscript development, writing and editing.
D. Tim Harwood	Helped with sample collection and editing of the manuscript.
Michael J. Boundy	Advised and helped with tetrodotoxin LCMS analyses and editing of the manuscript.
Ian Hawes	Advisor and main point of contact at Waikato University. Aided in overall study direction and manuscript preparation.
Susanna A. Wood	Advisor, helped with sample collection, data analysis, manuscript writing and editing.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
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Nature of contribution by PhD candidate	I was involved in sample collection, experimental design, sample processing, data analysis, and writing of the manuscript.
Extent of contribution by PhD candidate (%)	90%

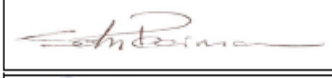
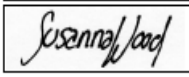
CO-AUTHORS

Name	Nature of Contribution
John K. Pearman	Helped with bioinformatics analysis and editing of the manuscript.
Kirsty F. Smith	Advisor, helped with manuscript development, writing and editing.
Ian Hawes	Advisor and aided in overall study direction and manuscript preparation.
Susanna A. Wood	Advisor, helped with results analysis, manuscript development, writing and editing.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
John K. Pearman		15/12/20
Kirsty F. Smith		15/12/20
Ian Hawes		17/12/20
Susanna A. Wood		16/12/20

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Nature of contribution by PhD candidate	I was involved in sample collection, experimental design, sample processing, data analysis, and writing of the manuscript.
Extent of contribution by PhD candidate (%)	80%

CO-AUTHORS

Name	Nature of Contribution
Kirsty F. Smith	Advisor, helped with results analysis, method development, manuscript writing and editing.
Susanna A. Wood	Advisor, helped with results analysis, method development, manuscript writing and editing.
Annabel Tidy	Helped with initial method development.
Roel van Ginkel	Helped with humic acid method development.
Joel R.D. Bowater	Helped with method development and reviewing of the manuscript.
Ian Hawes	Advisor and aided in overall method development, study direction and manuscript preparation.

Certification by Co-Authors

The undersigned hereby certify that:

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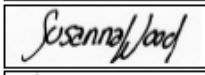
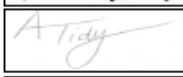
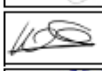
Name	Signature	Date
Kirsty F. Smith		15/12/20
Susanna A. Wood		16/12/20
Annabel Tidy		14/12/20
Roel van Ginkel		15/12/20
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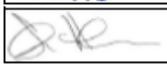
Ian Hawes		17/12/20

APPENDIX 6

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Review

Tetrodotoxin in marine bivalves and edible gastropods: A mini-review

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HIGHLIGHTS

- This review summarizes the current knowledge of TTX in bivalves and gastropods.
- TTX was reported in 11 gastropods and 10 bivalves in 5 and 7 countries respectively.
- Research should focus on assessing the variability and dynamics of TTX in seafood.

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ABSTRACT

Tetrodotoxin (TTX) is a potent neurotoxin responsible for countless human intoxications and deaths around the world. The distribution of TTX and its analogues is diverse and the toxin has been detected in organisms from both marine and terrestrial environments. Increasing detections seafood species, such as bivalves and gastropods, has drawn attention to the toxin, reinvigorating scientific interest and regulatory concerns. There have been reports of TTX in 21 species of bivalves and edible gastropods from ten countries since the 1980's. While TTX is structurally dissimilar to saxitoxin (STX), another neurotoxin detected in seafood, it has similar sodium channel blocking action and potency and both neurotoxins have been shown to have additive toxicities. The global regulatory level for the STX group toxins applied to shellfish is 800 μg/kg. The presence of TTX in shellfish is only regulated in one country; The Netherlands, with a regulatory level of 44 μg/kg. Due to the recent interest surrounding TTX in bivalves, the European Food Safety Authority established a panel to assess the risk and regulation of TTX in bivalves, and their final opinion was that a concentration below 44 μg of TTX per kg of shellfish would not result in adverse human effects. In this article, we review current knowledge on worldwide TTX levels in edible gastropods and bivalves over the last four decades, the different methods of detection used, and the current regulatory status. We suggest research needs that will assist with knowledge gaps and ultimately allow development of robust monitoring and management protocols.

Contents

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1. Introduction

Mollusca is one of the largest phyla in the animal kingdom with more than 100,000 existing species living in marine, freshwater and terrestrial habitats (Barnes, 1980). It is subdivided into seven classes with Bivalvia (more than 20,000 living species described) and Gastropoda constituting 95% of known mollusc species (Kantha, 1989). Bivalvia are a highly successful class of invertebrates found in aquatic habitats worldwide (Dame, 2011) and are an important food source for humans globally. The Food and Agriculture Organization estimated that 15 million tonnes of bivalves are traded each year (FAO and Department, 2012), representing around 14% of the total marine aquaculture production in the world (Wijsman et al., 2019). Bivalves are filter feeders, passing large quantities of water through their gills to capture particulate food such as phytoplankton (Arapov et al., 2010). The consumption of raw or insufficiently cooked shellfish can be associated with infectious diseases (Rippey, 1994). These are caused either by bacteria naturally present in the sea such as *Vibrio* spp. or by human pathogenic viruses and/or bacteria sourced from contaminated waters (Huss, 1997). In addition, organic and inorganic particles retained by bivalves can readily accumulate substances such as heavy metals (Landsberg, 2002; Deeds et al., 2008). A third possible risk for human consumers occurs when bivalves accumulate marine biotoxins as a result of ingesting toxic micro-algae. For example dinoflagellates from the genus *Alexandrium* (MacKenzie, 2014) can produce saxitoxin (STX) group toxins and, when concentrated in filter-feeding bivalves, these toxins are responsible for paralytic shellfish poisoning (PSP) events. Human and animal intoxications resulting from the ingestion of seafood contaminated with toxic micro-algae are recorded worldwide every year (Hallegraeff et al., 2003).

Gastropoda is the most diverse Molluscan class with over 75,000 existing species (30,000 are marine) and include both snail and slug species. They are highly abundant in marine ecosystems, playing important ecological roles as grazers, predators and major food sources for higher trophic levels (Holan et al., 2017). Marine gastropods are also an important source of animal protein to humans, especially in Asia (Hamli et al., 2013). Similar to bivalves, they are very susceptible to environmental contaminants and have been shown to accumulate metals (e.g., copper (Grosell et al., 2007); and marine biotoxins (e.g., ciguatoxins and STXs;Hwang et al., 1992a; Hwang et al., 2007; Luo et al., 2012)).

A marine biotoxin of increasing concern worldwide is tetrodotoxin (TTX). Tetrodotoxin is a potent toxin and its poisoning is described as one of the most violent forms of marine toxin intoxication (Bagnis et al., 1970). This toxin used to be reported as a threat only in Asian countries, mostly from pufferfish (Lago et al., 2015), but has now been reported in seafood in the Pacific and Mediterranean (Biessy et al., 2019; Katikou, 2019). Although there is increasing evidence that TTX is produced by microorganisms such as bacteria or marine micro-algae (Chau et al., 2011), there is still considerable uncertainty regarding its source and biosynthetic pathway. The evidence regarding the production of TTX by micro-algae is limited to one study that suggested the dinoflagellate *Alexandrium tamarense* produced this toxin (Kodama et al., 1996). However, the experimental conditions of this study are questionable as TTX was isolated from a large culture extract by Bio-Gel P-2 column which could also be used for isolation of other materials. In recent years, there has been an increase in reports of TTX in marine organisms, especially in commonly eaten bivalves like blue mussels (*Mytilus* spp.), but concentrations are not routinely monitored (Knutsen et al., 2017). Additionally, it has recently been demonstrated that TTX and STX toxicities are additive (Finch et al., 2018), making it appropriate to calculate TTX analogues as STX equivalents to determine a combined total toxicity of these toxin groups during monitoring. Most studies investigating TTX in edible shellfish report on the presence and concentrations of TTX in specific species, but very few explore toxin variability, location within organisms, or possible sources.

The aims of this review are; (1) to compile reports on the occurrence of TTX accumulation in edible bivalves and gastropods, and (2) evaluate the risk that TTX in edible shellfish poses to humans and assess whether it should be regulated in shellfish.

2. Tetrodotoxin

2.1. Overview

Tetrodotoxin is a potent neurotoxin (Campbell et al., 2013) named after the order of fish Tetraodontidae (Tetraodon pufferfish; Hwang and Noguchi, 2007). Tetrodotoxin has long been known as the causative agent in pufferfish poisoning events (Kodama et al., 1983; Noguchi et al., 2006). Records of pufferfish poisoning are documented in ancient literature particularly in Japan (200 AD) and China (at least 200 years ago; Miyazawa and Noguchi, 2001); and the poisoning was first described in 1774 in European literature by Captain James Cook who detailed the consumption of a local tropical fish from New Caledonia, now thought to be a pufferfish, and the resulting symptoms of weakness, numbness, and vomiting (Isbister et al., 2002).

Tetrodotoxin was first isolated and named in 1910 (Tahara, 1910). It is a low-molecular weight (319.2 g/mol), non-protein molecule with a highly unusual structure (Miyazawa and Noguchi, 2001). It is a zwitterion, with a positively charged guanidinium group, and a negatively charged hemiacetal alcohol group (Fig. 1A; Fuhrman, 1967). It was isolated as a crystal in the 1950s (Yokoo, 1950) and its structure was confirmed more than a decade later by several groups (Tsuda et al., 1964; Woodward, 1964; Goto et al., 1965). Although the structure of TTX has been confirmed,

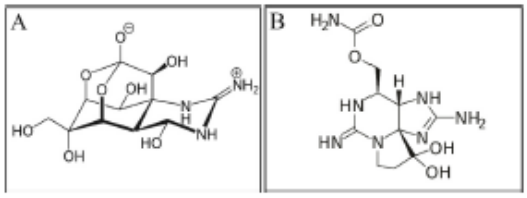

Fig. 1. Structures of tetrodotoxin (A) and saxitoxin (B).

its biosynthetic pathway remains unknown. Several proposed biosynthetic pathways have been published but have yet to be verified (Kotaki and Shimizu, 1993; Yotsu-Yamashita et al., 2013; Ueyama et al., 2018).

Tetrodotoxin is one of the most toxic natural substances known with a median lethal dose of 1–2 mg for a 50 kg human (Noguchi and Ebesu, 2001). The neurotoxin selectively binds and blocks voltage-gated sodium channels, hence inhibiting the propagation of action potentials in muscle and nerve cells often with fatal effects at extremely low doses. Following TTX ingestion, intoxication symptoms start rapidly with numbness and nausea, and can be followed by vomiting, breathing difficulties, paralysis, and at high enough concentrations, death by respiratory failure (Noguchi and Ebesu, 2001; Knutsen et al., 2017). The signs of intoxication by TTX are the same as those observed with STX which is also found in organisms containing TTX (Bane et al., 2014). Although the chemical structures of TTX and STX are considerably different (Fig. 1), both toxins exert their effects through an interaction with voltage-gated sodium channels resulting in inhibition of neuromuscular transmission (Narahashi, 1988). Both toxins are active on the α-subunit of the sodium channels although there are some differences in the affinities of TTX and STX for different sodium channel isoforms (Walker et al., 2012).

There is no known antidote for TTX (Soong and Venkatesh, 2006) and it has been responsible for numerous human fatalities, partly because the molecule is tasteless and heat-stable (Turner et al., 2015a). Most documented poisoning cases have occurred in Asian countries where pufferfish is regarded as a delicacy. Toda et al. (2012) reported 651 incidents of TTX poisoning including 56 fatalities in Japan due to pufferfish ingestion between 1989 and 2010. In 2008 in Bangladesh, three poisoning events resulted in 17 deaths (Islam et al., 2011) and Azanza et al. (2019) reported 93 cases of TTX poisoning with 21.6% leading to fatalities between 2005 and 2018 in the Philippines. Historically, incidence of intoxications by TTX were concentrated to Japan but in the last decade, TTX has been reported in a range of organisms from temperate environments, particularly in marine shellfish (Silva et al., 2012; Lago et al., 2015; Turner et al., 2015a, 2015b). Tetrodotoxin concentrations in bivalves are considerably lower than in pufferfish species (approximately 1000 times lower). However, the risk to humans cannot be ignored due to 1) the high amounts of bivalve consumed worldwide, and 2) the variability of TTX concentrations measured.

2.2. Tetrodotoxin methods of detection

A mouse bioassay (MBA) was the first method used for the detection of TTX in seafood. This involves aliquots of sample extract being injected into mice and the median death times used to calculate the toxicity (in mouse units; MU; Hungerford, 2006). It has been used for decades but aside from ethical concerns, the MBA is not specific to TTX and positive results could also be caused by the presence of STX-group toxins. Both classes of neurotoxin exhibit the same symptomology in mice, therefore some historical intoxications may have been incorrectly assigned to TTX or STX.

Another method used is the mouse neuro-2A (N2a) neuroblastoma cells assay (Kogure et al., 1988). In the presence of ouabain, there is an increase in sodium influx in the N2a cells, causing cellular swelling and death. When TTX is present, the sodium channels of the cells are blocked, enabling cell growth to continue. The N2a assay is a slow process with weeks required to culture the cells and cannot differentiate TTX from STX or other chemicals inhibiting sodium influx but the assay does not require multiple reference standards and is very sensitive. This technique has been reported to be used for monitoring below the European Food Safety Authority (EFSA) limit and is able to detect the toxin when present at 20 μg/kg (Gerssen et al., 2018).

Immunological assays also exist for the detection of TTX, with the enzyme-linked immunosorbent assay (ELISA), using an alkaline phosphatase-labelled monoclonal antibody being the most common (Raybould et al., 1992). ELISA kits are now commercially available although variability of performance and availability could hinder monitoring programmes relying on the assay for regular high-throughput testing.

Early chemical detection method of TTX was based on chemical conversion with alkali treatment of TTX and its analogues to fluorescent 2-amino-quinazoline derivatives and were able to be analysed in a fluorescence spectrophotometer (Nunez et al., 1976). This technique was utilised and further developed on a high-performance liquid chromatography (HPLC) system, by first separating the TTX analogues using on exchange or ion pairing chromatography then performing the derivatisation to this fluorescent C_9-base post-chromatographic separation continuously in line to the detector (Yasumoto et al., 1982; Yasumoto and Michishita, 1985). The analysis of TTX can also be undertaken by gas chromatography-mass spectrometry (GC-MS; Suenaga and Kotoku, 1980) using a similar derivatisation procedure to the HPLC but analysis requires an additional derivatisation process in order to get a GC-suitable product for analysis. The analysis of the C_9-base is, however, not specific to TTX, and therefore toxicity may be overestimated as less toxic analogues can be converted to the C_9-base and lead to false positives (Matsumura, 1995). To mitigate the issues of specificity, additional techniques such as thin layer chromatography (TLC), electrophoresis and nuclear magnetic resonance (NMR) have been used to confirm the presence of TTX (Noguchi et al., 1981).

Liquid chromatography-mass spectrometry (LC-MS) is now a well-established tool for the analysis of many different classes of marine toxins and has been used for over a decade as a regulatory monitoring of shellfish (McNabb et al., 2005; Stobo et al., 2005). This technique has been demonstrated to be very robust for the analysis of TTX and its analogues (Leung et al., 2011; Rodríguez et al., 2012; McNabb et al., 2014; Boundy et al., 2015). Tetrodotoxin and its analogues can be monitored directly with an excellent level of specificity, or alternatively may be derivatised and monitored as the C_9-base. The EFSA recommended that liquid chromatography with tandem mass spectroscopy (LC–MS/MS) methods were the most suitable for identification and quantification of TTX and its analogues (Knutsen et al., 2017).

3. Tetrodotoxin in edible shellfish

In this review, to enable a comparison between studies and to keep all units consistent, mouse unit (MU, the amount of toxin required to kill a 20 g female mouse in 30 min via intraperitoneal injection; Yu et al., 2004) was converted to μg/kg; the unit currently most commonly used to report biotoxins concentrations. The MU conversion calculation used in this review is approximate as some of the manuscripts did not specify the calibrated conversion factor

Table 1
Reports of tetrodotoxin in marine bivalves to date. DG = digestive gland; WF = whole flesh; HPLC = High performance liquid chromatography; MBA = Mouse Bioassay; LC-MS/MS = Liquid chromatography tandem mass spectrometry; LC-HRMS = liquid chromatography high resolution mass spectrometry; N2a = mouse neuro-2A neuroblastoma cell assay; TLC = Thin layer chromatography; * Recalculated from MU/g assuming 1 MU = 0.2 μg TTX.

Sampling dates	Country detected	Species	Maximum TTX concentration (μg/kg)	TTX localization	Detection methods	References
1993	Japan	*Patinopecten yessoensis*	8000*	DG	HPLC, MBA, TLC	Kodama et al. (1993)
2011	New Zealand	*Saccostrea commercialis* (synonym *Saccostrea glomerata*)	140	WF	LC-MS/MS	Ogilvie et al. (2012)
2011	New Zealand	*Crassostrea gigas*	80	WF	LC-MS/MS	Ogilvie et al. (2012)
2012	Greece	*Venus verrucosa*	176.5	DG	MBA, LC-MS/MS	Vlamis et al. (2015)
2012	Greece	*Mytilus edulis*	179.1	WF	MBA, LC-MS/MS	Vlamis et al. (2015)
			202.9	DG		
2013–2014	China	*Ruditapes philippinarum*	2.2	WF	LC-MS/MS	Han et al. (2018)
		Sinonovacula constricta	16	WF		
2013–2014	China	*Mytilus edulis*	2.7	WF	LC-MS/MS	Han et al. (2018)
		Mytilus coruscus	4.4	WF		
2014	New Zealand	*Paphies australis*	800	WF	LC-MS/MS	McNabb et al. (2014)
2013–2015	England	*Mytilus edulis*	120	WF	LC-MS/MS	Turner et al. (2015a)
2013–2015	England	*Crassostrea gigas*	5	WF	LCMS/MS	Turner et al. (2015a)
2015–2017	Netherlands	*Mytilus edulis*	33.3	WF	LC-MS/MS, N2a	Knutsen et al. (2017)
2015–2017	Netherlands	*Ostrea edulis*	124.1	DG	LC-MS/MS, N2a	Knutsen et al. (2017)
2015–2017	Italy	*Mytilus edulis*	64	WF	LC-HRMS	Dell'Aversano et al. (2019)
2017	New Zealand	*Perna canaliculus*	160	WF	LC-MS/MS	Boundy and Harwood (2017)

in their study. In these cases, a conservative estimate was used; 1 MU = 0.2 μg TTX, unless otherwise specified in the paper (for example, Luo et al. (2012) reported 1 MU = 0.220 μg TTX and Jen et al. (2007) reported 1 MU = 0.178 μg TTX). Some manuscripts only reported the amount of toxin in whole organisms (i.e., μg/organism) rather than a concentration (i.e., μg/kg) which makes it difficult to compare the toxicity across different studies.

3.1. Tetrodotoxin in marine bivalves

Tetrodotoxin has been identified in ten bivalve species from seven countries (Table 1; Fig. 2). The first report of TTX in marine bivalves was in Japan in 1993 when the toxin was found in the digestive gland of the scallop *Patinopecten yessoensis* following a bloom of the dinoflagellate *Alexandrium tamarense* (Kodama et al., 1993). Two decades later, TTX was detected in the endemic clam *Paphies australis* from New Zealand (max. 800 μg/kg; McNabb et al., 2014). The detection of TTX in edible bivalves like *P. australis* led to concerns about health risks for human consumers and prompted further research globally. Tetrodotoxin has now been detected in mussel species from five countries: England (*Mytilus edulis*), the Netherlands (*M. edulis*), China (*M. edulis* and *Mytilus coruscus*), New

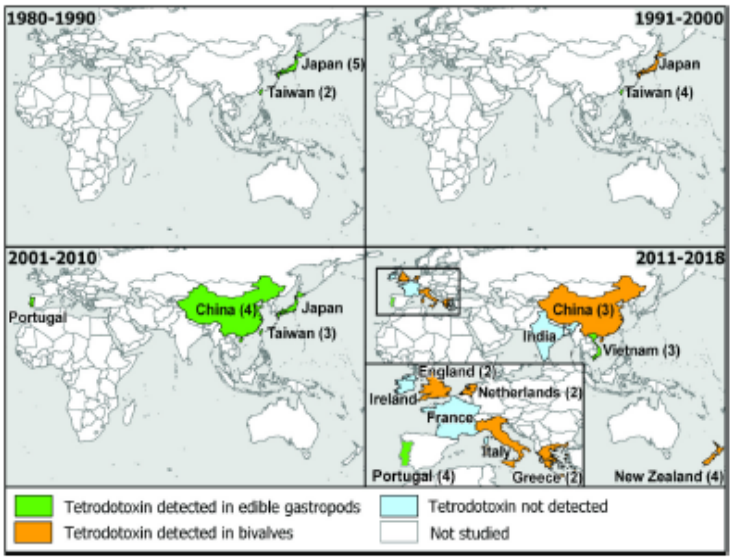

Fig. 2. Global detection of tetrodotoxin in edible gastropods and bivalves in the last four decades. When more than one species has been found to contain TTX in the same country, the number of species is given in brackets.

Zealand (*Perna canaliculus*), and Greece where the highest concentrations were detected (up to 203 µg/kg in *M. edulis*; Table 1). The neurotoxin has also been detected in oysters from Europe, New Zealand, and Asia with maximum concentrations of 140 µg/kg in the New Zealand rock oysters *Saccostrea commercialis*. Four species of clams have also been shown to contain TTX in five countries with the highest concentrations measured in the Venus clams *Venus verrucosa* in Greece (176 µg/kg; Table 1).

3.2. Tetrodotoxin in edible gastropods

Food poisoning cases caused by gastropod consumption have severe consequences to human health and the toxin responsible in most poisoning cases is TTX (Hwang et al., 2007). Tetrodotoxin has been reported in eleven marine gastropod genera from five countries, with the first detection occurring in 1980 (Table 2, Fig. 2; Shiomi et al., 1984).

Tetrodotoxin is generally found at the highest concentrations in the digestive glands (Table 2), possibly suggesting that accumulation is via dietary sources. For example, the trumpet shell *Charonia sauliae* was reported to accumulate TTX from starfish, a TTX-bearing food source of the gastropod (Noguchi et al., 1982). Rodriguez et al. (2008) found very high TTX concentrations (315,000 µg/kg) in the digestive glands of the predatory sea snail

Table 2
Reports of tetrodotoxin (TTX) in edible marine gastropods. † Reports linked to human intoxications; *Recalculated from MU/g assuming 1 MU = 0.2 µg TTX unless otherwise specified in the paper; # Average concentrations of tetrodotoxin reported in each paper; DG = digestive gland, WF = whole flesh; MBA = Mouse bioassay; GC-MS = Gas chromatography-mass spectrometry; HPLC = High performance liquid chromatography; LC-MS/MS = Liquid chromatography tandem mass spectrometry; NMR = Nuclear magnetic resonance; TLC = Thin layer chromatography.

Sampling dates	Country detected	Species	Maximum TTX concentration (µg/kg)	TTX localization	Detection methods	References
1980	Japan	*Babylonia japonica*	11,000*	DG	MBA, GC-MS, TLC	Noguchi et al. (1981)
1981	Japan	*Nassarius siquijorensis* (previously *Zeuxis siquijorensis*)	680*	Edible parts	MBA, GC-MS, TLC	Narita et al. (1984)
1979–1980 †	Japan	*Charonia lampas*	390,000*	DG	MBA, GC-MS, TLC, NMR	Noguchi et al. (1982)
1984	Japan	*Tutufa lissostoma*	140,000*	DG	MBA, GC-MS, TLC, NMR	Noguchi et al. (1984)
1988–1989	Taiwan	*Rapana rapiformis* *Rapana venosa venosa*	28,000* 2600*	DG DG	MBA, HPLC, TLC	Hwang et al. (1991a)
1988	Taiwan	*Natica lineata*	144,000* 2400* 5600*	Muscle DG Other parts	MBA, HPLC, TLC	Hwang et al. (1990)
1988–1989	Taiwan	*Natica vitellus* *Polinices didyma* *Polinices tumidus*	4500*# 1300*# 14,000*# 3600*# 800*	Muscle DG Muscle DG DG	MBA, HPLC	Hwang et al. (1991b)
1989–1990	Taiwan	*Nassarius conoidalis* (previously *Niotha clathrata*)	220,000*# 45,000*#	DG Other parts	MBA, HPLC	Hwang et al. (1992b)
1989–1990	Taiwan	*Nassarius scalaris* (previously *Zeuxis scalaris*) *Zeuxis castus-like*	28,000 µg/specimen* 2600 µg/specimen*	WF WF	MBA, HPLC, TLC	Hwang et al. (1992a)
2002	China	*Nassaris sinarum* (previously *Zeuxis samiplicatus*)	37,000 µg/specimen*	WF	MBA, HPLC, TLC	Sui et al. (2002)
2002 †	Taiwan	*Oliva miniacea* *Oliva mustelina* *Oliva mirasei*	3600* 3200* 5600*	Edible parts (no DG) Edible parts (no DG) Edible parts (no DG)	MBA, HPLC, GC-MS, LC-MS	Hwang et al. (2003)
2004 †	China	*Nassarius glans*	2,100,000* 400,000* 550,000*	WF DG Muscle	MBA, GC-MS	Yin et al. (2005)
2005 †	Taiwan	*Nassarius papillosus*	110,000* 96,000*	Muscle DG	MBA, HPLC, LC-MS/MS	Jen et al. (2007)
2007–2008	Japan	*Nassarius glans*	2,000,000* 470,000*	DG Muscle	MBA, LC-MS	Taniyama et al. (2009)
2007	China	*Nassarius sinarum* (previously *Nassarius semiplicatus*) *Nassarius variciferus*	190,000* 3800*	WF WF	MBA, LC-MS	Luo et al. (2012)
2007 †	Portugal	*Charonia lampas lampas*	315,000	DG	MBA, LC-MS	Rodriguez et al. (2008)
2007	China	*Nassarius nitidus*	1350	Edible parts	MBA, LC-MS	Huang et al. (2008)
2010	Portugal	*Charonia lampas lampas*	66.6 22.4	Muscle DG	LC-MS/MS	Nzoughet et al. (2013)
2011	Portugal	*Phorcus lineatus* *Charonia lampas* *Nucella lapillus* *Gibbula umbilicalis*	<5.46 <5.46 <5.46 <5.46	WF WF WF WF	HPLC	Silva et al. (2019)
2014	Vietnam	*Oliva hirasei* *Oliva ornata* (previously *Oliva lignata*) *Oliva annulata*	4800* (~5–6% TTX) 4600* (~5–6% TTX) 3400* (~5–6% TTX)	Muscle Muscle Muscle	MBA, LC-MS/MS	Jen et al. (2014)

Charonia lampas lampas harvested from the south coast of Portugal after reports of food poisoning. However, in some species, TTX was exclusively detected in the flesh and not in the digestive gland which may indicate a different binding mechanism or alternative source in those species (Table 2).

Human poisonings from gastropods mostly occur in Asian countries such as Taiwan, Japan and China, where the highly nutritious molluscs are a popular food (Hwang et al., 2007). TTX-poisoning from gastropod ingestions has occurred in every decade since the 1980's (Table 2), and from 1994 to 2006, nine food poisoning incidents occurred in Taiwan and three people died, mostly from eating gastropods of the Nassariidae (mud snails) family. Taiwanese studies have found nine species from this family contain TTX in their tissues and eight of these species have been associated with poisoning incidents (Hwang et al., 2007). There is currently no regulation or monitoring of TTX in edible marine gastropods.

Tetrodotoxin has also been reported at very high concentrations (up to 500 mg/kg, over 60 times higher than in bivalves) in marine worms and flatworms. These species are known to live inside other organisms, including shellfish (Carroll et al., 2003; Stokes et al., 2014; Salvitti et al., 2015; Turner et al., 2018). Although there are no confirmed poisoning cases from TTX-bearing worms, the presence of one toxic marine worm or flatworm in an edible shellfish could result in an adverse health effect to the consumer, and this risk should be investigated further.

3.3. Tetrodotoxin regulations in marine gastropods and bivalves

Japan, where pufferfish is a delicacy, is one of the only countries to have policies, guidance values and regulations for TTX to manage the risk of intoxication (Davis, 2000). This includes certification of chefs trained in handling the fish and identification of species and organs known to contain high levels of TTX. In Japan, a guidance value of 2 mg TTX eq/kg is used to classify pufferfish as high or low toxicity (Tani, 1945; Endo, 1984). In other areas such as Europe, fish belonging to families Tetraodontidae, Molidae, Diodontidae and Canthigasteridae (the main species containing TTX) are prohibited to enter the food market (Regulation, 2012). In New Zealand, the regulation allows the importation of Korean pufferfish as long as it is accompanied with certification that identifies the species, a guarantee that the product has been gutted and prepared by a certified person, and is therefore deemed fit for human consumption (Dansted, 2019). In the United States, illegal importations of fish containing TTX have been reported and often results in human poisoning (Cohen et al., 2009).

With the detection of TTX in edible seafood, the need to regulate the toxin in shellfish is currently under debate by regulatory agencies in affected countries. The EFSA published a scientific opinion on the evaluation of the toxicity of TTX and analogues in bivalve molluscs and marine gastropods, and determined that concentrations above 44 μg/kg would indicate a concern for consumers of a large portion of shellfish (400 g or larger) (Knutsen et al., 2017). Following the EFSA recommendation, The Netherlands adopted the concentration of 44 μg TTX/kg as the action limit after detecting TTX in mussels and oysters from their shellfish production areas (Gerssen et al., 2018). Some gastropods and bivalves analysed in different parts of the world have been shown to contain TTX at levels greater than the EFSA recommended level (Tables 1 and 2). A recent study demonstrated that TTX has similar potency to STX and that the toxicities of both toxins are additive when administered together (Finch et al., 2018). This indicates that TTX should potentially be considered together with the STX-group toxins for regulatory monitoring. Further studies are required to better understand the need for regulation and the effects of TTX on humans. This is further highlighted by results from Boente-Juncal et al. (2019) who demonstrated that chronic low oral doses of TTX might have deleterious effects on renal and cardiac tissues.

4. Possible sources of tetrodotoxin and its dynamics within edible shellfish

To manage the risk associated for TTX in edible seafood, it is important to understand its origin, the mechanism in which the toxin enters the food supply and how the concentrations observed are accumulated. For TTX, this is difficult as controversy remains regarding its origin. The two most common hypotheses are that it is produced by symbiotic bacteria (endogenous) or that it is accumulated through the diet (exogenous).

The evidence for bacteria producing TTX is contradictory. According to some literature, there are a number of bacterial species that can produce TTX and its analogues (Pratheepa and Vasconcelos, 2013). The most commonly reported bacterial genera associated with TTX production are; *Vibrio*, followed by *Bacillus*, *Pseudomonas*, *Actinomyces* and *Micrococcus* (Turner et al., 2018). However, in a review of bacteria reported to produce TTX, no evidence of production of TTX was found when using modern highly-specific analytical methods (Chau, 2013). This indicates that there may have been an overestimation of the number of bacteria that produce TTX due to analytical constraints. The two methods of analysis that have commonly been used to investigate the production of TTX in bacterial cultures are: 1) HPLC followed by fluorescence detection, which has low specificity and can give rise to false positives due to matrix interferences (Matsumura, 1995); and 2) GC-MS, which has also been shown to generate false positives from extraction of culture medias (Matsumura, 1995). The TTX concentrations reported in bacterial cultures are also usually very low, making it difficult to explain the extremely high levels found in higher trophic species and bioaccumulation in the food chain from TTX-producing bacteria. Furthermore, most of the publications on bacterial production do not provide controls to eliminate the possibility of contamination from TTX source material, and some demonstrate loss of production with further inoculations which could also indicate contamination from the starting material.

Knowledge of TTX production and accumulation in higher organisms is also hindered by an incomplete understanding of the biosynthetic pathways for TTX production (Chau et al., 2011). Katikou (2019) recently summarised the knowledge to date on the biosynthetic pathway of TTX and outlined that it is likely that TTX biosynthetic pathways were different between terrestrial and marine animals. This hypothesis was further strengthened by the recent work of Ueyama et al. (2018), reporting the discovery of seven novel spiro bicyclic guanidino compounds isolated from the pufferfish *Tetraodon biocellatus* that share the same carbon skeleton as TTX. These new compounds have not been detected in terrestrial species. It was further suggested that marine TTX and analogues are produced by marine microorganisms and then accumulate in marine animals in higher trophic levels. Further studies are required to investigate the presence of the new compounds in marine bivalves and gastropods, which would help confirm the proposed TTX biosynthetic pathway.

Limited research has been performed on the source of TTX in marine bivalves or edible gastropods, but available evidence indicates an exogenous source. Gammaproteobacteria, particularly *Vibrio* and *Pseudomonas* species, have been linked to the accumulation of TTX in bivalves. Two recent studies (Turner et al., 2017b; Leão et al., 2018) found a correlation between the presence of *Vibrio* and *Pseudomonas* and shellfish that contained TTX, but they were unable to culture any TTX-producing bacteria from these

samples. The hypothesis that bacteria or micro-algae are the source of TTX is fuelled by reports of toxic episodes in bivalves during warmer months, in particularly late spring in Europe (Gerssen et al., 2018; Leão et al., 2018) and New Zealand (Biessy et al., 2019). This may indicate the presence of a warm-water adapted TTX-producing microorganism.

No data is available regarding the toxin depuration in gastropods but bivalves have been shown to depurate TTX. In the field, Turner et al. (2017b) reported that blue mussels (*M. edulis*) depurated TTX over a period of several weeks. Tetrodotoxin-containing clams (*P. australis*) from New Zealand depurated the toxin over 150 days when kept in captivity and fed a non-toxic diet (Biessy et al., 2019). These authors also show significant variations in TTX concentrations between different sites across the country. Clams from warmer waters were significantly more toxic than the ones from colder waters and when collected in winter, adding further evidence for the producer being associated with warmer waters. Using LC-MS/MS combined with immunohistochemistry Biessy et al. (2018) demostrated that toxic *P. australis* mostly accumulate TTX in their siphons and organs used for feeding, supporting the exogenous toxin source hypothesis.

In New Zealand, *Pleurobranchaea maculata*, the grey side-gilled sea slug has been shown to lay eggs that contain relatively high TTX levels compared to other parts of the slug (Wood et al., 2012a). Small *P. maculata* larvae have also been shown to contain high concentrations of TTX. High densities of this sea slug have been found in aquaculture areas in New Zealand (Taylor et al., 2015). In England, a new invasive Nemertean species *Cephalothrix simula* has been shown to contain high levels of TTX (Turner et al., 2018). In Japan, highly toxic Nemertans of the genus Cephalothrix were also found on the shells of cultured oysters in Japan (Asakawa et al., 2013). *Cephalothrix simula* and the eggs and larvae of *P. maculata* are small enough to be filtered by shellfish and the presence of these organisms in shellfish aquaculture areas could serve as a possible vector or source of the toxin (Wood et al., 2012b).

5. Future directions

The concentrations of recent novel detections of TTX in gastropods and bivalves are significantly lower than the concentrations observed in historic detections which have been linked with intoxication in gastropods and pufferfish (Fig. 2). It is unclear if the increased detection is due to improved sensitivity of modern techniques, increased monitoring, or an increase in prevalence. However, there is an ongoing concern regarding the risk these toxins pose to human health. When TTX is observed in shellfish, its concentrations are typically significantly higher in edible gastropods than bivalves, with a few exceptional records in bivalves like the Japanese scallops *P. yessoensis* and the New Zealand clam *P. australis* (Tables 1 and 2). Its presence in bivalves or gastropods has not been thoroughly investigated and many aspects of its origin and distribution remain unclear. As climatic conditions change and anthropogenic pressures on waterways intensify, it seems likely that the prevalence of TTX in shellfish will increase. Tetrodotoxin-bearing organisms are also migrating with climate change and are been detected in more geographically diverse locations worldwide (Kalogirou, 2013; Turner et al., 2018). The presence, bioavailability and potency of marine toxins like TTX are predicted to intensify (Roggatz et al., 2019), making monitoring of toxic species important. Current knowledge gaps limit the ability to provide comprehensive advice to guide regulations and monitoring of TTX. Critical areas where further studies are needed are discussed below.

5.1. Monitoring programs and regulatory limits for tetrodotoxin in edible shellfish

There have been no confirmed reports of illness attributed to the consumption of TTX-bearing bivalves but five human poisoning have occurred in edible gastropods since the 1980's (Table 2). The most recent case of human poisoning was reported in Spain after consumption of the sea snail *Charonia lampas lampas* in 2007 (Rodriguez et al., 2008). The concentrations observed in shellfish are considerably lower than found in other marine organisms known to cause human intoxication (e.g. pufferfish). The levels reported in edible shellfish are all <2000 μg/kg, which represents the level used to classify non-toxic species of pufferfish in Japan. If these concentrations are representative of TTX levels generally present in bivalves consumed by humans, this could indicate that TTX should be regarded as a low food safety risk. However, the report of high TTX concentrations in Japanese scallops in the 1990s (ca. 8000 μg/kg) indicate the potential for TTX to accumulate in edible bivalves to concentrations that pose a genuine food safety concern. The source of TTX in edible shellfish remains uncertain, making it difficult to manage should regulation be required and enforced.

The presence of STX in commercial shellfish is tightly regulated worldwide to protect human health and facilitate international trade. Tetrodotoxin has been reported to co-occur with STX in bivalves and gastropods (Kodama et al., 1993; Jen et al., 2014) but is not included as part of this toxin group, although it would be detected along with STX when using the PSP mouse bioassay. Establishing monitoring for TTX in edible shellfish should be a priority, especially after a recent toxicology paper (Finch et al., 2018) demonstrated that STX and TTX have additive toxicological effects and the risk to human health. A recently developed multi-toxin LC-MS/MS method can simultaneously distinguish and quantify STXs and TTXs and offers significant potential to include the assessment of this toxin into monitoring without adding significant cost or time (Boundy et al., 2015; Turner et al., 2017a).

5.2. Research that explores tetrodotoxin analogues in edible bivalves and gastropods

Tetrodotoxin has 30 known structural analogues, yet only a limited number have been identified in edible bivalves and gastropods (Turner et al., 2015a; Bane et al., 2016). Tetrodotoxin is the most commonly reported analogue in shellfish but further studies are required to support this observation. One limitation to advancing these studies is that there are no reference materials available for other TTX analogues. There is also very limited knowledge on the toxicity of other analogues. For example, 11-oxo-TTX has been shown to be an important analogue, which may potentially be equipotent with TTX, and has been documented to be a dominant analogue in some crabs (Zheng et al., 2019). Other analogues have been shown to have relatively low concentration and/or low toxicities relative to TTX (Boundy and Harwood, 2017).

Although total synthesis has been reported for some TTX analogues (Nishikawa et al., 2001), this is a long and complex process with low yield. Obtaining purified TTX analogues for reference material and toxicology will likely need to come from naturally contaminated material (Endo et al., 1988; Yotsu et al., 1992; Jang and Yotsu-Yamashita, 2007). Assuming it is possible to source a sufficient quantity of contaminated material, further review of toxin profiles found in edible bivalves and gastropods will provide useful information as to which analogues are of significance, and practical for isolation, purification and toxicological evaluation.

5.3. Source, accumulation and depuration of tetrodotoxin in bivalves and gastropods

Understanding the source, mechanism of TTX accumulation and depuration in marine edible bivalves and gastropods is important for managing risk. For example, this information would allow health warnings to be removed following a contamination event.

Bivalves are easy to find, harvest, rear through life stages and as stationary filter feeders, they may prove a suitable model which may assist with elucidating the origin of TTX in the marine environment. Designing controlled studies on accumulation and depuration of TTX in bivalves and gastropods is critical and will help consumers, farmers and managers understand how much TTX can accumulate in different species, and how long it will take for them to be safe to eat again after a toxic event.

One of the difficulties with undertaking these studies is that in contrast to other marine biotoxins, where toxin-producing microalgae have been isolated and cultured and thus providing a feed source, this is not the case for TTX. An important first step would be to develop a method to feed TTX to filter feeding bivalves and grazing gastropods. Another important aspect of accumulation studies will be exploring differences in toxin uptake between closely related species. More experimental studies (i.e., spatial and seasonal variations) on known TTX-accumulating species are needed rather than just focusing on finding new species that accumulate the neurotoxin at low concentrations. These studies would provide insight into the source of TTX. For example, Biessy et al. (2019) observed that out of three clam species harvested from the same beach, only *P. australis* accumulated TTX and mostly in their siphons. It is possible that some species contain unique TTX-binding proteins in specific organs, similar to the one purified from the plasma of the pufferfish *Fugu pardalis* (Yotsu-Yamashita et al., 2001) or saxiphilin, the STX-binding protein in the bullfrog *Rana catesbeiana* (Mahar et al., 1991). Identifying species able to accumulate high amounts of TTX will be the first step towards investigating the hypothesis that TTX-binding proteins are present in certain mollusc species and are linked to toxin accumulation.

5.4. Has there been an increase in tetrodotoxin-detections worldwide and why?

This review has shown that there has been an increase in reports of TTX detection in edible bivalves and gastropods over the last four decades. It is unclear if this is due to an increase in surveillance or an actual increase in TTX in bivalves and gastropods. For the latter, this could be due to increases in sea water temperatures worldwide and TTX-bearing organisms expanding to new climates or to an increase in surveillance with the detection methods becoming more precise, available and cheaper. With the exceptions of India, France and Ireland, where all bivalves and gastropods tested were negative, all other studies have detected traces of TTX in edible shellfish (Turner et al., 2017c; Katikou, 2019; Fig. 2). To date, no studies have been carried out on TTX in bivalves and edible gastropods in Africa (except in Morocco (Silva et al., 2019)), Australia, North and South America. Further studies in these regions should be a priority.

A systematic worldwide survey is required to determine if TTX occurrence in edible seafood is increasing globally and if there is a risk to human health. This should be repeated at regular time intervals. Inclusion of TTX analysis in current shellfish monitoring programmes would add valuable data.

6. Conclusions

This review compiled reports on the occurrence of TTX in bivalves and edible gastropods and showed that there has been an increase in TTX and/or in the frequency of TTX reports in these organisms. Increased monitoring would help understand if this is due to more sensitive detection techniques or if the toxin prevalence is increasing with global environmental changes. Tetrodotoxin concentrations reported in some bivalves and edible gastropods are exceed the guidance level of 44 μg TTX/kg suggested by the European Food Safety Authority. However, this conservative concentration determined by EFSA is significantly lower than the current regulatory limit applied for STXs (800 μg/kg). Cases where human intoxication from gastropods was reported, the concentration of TTX was significantly higher than the regulatory limit for STX. This supports that the existing regulatory limit established for STX continues to be appropriate for food safety and inclusion of TTX within the existing regulation of the STX-group toxins would also be appropriate.

Declaration of interest

The authors declare no conflict of interest.

Authors' contributions

LB wrote the original draft, MJB and LB constructed the tables and all authors contributed, reviewed and edited the manuscript.

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APPENDIX 7

Table 0A7-0-1. Dates and coordinates of *Paphies australis* collection sites from around New Zealand.

Sites	Region	Collection Date	Coordinates	
			Latitude	Longitude
Hokianga Harbour	Northland (West Coast)	28 September 2017	35°47’28” S	173°40’23” E
Whangaruru Harbour	Northland (East Coast)	20 January 2018	35°36’18” S	174°34’03” E
Auckland *Whangateau Harbour*	Auckland	26 August 2018	36°18’35” S	174°46’44” E
Tauranga *Kauri Point*	Bay of Plenty Waikato	13 February 2018	37°51’56” S	175°97’78” E
Waihi Estuary	Bay of Plenty	28 August 2018	37°76’41” S	176°48’32” E
Mahia (Oraka)	Hawkes Bay	24 September 2018	39°07’23” S	177°89’96” E
Port Taranaki	New Plymouth	28 March 2018	39°05’82” S	174°05’01” E
Petone Beach	Wellington	22 March 2018	41°13’39” S	174°52’15” E
Marahau	Nelson Tasman	25 January 2018	41°00’88” S	173°00’96” E
Bleinhem *Wairau River*	Marlborough	24 January 2018	41°50’36” S	174°05’94” E
Akaroa *Children’s Bay*	Canterbury	18 March 2018	43°80’08” S	172°96’57” E
Dunedin *Karitane*	Otago	18 September 2018	45°63’48” S	170°65’51” E
Riverton	Southland	29 July 2018	46°21’38” S	168°12’98” E

Table A7-0-2. Pairwise *post hoc* comparisons (Tukey HSD test) of mean tetrodotoxin concentrations among New Zealand populations of *Paphies australis*. Bolded values represent statistically significant differences ($p < 0.05$).

Sites	Whangaruru	Auckland	Tauranga	Waihi	Mahia	New Plymouth	Wellington	Marahau	Blenheim	Dunedin	Riverton
Hokianga	0.098	**0.004**	0.940	**<0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Whangaruru		0.995	0.899	0.389	0.946	0.881	0.490	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Auckland			0.234	0.973	0.999	0.999	0.989	**<0.001**	**<0.001**	**0.008**	**<0.001**
Tauranga				**0.007**	0.103	0.064	**0.011**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Waihi					0.998	0.999	1	**<0.001**	**<0.001**	0.266	**<0.001**
Mahia						1	0.999	**<0.001**	**<0.001**	**0.025**	**<0.001**
New Plymouth							0.999	**<0.001**	**<0.001**	**0.042**	**<0.001**
Wellington								**<0.001**	**<0.001**	0.195	**<0.001**
Marahau									1	0.078	0.894
Blenheim										**0.035**	0.975
Dunedin											**<0.001**

APPENDIX 8

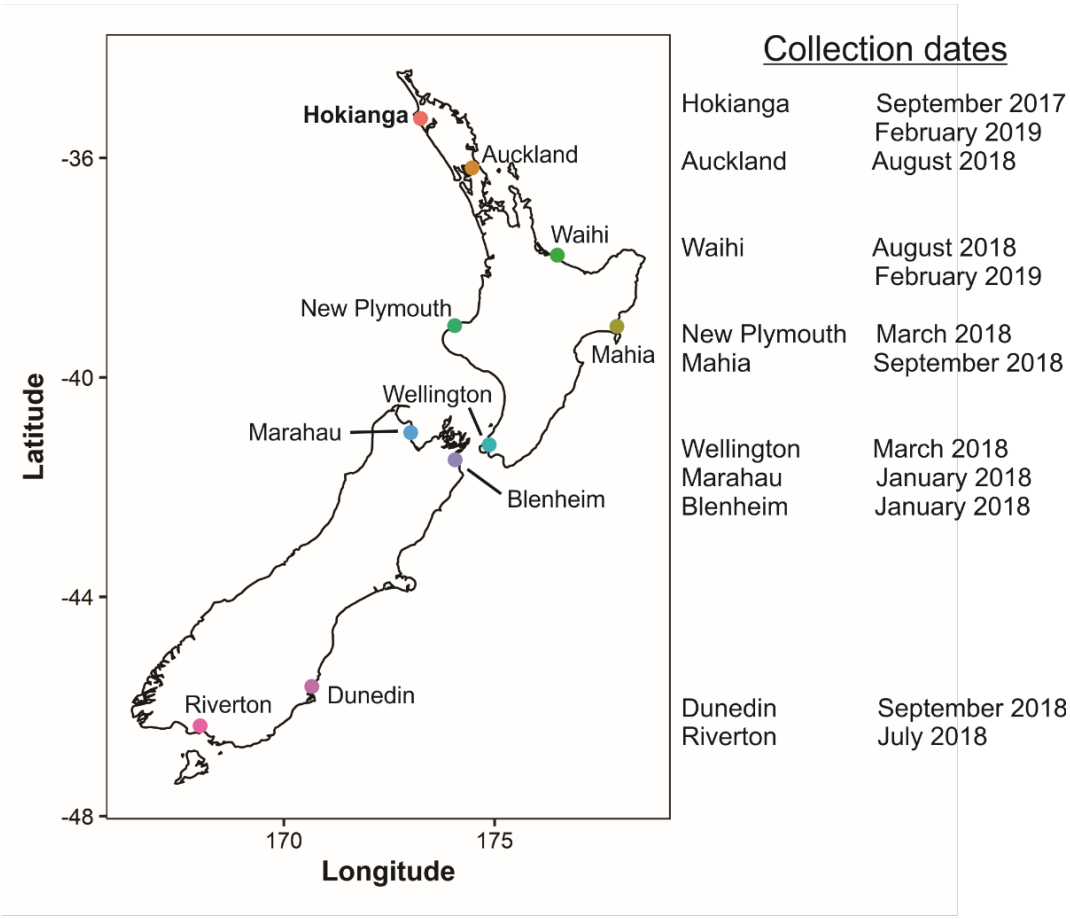

Figure 0A8-1. *Paphies australis* collection dates and locations around New Zealand.

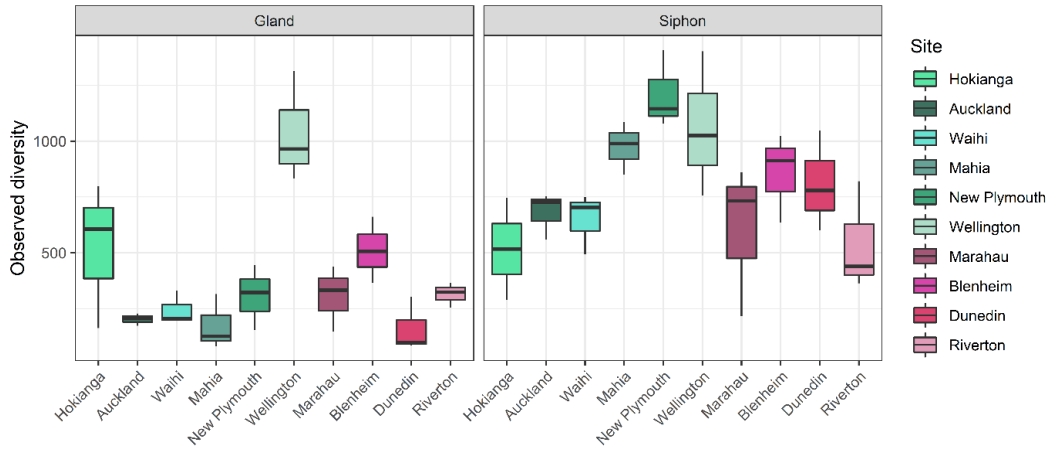

Figure A8-2. Boxplot displaying differences in community diversity between *Paphies australis* digestive glands and siphons from ten sites around New Zealand. Boxes display the first and third quartile spread of the data, with the line in the box indicating the median, the whiskers denoting the minimum and maximum values and the dots as outliers of the data. Sites are ordered by increasing latitude for each Island, the green colours represent the sites from the North Island and the pinks represent the sites of the South Island.

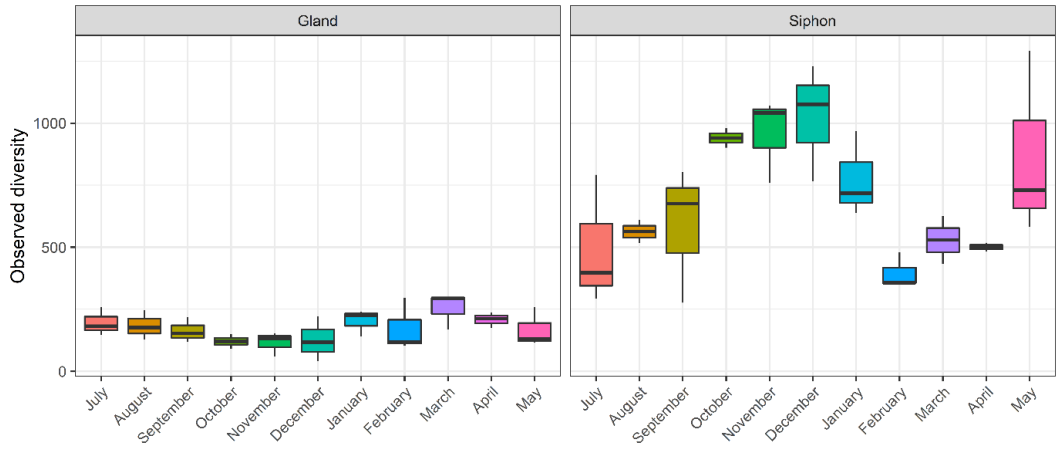

Figure 0A8-3. Boxplot displaying differences in community diversity between *Paphies australis* digestive glands and siphons from the Hokianga Harbour (Northland, New Zealand), sampled every month from July 2017 to May 2018. Boxes display the first and third quartile spread of the data, with the line in the box indicating the median, the whiskers denoting the minimum and maximum values and the dots as outliers of the data. Sites are ordered by increasing latitude for each Island, the green colours represent the sites from the North Island and the pinks represent the sites of the South Island.

Table A8-1. Summary of bacterial Amplicon Sequence Variants (ASVs) significantly correlated to higher tetrodotoxin concentrations**.** Unclass. = unclassified

Phylum	Class	Order	Family	Genus	ASVs	Linear Regression	
						R^2	*P* value
Acidobacteria	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	Subgroup_10	ASV3091	0.06	0.045
Acidobacteria	Blastocatellia	Blastocatellales	Blastocatellaceae	*Blastocatella*	ASV3444	0.21	<0.0001
Cyanobacteria	Oxyphotobacteria	Unclass.	Unclass.	Unclass.	ASV69	0.05	0.007
Cyanobacteria	Oxyphotobacteria	Synechococcales	Cyanobiaceae	*Synechococcus_CC9902*	ASV84	0.06	0.043
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	*Romboutsia*	ASV239	0.08	0.02
Marinimicrobia	SAR406_clade	Unclass.	Unclass.	Unclass.	ASV1911	0.23	<0.0001
Marinimicrobia	SAR406_clade	Unclass.	Unclass.	Unclass.	ASV48560	0.13	0.003
Planctomycetes	Planctomycetacia	Planctomycetales	Gimesiaceae	Unclass.	ASV188	0.23	<0.0001
Proteobacteria	Gammaproteobacteria	Unclass.	Unclass.	Unclass.	ASV12	0.16	0.001
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	*Mycoplasma*	ASV16	0.15	0.0021
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	*Mycoplasma*	ASV18	0.14	<0.0001
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Unclass.	ASV36	0.35	<0.0001
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	*Mycoplasma*	ASV47	0.17	<0.0001
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	*Roseibacillus*	ASV1308	0.13	0.003
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	*Rubritalea*	ASV208	0.12	0.005
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	Unclass.	ASV345	0.22	<0.0001
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	*Roseibacillus*	ASV541	0.24	<0.0001

Table A8-2. Summary of bacterial Amplicon Sequence Variants (ASV) corresponding to the core microbiome of *Austrovenus stutchburyi* digestive glands, define as present in at least 70% of all samples. In bold are the genera detected in more than 90% of all samples. Unclass. = unclassified.

Phylum	**Class**	**Order**	**Family**	**Genus**	**Number of ASVs**
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	***Mycoplasma***	3
Planctomycete	Planctomycetacia	Gemmatales	Gemmataceae	Unclass.	2
Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	***Psychrilyobacter***	1
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Unclass.	1
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Unclass.	1
Firmicutes	Clostridia	Clostridiales	Clostridiaceae_1	Unclass.	1
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	***Epulopiscium***	1
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	*Romboutsia*	1
Proteobacteria	Gammaproteobacteria	Cellvibrionales	Spongiibacteraceae	Unclass.	1

Table A8-3. Metabolic pathways and enzymes significantly more abundant in the tetrodotoxin (TTX)-bearing *Paphies australis* from the Hokianga Harbour compared to non-TTX-bearing *P. australis* from other sites.

Organs	log2Fold Change	*p*-value	Enzyme Codes	Enzyme Name	Potential KEGG pathways	Class
Digestive glands	-12.3	< 0.001	1.1.1.8	glycerol-3-phosphate dehydrogenase (NAD+)	Glycerophospholipid metabolism	Oxidoreductases
	-12.3	< 0.001	2.3.1.7	carnitine O-acetyltransferase		Transferases
	-11.2	< 0.001	3.1.31.1	micrococcal nuclease		Hydrolases
	-10.6	< 0.001	2.7.1.113	deoxyguanosine kinase	Purine metabolism	Transferases
	-8.1	< 0.001	4.1.1.85	3-dehydro-L-gulonate-6-phosphate decarboxylase	Pentose and glucuronate interconversions/ Ascorbate and aldarate metabolism	Lyases
	-6.9	< 0.001	1.1.1.17	mannitol-1-phosphate 5-dehydrogenase	Fructose and mannose metabolism	Oxidoreductases
	-6.9	< 0.001	1.1.1.298	3-hydroxypropionate dehydrogenase (NADP+)	Carbon fixation pathways	Oxidoreductases
	-6	< 0.001	2.4.1.230	kojibiose phosphorylase		Transferases
	-5.3	< 0.001	5.1.3.9	N-acylglucosamine-6-phosphate 2-epimerase	Amino sugar and nucleotide sugar metabolism	Isomerases
Siphon	-5.3	< 0.001	1.1.1.290	4-phosphoerythronate dehydrogenase	Vitamin B6 metabolism	Oxidoreductases
	-4.5	< 0.001	2.7.11.7	myosin-heavy-chain kinase		Transferases
	-4.4	< 0.001	3.10.1.1	N-sulfoglucosamine sulfohydrolase	Glycosaminoglycan degradation	Hydrolases
	-4.3	< 0.001	1.1.1.62	17beta-estradiol 17-dehydrogenase	Steroid hormone biosynthesis	Oxidoreductases
	-4.3	< 0.001	5.1.3.9	N-acylglucosamine-6-phosphate 2-epimerase	Amino sugar and nucleotide sugar metabolism	Isomerases
	-4.1	< 0.001	1.3.3.5	bilirubin oxidase	Porphyrin and chlorophyll metabolism	Oxidoreductases
	-4.1	< 0.001	1.1.1.91	Aryl-alcohol dehydrogenase (NADP+)		Oxidoreductases
	-4.1	< 0.001	3.5.1.49	formamidase	Cyanoamino acid metabolism/ Glyoxylate and dicarboxylate metabolism/ Nitrogen metabolism	Hydrolases

	-3.6	< 0.001	1.3.1.10	enoyl-[acyl-carrier-protein] reductase (NADPH)	Fatty acid biosynthesis/Biotin metabolism	Oxidoreductases
	-3.3	< 0.001	3.5.4.12	dCMP deaminase	Pyrimidine metabolism	Hydrolases
	-3.1	< 0.001	2.6.1.18	beta-alanine-pyruvate transaminase	Valine, leucine and isoleucine degradation/ beta-Alanine metabolism/ Propanoate metabolism	Transferases
	-3.1	< 0.001	2.3.1.101	formylmethanofuran-tetrahydromethanopterin N-formyltransferase	Methane metabolism	Transferases
	-2.7	< 0.001	5.4.2.8	phosphomannomutase		Oxidoreductases

APPENDIX 9

toxins

MDPI

Article

Survey of Tetrodotoxin in New Zealand Bivalve Molluscan Shellfish over a 16-Month Period

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Abstract: Tetrodotoxin (TTX) is a heat-stable neurotoxin typically associated with pufferfish intoxications. It has also been detected in shellfish from Japan, the United Kingdom, Greece, China, Italy, the Netherlands and New Zealand. A recent European Food Safety Authority (EFSA) scientific opinion concluded that a level of <0.044 mg TTX/kg in marine bivalves and gastropods, based on a 400 g portion size, does not result in adverse effects in humans. There have been no reports of human illness attributed to the consumption of New Zealand shellfish containing TTX. To obtain a greater understanding of its presence, a survey of non-commercial New Zealand shellfish was performed between December 2016 and March 2018. During this period, 766 samples were analysed from 8 different species. TTX levels were found to be low and similar to those observed in shellfish from other countries, except for pipi (*Paphies australis*), a clam species endemic to New Zealand. All pipi analysed as part of the survey were found to contain detectable levels of TTX, and pipi from a sampling site in Hokianga Harbour contained consistently elevated levels. In contrast, no TTX was observed in cockles from this same sampling site. No recreationally harvested shellfish species, including mussels, oysters, clams and tuatua, contained TTX levels above the recommended EFSA safe guidance level. The levels observed in shellfish were considerably lower than those reported in other marine organisms known to contain TTX and cause human intoxication (e.g., pufferfish). Despite significant effort, the source of TTX in shellfish, and indeed all animals, remains unresolved making it a difficult issue to understand and manage.

Keywords: HILIC-MS/MS; emerging marine toxin; saxitoxin; shellfish; tetrodotoxin

Key Contribution: Low or undetectable levels of tetrodotoxin observed in New Zealand shellfish. Toxin detected in all pipi (*Paphies australis*) included in study.

1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin that has been responsible for many human intoxications and deaths around the world, primarily from consumption of pufferfish (fugu). The distribution of TTX and its analogues in the environment is remarkably diverse, being found in a variety of organisms from both marine and terrestrial environments [1–3]. The source of TTX is still controversial and not definitively proven, although the dominant hypothesis is that it is of microbial origin [4,5]. There have been reports of TTX in 21 species of bivalves and edible gastropods from 10 countries since the 1980's [6]. Increasing reports of the detection of TTX in aquaculture species such as bivalve molluscs has drawn

considerable recent attention to the toxin, reinvigorating scientific interest and questioning whether regulation is required. TTX has similar sodium channel blocking action and potency to the paralytic shellfish toxin group (saxitoxin (STX) and analogues) but is structurally dissimilar. The maximum permissible level adopted in most countries for paralytic shellfish toxins in bivalve molluscs is 0.8 mg STX.2HCl eq/kg. In March 2017, the European Food Safety Authority (EFSA) published their scientific opinion on the risk to public health related to the presence of TTX and analogues in marine bivalves and gastropods. They concluded that a concentration below 0.044 mg TTX eq/kg, based on a large portion size of 400 g, was considered not to result in adverse effects in humans [7]. Liquid chromatography with tandem mass spectroscopy (LC–MS/MS) methods were determined to be the most suitable for the specific identification and quantification of TTX and its analogues. Japan used a maximum portion size of 1000 g, to determine that pufferfish are safe to consume below a TTX concentration of <10 MU/g (2 mg/kg) [8,9].

There have been reports of TTX in shellfish from Japan, New Zealand, the United Kingdom, Greece, China, Italy and the Netherlands. In contrast, several recent surveys looking for TTX in shellfish from the Iberian Peninsula did not find detectable levels. This included mussels (*Mytilus galloprovincialis*), oysters (*Crassostrea gigas*) and clams (*Ruditapes philippinarum* and *Donax* spp.) from Portugal [10], and various bivalve species from the Northwest and East of Spain [11,12]. In 1993, TTX was reported at concentrations of up to 40 MU/g (~8 mg TTX/kg) in the digestive glands of Japanese scallop (*Patinopecten yessoensis*). TTX was confirmed as being present using a variety of analytical techniques that included high-performance liquid chromatography with fluorescence detection and fast atom bombardment-mass spectrometry (FAB-MS) after the toxin was partially purified from scallop tissue [13]. In New Zealand, the first report was in pipi (*Paphies australis*), an endemic clam, collected from the North Island in 2011. Concentrations up to 0.8 mg/kg were reported in these samples with analyses being performed using two separate methods that employed LC-MS/MS [14]. One approach analysed intact toxin, while the other monitored a TTX-C9 base derivatisation product generated by TTX dehydration under highly alkaline conditions. More recent studies on TTX-contaminated pipi have identified organ level differences, with the siphon found to contain significantly higher TTX levels than other organs of the shellfish [15]. Depuration was observed in TTX-contaminated pipi maintained in captivity on a toxin-free diet and a concentration gradient has also been observed with higher levels observed in shellfish from the warmer northern latitudes of New Zealand [16]. In 2012 in Greece, during official shellfish monitoring for the presence of marine biotoxins, a series of unexplained positive mouse bioassay screens were observed. This was not attributed to any regulated toxin and analysis by LC-MS showed the presence of TTX at levels of up to 0.223 mg/kg in Mediterranean mussels (*Mytilus galloprovincialis*) [17]. Analysis of Greek library samples, mussels and *Venus verrucosa* (clams), between 2006–2012 showed TTX at levels between 0.061–0.194 mg/kg. In England, TTX was first reported in blue mussels (*Mytilus edulis*) and Pacific oysters (*Crassostrea gigas*) from southern England in 2014, with levels of up 0.12 mg/kg observed in the small sample set ($n = 29$) [18]. In this study, shellfish harvested from two sites on the south coast of England were screened for TTX using a hydrophilic interaction liquid chromatography (HILIC)-MS/MS method and its presence was confirmed using LC-MS/MS after derivatisation under alkaline conditions to the TTX-C9 base. Several TTX analogues were observed at low levels in the samples. A s more comprehensive study of various shellfish species collected from southern England between 2014 and 2016 reported a maximum level of 0.253 mg/kg a Pacific oyster sample [19]. In Italy (Sicily) in 2018, TTX was detected for the first time in 14 out of the 25 shellfish samples analysed. The species analysed were mussels (*Mytilus galloprovincialis*) and clams (*Venerupis decussata*) with a maximum level of only 0.0064 mg TTX eq/kg observed. TTX has also been reported in The Netherlands with a maximum level of 0.253 mg/kg in Pacific oysters (*Crassostrea gigas*) and 0.101 mg/kg in blue mussels (*Mytilus edulis*) from production areas [20]. TTX analogues were also monitored for using high-resolution LC-MS and were not observed, except for 4-*epi*-TTX in a single sample. In China, during 2015, as part of the validation for a new LC-MS method, Manila clams (*Ruditapes philippinarum*) purchased from markets in China were analysed for the presence of TTX and

trace levels were observed [21]. A similar observation was made in another Chinese study looking at TTX in aquatic products, with low µg/kg levels observed in clams (*R. philippinarum*), blue mussels (*M. edulis*), hard-shell mussels (*Mytilus corucus*) and Chinese razor clam (*Sinonovacula constricta*) [22]. The maximum level observed was 0.016 mg/kg in a Chinese razor clam.

In the present study, a survey on TTX in non-commercial New Zealand bivalves was performed over a 16-month period to determine the prevalence of this toxin and help determine if it represents a food safety risk to shellfish consumers. Samples analysed were collected as part of the non-commercial shellfish marine biotoxin monitoring programme administered by the New Zealand Ministry for Primary Industries (MPI), New Zealand Food Safety.

2. Results

2.1. Survey Results

In total, 766 shellfish samples were analysed over the 16-month period (Table 1). Sample matrices analysed comprised Greenshell™ mussels (*Perna canaliculus*; 63%), tuatua (*Paphies subtriangulata*; 28%) and pipi (*Paphies australis*; 6%), with fewer than 10 samples in total of blue mussels (*Mytilus edulis*), clams (unspecified), cockles (*Austrovenus stutchburyi*), Pacific oysters (*Crassostrea gigas*) and rock oysters (*Saccostrea glomerata*). There was no TTX detected in the majority of samples (69%, Table 1). A further 27% of samples had detectable TTX but at levels below the recommended safe guidance level reported in the 2017 EFSA scientific opinion (0.002–0.044 mg/kg). Another 4% of samples had TTX levels greater than the safe guidance level (≥0.044 mg/kg), with all of these being pipi. In fact, all pipi tested as part of this survey contained detectable TTX levels. These percentages are likely to be biased by the unequal numbers of samplings for each species but provide a valuable insight into TTX levels in many recreationally harvested shellfish species.

Table 1. Summary of non-commercial shellfish samples analysed for tetrodotoxin (TTX) (December 2016–March 2018).

Shellfish Species	TTX Level (Mg/Kg)			
	<0.002	0.002–0.044	≥0.044	Total
Blue mussel	4	3	0	7
Clams	1	0	0	1
Cockle	7	1	0	8
Greenshell™ mussel	318	162	0	480
Pacific oyster	0	1	0	1
Pipi	0	15	29	44
Rock oyster	3	6	0	9
Tuatua	194	22	0	216
Total	527 (69%)	210 (27%)	29 (4%)	766

Most samples were taken from sites located in the North Island as historical information shows these as the most at-risk areas for harmful algal blooms and hence why they are routinely monitored for regulated marine toxins. It was possible to overlay a map of New Zealand with the sampling location and number of shellfish samples tested for TTX (Figure 1).

Most pipi samples included in the survey came from Koutu Point, which is in the Hokianga Habour (Figure 2). Consistently high TTX levels were detected in pipi from this site over the time monitored (Figure 3A; blue dots). In contrast, cockles collected from the same site at the same time contained no detectable TTX (Figure 3A; black dots). To assess TTX level variability between individuals, pipi were collected from Tauranga harbour in February 2017 and 12 individual shellfish were analysed separately for TTX in addition to the usual pooled homogenate from a minimum of 12 individuals. Similar TTX levels were observed between individual shellfish with a precision <20% relative standard deviation (RSD) (Figure 3B). A pooled pipi homogenate generated gave a TTX level of 0.15 mg/kg, which is in line with the median level from the analysis of individuals.

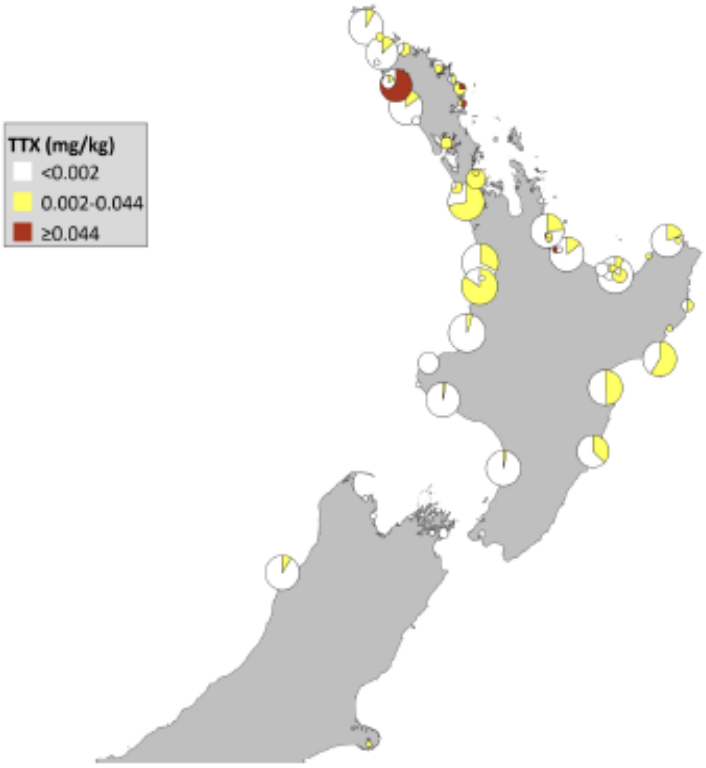


Figure 1. Location of sampling sites and tetrodotoxin levels observed in shellfish collected from around New Zealand between December 2016 and March 2018. The size of the circle corresponds to number of samples from a site (max = 41). Colouration within each circle shows the proportion of samples that fall within each of the three defined TTX levels. No samples were taken for the part of New Zealand not shown in the map.

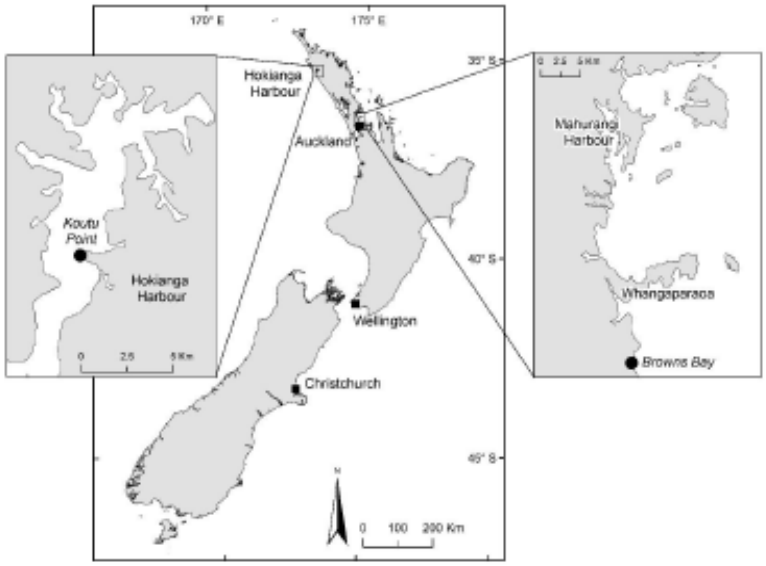
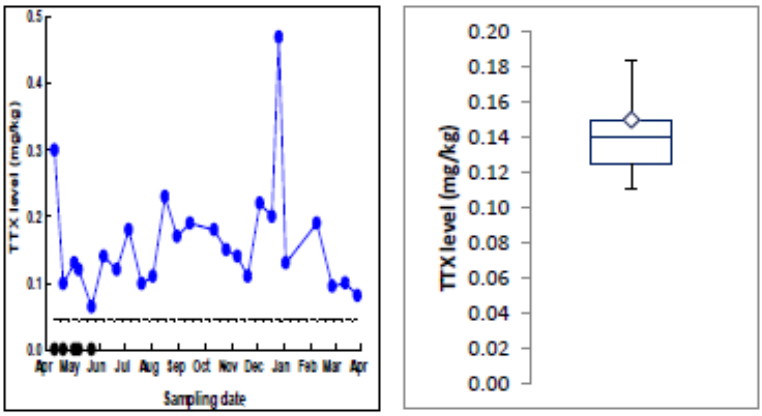


Figure 2. Location of Koutu Point and Browns Bay sampling sites in the north island of New Zealand.



(A) (B)

Figure 3. (**A**) Tetrodotoxin levels in pipi and cockles sourced from Koutu Point in the Hokianga Harbour between April 2017 and April 2018. Blue dots = pipi; black dots = cockles. Dotted line represents European Food Safety Authority (EFSA) safe guidance level (0.044 mg/kg). (**B**) Box and whisker plot showing tetrodotoxin levels measured in 12 individual pipi sourced from Tauranga harbour in February 2017. Shown is the median, interquartile range, 5th and 95th percentiles. The diamond represents the result from the pooled homogenate.

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

2.2. Analysis of Archive Shellfish Samples

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

2.3. Presence of Tetrodotoxin (TTX) Analogues

Selected shellfish samples that contained TTX above an arbitrary threshold level of 0.02 mg TTX/kg were re-analysed for the presence of known TTX analogues using a targeted TTX acquisition method. Quantitation of the other analogues was off TTX and assumed an equivalent response. In all cases TTX was found to be the most abundant analogue, accounting for >98% of the total TTX analogues present. Other TTX analogues were observed but in most cases were present at too low a concentration to allow quantitation. As a representative example, see Figure 4 showing the presence of TTX (0.19 mg TTX/kg) in a pipi sample and trace detections of structurally related analogues. The assignment of the TTX analogues was made based on comparison of retention time against published findings [23] and a naturally contaminated, and well characterized, flatworm quality control sample.

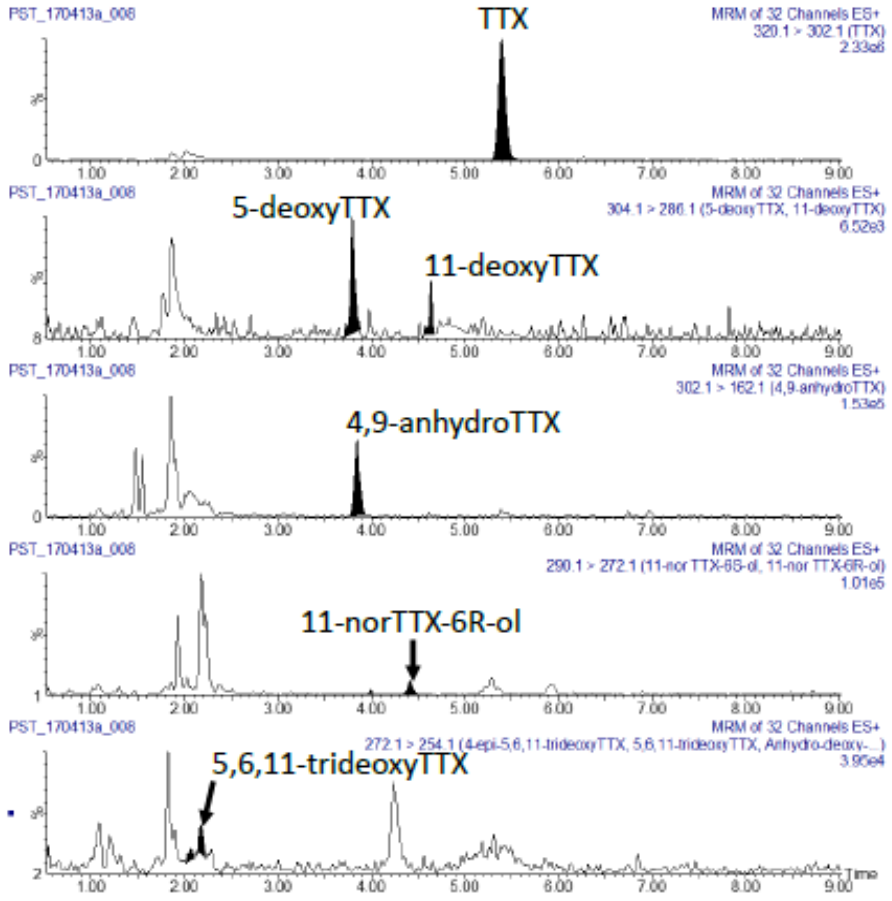


Figure 4. Observed TTX analogues from a Koutu point pipi sample (0.19 mg TTX/kg).

2.4. *Outlier Sample from Outside Survey Period*

Whilst establishing the methodology to monitor TTX in shellfish, several non-commercial samples being assayed for paralytic shellfish toxins were also monitored for TTX. A Greenshell™ mussel sample from Browns Bay in the Hauraki Gulf was found to contain TTX at a level of 1.6 mg/kg. This site had been sampled to determine the geographical spread of an *Alexandrium pacificum* paralytic shellfish toxin bloom event in the Mahurangi Harbour (Northland, Figure 2). Paralytic shellfish toxins were also present in this sample at 0.4 mg STX·2HCl eq/kg (the CODEX standard for reporting saxitoxin-group toxins is in saxitoxin dihydrochloride equivalents per kg), representing half the regulatory limit for this toxin class. To assess the contribution of TTX to the total toxicity of the sample, it was subjected to a mouse bioassay (AOAC959.08) that targets these toxins and was found to be above regulatory limit at 1.3 mg STX·2HCl eq/kg. The result from the mouse bioassay is consistent with the expected underestimation of TTX toxicity due to its slower death response time than saxitoxin [24]. Shellfish sampled from the same site pre- and post-this result contained only trace TTX levels, demonstrating the rapid appearance and disappearance of TTX in shellfish from this site (Table 2).

Table 2. TTX levels in samples taken from Browns Bay and nearby sites from May–July 2016.

Date Sampled	Shellfish	Distance from Browns Bay (Km)	TTX Level (Mg/Kg)
11 May 2016	Pacific oyster	11	0.002
1 June 2016	Greenshell™ mussel	11	0.02
6 June 2016	Greenshell™ mussel	11	0.01
6 June 2016	Greenshell™ mussel	5	<0.002
19 June 2016	Greenshell™ mussel	0	0.004
3 July 2016	Greenshell™ mussel	0	1.6
11 July 2016	Pacific oyster	11	0.004
17 July 2016	Rock oyster	11	0.003

3. Discussion

Low levels of TTX (>0.002 mg/kg) were detected in 31% of all non-commercial New Zealand shellfish tested during the survey period, with most samples being below the reporting limit of the method. No seasonal trends were observed at any sites sampled multiple times over the survey period. TTX analogues were observed in shellfish found to contain TTX, but only at much lower levels relative to TTX itself. No recreationally harvested shellfish that were tested during the survey, including mussels, oysters, clams and tuatua, contained TTX levels above the recommended EFSA safe guidance level of 0.044 mg/kg. The observation of TTX in New Zealand shellfish does not represent a new phenomenon as archive samples contained toxin at similar levels and frequency to shellfish tested during the survey.

TTX levels observed in individual pipi were similar to that of the pooled sample, indicating that TTX levels were similar in all pipi from that site. At times, pipi had levels that exceeded the recommended EFSA guidance level, but not the Japanese limit, including all samples taken from the Hokianga Harbour site. Cockles collected from the same site at the same time did not contain TTX. This represents a particularly interesting finding. Recent research on TTX-contaminated pipi identified tetrodotoxin in all the dissected organs but that the siphons contained significantly higher levels [16]. It has been hypothesized that *P. australis* contains unique TTX-binding proteins in the siphon similar to those found in the puffer fish (*Fugu pardalis*) [25] or the crab (*Hemigrapsus sanguineus*) [26], that allowed selective retention of the toxin within different organs. Hokianga Harbour is estuarine in nature and one of the northern most sampling sites, which means that it experiences warmer temperatures than the other sampling sites further south. Recent research has identified a latitudinal gradient for TTX levels in pipi, supporting the warmer water hypothesis [16]. The association of higher TTX levels in shellfish from warmer environments raises concerns that this toxin may become an increasing human health concern as the global climate warms.

TTX has been reported in many terrestrial and marine species, including bivalve shellfish, although its origin remains unclear. Accumulation from the diet, whether from bacteria or another source, is an attractive hypothesis supported by the observation that cultured pufferfish are found to be non-toxic and that pipi know to contain TTX depurate the toxin when fed a toxin-free diet. However, TTX levels found in bacteria and marine sediments are low, and production by bacterial species has still not been demonstrated. Many scientists believe bacteria are the source of TTX in shellfish, and this sentiment is reflected in recent EFSA opinion on the presence of TTX in bivalve shellfish, which begins: "TTX and its analogues are produced by marine bacteria and have been detected in marine bivalves and gastropods from European waters" [7]. Bacterial cultures have been reported to contain low TTX concentrations and are suggested to be the ultimate biosynthetic origin of the toxin. However, these results remain controversial and are disputed due to poor specificity of the methods of analysis used, or extremely low levels observed when more specific methods of analysis are employed. More conclusive data is needed to unequivocally determine the exogenous or endogenous source of TTX in shellfish.

When assessing sample toxicity, co-occurrence of TTX with paralytic shellfish toxins needs to be considered. This is because both TTX and STX bind to voltage-gated sodium channels and our research

has shown that they have additive toxicological effects [24]. The PSP mouse bioassay, which is still used for regulatory monitoring in some countries, is not able to distinguish TTX from saxitoxin group toxins (paralytic shellfish toxins). This is important as the presence of paralytic shellfish toxins in shellfish is regulated whereas the presence of TTX currently is not. Shellfish containing paralytic shellfish toxins below the regulatory action limit of 0.8 mg STX·2HCl eq/kg could be found to be above the regulatory threshold for this toxin class when using the PSP mouse bioassay, if TTX is also present. This situation, although likely a rare occurrence, has been observed in a non-commercial shellfish sample analysed prior to the main survey. A single mussel sample was found to contain 1.6 mg of TTX/kg and paralytic shellfish toxins at 0.4 mg STX·2HCl eq/kg (half the regulatory limit). When subjected to the PSP mouse bioassay, the toxicity of the sample was determined to be 7278 MU/kg or 1.3 mg STX·2HCl eq/kg, which is above the paralytic shellfish toxin regulatory limit of 0.8 mg STX.2HCl eq/kg. This demonstrates that even though TTX levels in New Zealand shellfish are typically low, there is potential for sporadic high levels. The factors that resulted in the high TTX level observation in this single sample are unresolved, although it is known that marine biota exists in NZ that contain elevated TTX and could be potential vectors (e.g., grey side-gilled sea slug [27] and flatworms [28]). In New Zealand, chemical analytical methods are used for all routine regulatory control of marine biotoxins in shellfish. For paralytic shellfish toxins, a LC-MS/MS method is used that allows specific identification of the various paralytic shellfish toxin analogues samples and simultaneous monitoring of TTX.

Having an accurate assessment of TTX toxicity and an understanding of the mechanism of TTX accumulation in marine foodstuffs is important for managing the potential risk to consumers. Existing data on TTX toxicity by oral administration are limited, with a wide range of results reported in the literature. Most of the information available relates to acute toxicity through intraperitoneal injection to mice, and this route of administration is of questionable relevance, given that seafood is consumed orally rather than injected. To address this we have recently generated robust toxicity information for TTX via oral administration (gavage and voluntary feeding) and demonstrated that the toxicities of STX and TTX are additive [24]. Data gaps still exist. For example, toxicity information for other TTX analogues (such as 11-oxo-TTX) is needed, as they are potentially equipotent with TTX and have been documented to be dominant analogues in some species of crab [29]. In New Zealand, the grey side-gilled sea slug (*Pleurobranchaea maculata*) is the most well-known TTX-containing organism and has been reported to lay highly toxic eggs [30]. The presence of these organisms, or their eggs, in shellfish harvesting areas could make them a possible vector of the toxin. In addition, many marine worm species also contain high TTX concentrations and they could potentially contaminate bivalve shellfish. This mechanism of toxin transfer represents a plausible explanation for the elevated TTX levels observed in the outlier mussel sample from Browns Bay. The likelihood of this possibility is heightened by the fact that the Browns Bay site is close to where toxic *P. maculata* have been found in the past.

4. Conclusions

TTX was detected in about one-third of samples tested during the survey of New Zealand shellfish. The levels were low and below the recommended EFSA safe limit, except for pipi, a common surf clam. All pipi tested during the survey contained TTX and a site was identified in the Hokianga Harbour where consistently elevated levels were observed. Recent research on pipi has identified that the siphon contains TTX levels significantly higher than other parts of the shellfish. Despite these findings it remains important to determine the source of TTX in bivalve shellfish, and indeed other marine and terrestrial organisms.

5. Materials and Methods

5.1. Shellfish Sampling

Shellfish samples received weekly (December 2016–March 2018; Cawthron Institute, Nelson) for paralytic shellfish toxin testing as part of the MPI administered non-commercial marine toxin shellfish monitoring programme were also analysed for TTX. In total there were 56 sampling sites from around New Zealand (Table 3) with a total of 766 samples analysed for TTX during the survey.

Table 3. Sampling site details and summary of TTX levels observed in non-commercial shellfish samples tested during the survey period.

Site	TTX Level (Mg/Kg)						
	Site Code	Average	Maximum	<0.002	0.002–0.044	> = 0.044	Total Sample #
Mangonui Harbour	SA006	0.007	0.025	2	3	0	5
The Bluff-90 Mile Beach	SA025	0.000	0.007	33	3	0	36
Waipapakauri	SA027	0.001	0.006	27	4	0	31
Tapeka Point	SA030	0.015	0.023	0	2	0	2
Taurua-Reef Point	SA036	0.000	0.000	1	0	0	1
Black Rocks (Bay of Islands)	SA040	0.000	0.000	3	0	0	3
Houhora Wharf	SA129	0.013	0.023	0	2	0	2
Oakura	SB001	0.001	0.002	1	1	0	2
Parua Bay	SB007	0.000	0.000	1	0	0	1
Pataua	SB008	0.038	0.045	0	1	1	2
Whananaki	SB032	0.028	0.045	0	3	1	4
Browns Bay	SC032D	0.009	0.033	0	11	0	11
Whangaparaoa Peninsula	SC032F	0.010	0.010	0	1	0	1
Tairua Harbour	SD012	0.000	0.000	1	0	0	1
Waihi Beach	SD017	0.001	0.010	25	8	0	33
Tauranga Harbour-Upper	SD018	0.000	0.000	1	0	0	1
Tauranga Harbour-Lower	SD021	0.048	0.048	0	0	1	1
Papamoa Beach	SD025	0.001	0.008	29	5	0	34
Pukehina Beach	SD028	0.000	0.000	36	0	0	36
Bowentown	SD030	0.013	0.013	0	1	0	1
Katikati-Tauranga Harbour	SD031	0.041	0.041	0	1	0	1
Katikati-Tauranga Harbour	SD031P	0.150	0.150	0	0	1	1
Kauri Point	SD031S	0.038	0.038	0	1	0	1
Whakatane Heads	SD032	0.001	0.002	5	2	0	7
Waiotahi	SD036	0.007	0.012	1	5	0	6
Ohope Beach	SD037	0.000	0.004	35	3	0	38
Whangaparaoa	SD041	0.001	0.007	25	6	0	31
Thornton	SD042	0.000	0.000	5	0	0	5
Te Kaha	SD050	0.002	0.002	0	1	0	1
Tolaga Bay Wharf	SE001	0.005	0.017	2	2	0	4
Mahia, Opoutama	SE006	0.003	0.018	14	20	0	34
Pania Reef	SE007	0.002	0.015	18	17	0	35
Taikorai Rocks-Porangahau	SE010A	0.002	0.013	19	11	0	30
Lottin Point	SE019	0.003	0.003	0	1	0	1
Gisborne Wharf	SE028	0.004	0.004	0	1	0	1
Muriwai, West Coast	SF009	0.006	0.015	1	3	0	4
Cornwallis (Manukau Hbr)	SF015	0.004	0.022	11	28	0	39
Raglan	SF016	0.001	0.007	27	13	0	40
Kawhia	SF017	0.006	0.024	6	32	0	38
Mohakatino	SF018	0.000	0.003	39	2	0	41
Oakura Beach	SF020	0.000	0.000	13	0	0	13
Koutu Point (Hokianga Hbr)	SF021	0.134	0.470	5	0	25	30
Tinopai (Kaipara Hbr)	SF026	0.006	0.010	0	4	0	4
Maunganui Bluff	SF029	0.001	0.007	29	5	0	34
Mitimiti	SF031	0.000	0.002	4	1	0	5
Aotea Harbour	SF033	0.000	0.000	1	0	0	1
Baylys Beach	SF156	0.000	0.000	2	0	0	2
Tapu Bay–Tasman Bay	SG006	0.000	0.000	1	0	0	1
Wedge Point	SG023	0.000	0.000	1	0	0	1
Onapua Bay	SG123	0.000	0.000	3	0	0	3
Pohara	SG313	0.000	0.000	1	0	0	1
Ohawe Beach	SH001	0.000	0.002	33	1	0	34
Foxton	SH002	0.000	0.003	34	1	0	35
Lower Kina Road	SH023	0.000	0.000	1	0	0	1
The Kaik	SI004	0.002	0.002	0	1	0	1
Cape Foulwind	SJ004	0.000	0.006	31	3	0	34

During the survey, pipi sourced from the Hokianga Harbour (Northland, New Zealand) were found to contain TTX levels well above shellfish from other areas. With this site not routinely being monitored for marine toxins, a request was made to increase the frequency of sampling for the duration of this study. From March 2017, fortnightly sampling of the pipi bed (Koutu Point, Hokianga Harbour) was performed. Individual pipis ($n = 12$) from one sampling event in February 2017 from the Tauranga Harbour (Bay of Plenty) were tested, in addition to a pooled sample, to determine TTX variability between individuals.

In addition, to assess whether the presence of TTX in New Zealand shellfish is a recent phenomenon a subset of 27 archived (2001–2003 $n = 18$, 2007–2009 $n = 9$) shellfish homogenate samples were retrieved from frozen storage and analysed for the presence of TTX. Samples that are in frozen storage are typically from routine monitoring activities and contain detectable levels of regulated marine toxins. Very few pipi samples were in the archive.

5.2. Sample Preparation and Analysis of TTX by HILIC-MS/MS

A HILIC-MS/MS method was used that was developed as a collaboration with the Cawthron Institute and the Centre for Environment, Fisheries and Aquaculture Science (Cefas; UK) scientists for routine regulatory monitoring of the paralytic shellfish toxin group [31,32], which could also be expanded to monitor TTX [33]. The limit of reporting for the method was 0.002 mg TTX/kg. Certified tetrodotoxin (TTX) material (6.75 ± 0.24 µg/g) was purchased from the National Research Council Canada (NRC, Halifax, NS, Canada). When TTX was observed in shellfish samples, it was possible to re-analyse the sample extract using a targeted TTX acquisition method to allow monitoring of a range of TTX analogues. As no reference material is currently available for the various known TTX analogues, it was not possible to accurately quantify them. Therefore, the concentration of each analogue was semi-quantified using an assumed relative response factor of 1. This will introduce a source of error, but in the absence of reference material it is the only option currently available to allow semi-quantification of TTX analogues.

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

5.3. Animals

Female Swiss albino mice (18–22 g) were bred at AgResearch, Ruakura, New Zealand. The mice were housed individually during the experiments and were allowed unrestricted access to food (Rat and Mouse Cubes, Speciality Feeds Ltd., Glen Forrest, Australia) and water. All experiments were approved by the Ruakura Animal Ethics Committee established under the Animal Protection (code of ethical conduct) Regulations Act., 1987 (New Zealand), Project Number 14005, approval date 6 October 2016.

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