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Studies on *Paphies australis* (Mesodesmatidae) in Whangārei, Aotearoa New Zealand: an investigation into their declining health.

A thesis

submitted in fulfilment

of the requirements for the degree

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at

The University of Waikato

by

Joanne Elizabeth Howells

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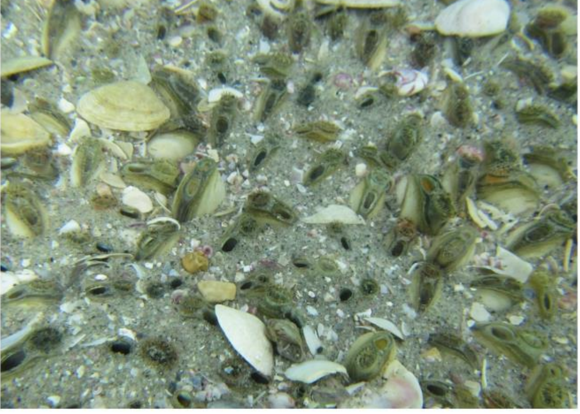

Abstract

Disease outbreaks in marine species can have devastating consequences, including increasing host mortality, destabilising commercial production, altering ecosystem functioning, and jeopardising human health. A changing ocean climate is expected to increase host stress and the risk of disease outbreaks. Particularly through new and emerging diseases and opportunistic infections.

Wild bivalve molluscs provide economic, ecological, and socio-cultural benefits. Healthy and sustainable kaimoana (shellfish/bivalves, seafood) are intrinsically linked to the wellbeing of many communities in New Zealand. Over the past decade there has been a noticeable decline in the general health of native marine bivalves, characterised by an increase in observation of mass die-offs. Reasons for this are not clear and could be due to a number of factors, such as sedimentation, harvesting pressures, or disease. Baseline health data on wild bivalve populations are very rare and therefore it is very difficult to identify the causation of ill health. There is a need to establish health baselines to help benchmark changes in infection prevalence and intensity and identify drivers of bivalve mortality events.

Wild bivalve populations are often located in remote and isolated parts of New Zealand. When a mortality event occurs, the logistics of obtaining quality samples for accurate diagnosis can be hindered due to time delays and sample degradation. To gain a better understanding of these limitations for diagnostics using bacteriology, in Chapter 2 I investigate typical sample collection methods (i.e., samples collected and sent to a testing laboratory) and sample collections immediately in the field to understand what effect different sample methods have on number and types of bacteria isolated. Although the results of Chapter 2 found there were limitations to both sampling methods, it did identify that typical sampling techniques were not wholly optimal, and the information gained by sampling immediately in the field retained additional information that could be essential for disease diagnostics. Field sampling for bacteriology was applied throughout this thesis research.

Pipi (*Paphies australis*) are native bivalves to New Zealand, inhabiting the shallow subtidal of estuaries and bays. Pipi have suffered serious population declines in an area of northern New Zealand (Whangārei) since 2009. I investigate possible drivers of the poor health of pipi in Whangārei, with the objective of identifying the mechanisms that have contributed to the decline, and prevention of the recovery of pipi in Whangārei, with a primary focus of disease as a causative agent. I establish a health baseline of pipi from Whangārei using histopathology, bacteriology, and qPCR of *Endozoicomonas* spp., a bacteria previously identified in mortality bivalves (Chapter 3). The health baseline was compared against samples collected during three mass mortality events to help identify drivers of mass mortality events (Chapter 4). Through laboratory experimentation, I test whether pipi exposed to stressors had higher infection intensity and prevalence of *Endozoicomonas* spp. (Chapter

5). Finally, using archived samples, I test whether the baseline data established was consistent through time and help to provide insights into considerations when analysing bivalve mass mortality events (Chapter 6).

I found that *Endozoicomonas* spp. make an important part of pipi host microbiome. Although *Endozoicomonas* spp. are highly prevalent in pipi collected during a mass mortality event, their infection intensity was significantly lower in moribund pipi than healthy pipi. The prevalence and infection intensity of *Endozoicomonas* spp. were not driven by environmental stressors. The stressors, however, did appear to impact host health, with high suspended sediment loads and water temperatures of 24°C appeared to be unfavourable to pipi. Overall, *Endozoicomonas* sp. does not appear to be a main driver in the declining population of pipi from Whangārei and is probably not important to bivalve health. The reduced prevalence and infection intensity of *Endozoicomonas* sp. in healthy pipi strongly suggests these bacteria are symbiotic. A relationship that has been reported for this bacterial group in species of coral and sponges.

The establishment of a health baseline represents a significant step forward towards biosecurity preparedness by being able to identify changes in infection prevalence and intensity against it. The operational use of these data when paired with historical data will be a powerful tool to identify patterns of disease dynamics and emerging disease.

Preface

This thesis comprises of five research chapters. Chapter 2 of this thesis has been submitted to a peer-reviewed journal and has been accepted. Chapters 4 and 5 have been submitted to peer reviewed journals and are currently under review. The co-authors on all publications provided constructive feedback during the design, data analysis, and text drafts of each study. The ideas and concepts introduced in this thesis were my own, unless otherwise referenced.

The work presented in this thesis was performed at the Animal Health Laboratory, Wallaceville, New Zealand from 2019 to 2023. All work was produced under the supervision of Dr Henry Somerset Lane at the National Institute of Water and Atmospheric Research Ltd. (NIWA), Wellington, Lisa Maria at Biosecurity New Zealand, Ministry for Primary Industries, Nelson, Dr Phil Ross and Dr Joanne Ellis, University of Waikato, Tauranga.

Chapter 2 has been accepted by Journal of Microbiological Methods:

Howells, J., & Brosnahan, C. (2022). Bacteriology & bivalves: Assessing diagnostic tools for geographically remote bivalve populations. *Journal of Microbiological Methods*, *202*, 106581. https://doi.org/10.1016/j.mimet.2022.106581

Chapter 4 (with data collected from Chapter 3) has been submitted to the Journal of Invertebrate Pathology:

Howells, J., Maria, L., Shirkey, T., Carrington, A., Lane, H.S. Testing a health baseline during a bivalve mollusc mortality event: an investigation into die-offs of pipi (*Paphies australis*) from Aotearoa New Zealand. Under review.

Chapter 5 has been submitted to Disease of Aquatic Organisms:

Howells, J., Maria, L., Ellis, J., Brosnahan, C., Lane, H.S. Unravelling the link between environmental factors and *Endozoicomonas* spp.: an experimental study on contributing factors to pipi (*Paphies australis*) mass mortality events in Whangārei, Aotearoa New Zealand. Under review.

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Photograph taken in 1964 by Ans Westra. Ref: AWM-0742-F 10. Alexander Turnbull Library, Wellington, New Zealand.

Global bivalve populations

Bivalves (Bivalvia; Mollusca) are a diverse group of animals characterised by hinged two-part shells enclosing a soft tissue body (Thain & Hickman 2004). Bivalves are filter-feeders and inhabit all marine ecosystems, including sheltered intertidal mudflats, rocky shores, deep gravel substrates affected by oceanic swells, and the deep ocean. They play critical roles in the marine ecosystem by facilitating nutrient cycling, improving water quality, acting as natural coastal defences, and increasing biodiversity by providing a hard substrate for settlement in soft substrate environments (Carss et al. 2020). Bivalves are important to commerce worldwide. Farmed and wild commercially harvested bivalves contribute to global seafood production and food security. Aquaculture accounts for 89% of global marine bivalve production, while the remaining 11% is sourced from wild fisheries (Smaal et al. 2019). Production from farmed bivalves have experienced rapid growth in recent decades to meet the rising demand for sustainable seafood. Total mollusc aquaculture production amounted to 17.7 million tonnes in 2018, consisting mainly of bivalves. This is a 30.5% increase from 13.1 million tonnes produced in 2008 (FAO 2008; FAO 2018). In contrast, wild fisheries of bivalve molluscs make nominally smaller contributions to global production and have declined over the same 10-year period (FAO 2022). Wild populations of molluscs have declined in some areas through overharvesting, habitat change, or disease (Carnegie & Burreson 2011; Morello et al. 2005). The benefits of healthy wild bivalve populations have led to numerous efforts globally to restore populations (Colsoul et al. 2021).

The close proximity of many bivalves to land (I.e., in nearshore coastal areas, rivers, and estuaries) mean they are particularly important to local communities. Bivalve shells have been used as jewellery and building materials (Morris et al. 2019) and their meat has been an important component of the diets of indigenous and colonial peoples for centuries (Small et al. 2019). Archaeological records from the Northwest Coast of North America have identified clam gardens, designed by indigenous people, as ancient mariculture systems dating back 3,500 years (Smith et al. 2019). Furthermore, bivalves have played a significant role in providing cultural ecosystem services. In many cultures they are sources of spiritual and religious connection to the environment, contributed to cultural heritage, and facilitated education in numerous societies worldwide (Kainamu-Murchie et al. 2018; Jackson-Bue et al. 2021). Bivalve populations are incredibly important and efforts to restore them have united communities through engagement with the environment, which can bring about a sense of place, social relations and inspirations which link directly to human social structures (Smaal et al. 2019). Monitoring the health and ensuring populations are sustained is crucial for their future conservation and use.

Bivalve health and a changing marine environment

Varying temperature, salinity, sedimentation, oxygen, and light availability characterize the distribution of species in the marine environment (Roberts et al. 2022). Each species has preferences for specific environmental conditions that enable them to thrive. However, the marine environment is undergoing significant changes due to climate change and human activities on land. Increased water temperature, acidification, pollution, eutrophication, and the introduction of species beyond their natural range through human intervention have reshaped marine environments, particularly in nearshore areas (Stephens et al. 1988). While marine organisms are generally well-adapted to natural environmental fluctuations, frequent and substantial changes that exceed their ability to adapt can lead to stress. Stress occurs when organisms encounter environmental conditions that are less favourable, moving them beyond their adaptive limits. Bivalves, with their closed, bivalved shells, can withstand periods of emersion and unfavourable conditions to some extent. However, not all bivalves possess broad environmental tolerances. The larval stages of bivalves, in particular, are vulnerable to stressors such as thermal and salinity variations. Stress can negatively impact the health of organisms (Sniezko, 1974) by directly affecting their physiology, immune function, growth, and reproductive capabilities (Balbi et al. 2021; Vázquez et al. 2021). These effects can have cascading consequences on bivalve populations and, in turn, marine ecosystems. The occurrence of mass mortality events and declining health conditions in bivalves can be attributed to a variety of factors, with environmental stressors playing a significant role. For this thesis I define a mass mortality event as an unexpected and unusual occurrence of dead and dying bivalves.

Temperature, both low and high, is an extrinsic factor associated with bivalve mass mortalities (Soon & Ransangan 2019). Thermal stress can cause physiological imbalances, reduced feeding activity, increased metabolic rates, and mortality. Thermal stress induced by elevated temperatures acts as a significant factor driving "summer mortalities" in farmed Pacific oysters (*Magallana gigas*, synonymous with *Crassostrea gigas*) in France, adversely impacting the health of the host (Delaporte et al. 2007). Temperature is implicated in a similar syndromic condition in farmed green-lipped mussels in New Zealand (Ericson et al. 2023). Oysters are particularly vulnerable to “summer mortality” when they are undergoing reproductive phases and their energy reserves are redirected into spawning activities (Rodríguez-Jaramillo et al. 2022). In Uruguay, a long-term study examining climate trends and surveying of a yellow clam (*Mesodesma mactroides*) population identified that increasing anomalies in sea surface temperatures impacted the population abundance over time and led to increased observations of gill lesions and weakened muscular tissue in the foot (Ortega et al. 2016).

Salinity profiles in estuarine systems can be altered by modifications to catchments and surface run-off (Rodriguez et al. 2001). Exposure to lower salinity over prolonged periods causes decreased

survivorship in two bivalve species from New Zealand, cockles (*Austrovenus stutchburyi*) and pipi (*Paphies australis*). Lower salinity was found to restructure populations, with more bivalves relocating to deeper waters where the salinity profile was preferable (McLeod & Wing 2008). Histopathological changes were observed in the digestive tissues of Pacific oysters in Tasmania, Australia, exposed to low salinity stress (Knowles et al. 2014). Expanded intercellular spaces and haemocyte infiltrate (diapedesis) in the walls of the stomach, digestive tubules and intestines compared to histological observation in Pacific oysters exposed to normal salinity strongly indicate physiological stress associated with low salinity and warmer water temperature (Knowles et al. 2014).

Limiting exposure to stressors is an optimal defence mechanism in bivalves in the short term, however, closing their shells for extended periods of time can lead to anoxic conditions and ultimately increased mortality (Mohamed 2003; Cheney et al. 2000; Matthews & McMahon 1999). Low oxygen levels in aquatic environments are known as anoxia/hypoxia. Prolonged exposure to hypoxia can lead to tissue damage, reduced growth, and increased mortality rates. Despite a bivalve's ability to withstand anoxic environments, these conditions can compromise the host microbiome, accelerating mortality. Coffin et al. (2021) studied the interactions between American oysters (*Crassostrea virginica*) and their resident bacterial communities in the context of environmental stress. Their results showed that certain endogenous bacteria can contribute to the accelerated mortality of oysters during sustained anoxia, highlighting the complex interplay between the host and its microbial community under environmental stress (Coffin et al. 2021).

The close proximity to land of many bivalve populations exposes them to higher amounts of terrestrial run-off, including high nutrient inputs that can lead to eutrophication and increased sedimentation (Fredston-Hermann et al. 2016; Tuttle et al. 2020). High levels of suspended solids can cause abrasion to the gills, causing physiological stress to the bivalve (Ellis et al. 2002). Gill abrasions can affect respiration and filtration, leading to an overall decline in health (Soon & Ransangan 2019). In addition to directly affecting feeding rates through abrasion, it may also result in a reduction in ingested food, thereby impacting the energy resources necessary for growth and reproduction (Ellis et al. 2002). Deposits of terrigenous sediments impact the settlement of juvenile bivalves, changes in the substrate may cause bivalves to reject the substrate and disperse to another preferable location, impacting the community location and structure (Cummings et al. 2009).

Environmental stressors on bivalves can lead to increased susceptibility to disease, particularly from opportunistic infections (Costello et al. 2021). Some environmental conditions can create the perfect environmental conditions for pathogens to grow and thrive and therefore posing an increasing threat to the host health (Lane et al. 2022). In humans, we are familiar with wounds that become infected under unsanitary conditions by opportunistic bacteria that are otherwise benign when a person is healthy. Opportunistic pathogens are dependent on environmental factors to cause disease, and the

emergence of opportunistic parasites during climate instability is an important area of future focus and main theme running through this thesis. Of course, environmental stressors do not always lead to increased infections or disease because pathogens have optimal ranges of performance. If the environment becomes unfavourable for a particular pathogen, we might see a decrease in infection and disease.

Disease in bivalves

Disease is a negative deviation from normal health. Disease can be caused by a number of agents such as bacteria, viruses, fungi, helminth parasites and protozoans (Gosling 2015; Potasman et al. 2002). The presence of microorganisms does not necessarily equate to disease. The outbreak of disease in marine organisms is the result of complex shifting interactions between the host, the pathogen, and the environment (Figure 1.1) (King et al. 2019). A shift may come in the form of an increased pathogenicity or virulence of the parasite, or increased susceptibility to infection. For example, infection prevalence, intensity, and host mortality increase with water temperature in Pacific oysters infected with the ostreid herpesvirus 1 microvariant (OsHV-1 µvar) (de Kantzow et al. 2017).

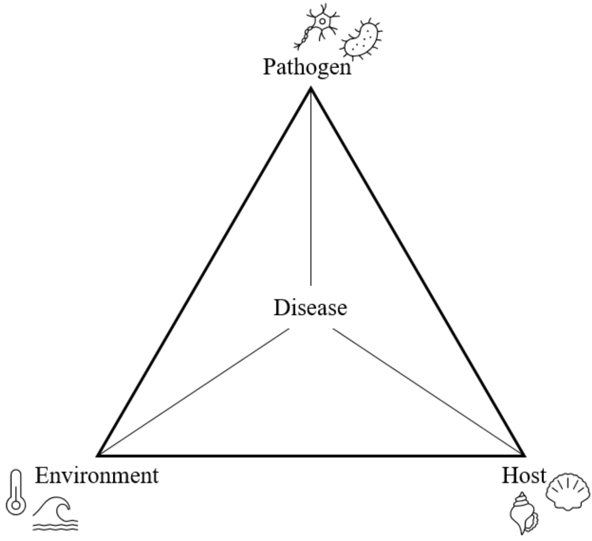

Figure 1.1 Epidemiological triangle, used to model the interacting elements that result in disease (Harvell 2004).

Bivalves have experienced numerous consequential disease events in farmed and wild populations, which can result in ill health and mass mortality events. A haplosporidian-like parasite was responsible for extensive mass mortality events in fan mussels (*Pinna nobilis*) within the Spanish Mediterranean Sea. These events resulted in some areas experiencing 100% mortality, making them likely the largest mass mortality events recorded in fan mussels (Vázquez-Luis et al. 2017). Two well known cases of disease in bivalves have occurred in New Zealand. The haplosporidian parasite *Bonamia exitiosa* was first reported in New Zealand flat oysters (*Ostrea chilensis*) in 1987 (Dinamani et al. 1987) where it caused >90% mortality. Recurrent outbreaks of disease mean that *B. exitiosa* is

the main driver of oyster population size within the fishery (Michael &Shima 2018). In 2010-2011, the OsHV-1 µvar caused >70% mortality in farmed populations of Pacific oysters in northern New Zealand (Bingham et al. 2013). Both *B. exitiosa* and OsHV-1 µvar are well known globally for their impact on bivalve populations; *B. exitiosa* is notifiable to the World Organization for Animal Health (WOAH). Surveillance for these pathogens and other globally notifiable pathogens (see WOAH Chapter 1.4, OIE 2022) are important to understanding disease distribution, disease dynamics, and preventing their transboundary spread. The changing marine environment, however, could mean that the next threat could emerge from an organism not previously known. It is important to be prepared for this.

Much research effort on bivalve diseases has been on farmed populations. Disease is a significant constraint to farmed production and there is typically more financial incentive to monitor and manage diseases in aquaculture. There are of course exceptions such as the *B. exitiosa-O. chilensis* research in New Zealand, where the fishery is surveyed annually as part of its fisheries management (Michael et al. 2021). Consequently, disease research on wild bivalve populations is comparatively less than farmed bivalves. A Web of Science search using keywords "aquaculture and bivalves and disease" returned 1,124 results, whereas keywords "wild and bivalves and disease" returned 648 results. Based on this search result, 50% less research has been published on wild populations than farmed populations. Wild populations are often "out of sight" compared to farmed populations, which undergo regular inspections, and are typically located in rural and isolated areas. This makes it logistically challenging and costly to study wild populations (Howells & Brosnahan 2022). Despite the increasing recognition of the overall services – ecological, cultural and economic – provided by bivalves, disease threats and drivers of poor health in wild bivalve populations are understudied.

A requirement for future research is to shift from reactive research to pro-active research, which would broaden the identification of potential pathogens within the marine environment (Wood et al. 2013). Data on baseline health for aquatic animals are scarce (Ward et al. 2004). The lack of baseline health data makes it difficult to understand the significance of different bacteria, viruses, or parasites detected in marine species. Many bacteria may constitute as part of the normal biota of bivalve but could be opportunistic leading to disease in stressed hosts. Overall, there is very little research available on what constitutes "normal" health for wild bivalve populations. Diagnosing the cause of die-offs in wild populations is extremely difficult when no baseline health data is available to provide clues on any divergence from normal health (Ward et al. 2004; Tracy et al. 2019).

Bacterial disease

In the marine environment, microorganisms make up 90% of living biomass with bacteria making up large proportion of them (Cavicchioli et al. 2019). As bivalves filter feed, they are exposed to an

abundance of bacteria present in the water column, which can be ingested (Rippey, 1994). Bacteria are frequently isolated from bivalves, including species belonging to the genus *Vibrio* (Travers et al. 2015). However, their exact role in disease is less well known because bacteria are environmentally ubiquitous and what is isolated might represent either a normal part of the host microbiome or environmental bacteria. Whilst most bacteria are not harmful, some are known to be pathogenic. For example, *Vibrio parahaemolyticus* and *Photobacterium damselae* subsp. *damselae* (*Pdd*), and the high accumulation of them in bivalves can result in disease and mortality (Gosling 2015). Marine bacterial diseases are complex, and the behaviour and characteristics of some bacteria can be unpredictable, with the ability to jump from host to host, emerging and re-emerging (Hunter-Cevera 2005; Travers et al. 2015). Understanding the epidemiology is central to predicting disease emergence. A few examples of common aquatic bacteria found in bivalves are described below to highlight the range and complexity of these bacteria, and how much work is still required to understand their role in the health of the aquatic environment.

Vibrio species

Bacteria belonging to the genus *Vibrio* are gram-negative, rod-shaped heterotrophic bacteria that naturally occur in a wide range of marine environments including sediments, free-swimming in the water column, and can be found attached to or living within other marine organisms (Takemura et al. 2014). Members of the *Vibrionaceae* family have the potential to induce severe diseases or persistent low-grade infections that gradually diminish productivity over time (Le Roux et al. 2009). Mollusc diseases linked to *Vibrionaceae* are often characterized by opportunistic infections. In many cases, the pathogen may exist as part of the normal microbial community in the host, only causing disease under specific circumstances. Consequently, determining whether a bacterium is endemic in New Zealand or represents an incursion can be challenging (refer to Carson et al. 2020, for a preliminary list of *Vibrionaceae* reported in New Zealand). This complexity is further compounded by the fact that a single species can exhibit different variants, some of which may be harmless while others possess the capacity to induce disease, as exemplified by *V. harveyi*. Disease outbreaks associated with *Vibrionaceae* can manifest during periods of heightened stress or due to the emergence of pathogenic and virulent strains within a population (Destoumieux-Garzón et al. 2021). Lorgeril et al. (2018) describes that Pacific Oyster Mortality Syndrome (POMs) in Pacific oysters where OsHV-1 µvar triggers an immune-compromised state in the host resulting in opportunistic pathogenic bacteria, such as *V. crassostreae*, to colonise and eventually result in mortality. Pathogenicity is frequently influenced by factors such as host species, life stage and environmental factors (Zannella et al. 2017). *Vibrio alginolyticus* was first described as a bivalve pathogen in 1965 by Tubiash et al. (1965). Studies described the virulence of this bacteria in a number of bivalve species at different life stages including carpet shell clam (*Ruditapes decussatus*), scallops (*Argopecten ventricosus* and *Nodipecten*

subnodosus) and Pacific oysters (Dubert et al. 2017). Temperature is considered a key driver in some bacterial diseases, including vibriosis, and it has been reported that temperatures above 17°C create favourable conditions which can increase replication of bacteria such as pathogenic species i.e., *V. parahaemolyticus* (Cruz et al. 2015; Destoumieux-Garzón et al. 2021).

Endozoicomonas species

Endozoicomonas spp. are intracellular bacteria that have been detected and reported from several bivalve species over the past five years (Cano et al. 2018; Bennion et al. 2021 Howells et al. 2021). The genus *Endozoicomonas* was first described by Kurahashi & Yokota (2007) but is relatively new to bivalve disease work. Previously, bacteria belonging to this group were classified as *Rickettsia*-like organisms (RLOs) when observed in histopathology in bivalves. This classification was based on phenotypic characteristics, which included being gram-negative, intracellular and forming intracytoplasmic inclusions within the epithelial cells of gills and digestive tissues (Figure 1.2) (see Cano et al. 2018). RLOs were first reported in 1977 in Chesapeake Bay, USA, in soft shell clams (*Mya arenaria*) (Harshbarger & Chang 1977) and were subsequently observed in 60 marine molluscs around the world (Cruz-Flores & Caceres-Martınez 2020).

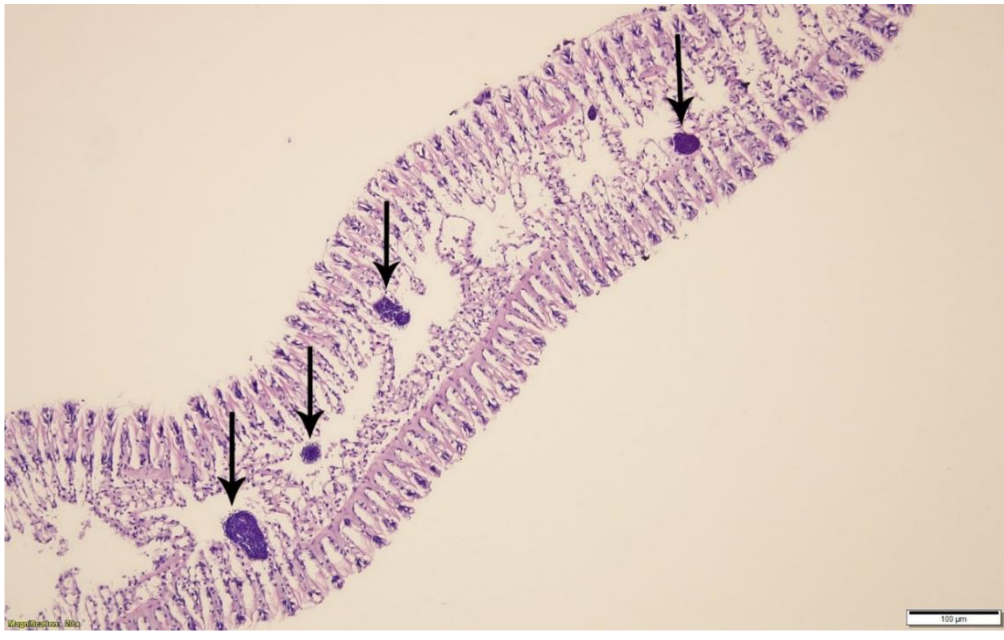

Figure 1.2 Photomicrographs of *Endozoicomonas* sp., previously termed *Rickettsia*-like organism (RLO) inclusions, in New Zealand bivalves. Inclusions are shown by black arrows - *Dosinia anus* gill tissue scale bar = 100 µm (Howells et al. 2021).

Several attempts to identify RLOs and clarify their role in bivalve health led to them being identified as belonging to the genus *Endozoicomonas* (Figure 1.2) (Howells et al. 2021). RLOs observed in king scallops (*Pecten maximus*) in the United Kingdom were identified as *Endozoicomonas* sp. by 16S rRNA gene sequencing which was confirmed by *in*-situ hybridization (ISH) (Cano et al. 2018). In

New Zealand, *Endozoicomonas* spp. have been identified in eight species of bivalve presenting the same histological lesions (Howells et al. 2021). Cano et al. (2020) identified DNA sequences belonging to the *Endozoicomonadaceae* in 22 mollusc species from 10 different countries. These results suggest that these bacteria have a cosmopolitan distribution. Both Howells et al. (2021) and Cano et al. (2020) showed *Endozoicomonas* sp. was a common low abundant component of the bacterial community of healthy bivalves that significantly increased in abundance in times of poor health.

The diversity of *Endozoicomonas* spp. is far reaching and has been described in several marine organisms including molluscs, poriferans, cnidarians and fish (Jensen et al. 2010). *Endozoicomonas* spp. has a global distribution yet the functional role of *Endozoicomonas* spp. is unclear (Neave et al. 2016). The role of this bacteria has been reported to be both a beneficial symbiont and pathogenic to their host (Bourne et al. 2013; Katharios et al. 2015). The high host range and range of symbiosis suggests these bacteria are highly adaptable (Neave et al. 2017).

Diagnostic methods for bacterial diseases

Understanding bacteria and the outbreak of diseases requires accurate diagnostic methods (Palliard et al. 2004). Bacterial culture is a gold standard method for isolating and identifying bacteria, but the advent of genetic tools has enabled a greater understanding of the diversity of marine bacteria, especially those unable to be cultured, such as intracellular bacteria like *Endozoicomonas* spp. (Howells et al. 2021). Developments in diagnostic tools enable better detection and understanding of epidemiology which can inform disease tracking and monitoring.

Histopathology is a classic tool for disease diagnosis in aquatic animals (Aranguren & Figueras 2016). Histopathology is the microscopic examination of cells and tissue. Histology provides detection of a wide range of pathogens, assessment of host immune response, reproductive state, and an overview of its general health status. It is a great tool for long and short-term studies because alterations within the cells/tissue are clear biomarkers that can be correlated with symptoms and disease outcomes (Yancheva et al. 2015). Up until the 1990s, histology was a common tool of disease diagnosis and for many WOAH-notifiable diseases remains a requisite for reporting a positive detection (Aranguren & Figueras 2016). Histology, however, requires professional training, is time consuming and providing definitive diagnosis can be difficult. When combined with molecular tools, however, a more streamlined and accurate diagnosis can be achieved (Buss et al. 2018).

The number of bacteria able to be grown in the laboratory is only a small fraction of the total number of bacterial species. Some bacteria are fastidious and require particular nutrients, atmospheres, and temperatures to be grown (Rodrigues & de Carvalho 2022). Cultivating bacteria enables a better

understanding of their physiology, ecology, and interactions with host health (Stewart 2012). Bacteria that are not able to be cultured require other means of diagnosis. Intracellular bacteria, such as *Endozoicomonas* spp. and *Xenohaliotis californiensis*, the causative agent of withering syndrome in abalone (*Haliotis* spp.), are usually detected using histopathology and confirmed by polymerase chain reaction (PCR). Isolating intracellular bacteria is a challenge. Culture methods do exist for some intracellular bacteria. For example, RLO in Chinook salmon (*Oncorhynchus tshawytscha*) can be grown in vivo in Chinook salmon embryo cells and *Epithelioma papulosum cyprini* cells (Gias et al. 2018). Alternate methods for isolation bacteria could be achieved through purifying bacteria from heavily infected host tissue using centrifugation (e.g., Mialhe et al. 1988). The inability to grow intracellular bacteria makes experimentation difficult (Crosson et al. 2020). Infection experiments often rely on assuming a host is infected or macerating tissue from known infected animals (Crosson et al. 2020)

Since the 1990 and the advent of molecular tools, detecting pathogens has become more efficient, sensitive and specific. PCRs are commonly used as a tool for the detection of bacterial pathogens. Unlike histology and culturing, PCR removes the need to first identify and isolate the pathogen of interest (Pinto et al. 2017). This can also be a drawback of PCR, however, because it is very difficult to know if the DNA amplified is of a viable organism and not from genetic material of an organism that is no longer present or alive (see Brosnahan et al. 2019). PCRs require careful optimisation and validation to understand the specificity and sensitivity of the test for its intended target gene (Chatterjee & Haldar 2012).

The quality of samples used for diagnosis is critical (Join et al. 2010). Obtaining high-quality samples ensures that the diagnostic tests yield valid results and provide valuable insights into the presence or absence of pathogens or abnormalities associated with the disease (OIE 2019). Sample quality encompasses various factors, including proper collection techniques, appropriate preservation methods, and ensuring minimal contamination or degradation during handling and transportation (Coan & Valentich-Scott 2016; Howells & Brosnahan 2022). The integrity of the sample directly impacts the sensitivity and specificity of diagnostic assays used. Therefore, maintaining strict standards for sample quality is essential for effective disease diagnosis and management.

Bivalve host response to disease

Bivalves do not have an adaptive immune system like vertebrates, relying on innate cellular and immune memory (Song et al. 2010). They do, however, still possess a sophisticated immune system, with immune receptors, regulators, and effectors (Guo & Ford 2016). To understand the cause of mass mortalities requires an understanding of the host immune system response and what factors impact the

immune system's functioning. Histopathology is a useful method to assess immune responses while simultaneously observing any pathogens that could be responsible for the response.

Haemocytes are cells circulating within the haemolymph that play a key role in the immune response of bivalves (Allam & Raftos 2015). Haemocytes can recognise, locate, ingest, transport, and digest foreign particles. This makes them particularly useful and are involved in several natural functions, such as shell repair, transportation of nutrients and reabsorbing gonad material post-spawning (Andreyeva et al. 2019). Under histology, haemocytosis is a non-specific response and can indicate stress related to poor water quality, pollutants, or a pathogen (Gorbi et al. 2013). The histological appearance of haemocyte reaction in bivalves may vary depending on the severity and distribution of the infection. Animals may exhibit diffuse infiltrative haemocytosis in severe systemic infections or focal accumulations of haemocytes where the distribution of the pathogen is restricted (Webb & Duncan 2019).

Diseases in bivalves do not always end in a mass mortality. There can, however, be behavioural changes that can indicate disease or stress in bivalves. For example, changes in community structure, or visual mechanical impairment is observed (Cummings & Thrush 2004). A well-known example is inability of infected cockles to burrow, because larval life stages of a digenean trematode encyst in the shell hinge or foot musculature (Longshaw & Malham, 2013). In the case of cockles, both bacteria (Blanchet et al. 2003) and various digenean trematodes can cause cockles to surface instead of being buried when exposed to the tide. Bivalves do not typically exhibit clinical signs of disease. Often the discovery of a mass mortality event serves as the initial indication that something is wrong (Lane et al. 2022). Some diseases can cause gross pathology. For instance, the parasite *Mikrocytos mackini* can lead to the formation of yellow pustules in infected Pacific oysters (Bower et al. 2005) and the parasite *Marteilioides chungmuensis* can cause infected individuals to exhibit nodules in Pacific oyster gonads (Yanin et al. 2016).

Identification of the main cause of bivalve mass mortalities is extremely difficult due to the complex interactions between the bivalve/host physiological condition, the environment, and pathogens. Cause of death cannot be determined by one single factor and a multidisciplined approach needs to be taken to understand these complex relationships. By understanding which variables cause stress within the host then patterns can be built to help predict the emerging risk on host health.

Biosecurity risks to New Zealand bivalves

The Ministry for Primary Industries (MPI) is a governmental agency in New Zealand that was established in 2012 through the amalgamation of the Ministry of Fisheries, Ministry of Agriculture and Forestry, and NZ Food Safety Authority. MPI holds a comprehensive mandate concerning the

nation's primary industries, encompassing agriculture, fisheries, forestry, and food safety. Biosecurity New Zealand (BNZ) is a specific division within MPI that focuses on managing and protecting New Zealand from biosecurity threats. The marine biosecurity team at MPI, previously Ministry of Fisheries, was established in 1998 with the aim of implementing measures and strategies to safeguard New Zealand's marine environment against the introduction and spread of invasive species, diseases, and other harmful organisms (Hewitt et al. 2004). Over time, the marine biosecurity system has developed three key components of protection: pre-border, border, and post-border. These measures are designed to minimize the entry of unwanted pests and diseases and identify any potential incursions that may have bypassed the pre-border and border biosecurity measures. Adhering to the guidelines of WOAH as a member country, New Zealand has specific concerns regarding unwanted pests and diseases due to its isolated nature and unique biodiversity. The Biosecurity Act of 1993 also includes additional diseases of national concern for New Zealand, in addition to those listed by WOAH (see Biosecurity Act 1993).

Marine disease is a significant concern in New Zealand and MPI has been actively monitoring disease in the marine environment since the establishment of the marine biosecurity team. One of the post-border efforts of BNZ is the general surveillance program, which relies on the general public to report unusual observations that might represent a new incursion (Figure 1.3). Reports include observations of ill health or mass mortalities in marine species and sightings of aquatic pests. This approach ensures that if a pest or disease is detected, appropriate management processes can be implemented swiftly to minimize the impacts and aim for eradication of the incursion.

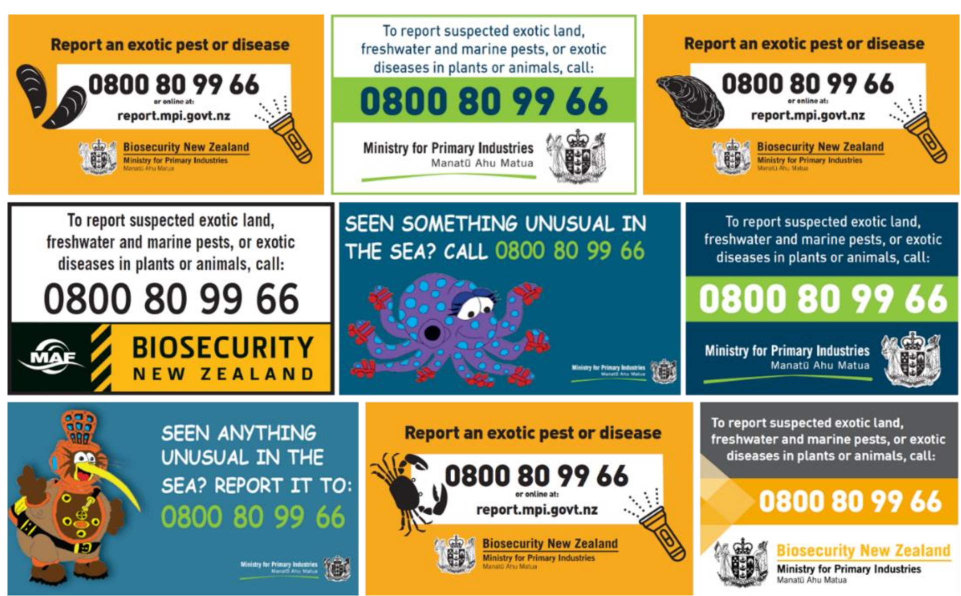

Figure 1.3 Biosecurity NZ marine magnet campaigning through the years 2015 - 2023.

MPI has been receiving reports from the public through the general surveillance program concerning the marine environment, with the numbers of reports steadily increasing since 2002 (Figure 1.4). MPI publicly shares the outcomes of its general surveillance on a quarterly basis (see Surveillance magazine, MPI). Prior to 2010, the marine environment surveillance reports were combined with those related to terrestrial animals. However, due to the rising number of reports, a dedicated section for the marine and freshwater environment was established in the MPI surveillance magazines (Williams 2010). Reports of ill health and mass mortality events in bivalves are commonly sent to the National Animal Health Laboratory (AHL) to investigate the occurrence of exotic and emerging diseases (O'Keefe 2022). A search through the MPI surveillance magazines provides an overview of mollusc disease investigations that have been carried out from 2009-2022 (Figure 1.4) (see Surveillance magazine, Ministry for Primary Industries). These investigations have included submission from both the aquaculture industry and wild populations from around the country, with a higher overall percentage from wild populations (Figure 1.4). Mollusc reported incidences include 16 different species including, wild gastropods (e.g., *Haliotis* spp.), commonly cultured bivalve species (e.g., *Perna canaliculus*, *M. gigas*), wild bivalve populations from important fisheries (e.g., *Ostrea chilensis*) and all other wild bivalve populations (e.g., *Austrovenus stutchburyi*, *Paphies australis*, *Paphies subtriangulata*, *Paphies ventricosa*, *Pecten novaezelandiae*) (Taylor 2019a; Taylor 2019b).

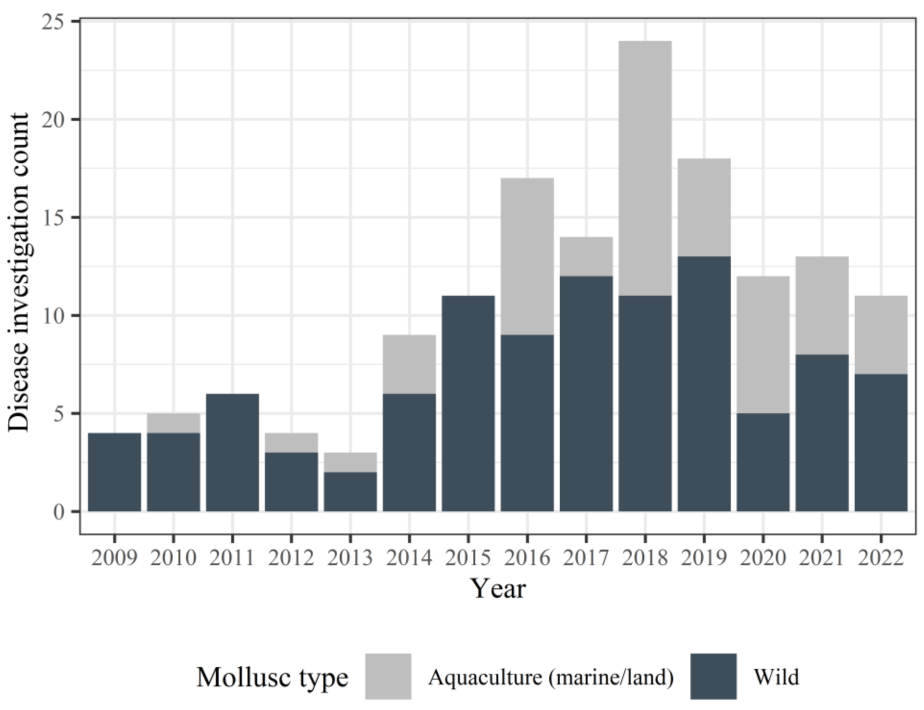

Figure 1.4 Overview of mollusc (bivalve & gastropod) cases investigated at AHL for exotic diseases. This plot does not include samples that were collected under responses or surveillance purposes. A drop in numbers from 2020 likely due to covid-19.

Bivalves in New Zealand

New Zealand's coastal waters are home to 5,486 described species belonging to the phylum Mollusca, with 765 making up the class Bivalvia (Spencer et al. 2016). These values are likely an underestimation as many species are still undescribed and undiscovered. Wild bivalve populations provide economic (commercial harvesting), ecological (biodiversity and water quality), and social (recreational and cultural practices). In New Zealand the most commonly harvested bivalves inhabit either sandy sea floor (e.g., scallops), burrow in sand/substrate (e.g., pipi or cockle), and anchor onto rocks (e.g., Pacific oysters or mussels).

Kaimoana (shellfish/bivalves, seafood) is a significant social and cultural component of many New Zealanders: a common sight at low tide on New Zealand beaches is recreational gatherers of tuatua or pipi; indigenous Māori are kaitiaki (guardians) of kaimoana ensuring mana (respect) and prosperity for their iwi (tribe); and commercially caught bivalves provide socio-economic benefits to many communities, especially rural. The existence of healthy kaimoana is intrinsically linked to the wellbeing of all communities that rely on them (Hinemihi Rāwiri, 2018). Over the past decade there has been a noticeable decline in the general health of many bivalve populations in New Zealand. Reasons for this are not clear and could be due to a number of factors, including anthropogenic pressures, such as sedimentation (Tricklebank et al. 2021), harvest pressure (MPI, 2015), disease (Ross et al. 2017) or increased organic matter (Coffin et al. 2021).

Most commercial, recreational and customary fisheries are managed under the MPI Quota Management System (QMS) to ensure the sustainable use of New Zealand's key fisheries. As of 2023 there were 98 species (or species groups) under QMS, with bivalves making up 17 of them. Under the QMS, each species has an annual catch limit known as the total allowable catch (TAC). The TAC of each species comprises commercial catch, recreational catch, and customary catch. The Treaty of Waitangi recognises Māori custody and use of fisheries, and since 1992 have been allocated 20% of the commercial quota for each species managed under the QMS. Customary fisheries are recognised rights of tangata whenua (local people) for traditional and customary practices and customary non-commercial harvesting. Under regulations, guardians can be appointed for a specific rohe moana (a defined customary fishing area). Tangata kaitiaki (local guardian or trustee of a specific area) authorise and manage customary activities, enabling customary fishing and management traditions to continue in the rohe moana. The complexities of managing NZ fisheries encompasses mātaitai reserves (closed to commercial fishing); taiāpure (local fisheries of special significance); temporary closures; rohe moana; and South Island Fisheries Waters.

Bivalves belonging to the genus *Paphies* (Mesodesmatidae) are culturally significant. There are four species within the genus *Paphies* that are native to New Zealand: two species of tuatua (*Paphies*

subtriangulata and *P. donacina*), toheroa (*P. ventricosa*) and pipi (*P. australis*). These bivalves have fed generations of New Zealanders, especially the indigenous Māori. All four species within this genus have common names that differ between areas of New Zealand. The common name used is determined by the area and also can change between generation even in one area. For example, on the south west coast of New Zealand tuatua are known as 'pipi', and in Northland the younger generation refer to pipi as 'pipi' but the older generation as 'kōkota'. Iwi (Māori community or people) located near populations of tuatua, toheroa and pipi use them in local and customary practices. As such, all four of these species are culturally significant throughout New Zealand. Customary practices include ceremonies of welcoming, communication, and mourning.

Not only are these four species of cultural significance but play an important part of New Zealand's marine ecosystem. All four species are endemic to New Zealand, yet all are under increased pressure from various anthropogenic stressors threatening population longevity. Howells et al. (2021) reported increasing observations of bivalve mass mortalities in New Zealand including of pipi, toheroa, and tuatua (see also Figure 1.4). The toheroa, in particular, had a population crash in the mid-1900s due to overfishing from recreational and commercial harvesting. Bans have been in places since 1969 to try to aid in the recovery of this species of bivalve (Williams et al. 2013). Unfortunately, for this species of bivalve they have not been able to recover to the biomass that they once were (Ross et al. 2017). Recently there has been an increased research effort looking into the health of toheroa (Bennion et al. 2021; Ross et al. 2018). Many toheroa populations are beyond the point of population recovery, with many restoration efforts being unsuccessful. Other measures such as introducing catch bans and creating catch-free zones have been similarly ineffective in preventing population decline (Marsden & Adkins 2009).

Toheroa are an example of an endemic species in New Zealand that has undergone a catastrophic population decline that may be irreversible. Unfortunately, as Howells et al. (2021) reported, they are not the only species of endemic bivalves that are at risk. The causes of declining health can be multifactorial and can include natural die-off, over harvesting, anthropogenic influences (e.g., change in land use, run-off from farming), environmental conditions (e.g., extremes in water temperature), and disease. Identifying the cause or causes of bivalve mortality events is challenging, primarily due to the absence of ongoing monitoring of population health. This lack of baseline data makes it difficult to benchmark mortality events and identify potential causes.

Pipi - *Paphies australis*

Biology & ecology

Pipi (*Paphies australis*) are a bivalve mollusc in the Mesodesmatidae family and are endemic to New Zealand. Pipi are a burrowing surf clam that are distributed around New Zealand coasts and reside in sheltered beaches, bay, and estuaries (Morton & Miller 1968). Despite being characteristic of sheltered areas, pipi are tolerant of high wave action and can be found in areas of fast flowing water (Morton & Miller 1968). Pipi are reported to be both intertidal and subtidal, having been recorded at a depth of at least 7 m (Hooker 1995a). Pipi are often found to be densely populated into clusters that make up a population. The population of pipi at a site can spread over large areas and densities can exceed that of 1000 individuals per m^2 (Morton & Miller 1973, Hooker 1995a). They bury into the substrate using their muscular foot, and are normally found in sandy substrates, but are also known to reside in coarse shell and sand substrate as well (Williams et al. 2007). Pipi reproduce by broadcast spawning, developing into planktotrophic larvae that after around 3 weeks settle into the substrate (Hooker 1997). Once settled, metamorphosis occurs into post-larvel spat usually in the mid intertidal zone (Williams et al. 2017). The juvenile and adult migrate down shore towards and into the subtidal zone with age (Hooker 1995b; Williams & Hume 2014). While there have been reports that pipi can use water currents to disperse within a harbour, the triggers or reasons behind this motility are unknown (Hooker 1995b). There is very little knowledge known about pipi in their planktonic and “adult” stage. Studies have reported that they are sexually mature when the length of the shell is ~40mm, though sexual maturity has also been observed in pipi between 30–40 mm. Hooker (1995a) reported that pipi in Whangateau Harbour, Northland, grow to about 30 mm in one year and reach 50 mm within three years, with the greatest growth occurring in the austral summer and spring.

Once settled, the pipi uses a foot to anchor into the substrate, this foot allows for burying securely into the substrate and propulsion out of the substrate for migration (Figure 1.5). The pipi can open and close its shell by using two adductor muscles that sit either side of the shell beak. Pipi are filter-feeders, drawing the water into their bodies using an inflowing siphon and feeding off the particles in the water using their gills.

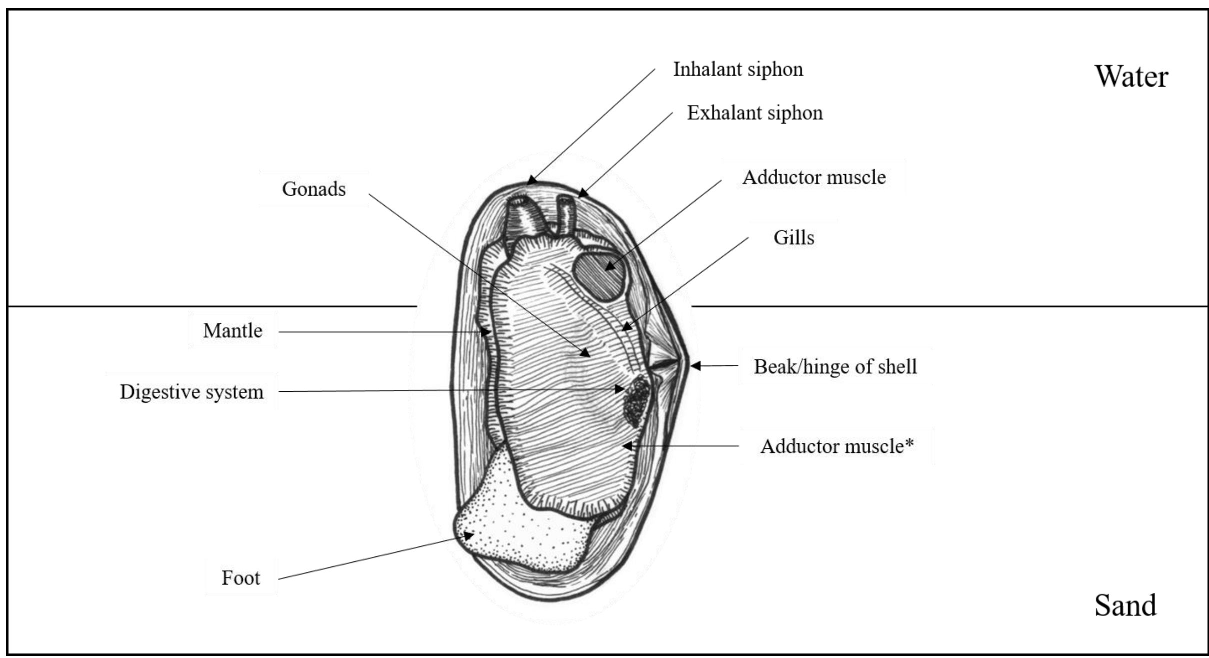

Figure 1.5 Anatomy of pipi (*Paphies australis*). * denotes the position of the second adductor muscle - it has been removed to show other organs more clearly.

Little is known about the overall life cycle of pipi. Haddon (1989) postulated that pipi are unlikely to live longer than 10 years. Estimations of natural mortality for pipi is hard to predict, owing to the migration from mid-intertidal to subtidal with age, and to increased wave actions and tidal currents contributing to the constant shifting of the substrate that can concomitantly move pipi (Hooker 1995a). Whether the observation of mass mortality events of pipi (Howells et al. 2021) are in fact natural mortality and the population is made up of an older cohort reaching the natural end of the life or whether there is another factor contributing to the occurrence of these observed mass mortalities is still unknown.

Pipi make up an important part of a marine system, contributing numerous ecosystem functions. For instance, as filter feeders they can improve water quality by removing phytoplankton, detritus, and bacteria. They also impact benthic nutrient flows through excretion and burrowing, bioturbation and upwelling the substrate they inhabit (Vaughn & Hakenkamp 2001). Pipi also provides recreational services. As a locally plentiful resource many New Zealanders harvest pipi for food and they are a significant part of the local customary practices of iwi. Pipi also supports a commercial fishery, although the fishery has fluctuated through the years and has in more recent years all but ceased (see section below).

In New Zealand, much of the evidence for declining health and population density is based on anecdotal observations which is accepted on the basis of the longevity of traditional cultural knowledge. As pipi reside in estuarine sand flats their environment is subject to a dynamic array of

environmental variables, including changes in sea temperature, instability in water salinity from freshwater outlets or storms and a multitude of pressure from anthropogenic variables including industry, recreational and many more factors (Elliott & Quintino 2007). Ultimately, this means that pipi are exposed to a huge diversity of particulates, contaminants and microbes present within the water column from the various sources.

Fisheries

Commercial harvesting records of pipi date back to 1986–1987 (MPI 2016). Up until 2004-2005, pipi fisheries were not under the QMS. In 2004–2005, commercial and non-commercial (recreational & customary catch) pipi fisheries were introduced into the QMS, containing 10 pipi fish stocking areas. The TAC amounted to 713 t. Between 10 pipi fish stocks area this amounted to 204 t for total allowable commercial catch, 242 t for both recreational and customary allowance, and 25 t for other sources of mortality. The largest commercial fishery was Whangārei Harbour, with a total allowable commercial catch 200 t, the second largest Hauraki Gulf and the Bay of Plenty with a commercial catch of 3 t (MPI 2017). However, no commercial landings have been reported since 2010 for Hauraki Gulf and the Bay of Plenty and 2014 for Whangārei Harbour (MPI 2022).

Pipi can be harvested all year round. Both recreational and commercial harvest are restricted under regulations to be gathered by hand, though commercial fishers typically use a mask and snorkel. Under current MPI fishing rules, there is no minimum catch size for pipi, though larger pipi are preferred. In customary harvesting, smaller pipi are returned to the sea, on the knowledge that pipi have more years of spawning left in them to reseed the population (*pers. comms.* Patuharakeke Te Iwi Trust Board (PTB)). As commercial harvesting has been retained mostly to Whangārei Harbour, the commercial landing reports have been well documented since 1986 to 2013. Overall permit holders within this region reported considerably fewer harvesting quantities than the TACC. Since 2014 no commercial harvesting has taken place in this region.

Reports on harvesting quantities for recreational and customary fisheries are poorly documented. Creating estimates from the data available is problematic due to lack of regulatory and inconsistencies with documentation. The current recreational daily limits for pipi are 150 and 50 for Auckland/Coromandel respectively. Some areas have further specific restrictions on fishing/harvesting, such as Mair and Marsden Bank which are currently no take zones for all bivalves.

Current research on pipi health

There are very few dedicated studies available on pipi. Pipi stock assessments demonstrate a decline in biomass in certain regions of New Zealand (Williams et al. 2017). For instance, a comparison of the Mair Bank stock assessment between 2005 and 2010 revealed a reduction of over 50% in biomass

(Pawley et al. 2013). A report from 2016–2017 discussed the changing uniformity of pipi in Mair and Marsden Bank due to substrate changes to the pipi beds (Williams et al. 2017). Sloping beaches in the mid-tidal zone are characteristic of areas where pipi were once abundant, but now many of these areas are devoid of pipi with the beach morphology having changed to elevated banks and deep sand depressions (Berkenbusch & Neubauer 2018). Although studies looking at stock assessments provide indicators of the abundance of pipi in some areas of New Zealand, pipi are not always the primary target species of interest, and methodologies and frequencies of testing vary from location to location making the data difficult to compare due to the significant time gaps (Pawley et al. 2013). Estimates of stock assessment abundance alone do not always provide any information on the biological process such as reproductive rates, spawning cycles in time and instead makes assumptions of this information based on prior knowledge and is not a holistic approach to understanding the health of a population in real-time.

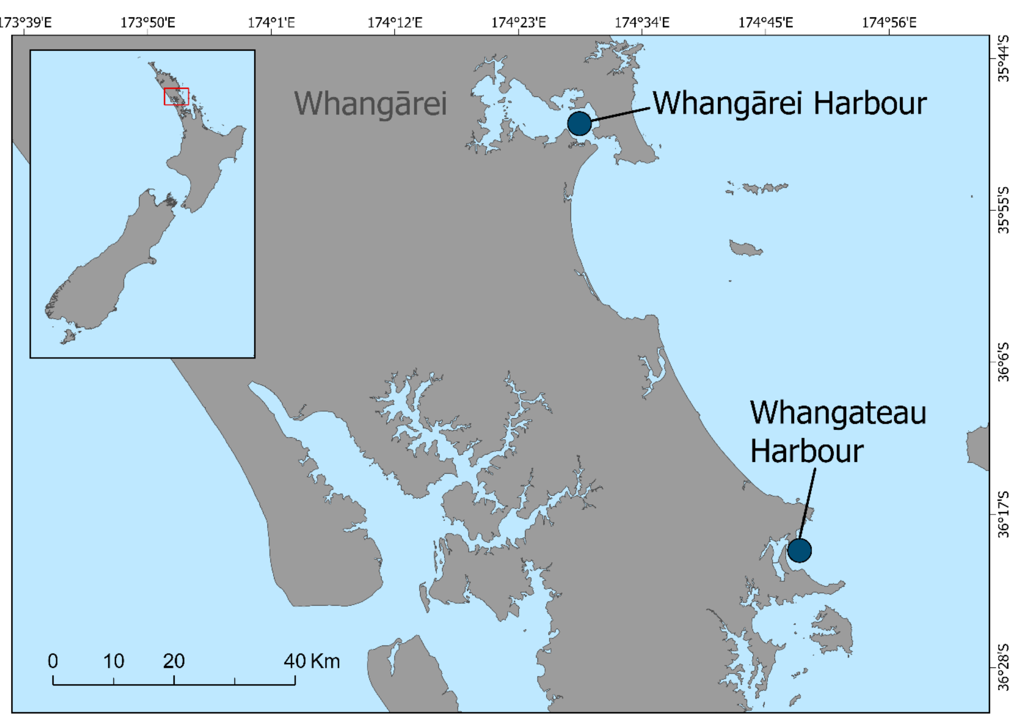

Figure 1.6 Map of New Zealand in the top left corner. Close-up localised map showing harbours in Northland, Whangārei Harbour (north icon), and Whangateau Harbour (south icon).

The most significant study to date was carried out in Whangataeu Harbour (Figure 1.6), looking at the life history and demography of pipi in this region (Hooker 1995a). Hooker (1995a) identified that pipi in this region showed a population maximum size of 55–60 mm, and the localised movement of pipi showed them gradually moving into deeper waters. A peculiar observation was that both adult and juvenile pipi had the ability to drift in the mid-water zone using mucus thread which extended out from their siphons, though a higher ratio of this mechanism of buoyancy was observed in juvenile pipi (<15 mm). Hooker (1995b) hypothesised that pipi adopted this buoyancy technique to overcome areas

of high density, i.e., over 4000 m^2. This study provides a detailed description of not only the distribution and abundance of pipi in this region, but also provides an insight to the ecology of pipi population in this region. This research also described the variations seen in one harbour, with pipi showing different characteristics, i.e., shell shape, and population size structures, characteristically different despite the proximity.

Another report incidentally looks at bacterial impacts on pipi health, reporting *Endozoicomonas* spp. as a potential causative agent in wild bivalve mass mortality events (Howells et al. 2021). The true effects of *Endozoicomonas* spp. have only been postulated and will be tested in this thesis. The development of molecular tools in Howells et al. (2021) allows for a more in-depth analysis of the role that this bacteria may have on the health of New Zealand bivalve. Howells et al. (2021) does indicate that the bacteria may have a negative impact on bivalve health within New Zealand, including pipi, however, more targeted research needs to be carried out. Biessy et al. (2020) reported the high biodiversity of bacteria isolated from different tissues from ten pipi populations around New Zealand, including the *Endozoicomonadaceae* family showing a nationwide dispersal of bacteria from this family. Whether it is the same genus isolated from the mass mortality events from Howells et al. (2021) is unknown.

Cummings & Thrush (2004) identified behavioural changes in pipi that were exposed to terrestrial sediment. Harbours around New Zealand are seeing a rapid increase in development, this poses many risks to the biodiversity that inhabit these areas from run-off, pollutants and changing bathymetry. Much of the run-off deposits terrestrial sediment into harbours changing the water column dynamics and substrate type. Cummings & Thrush (2004) observed reduced abilities to burrow in terrestrial sediments in pipi, and although this species can re-disperse to a new area to re-burrow, there is no guarantee that the area won’t be similarly affected. Re-dispersing to search for more favourable conditions will have direct impacts on the distribution and demography of pipi populations in affected areas ultimately impacting the abundance and survivability of the population.

Pipi in Whangārei Harbour

The Whangārei area is home to several pipi populations (Figure 1.6). The bathymetry of Whangārei Harbour itself creates a perfect substrate for these pipi to settle and reside for their adult life, with the most significant population of pipi residing at Mair Bank. Mair Bank is a large reach of sand tightly packed with shell mass forming an ebb-tide delta, the shell ridge lining the edge of the mouth of the estuary creating stability from erosion (Figure 1.7) (William & Hume 2014).

Figure 1.7 Masses of pipi shell deposition that covers seabed at Mair Bank, Whangārei Harbour.

Pipi in this region are known as kōkota and are an important part of the history of this region (Patuharakeke Te Iwi pers. comm.). The Patuharakeke hapū are kaitiaki of pipi, which are considered taonga (an object or natural resource which is treasured). The banks of pipi that are exposed during low tide line the edges of the estuary. These pipi have provided not only ecological and economic services, but also social services to the Patuharakeke hapū (Williams et al. 2017). The social services of the kaimoana outweigh any other service, and the social impact loss of this kaimoana is incomprehensible to anyone outside this hapū. Taonga, such as pipi, are a huge part of local custom and local practices of the iwi. The Patuharkeke hapū have a wealth of knowledge that can only be obtained through the close spiritual, social and cultural practices they share with their environment. The knowledge of the Patuharkeke hapū steps outside the classic scientific textbook and is enriched by their experiences. For example, a classic textbook description describes pipi as the smallest of the *Paphies* species ranging from 40–60 mm (Armitage et al. 1981). However, cultural knowledge tells us that this is an understatement and different populations around the country have very different characteristics e.g., an archive pipi shell from 1961 in Whangārei Heads, Urquharts Bay (35° 51.00 S, 174° 32.00 E), measured to over 80 mm in length (Figure 1.8). This knowledge that the Māori people possess about their taonga encompasses the living history of the land itself. As witnesses to their changing environment, they can account for social and ecological losses they are experiencing in the rohe. And the Patuharkeke hapū are no strangers to a changing environment.

Figure 1.8 Archive pipi shell from Whangārei Heads, Urquharts Bay (35 51.00 S - 174 32.00 E) collected in 1961. BY 4.0 Museum of New Zealand Te Papa Tongarewa.

Whangārei Harbour is known for its rich abundance of pipi, so much so that from 1986–2004 Mair Bank supported New Zealand's largest commercial pipi fishery which accounted for 99% of all commercial landings. On 1st October 2004 Mair Bank was introduced into the QMS. Around 2009/2010 Mair Bank and the surrounding populations of Marsden Bank saw a considerable decline in pipi abundance. For Mair Banks stock assessments revealed 10152 tonnes (t) in 2005, 4450 t in 2010 and 73.5 tonnes in 2014, similar for Marsden Bank population saw 208.8 million pipis in 2010 which declined to 60.0 million pipis in 2012 (MPI 2014). The cause of this decline in abundance is uncertain with a number of speculative causes ranging from high natural mortality, low recruitment, and the changing morphology of the banks in which the populations reside (Williams & Hume 2014). Soon after this observed decline, the Patuharakeke hapū put in place a rāhui, prohibiting the taking of pipi from these populations in 2009–2010. In 2011, the Ministry of Fisheries introduced a two-year temporary closure under section 186A of the Fisheries Act 1996. This closure was extended to 2013, followed by an indefinite closure of both Marsden and Mair Banks to the take of pipi from 1st October 2014. This was then followed by an application from the Patuharakeke hapū for a further two-year temporary closure of the two banks of the take of all shellfish (Figure 1.9). On 20th April 2020 the Patuharakeke hapū requested a further temporary closure at Mair Bank and Marsden Bank of the take of all shellfish for a further two-year period.

Figure 1.9 Map of New Zealand in the top left corner. Close-up localised ma showing closure area for pipi and shellfish in Whangārei Harbour (Fisheries New Zealand 2021).

These closures have been initiated by the Patuharakeke hapū in an effort to restore the rich biomass of bivalves, in particular pipi, that once dominated this region. Pipi in Whangārei Harbour have to a lesser or greater extent been impacted by the commercial harvesting of bivalves, changing land use, dredging to the harbour and the development of an oil refinery which sits aside these pipi banks (Williams et al. 2017). These are some of the more visually obvious factors that might be harming bivalves in the harbour, but there are many other known factors that interact with bivalve health that are less obvious to the eye (refer to section 1.2 & 1.3). This thesis will address disease and whether microorganisms are a potential factor in the declining health of pipi in this region. The Patuharakeke Te Iwi Trust Board work in collaboration with many stakeholders and scientific institutes, such as National Institute for Water and Atmosphere (NIWA), Cawthron Institute and BNZ, to combine their knowledge with different organisations, with a number of studies looking into the pipi populations present in this area.

There is an increasing need for the combined efforts of ecological science, microbiological science and mātauranga Māori (Māori knowledge), to combine their knowledge to support restoration and management efforts for a range of species and environments. With a continuously shifting environment there is greater need to combine and reinforce the history and the significance of these native species in New Zealand.

Thesis structure

The overall aim of this thesis research is to start filling knowledge on the health of *P. australis* in the Whangārei Harbour. This will be achieved through a series of studies focused on establishing a health baseline of pipi, determining whether the intracellular bacteria *Endozoicomonas* spp. has any measurable impact on the health of pipi from the area, and identifying through experimentation what role varying environmental conditions may have on pipi heath and opportunistic infections. This thesis is intended to extend and deepen our knowledge of health on important wild bivalve fisheries and provide data to help track and forecast marine diseases. Very little work has been carried out on identifying the baseline microbial community of wild surf clams in New Zealand, which precludes accurate and rapid diagnosis. More broadly, I hope to begin moving marine disease diagnosis beyond looking for a single cause and identify the multiple factors which can provide a more realistic picture of the complex processes shaping marine disease in native bivalve populations around New Zealand waters.

Included in this thesis are five data chapters that are bookended by a general introduction and a general discussion (Chapter 7); the latter identifies key areas of future research, summarises the findings of this thesis and discusses them in a broader context. All data chapters included in this thesis are written in manuscript format for publication in scientific journals. Consequently, there may be minor elements of repetition among chapters although effort has been made to minimise this.

The five data chapters are as follows:

Chapter 2: Bacteriology & Bivalves: Improving diagnostic tools for more reliable results of remote bivalve populations.

Chapter 3: A longitudinal study of the baseline health of pipi (*Paphies australis*) in Whangārei.

Chapter 4: Testing baseline health data to investigate pipi (*Paphies australis*) mortality events in Whangārei.

Chapter 5: Unravelling the link between environmental factors and *Endozoicomonas* spp.: an experimental study on contributing factors to pipi mass mortality events in Whangārei.

Chapter 6: A retrospective analysis of mortality events of culturally significant bivalve pipi (*Paphies australis*) in Whangārei.

Chapter 2 : Bacteriology & Bivalves: Improving diagnostic tools for more reliable results of remote bivalve populations

Māori Women preparing potatoes with pipi shells. Rotorua, Auckland Province. Credit: National Publicity Studios, Wellington, New Zealand.

Introduction

Bivalve molluscs inhabit all aquatic ecosystems and are particularly abundant in nearshore coastal areas (Smaal et al. 2019). Due to this proximity with land, bivalves are an important human food source, providing valuable economic, cultural, and recreational services (Grant & Strand 2019). Bivalves also support healthy ecosystem functioning, by improving water quality and providing habitat for other marine organisms. Bivalves are filter feeders, straining microscopic particles such as plant detritus, algae, and bacteria from the water column (Jørgensen 1993; Wang et al. 2005; Burge et al. 2016). Bivalves are exposed to an abundance of microorganisms, including bacteria, from both their surrounding environment and through the process of filter feeding (Rippey 1994; Zannella et al. 2017). The surrounding environment and water quality conditions often determine the composition of microorganisms that are present in the bivalve microbiome, for example warmer temperatures have been reported to increase proliferation of certain bacteria (e.g., *Vibrio*) (Pierce & Ward 2018; Paillard et al. 2022). Most aquatic bacteria are harmless and contribute to the healthy microbiome of bivalves which helps in nutrient processing, and immune service responses (Lokmer & Wegner 2015). However, some bacteria are opportunistic or pathogenic and can cause disease, significantly impacting bivalve health, or human health through ingestion of infected animals (Potasman et al. 2002).

Classical bacteriology is a common tool for disease diagnosis in aquatic animal health. Classical bacteriology involves growing bacteria on specialised media followed by taxonomic identification by biochemical methods. Classical culture methods still play an important role in disease diagnostics, despite becoming superseded by modern technologies such as 16S rRNA gene metabarcoding and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Austin 2019). Through cultivating bacteria, a better understanding of bacterial physiology, ecology, natural production, and their interaction with host health can be deduced (Stewart 2012). Additionally, a pure bacterial culture supports further experimental work such as whole genome sequencing, vaccine creation, and disease challenge trials (Brock 1999; Barnes et al. 2016; Brosnahan et al. 2019). The disadvantage of classical bacteriology, however, is that it is time consuming with initial culture and identification taking approximately two days to four weeks (Ferone et al. 2020). Despite this disadvantage, classical bacteriology is still widely considered a gold standard and the preferred international diagnostic tool for confirmation of disease by organisations such as the International Organization for Standardization (IOS).

Classical bacteriology can be applied in disease diagnosis, during acute mortality events or during surveillance operations, and the generation of baseline health data. Both general surveillance and baseline health studies are important tools for assessing general health and can be used for early disease detection (OIE 2015). Creating reliable data is essential for long-term health evaluations, key

reference points and can also inform on potential risk factors. Bivalves are often located in remote areas (Bennion et al. 2022), slowing sample collection and transportation to a diagnostic laboratory, impacting sample quality and an ability to produce useful test results (Buller 2004). Delayed sample processing can lead to changes in the bacterial diversity of the animal from fluctuating temperatures during transportation, and stress experienced by the animal being transported. An overgrowth of environmental or spoilage bacteria can occur without timely collection, sampling, and transport conditions, possibly masking the identification of important bacteria (Join et al. 2010). Moreover, some bacterial species may go into a state of being viable but non-culturable during unfavourable transportation (Xu et al. 1982). Importantly, this can all lead to the potential for misdiagnosing a bacterial agent. With reports of bivalve mortalities increasing in New Zealand (Howells et al. 2021) and marine disease expected to increase because of climate change, ensuring procedures for processing samples accurately and timely is important for future surveillance operations, baseline health studies and accurate disease detection.

A longitudinal study was carried out comparing two different sampling approaches for classical bacteriology of two bivalve species *Paphies subtriangulata* (tuatua) and *P. australis* (pipi) from two locations, Ōtaki Beach and Whangārei Harbour, in New Zealand. Tuatua and pipi are two native New Zealand bivalves frequently reported as suffering mass mortality events (Howells et al. 2021). The two bacteriology sample approaches were: (1) processing bivalves in the field immediately after collection, and (2) processing bivalves at the laboratory at least 24 hours after collection and transportation, mimicking current practices. The aim of this study was to understand if delaying processing for 24 h after sample collection would alter the bacteria isolated, which could impact on general surveillance operations, baseline health studies, and bacterial disease diagnosis.

Materials & Methods

Sample collection

A comparison of two sampling approaches were used in this study: (1) samples collected and processed immediately in the field (field samples), and (2) samples processed in the laboratory 24 h after collection (laboratory samples).

Tuatua (Paphies subtriangulata)

Five collections of tuatua were made by hand from Ōtaki Beach (Figure 2.1) over a period of two weeks in June 2020 (austral winter). During each collection, 20 animals were sampled: 10 animals for field samples and 10 animals for laboratory samples (total n = 100). Tuatua were gathered by hand

using practices traditional to the local community, with the acknowledgement from Ngāti Raukawa (local Māori iwi - community of indigenous people of New Zealand).

Pipi (Paphies australis)

Pipi were collected from five sites in, and adjacent to Whangārei Harbour (Figure 2.1) in February (all sites), August (two sites) and November (four sites) 2020 (Table 2.1). For each sampling event at each site, 10 animals were collected: 5 animals for field samples and 5 animals for laboratory samples (total of eleven collections with n = 110). Pipi were gathered by hand with the Patuharakeke Te Iwi Trust Board, Te Pou Taiao under a customary fishery allocation as custodians of pipi in this region.

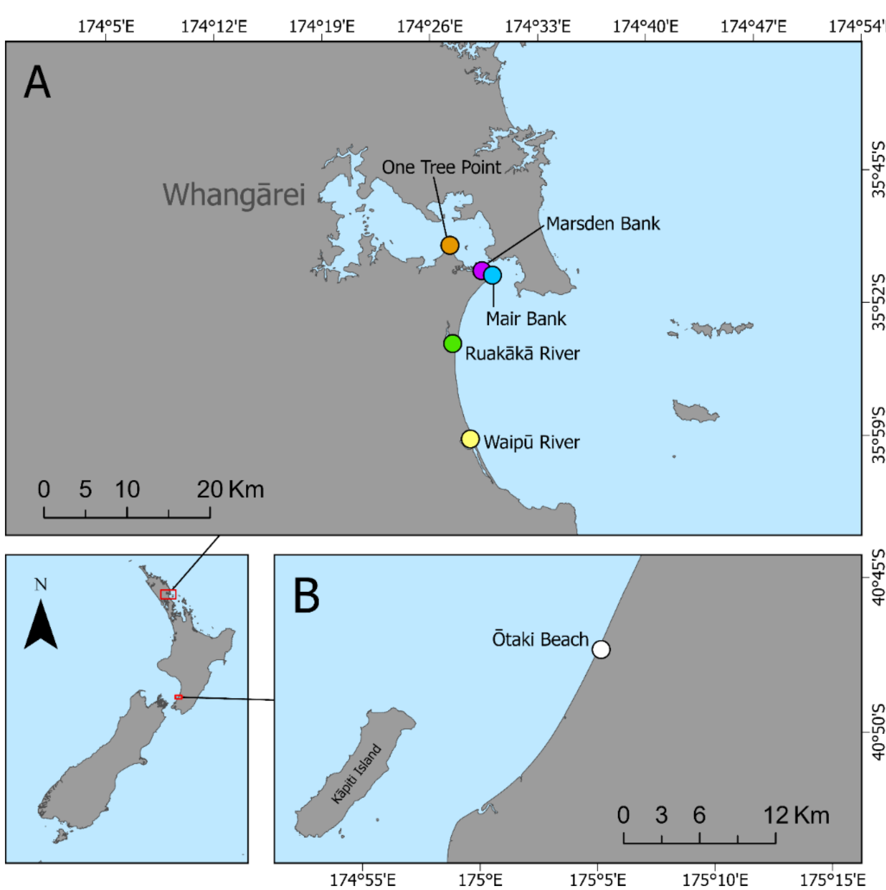

Figure 2.1 Map of New Zealand in the bottom left corner. Close-up localised maps showing A) Whangārei Harbour sampling sites B) Ōtaki Beach sampling site.

Water temperature, salinity, and other field observations were gathered during each collection to monitor environmental conditions over the sampling period (Table 2.1). Temperature and salinity were measured using an 837-2_SOL Salinity & Temperature Meter (Gain Express Holding Ltd.). Other field observations included a presence of algal blooms, elevated influxes of fresh water from outlets or heavy rainfall.

Table 2.1 Environmental observations gathered at each sample collection.

Site	Site characteristics	Subsite	*n* of bivalves collected	Date	Water Temperature °C	Water Salinity ppt	Observations
Ōtaki Beach	High energy surf beach	-	20	Jun 2020	13.6	31.6	Light rain during 24h period of collection.
		-	20	Jun 2020	14	30.6	Rain during 24h period of collection. Heavy freshwater flow from Ōtaki River 1km south of sampling site.
		-	20	Jun 2020	14.2	32.7	No observation of note.
		-	20	Jun 2020	13.4	31.7	Lots of debris brought up in the surf i.e., branches, wood, substrate.
		-	20	Jun 2020	13.3	32.5	Algal blooms present in patches. Light rain on/off over last 24 hrs period.
Whangārei Harbour	Estuarine/river	Waipū River	10	Feb 2020	22.5	19.2	No observations of note.
		Ruakākā River	10	Feb 2020	23.1	11.1	No observation of note.
		Mair Bank	10	Feb 2020	24.8	34.5	No observation of note.
		Marsden Point	10	Feb 2020	24.8	34.5	No observations of note.
		One Tree Point	10	Feb 2020	25	34.1	Black sand with lots of seagrass present.
		Waipū River	10	Aug 2020	15	18.9	Yellow tinge to substrate. Recent flooding with storm.
		Ruakākā River	10	Aug 2020	14.7	10.4	Recent flooding over the edge of the channel 2 weeks prior to collection.
		Waipū River	10	Nov 2020	23.1	27.8	Heavy rain on/off week prior to collection.
		Ruakākā River	10	Nov 2020	20.6	25.9	Heavy rain on/off week prior to collection.
		Mair Bank	10	Nov 2020	21.6	34	Heavy rain on/off week prior to collection.
		One Tree Point	10	Nov 2020	26	34.6	Heavy rain on/off week prior to collection.

Sample processing

Field samples

Field samples were processed within 1 h of collection (Figure 2.2) (Appendix A, Figure A.1). Animals were opened, exposing the adductor muscle. A sterile swab dipped in 70% ethanol was used to clean the adductor muscle. An incision was then made with a sterile scalpel through the middle of the adductor muscle. A separate sterile swab was then inserted into the sterile incision, to sample the haemolymph, and placed into the amies agar gel. Amies agar gel without charcoal (Copan, Brescia Italy) was used for transportation. This transport media was chosen to maintain bacteria on the swab during transport to the laboratory. Swabs in the agar gel were transported to the laboratory in a polystyrene container with ice packs, to keep ambient temperature around swabs cool, which was then placed at room temperature for 24 h until the swabs were removed from the polystyrene container and plated onto growth media. A time interval of 24 h was chosen as during diagnostic investigations this is the most common time delay in which sampling occurs with New Zealand's current bivalve mortality response practices.

Laboratory samples

Animals collected for laboratory samples were placed into a watertight screw top container containing seawater after collection (Figure 2.2). These screw top containers were then placed into a polystyrene container with ice packs and transported back to the laboratory. Laboratory samples, within the polystyrene container, were left at room temperature for ~24 h until processing to mimic conditions similar to those experienced during transportation. A swab of haemolymph was sampled via the adductor muscle as described above and immediately plated onto growth media.

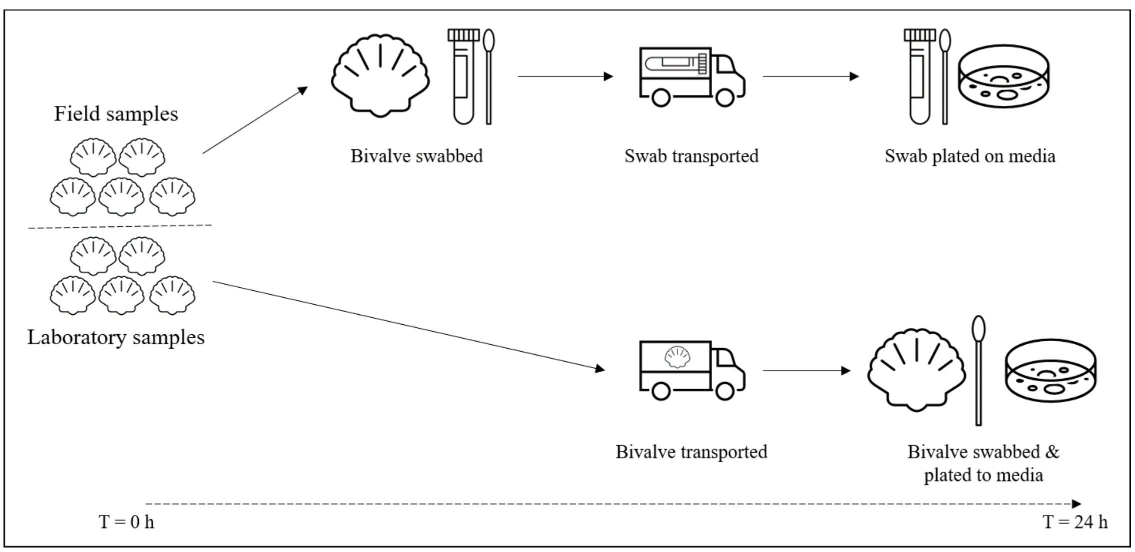

Figure 2.2 Infographic of field and laboratory sampling technique used in study.

Bacteriology and DNA sequencing

Swabs from field (stored in AIMES agar gel) and laboratory samples were plated onto two types of solid media, tryptic soy agar with 3% sodium chloride (TSA+3%) (Fort Richard) and thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Fort Richard) in the laboratory. Swabs were used to inoculate a small area of the selected media and standard streak plating was carried out. The plates were incubated at 22°C for a total of seven days. Incubation temperature of 22°C was chosen to ensure bacteria from all sites would be able to grow as the average temperature from Ōtaki Beach was 13.7°C and the average temperature from Whangārei Harbour was 21.6°C. The range of temperatures that marine bacteria can grow is wide and includes 22°C. Growth was recorded at three days and a final read at seven days.

Bacterial growth on the plate was recorded as a score depending on the quadrant of growth displayed on the plate (Table 2.2). As well as quantifying the bacterial growth, bacteria were also grouped into categories: no bacterial growth, mixed bacterial growth (no common or dominant bacteria), and common or dominant bacterial growth. Common or dominant bacterial growth was the main group of interest and were characterised by bacterial isolates that were either common across multiple samples or were the dominant growth on any singular plate. At both plate readings, any common or dominant isolates were sub-cultured onto the initial growth media and Columbia Sheep's blood agar (BA) to assess purity and for identification.

Table 2.2 Categorisation of bacterial growth and the relative scoring associated.

Inoculum area	**Relative growth score**
No growth	0
Primary inoculum	1
Second quadrant	2
Third quadrant	3
Fourth quadrant	4

Basic biochemical tests were carried out on all common or dominant isolates (Gram stain, indole, oxidase, and catalase) followed by MALDI-TOF MS identification. Isolates that were gram negative rods and oxidase positive were tested for sensitivity to O/129 (150 µg) (Oxoid Ltd.). Where basic biochemical results and MALDI-TOF MS did not provide either genus or species identification, PCR and sequencing analysis was carried out. Isolates sensitive to O/129 were suspected *Vibrio* species and were subjected to PCR and nucleotide sequencing of the atpA gene. Isolates resistant to O/129 (150 µg) were non-suspect *Vibrio* spp. and were subjected to PCR and nucleotide sequencing of the 16S rRNA gene.

DNA extraction

Pure isolates were extracted separately using InstaGene™ Matrix (BIO-RAD). Briefly for each isolate, five individual colonies were scraped off the agar and homogenised in 200 µL by pulse vortexing for 15s in InstaGene™ Matrix in a centrifuge tube. The tube was then heated at 56°C for 30 mins, pulse vortexed, and heated at 72°C for 10 mins before being pulse vortexed again then centrifuged at 3,000 g for 3 minutes. The supernatant was then removed for downstream applications and quantified (ng/µL) using QubitTM fluorometer (Life Technologies).

16S rRNA gene PCR

Isolates not suspected to be *Vibrio* spp. were subjected to a bacterial species PCR targeting ~1498 bp of the 16S rRNA gene (Lane 1991). Template DNA concentration 2.5 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer 27f and 1525r to a total volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 50°C for 15 sec, 72°C for 1 sec. For every PCR performed, two no template controls (NTC) and a positive control were used. For the NTC, molecular-grade water was added to the reagent mix instead of nucleic acid template. For the positive control, *Escherichia coli* (ATCC 25922) DNA was used at 0.1 ng/µL.

Isolates suspected to be *Pseudomonas* spp. (based on MALDI-TOF MS identification) were subjected to a *Pseudomonas*-specific PCR targeting 618 bp of the 16S rRNA gene (Ardura et al. 2013). This additional analysis was carried out as *Pseudomonas* spp. are known to cause bacterial disease in bivalves (Zanella et al. 2017) and were not able to be speciated using 16S rRNA gene or atpA gene assays. DNA of concentration 1 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer PA-GSF and PA-GSR to a total volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 54°C for 15 sec, 72°C for 30 sec. Each PCR had two NTC and a positive control of *Pseudomonas aeruginosa* (ATCC 27853) at 1 ng/µL.

Vibrio atpA gene PCR

Suspect *Vibrio* spp. were subjected to a PCR targeting 1500 bp of the atpA gene (Thompson et al. 2007). DNA of concentration 1 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer atpA-06F and atpA-04R to a total volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 58°C for 15 sec, 72°C for 30 sec. Each PCR had two NTC and a positive control of *Vibrio anguillarum* (ATCC 19264) at 1 ng/µL. One suspect *Vibrio* species did not amplify from the atpA gene PCR so was ran on the 16S rRNA gene PCR to obtain an identification.

DNA sequencing

All PCR amplicons were visualised by electrophoresis in a 1.5% agarose gel and stained with GelRed™ (Biotium). Amplicons of the correct size were purified using a Zymoclean Gel DNA Recovery Kit (Zymogen Research). Purified amplicons were nucleotide sequenced in the forward and reverse direction using the PCR primers for 16S rRNA gene (27f with 1525r, and PA-GSF with PA-GSR) and atpA gene (atpA-06F, atpA-04R, atpA-02F, and atpA-03R). DNA sequencing was carried out on Sanger sequencing platform by Ecogene Landcare Research. DNA sequences generated were imported into Geneious (Biomatters, Auckland), assembled into contigs and manually edited and trimmed. Consensus sequences were queried against the National Centre for BioTechnology Information (NCBI) nucleotide database via BLASTn using default settings for closest isolate identification.

Statistical analysis

Bacterial growth was categorised into four descriptions and given a relative score (Table 2.2). This score was used to compare the relative growth of bacteria between the two sampling approaches. Statistical analyses were performed in R Studio (version 3.6.1, R Core Team 2019). A one-way ANOVA was used to compare the average relative growth scores between the two sampling approaches, using Tukey contrasts and p-values adjusted for multiple testing using Benjamini-Hochbergs false discovery rate (FDR) (Benjamini & Hochberg 1995, Hothorn et al. 2008). A separate comparison was carried out for samples taken from tuatua and pipi. A one-way ANOVA was also used to compare the different levels of bacterial growth, incidence of common or dominant growth, mixed growth, and no growth between field sampling and laboratory sampling. A final comparison was carried out to determine if the common or dominant bacteria, at a genus level, were isolated more frequently from those processed in the field compared to those in the laboratory.

Results

Environmental observations

A few notable observations were recorded in the field at the time of each collection (Table 2.1). The average temperature and salinity for tuatua samples were 13.7 °C and 31.8 ppt, and for pipi samples 21.6 °C and 25 ppt respectively.

Bacterial growth

Tuatua

Field samples had a significantly higher average amount of bacterial growth than the laboratory samples based on the relative growth score (p-value < 0.05) (Figure 2.3).

A significant difference in the type of bacterial growth was detected (p-value < 0.05) (Figure 2.3). Significantly more laboratory samples had no growth compared to field samples. Field samples had significantly more samples with common or dominant bacterial growth.

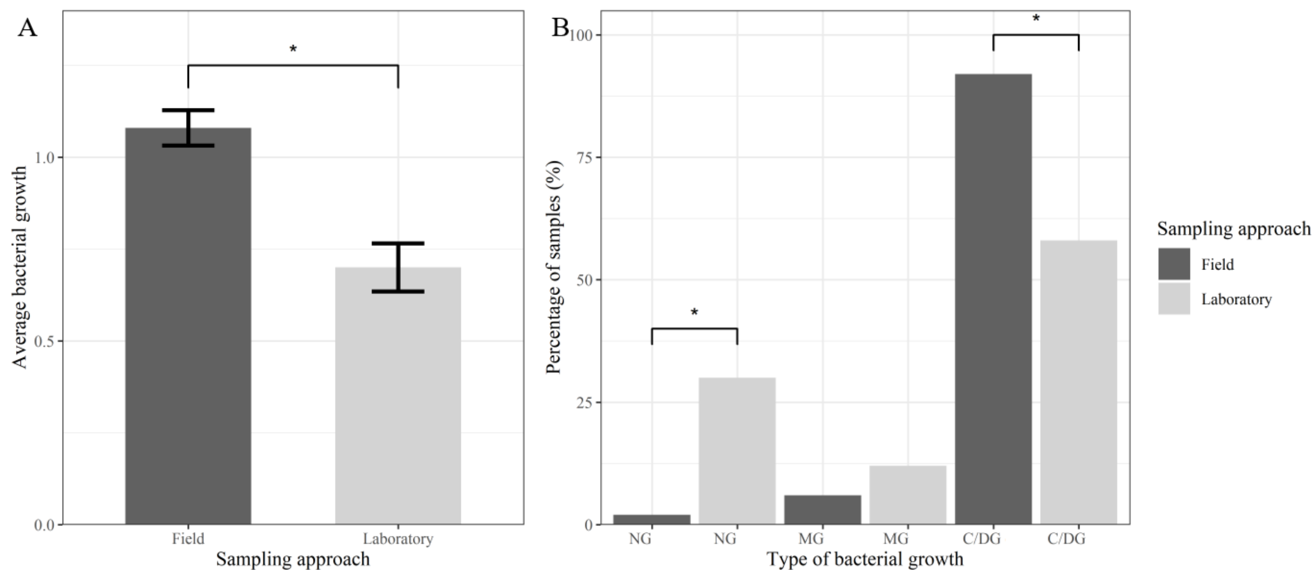

Figure 2.3 Overview of bacterial growth from tuatua samples A) Average relative bacterial growth score observed on plates between field and laboratory sampling approach. Bar chart with standard error of mean. B) Percentage of samples categorised into the type of bacterial growth present. Key: NG – No bacterial growth, MG – Mixed (no common or dominant) bacterial growth, C/DG – Common or dominant bacterial growth. * represents significant difference measured.

Pipi

There was no significant difference in the amount of bacterial growth between samples processed in the field and the laboratory (p-value > 0.05) (Figure 2.4).

Significant differences were observed in the type of growth. Pipi processed in the field had significantly more samples with no growth (p-value < 0.05). Pipi processed in the laboratory had significantly more samples with mixed growth (p-value < 0.05) (Figure 2.4).

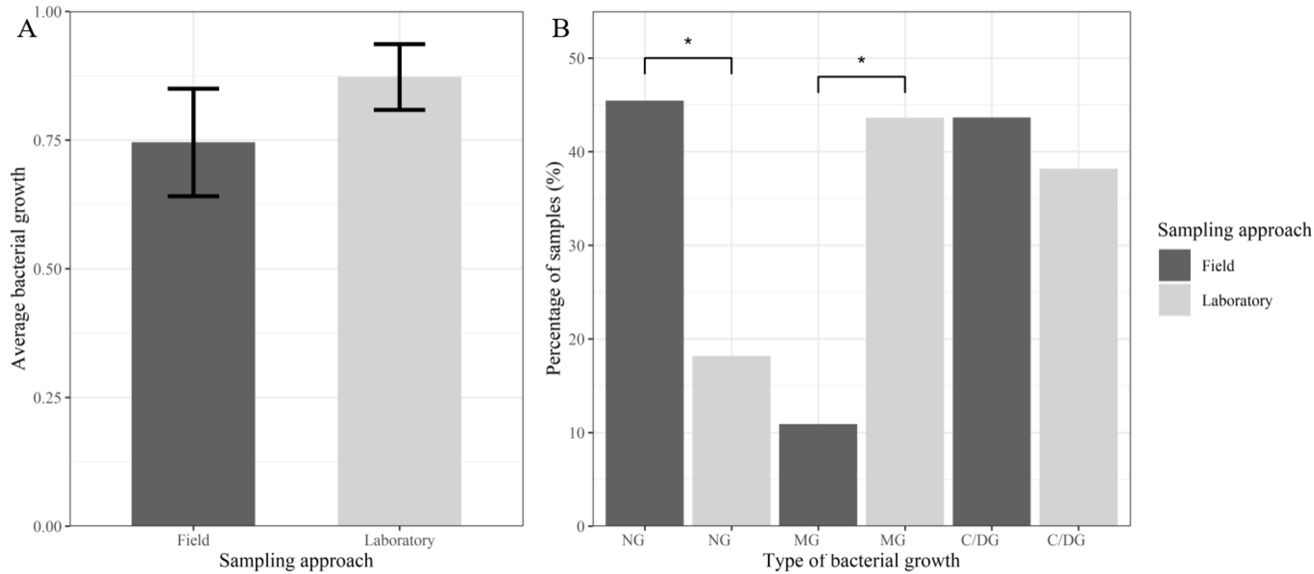

Figure 2.4 Overview of bacterial growth from pipi samples A) Average relative bacterial growth score observed on plates between field and laboratory sampling approach., Bar chart with standard error of mean. B) Percentage of samples categorised into the type of bacterial growth present. Key: NG – No bacterial growth, MG – Mixed (no common or dominant) bacterial growth, C/DG – Common or dominant bacterial growth. * represents significant difference measured.

Identification of bacteria

The majority of bacteria isolated consisted of gram-negative bacteria (n = 22) and a small portion of gram-positive bacteria (n = 5) (Table 2.3). Bacterial identification by MALDI-TOF MS provided limited level identification on the majority of bacteria, providing the best organism match to ‘No Organism Identification Possible’. DNA sequencing was therefore carried out on all isolates to obtain a more conclusive level of identification.

Table 2.3 Summary of common or dominant bacteria isolated from pipi and tuatua. Bacteria are shown to closest identification level*, with biochemical test results and gram stain. * Genera identified via sequence analysis as biochemical and MALDI-TOF MS were inconclusive. The number of different species within that genus is shown in Table 2.4.

Species isolated from	**Closest identification**	**Biochemical tests**			
		Gram	**Indole**	**Oxidase**	**Catalase**
Pipi only	*Kistimonas**	-	-	+	+
	Pseudomonas	-	-	+	+
	Shewenella	-	-	+	+
	*Uncultured bacterium**	-	-	+	+
Pipi & tuatua	*Vibrio*	-	-/+	+	+
	Staphylococcus	+	-	-	+
	*Marinomonas**	-	-	+	+
Tuatua only	*Marine bacterium**	-	-	-	+
	*Pseudoalteromonas**	-	-	+	+
	*Sphingobium**	-	-	+	+
	*Tenacibaculum**	-	-	+	+

Twenty-seven different bacterial species were identified through atpA gene and 16S rRNA gene sequencing from pipi and tuatua. Three genera were common to both host species: *Vibrio* spp., *Staphylococcus* spp., and *Marinomonas* spp. (Table 2.3). A higher number of *Vibrio* spp. were isolated from pipi (n = 9) compared to tuatua (n = 1) (Table 2.4).

Four genera were exclusively isolated from tuatua: *Marine bacterium*, *Pseudoalteromonas* sp., *Sphingobium* sp., and *Tenacibaculum* sp. (Table 2.3). *Vibrio* spp. were the only genus that were isolated solely from field samples. All others were isolated from both field and laboratory samples (Table 2.4).

Four genera were isolated exclusively from pipi samples: *Kistimonas* sp., *Pseudomonas* spp., *Shewenella* spp., *and Uncultured bacterium* (Table 2.3). Two of these were only isolated from samples processed in the field (*Staphylococcus* species and *Pseudomonas* species) and two only isolated from samples processed in the laboratory (*Marinomonas* species and uncultured bacterium). All other genera were isolated from both field and laboratory samples (Table 2.4).

Table 2.4 Common or dominant bacterial species identified from DNA sequencing of the atpA gene and 16S rRNA gene from each sampling approach from tuatua and pipi (see appendix A, Table A.1 for GenBank reference ID's). *same BLAST ID - geneious alignment confirmed different species.

Genus	Species (closest BLAST ID)	Tuatua		Pipi	
		Field samples	Laboratory samples	Field samples	Laboratory samples
Marine bacterium	*Marine bacterium*	+	+	-	-
Pseudoalteromonas	*P. agarivorans*	+	+	-	-
Sphingobium	*S.naphthae*	+	+	-	-
Tenacibaculum	*Tenacibaculum sp.*	+	+	-	-
Vibrio	*Vibrio sp.**	+	-	-	-
	*Vibrio sp.**	-	-	+	+
	V. splendidus	-	-	+	+
	V. tetraodonis	-	-	+	+
	V. artabrorum	-	-	+	-
	V. kanaloae	-	-	+	-
	V. genomosp.	-	-	+	-
Staphylococcus	*S. warneri**	+	+	-	-
	*S. pasteuri**	+	+	-	-
	*S. pasteuri**	-	-	+	-
	*S. warneri**	-	-	+	-
	S. epidermidis	+	+	-	-
Marinomonas	*M. profundimaris*	+	+	-	-
	M. pontica	-	-	-	+
Kistimonas	*Kistimonas sp.*	-	-	+	+
Pseudomonas	*Pseudomonas sp.*	-	-	+	-
	P. lurida	-	-	+	-
	P. poae	-	-	+	-
Shewenella	*S. colwelliana**	-	-	+	+
	*S. colwelliana**	-	-	-	+
	S. hafniensis	-	-	+	-
	S. baltica	-	-	+	-
Unculturedbacterium	*uncultured bacterium*	-	-	-	+
Total *n* of taxa:	27	9	8	15	8

Once genera were identified from DNA sequencing, a final statistical comparison was carried out to identify whether each of the common or dominant bacteria grew on more samples tested in the field or the laboratory. For tuatua samples, *Staphylococcus* spp. grew in significantly more samples processed in the field (p-value < 0.05). Conversely, *Tenacibaculum* sp. grew in significantly more samples

processed in the laboratory (p-value < 0.05). No significant difference was observed between the sampling methods for any other common or dominant bacteria isolated from tuatua.

For pipi samples, *Pseudomonas* spp., *Shewanella* spp. and *Staphylococcus* spp. were isolated in significantly more samples processed in the field (p-value < 0.05). *Marinomonas* sp. and *Vibrio* spp. were isolated in significantly more samples processed in the laboratory (p-value < 0.05).

Discussion

Bacteriology of haemolymph from two bivalve species over time has allowed for an assessment of two different sampling approaches. This study investigated the amount of bacterial growth and diversity between two sampling approaches to understand limitations when diagnosing disease, carrying out surveillance or baseline health studies on remote bivalve populations. The results demonstrate that field sampling does generate a different characterisation on the types of bacterial growth and species diversity than samples processed in the laboratory after a 24 h delay. Differences in amount and type of growth between the two sampling approaches were observed. Samples processed in the field from tuatua had significantly more bacterial growth, and a higher diversity of bacteria, including more common or dominant bacterial species. Laboratory samples had a higher proportion of samples with no growth, however common or dominant bacteria were still isolated from these samples. For pipi, field samples more often had no bacterial growth and laboratory samples had a significantly higher number of common or dominant growth present. Field samples did however contain a higher diversity of bacteria.

Factors effecting the results of this study

Temperature can influence the type and rate of bacterial growth (Shiah & Ducklow 1994; Kirchman et al. 2005; Rahman et al. 2019). Transportation of samples from field to laboratory pose difficulties around maintaining a constant temperature that may directly impact the sample integrity or compromise the bacterial community present. In this study, transport media was used to assess whether waiting to sample for bacteriology at the laboratory impacted the community of bacteria present compared to sampling immediately after collection in the field. The results did indicate that the growth and community of bacteria differed between the two sampling approaches and may indicate that the community of bacteria could be compromised when only using the laboratory sampling approach. The results demonstrated that if the animals were processed in the field directly after collection, they retained additional information and therefore the approach may be a more useful tool to investigate bivalve health.

Logistically it is very difficult to process samples for bacteriology directly in the field using the same techniques as in the laboratory. Transport media were designed to preserve specimens and minimise

bacterial overgrowth. Transport media were developed as a tool in human health surveillance and does not appear to be optimised for the transport of marine bacteria (Stuart 1959; Amies 1967). Transport media has evolved over the years to be optimised for the users' needs, and no single transport media fits all purposes (Rosa-Fraile et al. 2005; Druce et al. 2012). It would be worth exploring different options of transport media, including the one used in this study, to optimise this tool for bacteriological analysis of bivalves that may greatly benefit diagnosis. While the results from the two bivalve species cannot be compared as bacterial presence, diversity and pathogenicity will depend on the geography, host species, environment, and life stage, it is interesting to note their differences. Overall, there are contrasting results observed in tuatua and pipi between bacterial growth score and the diversity of bacteria isolated. The higher overall species diversity of bacteria isolated from pipi could have been due to the range of environmental conditions experienced in this collection series – temperature and salinity had a variable range which is likely to contribute to the diversity of bacteria isolated at this location. Whereas tuatua samples were collected over a shorter time-period and smaller geographical range experiencing less variable environmental conditions. Mo et al. (2021) found that even small incremental changes to salinity altered the bacterial community. Despite pipi being well adapted to fluctuations in salinity as they reside in estuarine environments (McLeod & Wing 2008), heavy rainfall and flooding was experienced in these areas prior to the collection of samples (Table 2.1). Additionally, heavy rainfall and flooding would have resulted in land runoff and this bacteria from terrestrial environment may have been pushed into the water where pipi filter-feed, altering the community of bacteria present (Le et al. 2016).

In addition to the environmental observations, the contrasting results could also be due to differences between species, geographical location and overall "health" status. There have been numerous anecdotal reports from the Whangārei region reporting an overall loss of health of pipi in the region in the last few decades (Williams et al. 2017). The impacted health of pipi in this region could be due to changes to their surrounding habitat, such as changes to substrate morphology or structure from surrounding activity (Williams & Hume 2014). These changes to the pipi's habitat cause stress on the animals and may reduce their physiological condition (Ellis et al. 2002), which could cause an imbalance of the growth and diversity of bacteria present. Future work should be carried out to look at different species, different health statuses and different geographic ranges to see whether the limitations observed are characteristic of the test or the species.

Bacterial growth

Many factors need to be considered when interpreting classical bacteriology techniques, fundamental elements such as bacterial diversity and growth need to be reviewed (Buller 2004). The results from our study show that bacterial growth is supported even when testing in the field using transport media.

Tuatua field samples revealed common or dominant bacterial species were isolated more often than those processed in the laboratory. Most of this growth was *Staphylococcus* spp. This genus was also isolated from samples processed in the laboratory, but in a smaller number. *Staphylococcus* spp. are ubiquitous and often cultured from food or environmental samples, including seafood. Three *Staphylococcus* species were isolated from tuatua (*S. pasteuri, S. warneri* and *S. epidermidis*), these species have been associated with fish or shellfish disease (Metin et al. 2014), as well as being found in healthy populations (Luis-Villaseñor et al. 2017). The significance of the isolation of *Staphylococcus* spp. in this population of apparently healthy bivalves is uncertain. Samples processed in the field are inherently less aseptic than samples processed in the laboratory by nature of them being processed in the open environment. However, recent work carried out on this population of bivalves in another study analysing the baseline bacterial population using a shotgun metagenomics approach (MPI unpublished) revealed a large proportion of *Staphylococcus* spp., (1-6% of all bacterial species identified). These samples were processed within the laboratory where aseptic technique was possible, providing more confidence that results from the present study are a true finding rather than a limitation of sampling in the field. Bacterial diversity can be altered through environmental changes which then allow for transient bacteria to gain an advantage and the potential to cause disease (Moriarty, 1990). The identification of this baseline bacterial population, using tools such as metagenomics, is useful for interpretation of the importance of such cultured bacteria.

There was no significant difference in the amount of bacterial growth from pipi samples between the field and laboratory samples. However, the laboratory samples exhibited significantly more samples with mixed bacterial growth across all the animals at each sampling event. Additionally, a higher proportion of the field samples produced no bacterial growth. As this population of bivalves were already thought to be under stress, a possible explanation for what was observed in this study, is that the shock in the change of environment during transportation led to a suppressed immune system being overcome by any bacteria that were circulating within the animal (Pruzzo et al. 2005) resulting in bacterial growth once the animals were sampled at the laboratory. Contrastingly, animals that were sampled directly in the field, without the shock of prolonged transport, may have been more capable of managing the bacteria resulting in more samples processed in the field with no bacterial growth as it did not proliferate to the haemolymph. As this study did not monitor the conditions during transportation, we cannot rule out that transport did not compromise the bacterial community hence the variation in the type of bacterial growth between field and laboratory sampling. It is therefore beneficial to use transport media as a way of aiding in the interpretation of result diagnosis.

Bacterial diversity

The diversity of bacterial genus isolated between the two sampling groups for each species is very similar. Gram-negative bacteria such as *Tenacibaculum*, *Vibrio* and *Pseudomonas* have all been

identified as disease-causing bacteria in marine species (Austin & Austin 2012). Both field and laboratory sampling isolated *Vibrio* spp. and *Tenacibaculum* sp., however, *Pseudomonas* spp. were only isolated in samples processed at the laboratory. During general surveillance early detection of important bacteria is critical (OIE 2015). Therefore, the application of both sampling techniques would be recommended to ensure important bacteria do not go undetected. It is worth noting that although the above-mentioned bacteria are known to cause disease in bivalves, they may also have no impact on the health of bivalve and may be a part of the normal microbiota at that time (Olafsen et al. 1993; Vezzulli et al. 2017; Nowlan et al. 2020). Not only is it important that sampling techniques should support bacteria that may be critical to bivalve health, but it is important that the sampling techniques support other bacteria that make up a part of the animal's normal health. Information on healthy bivalves can provide reference points in future surveillance and a record of the baseline health of the species. Collating information on the bacteria that make up the normal microbiota of healthy bivalves will help detect shifts from the baseline in the future that may be critical to identify risk factors and help inform future management and protection. Both techniques in this study show that they support a wide diversity of bacteria, including some that may be critical to bivalve health and some that are part of the bivalve normal microbiota.

From the common or dominant bacteria isolated, more species were isolated in samples processed in the field compared to the laboratory, particularly in pipi. As this sampling method is biased in that it will only detect culturable bacteria and not all bacterial growth was purified and identified, the possibility of common or dominant bacteria isolated in the field samples being present in the laboratory samples cannot be excluded. These species may have been present in lower numbers in the laboratory samples and therefore not considered dominant or common bacteria in the analysis.

In most cases, the field samples were isolating the same bacterial genus as the laboratory samples. However, in some cases, more bacterial species were present in the field samples (Table 2.4). For example, three *Vibrio* spp. were isolated from pipi processed in both the laboratory and the field, but three additional *Vibrio* spp. were isolated in field samples only. Despite field samples having a higher diversity of *Vibrio* spp. present, *Vibrio* spp. were isolated in significantly more pipi samples processed in the laboratory than field samples. The number of culturable and live *Vibrio* spp. has been reported to decrease with time following sampling (Burgents et al. 2005; de Souza Valente & Wan 2021). The significantly higher amount of growth of *Vibrio* spp. (not species) in the laboratory samples therefore could be a bias of the more robust *Vibrio* spp. that survived and outcompeted other *Vibrio* spp. during the prolonged transportation period (~24 h). Therefore, an overall bias towards more robust bacteria in the final diagnosis may be observed if animals are only sampled in the laboratory. Another possibility is that certain bacterial species could be outcompeted, or conversely stimulated, by others during transport. Rami et al. (2014) demonstrated that *Staphylococcus warneri* can stimulate *V.*

anguillarum growth. The interactions between different bacterial species during sample transport may alter the significance of identifying potential disease-causing bacteria when only sampling post-transportation.

Buller (2004) discusses the reduced effectiveness of transport media with time and suggests that some bacteria are not viable on transport media after 24 h. When diagnosing the cause of bivalve mass mortalities, in particular wild or remote populations, there is very little background information known about these populations (Ward & Lafferty 2004). Therefore, it is difficult to determine which bacteria are involved prior to collection and it may not always be possible to apply the right techniques to maintain viability of those common or dominant species. Retaining sample integrity and testing before the bacterial community is compromised is essential for accurate diagnosis (Gumedea et al. 2017). To retain sample or product integrity, commercially sensitive products are often transported with temperature loggers which monitor conditions during transportation or meat temperature is checked upon arrival (Cruz et al. 2015). Unfortunately, this is not always possible or feasible when investigating the health of wild or remote bivalve populations. Despite these potential limitations, transport media may be the best option for preserving common or dominant bacteria. The results from this research show that overall transport media used in the field are isolating the same bacterial genus and species that were isolated in the laboratory samples (Table 2.4). However, bacteria from the genus *Marinomonas* were not supported in field samples from pipi whereas it was isolated in the laboratory samples. This could have been due to a delay in testing the swabs, resulting in reduced viability of the bacteria or was not detected because the animal sample size was too small. The genus *Marinomonas* was supported in both field and laboratory samples in tuatua. As this was a proof-of-concept investigation it is recommended when applying this tool in the field to test a larger number of animals to reduce chances of missing common or dominant bacteria. Additionally, it is worth noting that swabs were only taken from the adductor muscle and not from other organs. Taking bacteriology samples from the adductor muscle haemolymph is a common practice in bivalve disease diagnostics, as it provides a good representation of the microbes present in the entire bivalve (OIE 2019). The techniques tested in this study were only carried out on one organ type and could be similarly applied to other more targeted organs, depending on the researchers' objectives.

Reporting of bivalve mass mortality events have increased in recent years (Capelle et al. 2021). There are a range of factors that can cause bivalve mass mortalities, one of these factors being disease (Burdon et al. 2014). Quick identification of potential disease agents, including exotic disease agents, is essential so that management and protection protocols can be put in place in a timely fashion to reduce significant ecological, economic or social damage (Groner et al. 2016). Bacteriology is a widely used disease diagnostic tool that is commonly applied and will continue to be used. However, due to increasing pressures on the marine environment and the need for rapid and accurate diagnosis,

classical techniques used may need to be optimised. Further exploration into the techniques highlighted in this paper would help streamline this diagnostic tool to be specific to the user's needs. It would be beneficial to apply the techniques explored in this paper to multiple bivalve species within the same location across multiple seasons. By applying different media options, incubation temperatures and transportation methods this tool could be optimised to different species, habitats, and times of year to ensure sample integrity and accurate diagnosis.

Conclusion

The present study found overall, field and laboratory testing supported the same genera of bacteria with field sampling providing additional information on the type of bacterial growth and species diversity that was not captured in laboratory sampling alone. Many of the bacterial genera isolated in this study, such as *Tenacibaculum*, *Vibrio* and *Pseudomonas*, are important bacteria in aquatic animals' health (Lopez et al. 2012). Some species within these genera can have a range of impacts on host health, and some can be harmful to human health when consumed. Retaining sample integrity and processing before the bacterial community is compromised is essential for accurate diagnosis. This study shows that the existing process involved in sampling post transportation may not be optimal for diagnosis; however, this tool still needs further development and is not a replacement for existing protocols. It is therefore recommended where practically possible to apply both approaches from this study when assessing bivalve health to get a full profile of bacteria for surveillance operations, baseline health studies and disease detection. The addition of this technique will be applied wherever necessary throughout the research in this thesis to ensure the retention of important bacteria.

Chapter 3 : A longitudinal study of the baseline health of pipi (*Paphies australis*) in Whangārei

Whangārei Heads and Marsden Point, Northland Region. 1959. Alexander Turnbull Library, Wellington, New Zealand. (BY CC 4.0).

Introduction

Bivalve mortalities are a common occurrence in our world's oceans (Tricklebank et al. 2020). The cause of these mortalities can range from natural die-off, pollution, environmental factors (e.g., reduced salinity), and disease (Burdon et al. 2014). Globally, and particularly within Aotearoa New Zealand, bivalve mortality events are being recorded with increasing frequency (Fey et al. 2015; Capelle et al. 2021; Howells et al. 2021). This may be due to an increase in the reporting and research into events, or alternatively, a true increase in these episodic events (Burdon et al. 2014; Fey et al. 2015). Although there may be a potential bias in reporting, there is a growing concern that some bivalve mortality events are not simply a natural part of the population cycle. Rather, external pressures are believed to be exerting an impact on wild bivalve populations. There is concern about the impact these mortalities will have on native bivalve species that are not only ecologically significant but culturally significant (Ross et al. 2018; Patuharakeke iwi pers. comms. 2020).

Pipi (*Paphies australis*), locally known as kōkota in the Whangārei area, are a bivalve surf clam native to New Zealand. Pipi inhabit rivers, harbours, estuaries, and small bays, typically preferring areas with fast flowing water (Morton & Miller 1968). Pipi are shallow burrowing filter feeders, found in intertidal and subtidal areas throughout New Zealand coastlines (Hooker 1995). Populations of pipi are often found in densely populated clusters (> 1000 individuals per m^2) that can spread over large areas (Morton & Miller 1968; Hooker 1995). Like many bivalve species, pipi are an important food source for the communities who live close to them (Kainamu-Murchie et al. 2018). In Whangārei, northern New Zealand, pipi are taonga (treasure) to the local community but have undergone a dramatic population shift over the last few decades (Pawley et al. 2013; Williams et al. 2017). Since 2009/2010 there has been an anecdotal decline in the health of pipi in the Whangārei Harbour, represented by increased mass mortality events and a decrease in population size (MPI, unpubl. data; Pawley et al. 2013; Williams et al. 2017). Between 2013 to 2019, there were six reported pipi mass mortalities from Whangārei Harbour (Mair Bank and One Tree Point) and its surrounding areas, for which samples were submitted for disease investigation, to Biosecurity New Zealand Animal Health Laboratory (BNZ AHL) (BNZ AHL, unpubl. data). Exotic diseases were ruled out in each case, but of interest was the presence of non-exotic bacteria including *Endozoicomonas* spp. and *Vibrio* spp., that have previously been implicated in bivalve mortalities (Cruz et al. 2015; Cano et al. 2018). Unfortunately, data are scarce on the baseline health of wild bivalve populations around New Zealand. Consequently, we have no insight into whether the bacteria isolated from these previous Whangārei mass mortality events are representative of an increase in infection prevalence, are new introductions to these populations, or are an incidental finding and represent the common microbial flora. Baseline studies on the microbial community of wild bivalve

populations are practically non-existent within New Zealand. This is a problem because an absence of baseline data can hinder effective disease diagnosis, forecasting and mitigation (Lane et al. 2020).

Baseline health data is helpful when identifying causation (Callaway et al. 2013). Without baseline health assessments it is hard to identify whether the presence of bacteria or pathogens are in fact an emerging risk or are normal part of health. Baseline health data is required to understand the relationship between the microbiota, the host, and external stressors (Pierce & Ward 2018). Without knowledge on what has happened over time we cannot identify how the ecosystem has changed and therefore it is hard to pinpoint causes of population decline (Compton et al. 2017). The pipi in Whangārei are a good demonstration of this. Without baseline information we cannot infer what changes have occurred and what may be contributing to the declining population. Without these data, identification into causation, ecological modelling, and future management cannot be employed strategically.

The main goals of this study are to create baseline health of pipi in the Whangārei area that can be applied in future diagnosis of pipi mortality events to identify causation. To accomplish this goal, pipi were collected from four different populations within the Whangārei area to determine their overall health status using different observational methods and diagnostic tools. By compiling a concise, broad and readily accessible overview of longitudinal data, I will provide a practical guide for investigating the cause of future bivalve mass mortality events in the Whangārei area and extrapolated to the rest of New Zealand. The information in this article can be used as a benchmark for pipi health within the Whangārei area from 2020-2021.

Materials & Methods

Sample collection

Pipi (n = 20) were collected from four populations in the Whangārei area every three months for two years (February, May, August, and November 2020 & 2021) (Figure 3.1) (Table 3.1). The four populations consisted of two river populations, Waipū River and Ruakākā River, a lower estuarine population, Mair Bank, and an estuarine population, One Tree Point. All collections were carried out during low tide. Pipi were collected by hand and transported from the field in chilled (~4°C) seawater to the laboratory (BNZ AHL, Upper Hutt Wellington) where they were processed within 24 h of collection. Pipi of around 40 mm were collected, because pipi at this size are sexually mature and there is enough tissue to perform all the required testing for this study. If mortality or gross pathology in pipi were observed in any population during this study, protocol was to notify BNZ via the exotic disease hotline and collect samples for disease investigation.

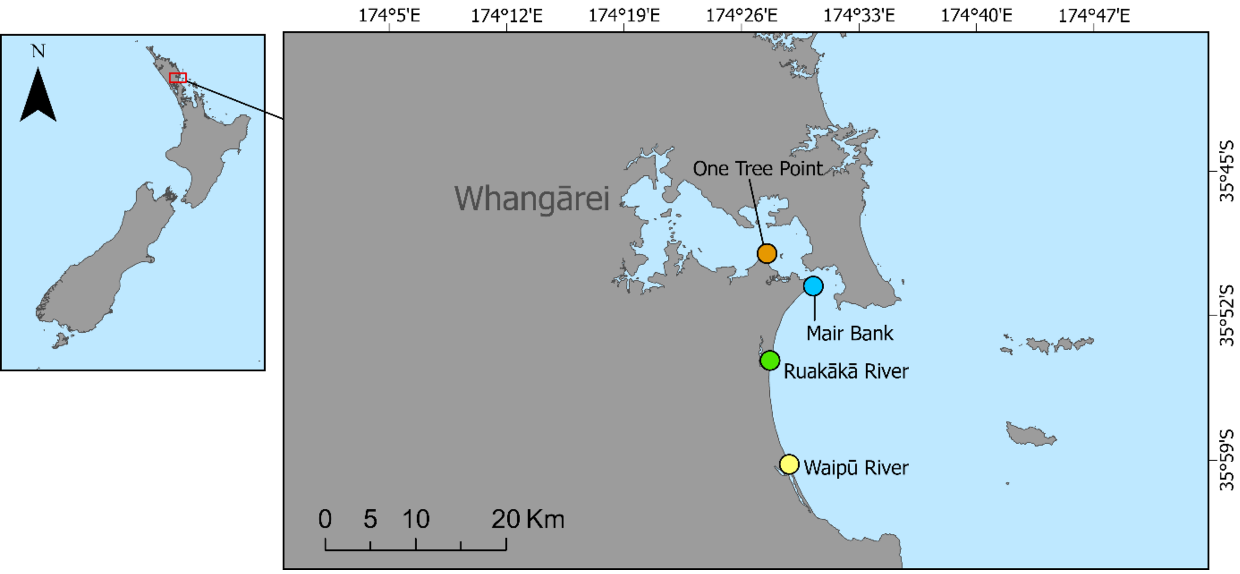

Figure 3.1 Map of New Zealand in the top left corner. Close-up localised map showing the Whangārei area and locations of sample collections. From North to South; orange icon: One Tree Point, blue icon: Mair Bank, green icon: Ruakākā River, yellow icon: Waipū River.

Table 3.1 *Paphies australis* location and specimen collection related information. *additional samples were taken for Chapter 2.

Collection Population	Date	*n*	Water temperature (°C)	Water salinity (ppt)	Average length (mm)	Average weight (g)	*n* per test Histology	Bacteriology	*Endozoicomonas* spp. qPCR
Waipū River	Feb-20	25*	22.5	33.07	42.5	NA	20	10	20
	May-20	20	15.5	34.52	42.9	NA	20	5	20
	Aug-20	25*	15	18.9	45.9	NA	20	10	20
	Nov-20	25*	23.1	27.8	43.6	8.6	20	10	20
	Feb-21	20	20.1	31.6	43.9	8.9	20	5	20
	May-21	25*	16.7	27.6	41.6	7.8	20	5	20
	Aug-21	20	13.4	16.1	44.1	10.4	20	5	20
	Nov-21	25*	20.8	25.5	45.7	10.9	20	5	20
Ruakākā River	Feb-20	25*	23.1	32.01	35.7	NA	20	10	20
	May-20	25*	14.8	32.79	40.4	NA	20	5	20
	Aug-20	25*	14.7	10.4	38.7	NA	20	10	20
	Nov-20	25*	23.1	27.8	41.7	6.2	20	10	20
	Feb-21	20	21.1	30.7	39.9	5.6	20	5	20
	May-21	25*	15.1	26.5	38.6	5.4	20	5	20
	Aug-21	20	13.8	4.6	40.5	6.8	20	5	20
	Nov-21	25*	20.7	18.6	41.5	6.7	20	5	20
Mair Bank	Feb-20	25*	24.3	35.05	54.7	NA	20	10	20
	May-20	20	15.2	34.82	59.9	NA	20	5	20
	Aug-20	20	16.5	34.5	56.6	NA	20	5	20
	Nov-20	25*	21.6	34	59.9	25.6	20	10	20
	Feb-21	20	21.8	34.9	53.0	17.9	20	5	20
	May-21	25*	18.1	33	56.9	22.8	20	5	20
	Aug-21	20	14.4	33.2	52.4	19.9	20	5	20
	Nov-21	20	21.9	32.2	61.1	30.2	20	5	20
One Tree Point	Feb-20	25*	25	35.35	39.9	NA	20	10	20
	May-20	20	15.3	34.74	45.9	NA	20	5	20
	Aug-20	20	19.4	34.1	44.4	NA	20	5	20
	Nov-20	25*	26	34.6	47.0	13.9	20	10	20
	Feb-21	20	22.8	35.1	45.3	11.8	20	5	20
	May-21	25*	16.3	34.2	43.7	10.4	20	5	20
	Aug-21	20	15.1	32.3	45.6	12.2	20	5	20
	Nov-21	25*	22.8	10	46.0	12.8	20	5	20

Environmental data

Temperature and salinity were recorded during each collection at all populations to monitor any fluctuations over the study period that may interact with pipi health (Table 3.1). Temperature and salinity measurements were taken using an 837-2_SOL Salinity & Temperature Meter (Gain Express Holding Ltd.). Additional environmental parameters such as overall weather condition, algal bloom presence, moon phase, and tidal phase or range were also recorded (Appendix B, Table B.1). Field

observations were made and recorded when sampling to identify any potential variables that may affect the growth of bacteria i.e., algal blooms, increased freshwater flow from outlets or heavy rainfall. Moon phase and tidal phase can impact biological cues in bivalves and are important conditions to note in terms of traditional customary harvesting approaches (Payton & Tran 2019).

External observations

The external shell surfaces of all collected pipi were assessed for signs of shell damage or unusual growth that may be harmful to the health of the pipi. Each pipi was then opened by severing the adductor muscles with a sterile knife. The tissue of each pipi was used for histology, bacteriology, and molecular testing (see relevant sections below for details). Shell length and weight (shell and animal) were taken for each individual across the four populations. All samples were measured to the nearest 0.1 mm with vernier callipers and weighed to the nearest 0.1 g using electronic scales. Correlation analysis and correlation coefficient between shell length and weight was carried out on each population. This method does not provide information on condition index between the populations but allows for 1) comparability across the four populations; 2) least variation for method accuracy; and 3) easy measurement method for convenience and repeatability.

Identification of hydrozoans

In November 2020, an abundance of organisms (size 250 µm – 500 µm) was observed under the mantle tissue of pipi from Mair Bank. Organisms were aspirated off onto a microscope slide, cover slipped and examined under a microscope by the NIWA invertebrate collection taxonomist that identified the bodies as a hydroid. A scraping of hydroids (taken using a scalpel) was taken for genetic identification. Approximately 30 mg of planktonic bodies was aseptically excised for DNA extraction (see section Molecular analysis for protocol). Suspect-hydroids species were run with a PCR targeting ~ 600 bp of the 16S rRNA gene (Cunningham & Buss 1993) with minor modifications. DNA of concentration 1 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer 16SAR (5'- TCG ACT GTT TAC CAA AAA CAT AGC -3') and 16SBR (5'- ACG GAA TGA ACT CAA ATC ATG TAA G-3') to a total volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 52°C for 15 sec, 72°C for 30 sec. For every PCR performed, two no-template controls (NTC) were run. All amplicons from the conventional PCR were visualised by electrophoresis in a 1.5% agarose gel and stained with GelRed (Biotium). Amplicons of the correct size were purified using a Zymoclean Gel DNA Recovery Kit (Zymogen Research) and nucleotide sequenced (Landcare Research). DNA sequences generated were imported into Geneious (Biomatters, Auckland), assembled into contigs, manually edited and trimmed. Consensus sequences were queried

against the National Centre for BioTechnology Information (NCBI) nucleotide database via BLASTn using default settings.

Histology

Histological sections were made from every pipi collected (Table 3.1). A standard histological section was taken from each animal and placed in 10% formalin for a minimum of 24 h for histopathology. A standard histological section included a cross section from each individual that captures all major organs, including gill, mantle, digestive gland, gonad, connective tissue, and foot (Howard 2004). Fixed tissues were processed using standard histological techniques, stained with hematoxylin and eosin (H&E), and cover slipped. Each sample was examined for general health status using an Olympus BX51 microscope. Records were made for each individual, including sex, reproductive phase, haemocyte response (if present), parasites observed and their identification to the lowest possible taxonomic level, *Endozoicomonas* spp. presence, and significant bacteria observed i.e., common or dominant within or across samples.

Bacteriology

Sample processing

Five pipi from every collection from each population were tested for bacteriology (Table 3.1). Using a sterile swab, the adductor muscle was cleaned with 70% ethanol, with a new scalpel an incision was made through the middle of the muscle. A sterile swab was used to take a sample from the animal and plated onto growth media immediately. On several collections additional bacteriology samples were collected immediately in the field using transport media swabs and transported to the laboratory for testing (Table 3.1).

Culture

Swabs, including transport media swabs, were plated onto tryptic soy agar with 3% sodium chloride (TSA+3%) (Fort Richard) and thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Fort Richard) in the laboratory. Swabs were used to inoculate a small area of the selected media and standard streak plating was carried out. The plates were incubated at 22°C for a total of seven days. Growth was recorded at three days and a final read at seven days.

Bacterial growth on the plate was recorded as a score depending on the quadrant of growth displayed on the plate (Table 3.2). As well as quantifying the bacterial growth, bacteria were also grouped into categories: no bacterial growth, mixed bacterial growth (no common or dominant bacteria), and common or dominant bacterial growth. Common or dominant bacterial growth was the main group of

interest and were characterised by bacterial isolates that were either common across multiple samples or were the dominant growth on any singular plate. At both plate readings, any common or dominant isolates were sub-cultured onto the initial growth media and Columbia Sheep's blood agar (BA) to assess purity and for identification.

Table 3.2 Categorisation of bacterial growth and the relative scoring associated.

Inoculum area	Relative growth score
No growth	0
Primary inoculum	1
Second quadrant	2
Third quadrant	3
Fourth quadrant	4

Basic biochemical tests were carried out on all common or dominant isolates (Gram stain, indole, oxidase, and catalase) followed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) identification. Isolates that were gram negative rods and oxidase positive were tested for sensitivity to O/129 (150 µg) (Oxoid Ltd.). Isolates sensitive to O/129 were suspected *Vibrio* spp. and were subjected to PCR and nucleotide sequencing of the atpA gene. Isolates resistant to O/129 (150 µg) were non-suspect *Vibrio* spp. and were subjected to PCR and nucleotide sequencing of the 16S rRNA gene.

DNA sequencing

DNA was extracted from separate pure isolates using InstaGene™ Matrix (BIO-RAD). Briefly for each isolate, five individual colonies were scraped off the agar and homogenised in 200 µL vortexed InstaGene™ Matrix in a centrifuge tube. The tube was then heated at 56°C for 30 mins, vortexed, and heated at 72°C for 10 mins before being vortexed again then centrifuged at 3,000 g for 3 minutes. The resulting DNA was quantified (ng/µL) using QubitTM fluorometer (Life Technologies).

Isolates not suspected to be *Vibrio* spp. were subjected to a bacterial species PCR targeting ~1498 bp of the 16S rRNA gene (Lane 1991). Template DNA concentration 2.5 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer 27f and 1525r to a total volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 50°C for 15 sec, 72°C for 1 sec. For every PCR performed, two NTCs and a positive control were used. For the NTC, molecular-grade water was added to the reagent mix instead of nucleic acid template. For the positive control, *Escherichia coli* (ATCC 25922) DNA was used at 0.1 ng/µL.

Isolates suspected to be *Pseudomonas* spp. (based on MALDI-TOF MS identification) were subjected to a *Pseudomonas*-specific PCR targeting 618 bp of the 16S rRNA gene (Ardura et al. 2013). This additional analysis was carried out as *Pseudomonas* spp. are known to cause bacterial disease in bivalves (Zannella et al. 2017) and were not able to be speciated using 16S rRNA gene or atpA gene assays. DNA of concentration 1 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer PA-GSF and PA-GSR to a total volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 54°C for 15 sec, 72°C for 30 sec. Each PCR had two NTC and a positive control of *Pseudomonas aeruginosa* (ATCC 27853) at 1 ng/µL.

Suspect *Vibrio* spp. were subjected to a PCR targeting 1500 bp of the atpA gene (Thompson et al. 2007). DNA of concentration 1 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer atpA-06F and atpA-04R to a total volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 58°C for 15 sec, 72°C for 30 sec. Each PCR had two NTC and a positive control of *Vibrio anguillarum* (ATCC 19264) at 1 ng/µL. One suspect *Vibrio* species did not amplify from the atpA gene PCR so was ran on the 16S rRNA gene PCR to obtain an identification.

All PCR amplicons were visualised by electrophoresis in a 1.5% agarose gel and stained with GelRed™ (Biotium). Amplicons of the correct size were purified using a Zymoclean Gel DNA Recovery Kit (Zymogen Research). Purified amplicons were nucleotide sequenced in the forward and reverse direction using the PCR primers for 16S rRNA gene (27f with 1525r, and PA-GSF with PA-GSR) and atpA gene (atpA-06F, atpA-04R, atpA-02F, and atpA-03R). DNA sequencing was carried out on Sanger sequencing platform by Ecogene Landcare Research. DNA sequences generated were imported into Geneious (Biomatters, Auckland), assembled into contigs and manually edited and trimmed. Consensus sequences were queried against the National Centre for BioTechnology Information (NCBI) nucleotide database via BLASTn using default settings.

Statistical analysis

Bacterial growth was categorised into four descriptions and given a relative score (Table 3.2). This score was used to compare the relative growth of bacteria between the four populations and across all eight collections. Statistical analyses were performed in R Studio (version 3.6.1, R Core Team 2019). A one-way ANOVA was used to compare the average relative growth scores between the four pipi populations, using Tukey contrasts and p-values adjusted for multiple testing using Benjamini-Hochbergs false discovery rate (FDR) (Benjamini & Hochberg 1995, Hothorn et al. 2008). A separate comparison was carried out for each population across the eight collections.

Molecular analysis

DNA extraction

DNA was extracted from gill and digestive tissue of every pipi at each collection to measure the intensity of *Endozoicomonas* spp. across the collections (Table 3.1). Approximately 30 mg of gill and digestive tissue was aseptically excised, combined in a tube containing 180 µl tissue lysis buffer (ATL) and 20 µl proteinase K (Qiagen). The tube was incubated at 56°C overnight for tissue lysis. DNA extraction was then carried out using the QIAcube automated extraction robot using the QIAamp HT kit (Qiagen). 18S rRNA gene qPCR (TaqManR Ribosomal RNA Control Reagents VIC™ Probe) was performed on all extracted DNA to check that the DNA was amplifiable. A qPCR cycle threshold between 15 and 25 was considered acceptable.

Endozoicomonas spp. qPCR & statistical analysis

All DNA extracts were tested using a specific qPCR targeting a 118 bp region of the *Endozoicomonas* spp. 16S rRNA gene. The PCR primers NZELO-F (5'- AAG GAA CAC CAG TGG CGA A-3'), NZELO-R (5'- TAG TAG ACA TCG TTT ACG GCG T-3') and NZELO-Pr (5'-56-FAM- TCA GCG TCA GTG TCA GAC CAG AGT GT-3BHQ_1-3') as per Howells et al. (2020). Two NTC and a dilution series of a gblock positive control were included with every qPCR. A gBlock ® gene fragment was designed from the sequences *Endozoicomonas elysicola* (Genbank: NR_041264). A series of dilution ($1{\times}10^{-4}$ ng μl^{-1}; $1{\times}10^{-5}$ ng μl^{-1}; $1{\times}10^{-6}$ ng μl^{-1}) were ran in duplicate to create a standard curve to estimate the gene copies per sample.

Gene copy numbers from the qPCR results were used as a proxy to compare infection intensity between the populations and across the seasons. Using a GLM, comparisons between the populations and season were tested using Tukey contrasts, and p-values were adjusted for multiple testing using Benjamini-Hochbergs false discovery rate (FDR) (Benjamini & Hochberg 1995, Hothorn et al. 2008). A p-value <0.05 was considered statistically significant. Statistical analyses were performed in R Studio (version 3.6.1, R Core Team 2019).

Results

Environmental & external observations

Environmental data

Water temperature and salinity measurements were taken at every collections (Table 3.1). Environmental observations were taken during sample collections and other key findings are presented in Table 3.3 (all observations Appendix B, Table B.1).

External observations

All four populations had different external characteristics, which are presented in Table 3.3.

Table 3.3 Pipi image showing characteristic shell features, population characteristics and key observations during the collection period.

Population	Image	Observations
Waipū River		• **Shell characteristics:** A mainly green and orange discolouration to the outer shell. Algae and barnacles were attached to the upper end of the pipi shell. • **Population characteristics:** River, containing fine sand and fast water flow. Residential surroundings. • **Key observations:** Yellow tinge muddy sand; Nov-20 heavy storms; Feb-20 lots of red algae in the water, identified as *Spyridia filamentosa* (NIWA 2018).
Ruakākā River		• **Shell characteristics:** Black and orange discolouration to the outer shell. Some pipi had algae and barnacles attached to the outer shell. Pipi from this population were characteristic for having thin and very brittle shells. • **Population characteristics:** River, fine sand and fast water flow. Residential surroundings. • **Key observations:** Yellow and black tinge to sand, with eggy smell; Nov-20 heavy storms; removal of surrounding mangrove ongoing in the area.
Mair Bank		• **Shell characteristics:** Black and orange speckling to outer shell. Pipi from this population had very thick shells. • **Population characteristics:** Harbour entrance, fast water flow. Pipi reside in sand and densely packed shell substrate. The surrounding area is industrial. • **Key observations:** Aug-20 gas-bubble disease was also observed on pipi shells; Nov-20 heavy storms; Pearls under mantle were observed on 5 animals collected from this population (Appendix B, Figure B.1).
One Tree Point		• **Shell characteristics:** Black discolouration to the outer shell and black discolouring to the tissue near the siphons and foot. Barnacles and small anemones were attached to the upper shell of the animals. • **Population characteristics:** Harbour, sandy/muddy substrate, substrate blackened and smelly. Slow water flow. The surrounding area is residential. • **Key observations:** Nov-20 heavy storms; Increased presence of seagrass and cockles across the sampling period; Extensive ongoing development to marina in close proximity to pipi population.

Brown/red-coloured organisms were observed under the mantle and gills in the cavity spaces of pipi from all populations in Aug-20, Nov-20 and Nov-21 (Figure 3.2). Brown/red-coloured organisms were observed in wet mounts when examined using a microscope and were identified as suspect hydroid-like organisms. The hydroid-like organisms had clear medusoid bud and polyp morphology (Figure 3.2). Hydroid-like organisms were also observed under histological slides of the same samples (Figure 3.2). DNA sequencing of a 628 bp fragment of the 16S rRNA gene revealed a 99.68% identification to *Eutima* sp. BMK-2020 mitochondrion (NCBI accession number MW066348.1). No gross lesions were associated with the hydroids and no haemocyte response was observed in the tissue areas where the hydroids accumulated, suggesting these organisms were not harming the pipi at this time.

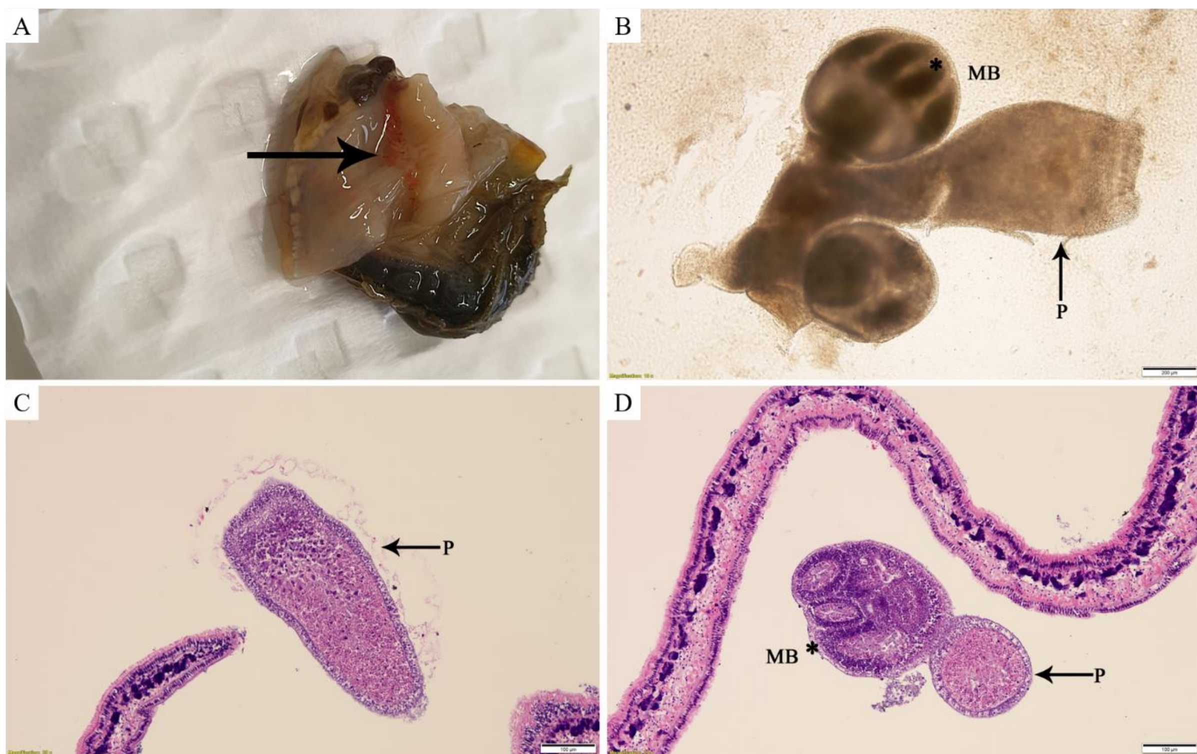

Figure 3.2 Hydroid-like organisms observed in the mantle cavity space A) Gross observation of hydroid-like organisms accumulating between the gills of the pipi (arrow). B) wet-mount of hydroid-like organisms showing * (medusoid body; MB) and a polyp (P), scale bar = 200 µm C) H&E stained histological slide of hydroid-like organisms presenting a polyp cross-section (P), scale bar = 100 µm. D) H&E stained histological slide of hydroid-like organisms with hydroid polyp body (arrow) and developing MB (*), scale bar = 100 µm.

Length-weight correlation

Length-weight index was only carried out from Nov-20 (collection 4) onwards (Table 3.1). Reproductive phase was noted from the histological examination for each sample to identify any differences in reproductive state that may bias animal weight. All populations were undergoing similar reproductive phases and therefore the analysis is considered comparative (Figure 3.4).

Correlation analysis and Pearson's correlation between length and weight of pipi from each population is shown in Figure 3.3. Although not every size cohort is represented, the correlation coefficient provided an insight of what to expect for different sized pipi. Pipi from Mair Bank had the strongest relationship between length and weight and the weakest positive correlation was for pipi from Ruakākā River.

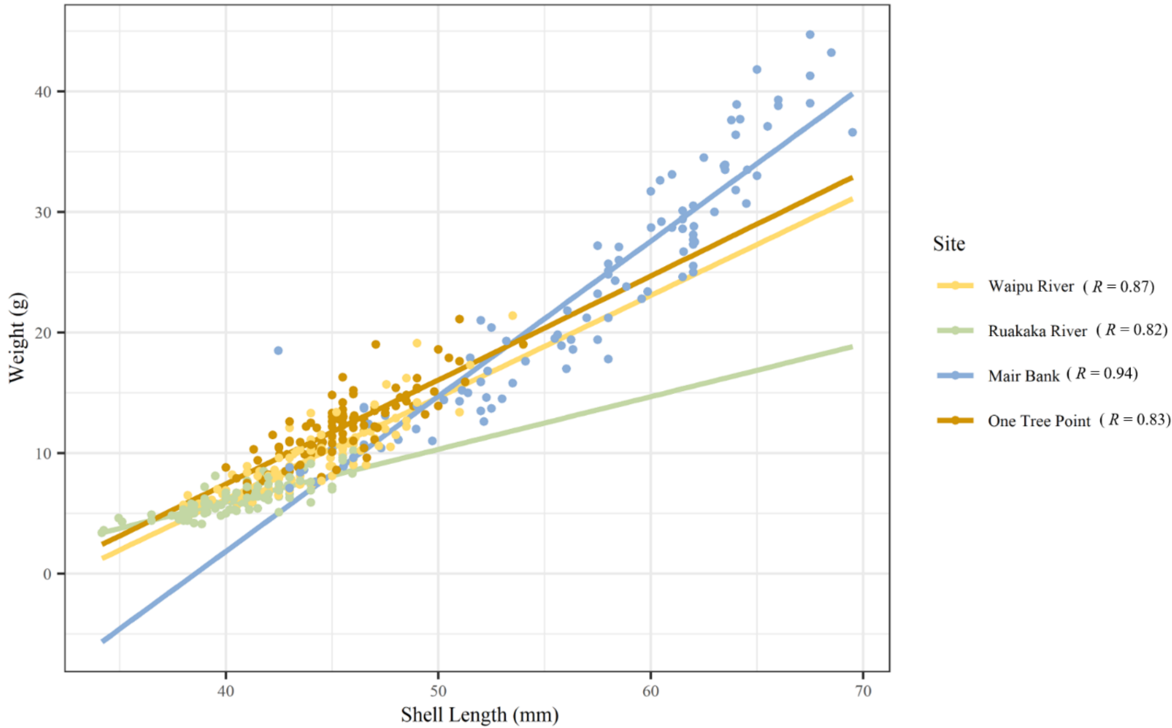

Figure 3.3 Scatterplot of weight of entitre animal and the shell length for each of the four populations. A regression line showing the linear relationship has been indicated for each population. Pearson's correlation of shell weight and shell length for the four pipi populations studied in legend (R).

Histological observations

Pipi sex & reproduction phase

From the results there was an even distribution of male to female pipi at each population. A number of individuals were indeterminate either due to the presence of encysted metacercariae in gonadal area or pipi were undergoing recovery and no sex cells were observed to aid in sex determination (Figure 3.4). Reproductive patterns were undergoing the same processes for both male and female across the course of the collection and have been combined in the analysis (Figure 3.5).

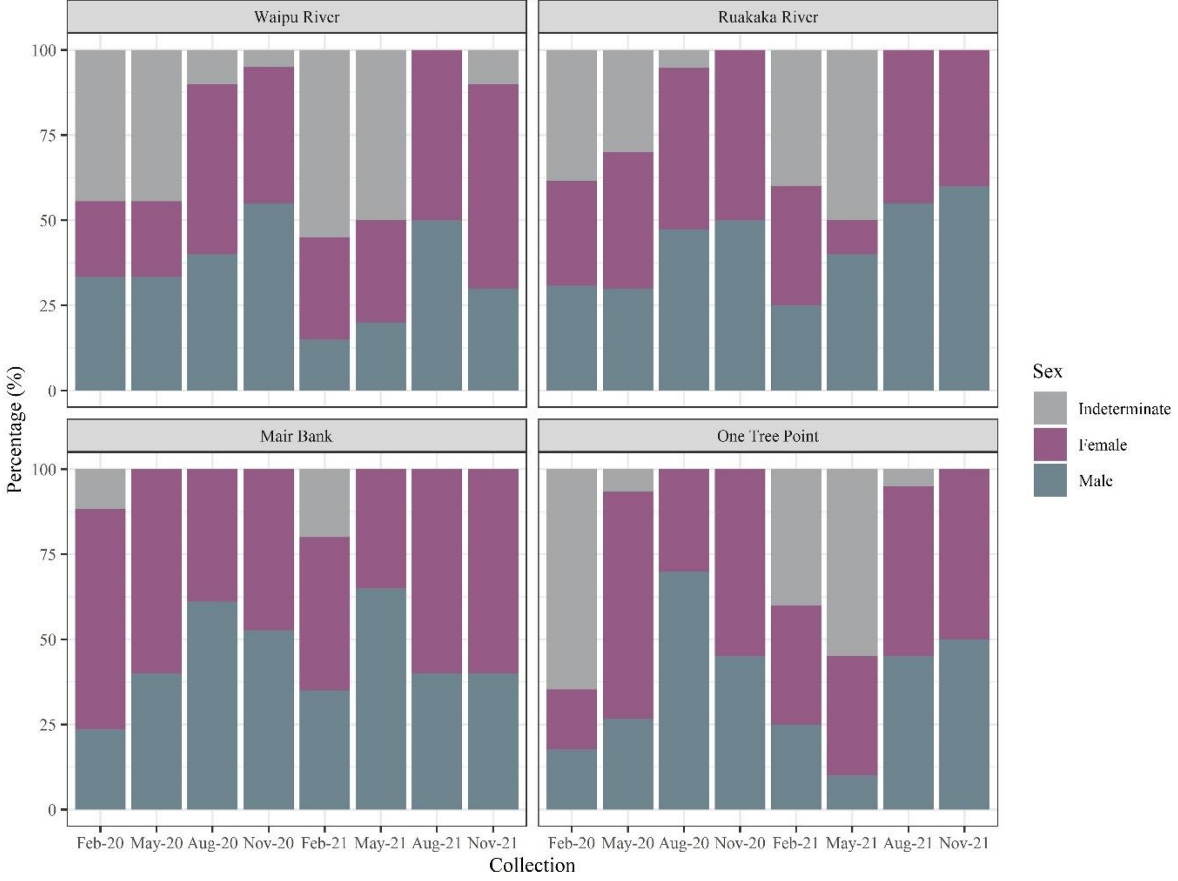

Figure 3.4 Stacked bar plot showing the proportion of individuals that are male, female, or indeterminate across the two -year collection.

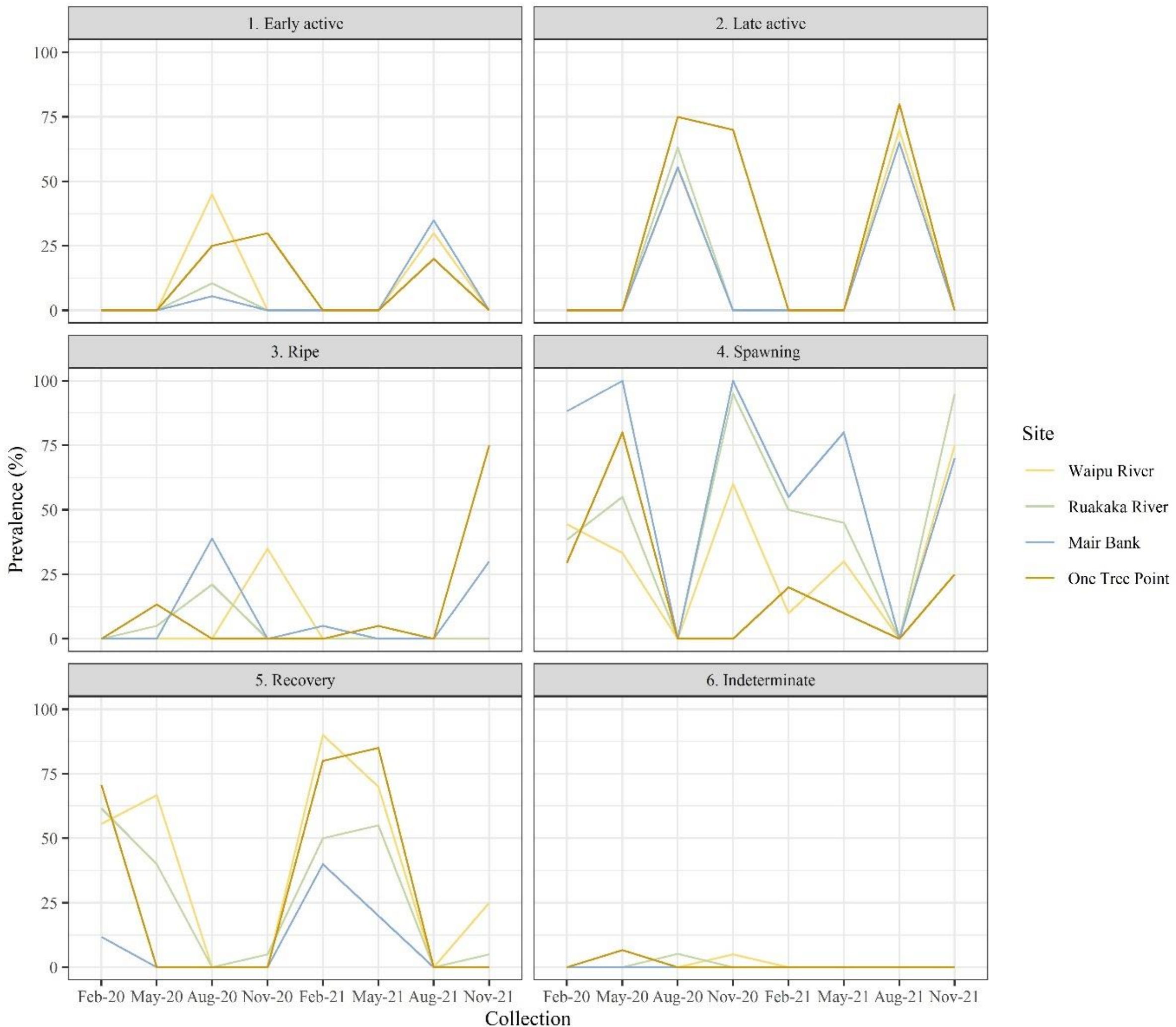

Figure 3.5 Line graph showing the proportion of individuals in each of the six reproductive phases for each pipi population across the two-year collection.

Trematodes & hydroids

Trematodes were observed within pipi from all four populations in various organs including superbranchial, gonads, gills, mantle and foot (Figure 3.6). Trematodes within the superbranchial, mantle and foot shared the same morphology (Figure 3.7), trematodes in the gonads and gills shared a different morphology. Hydroids were observed in the cavity spaces between the gills and the body of the animal and between the gills and the mantle (Figure 3.2). Trematodes and hydroids were observed at all times of the year. No obvious haemocyte response was associated with the presence of trematodes or hydroids.

Endozoicomonas spp.

Endozoicomonas spp., an intracellular bacteria that appear basophilic under H&E (previously confirmed as *Endozoicomonas* spp. in pipi from this region through *in situ* hybridization, see Howells

et al. 2021), were observed in the gill epithelium and the digestive epithelium of pipi from all four pipi populations (Figure 3.7). Overall *Endozoicomonas* spp. were observed more commonly in gill tissue than digestive tissues (Figure 3.6). Varying intensity of *Endozoicomonas* spp. was observed between the tissue types, with a higher intensity infection observed in the gills. No haemocyte response was associated with the bacteria.

Mucus cell proliferations in gills

A constant presence of mucus cells (basophilic) was observed in the water channel of the gills in pipi from all four pipi populations (Figure 3.6). Pipi from Waipū River, Ruakākā River and One Tree Point had mucus cells lining the edge of the water channel (Figure 3.7). Whereas pipi from Mair Bank had a higher proliferation of mucus cells, classified here as mucus hyperplasia, that filled the entire water channel. This was a consistent finding for pipi at Mair Bank and was more commonly seen within the inner gill (closest to the animal body) (Figure 3.7). Mucus cells were observed at all four populations with no difference in presence between time of year (Figure 3.6).

Mantle-skirt accumulation

Accumulation of particles from the water collected at mantle skirt were observed on pipi from all four populations at all collections over the two-year period (Figure 3.6). The particles had very little morphological definition and were predominantly observed accumulating at the mantle where new shell was being secreted (Figure 3.7). On a few occasions across the collection, clear bodies could be discerned within the mass with hyphae and spore structures, indicating the presence of fungi within the masses (Figure 3.7). There were no signs of damage to mantle epithelium that would indicate infiltration of particles internally.

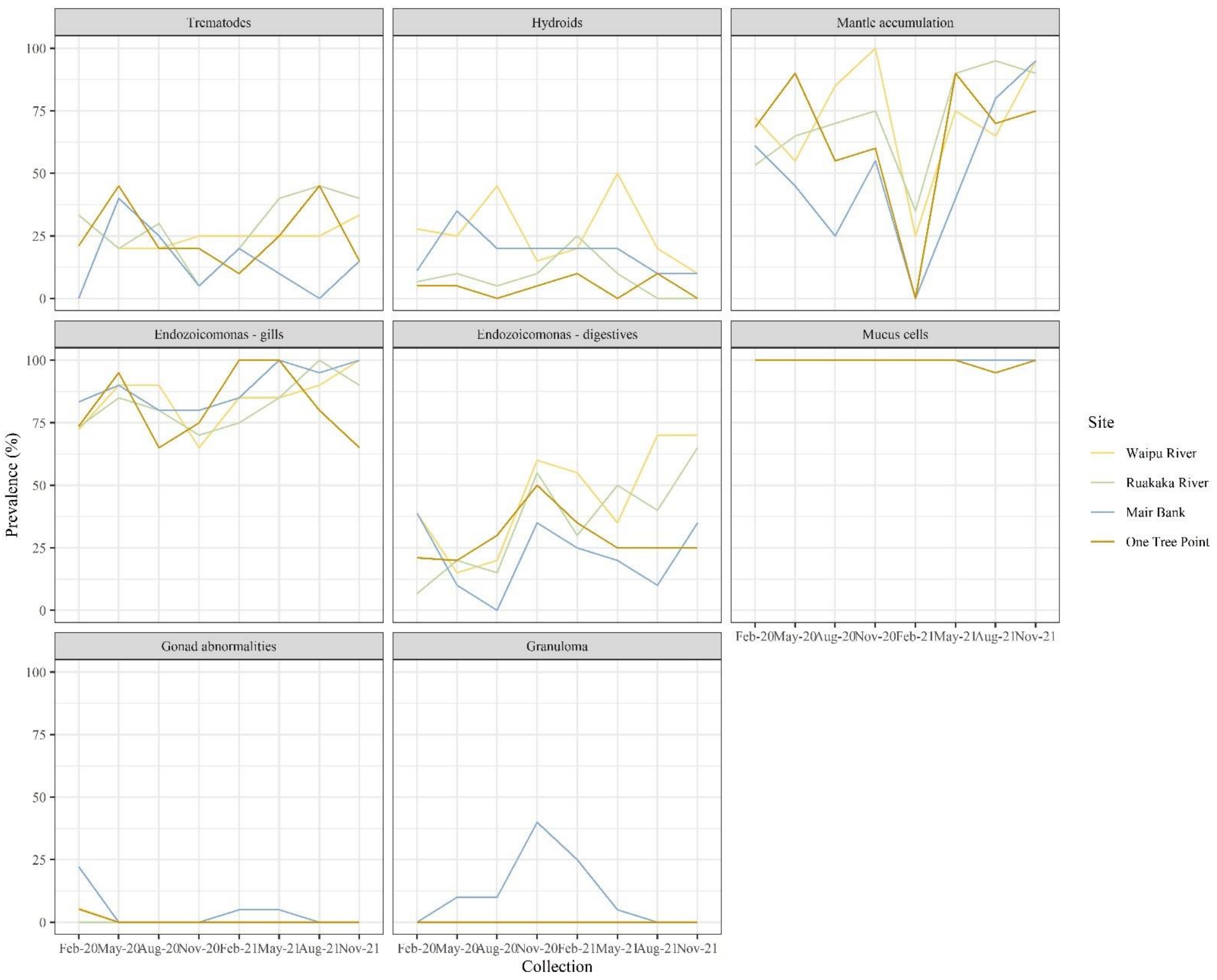

Figure 3.6 Line graph showing the proportion of individuals with certain characteristics under histological analysis across the two -year collection.

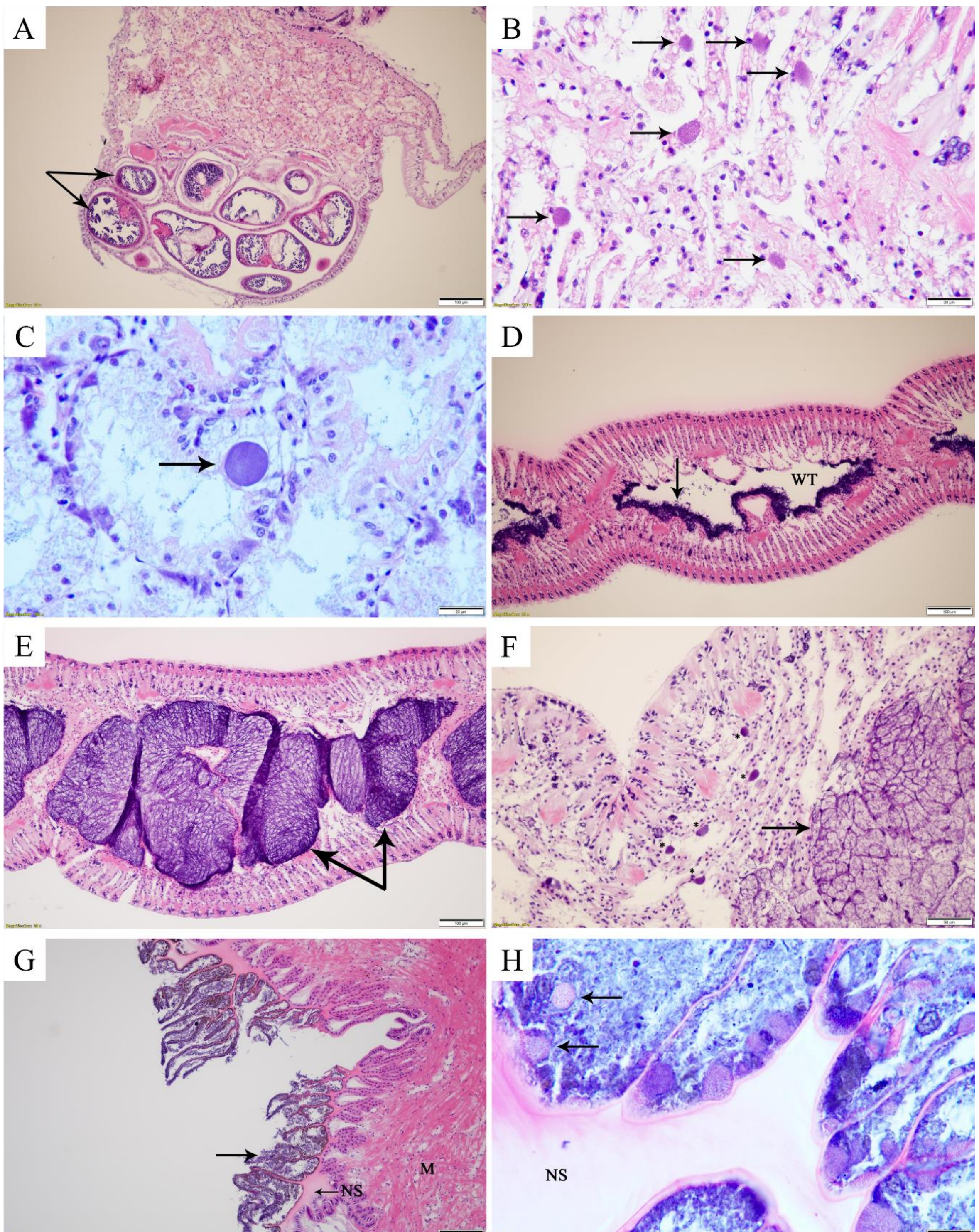

Figure 3.7 H&E photomicrographs of pipi observations that were common across the four pipi populations. A) Trematodes (arrows), digenean metacarcariae, sitting in suprabranchial chamber, scale bar = 100 µm B) *Endozoicomonas* spp. (arrows) bacteria in gills, scale bar = 20 µm C) *Endozoicomonas* spp. (arrows) bacteria in digestive gland, scale bar = 20 µm D) Mucus cells (arrow) lining water channel of gill, scale bar = 100 µm E) Mucus hyperplasia (arrow) filling water channel of gill, scale bar = 100 µm F) Close up mucus hyperplasia in gill water channel (arrow), *Endozoicomonas* spp. (asterisks) bacteria in gills, scale bar = 50 µm E) Pipi mantle (M) where epithelial meet the secretion of new shell (NS) a build-up of particles accumulate (arrow), scale bar = 100 µm F) spore-like bodies (arrows) accumulating at the mantle where new shell is developed, scale bar = 20 µm.

Male gonad abnormalities

Abnormalities within male gonads were observed within Mair Bank, Waipū River and One Tree Point pipi (Figure 3.6), these abnormalities were observed in Feb-20, Feb-21 and May-21. Although overall prevalence was low over the two years, appearance of loss of spermatozoa were observed with male gonads with defined areas of clearing around the abnormal aggregates (Figure 3.8). No haemocyte response was associated.

Granuloma

An increased haemocyte response was observed in the digestive system of pipi at Mair Bank (95%). This was commonly seen across the two-year period; however, no bacteria or pathogens were associated with this increased haemocyte response. On several collections, granulomas were observed within pipi from Mair Bank (Figure 3.6). Granulomas were observed mainly within the digestive system, closer to the gonads, from pipi at Mair Bank from May-20 to May-21 (Figure 3.6 & 3.8). Within these granulomas, irregular cells were observed, with morphology very similar to haemocytes, but were more rounded and donut-shaped (Figure 3.8).

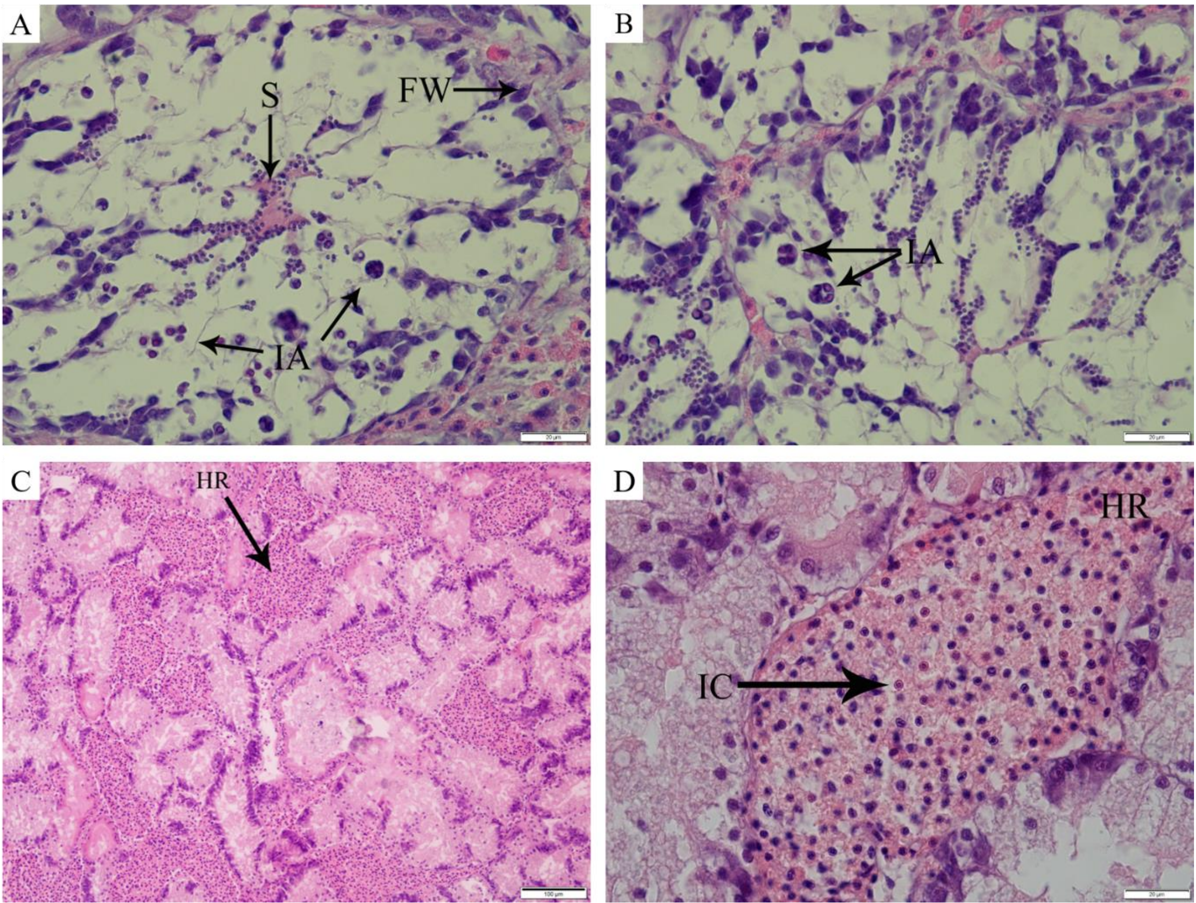

Figure 3.8 H&E photomicrographs of pipi observations from Mair Bank pipi that were irregular. A) Male gonad follicle, spermatozoan (S) centred in follicle wall (FW) with irregular aggregates (IA) with defined clearing zone throughout follicle, scale bar = 20 µm B) irregular aggregates (arrow) within male gonad follicle, scale bar = 20 µm. C) Digestive system with digestive tubes completely swarmed in haemocyte response (HR with arrow), scale bar = 100 µm D) Irregular cells (IC) observed within haemocyte response surrounding digestive tubules, scale bar = 20 µm.

Bacteriology

A summary of bacteriology results are shown in Table 3.4. A similar proportion of samples from each population produced no bacterial growth (40-54%; Table 3.4). One Tree Point had 50% of samples with common or dominant bacterial growth, with 15 different dominant or common bacterial isolates from four bacterial genera. Whereas Mair Bank had the fewest samples (25%) with common or dominant bacterial growth and only eight common or dominant bacterial isolates from two bacterial genera. Most bacteria isolated from the four populations were Gram-negative. There was no significant difference in average bacteria growth between the four populations across the two years.

Table 3.4 Summary table of bacteriology results for each population. Percentages of the type of bacterial growth for all samples collected are shown for each population. NG = no growth; MG = mixed (no common or dominant) bacterial growth;

C/DG = common or dominant bacterial growth. C/D = common or dominant isolates; number of bacteria isolated, number of genera of bacteria and the type of bacteria isolated (i.e., Gram-negative and/or Gram-positive) present (+) or absent (-).

Population	Type of growth			C/D isolates		Type of bacteria present (+/-)	
	NG	MG	C/DG	*n* isolates	*n* genera	Gram -ve	Gram +ve
Waipū River	40%	23%	37%	14	5	+	-
Ruakākā River	46%	21.5%	32.5%	13	5	+	-
Mair Bank	53.3%	31.7%	25%	8	2	+	+
One Tree Point	41.7%	8.3%	50%	15	4	+	+

Higher bacterial growth was observed in 2020 compared to 2021 across the region and between the populations (Figure 3.9), with May-20 having significantly higher overall growth (p-value <0.05), especially at Waipū River and Mair Bank (Figure 3.9). All populations presented similar skewed patterns of bacterial growth, with the highest growth recovered in Feb-20 and May-20 and a gradual decline and plateauing of low growth recovered for all remaining collections. Specifically, Waipū River had an increase in growth in Aug-21 and Nov-21 but was still less than Feb-20 and May-20, whereas One Tree Point had consistently low bacterial growth compared to the other populations.

DNA sequencing of atpA and 16S genes of common or dominant bacteria revealed 31 different species (Table 3.5 & 3.6). The majority of species identified belonged to the *Vibrio* genus (46.6%), followed by *Shewanella* (16.6%) and *Kistimonas* (13.3%) (Table 3.5 & 3.6). The highest species diversity was observed at One Tree Point and the lowest at Mair Bank (Table 3.5 & 3.6). Bacteria belonging to the genus *Vibrio* were common across all four populations. *Vibrio* spp. were always present whenever common or dominant growth was isolated at Mair Bank. Several collections across the four populations had no common or dominant bacterial growth. Waipū River had no common or dominant bacterial growth in Feb-21 and May-21. Ruakākā River had no common or dominant bacterial growth in Feb-21, May-21, and Aug-21. Mair Bank had the lowest diversity of bacterial growth, with no common or dominant bacterial growth recovered on Aug-20, Feb-21 and Aug-21. No common or dominant bacterial growth was observed in Aug-20 at One Tree Point. The most similar bacterial growth across the four populations was detected in Feb-20 and May-20 where nearly all growth isolated was from the genus *Vibrio*.

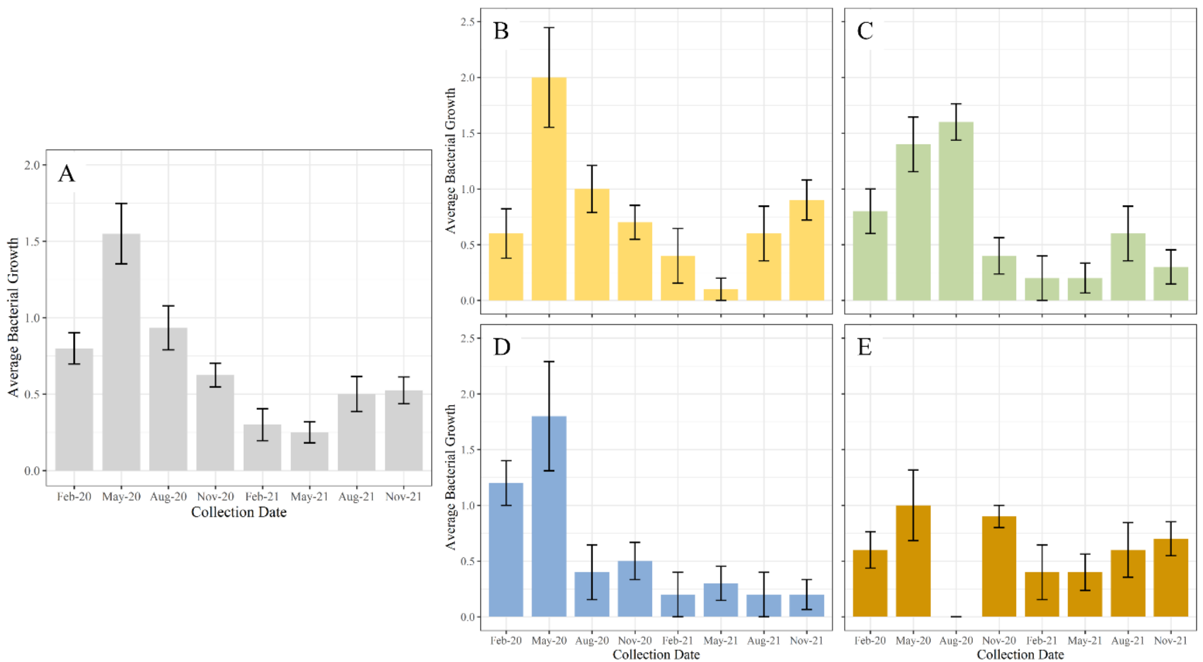

Figure 3.9 Seasonal average bacterial growth for each collection at the four populations. Bar chart with standard error of mean. A) Combined bacterial growth present for four populations, B) Bacterial growth from Waipū River, C) Bacterial growth from Ruakākā River, D) Bacterial growth from Mair Bank, E) Bacterial growth from One Tree Point.

Table 3.5 Non-suspect *Vibrio* spp.isolated using 16S rRNA gene and *Pseudomonas* spp. PCRs from pipi at the four populations across each sampling collection, bacteria recorded as either present (+) or absent (-) for every collection. Identification based on closest BLAST ID (see appendix B, Table B.2 for GenBank reference ID's). *same BLAST ID - geneious alignment confirmed different species. Key: WR = Waipū River, RR = Ruakākā River, MB = Mair Bank, OTP = One Tree Point. *same BLAST ID - geneious alignment confirmed different species.

Genus	Closest level identification	Feb-20				May-20				Aug-20				Nov-20				Feb-21				May-21				Aug-21				Nov-21			
		WR	RR	MB	OTP	WR	RR	MB	OTP	WR	RR	MB	OTP	WR	RR	MB	OTP	WR	RR	MB	OTP	WR	RR	MB	OTP	WR	RR	MB	OTP	WR	RR	MB	OTP
Kistimonas	*Kistimonas* sp.*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	Kistimonas sp.*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	Kistimonas sp.*	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+
	Kistimonas sp.*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Marinomonas	*Marinomonas pontica*	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pectobacterium	*Pectobacterium brasiliense*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Pseudomonas	*Pseudomonas lurida*	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pseudomonas orientalis	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pseudomonas sp.	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella	*Shewanella baltica*	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	*Shewanella colwelliana**	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	*Shewanella colwelliana**	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	*Shewanella colwelliana**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	+
	Shewanella hafniensis	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus	*Staphylococcus pasteuri*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Staphylococcus warneri	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Uncultured bacteria	uncultured bacterium	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	**17**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**3**	**6**	**0**	**0**	**2**	**1**	**1**	**3**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**2**	**1**	**0**	**0**	**1**	**3**	**1**	**0**	**2**

Table 3.6 Suspect *Vibrio* spp. isolated using atpA gene PCR from pipi at the four populations across each sampling collection, bacteria recorded as either present (+) or absent (-) for every collection. Identification based on closest BLAST ID (see appendix B, Table B.2 for GenBank reference ID's). *same BLAST ID - geneious alignment confirmed different species. Key: WR = Waipū River, RR = Ruakākā River, MB = Mair Bank, OTP = One Tree Point. *same BLAST ID - geneious alignment confirmed different species.

Genus	**Closest level identification**	**Feb-20**				**May-20**				**Aug-20**				**Nov-20**				**Feb-21**				**May-21**				**Aug-21**				**Nov-21**			
		WR	**RR**	**MB**	**OTP**	**WR**	**RR**	**MB**	**OTP**	**WR**	**RR**	**MB**	**OTP**	**WR**	**RR**	**MB**	**OTP**	**WR**	**RR**	**MB**	**OTP**	**WR**	**RR**	**MB**	**OTP**	**WR**	**RR**	**MB**	**OTP**	**WR**	**RR**	**MB**	**OTP**
Vibrio	*Vibrio artabrorum*	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
	Vibrio breoganii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	Vibrio chagasii	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vibrio cortegadensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+
	Vibrio genomo*	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vibrio genomo*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	Vibrio kanaloae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	Vibrio maritimus	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vibrio owensii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	Vibrio rumoiensis	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vibrio sp. Scap24*	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vibrio sp. Scap24*	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vibrio splendidus	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+
	Vibrio tetraodonis	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	**14**	**1**	**3**	**4**	**1**	**3**	**3**	**2**	**2**	**0**	**0**	**0**	**0**	**2**	**1**	**1**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**2**	**3**	**0**	**0**	**0**	**0**	**2**	**2**	**3**	**3**

Endozoicomonas spp. qPCR

The number of gene copies of the 16S rRNA *Endozoicomonas* spp. detected across all populations were similar for the sampling period (see Histology observations section, Figure 3.7 for visual of bacteria under histology). Statistical significantly differences in gene copies of *Endozoicomonas* spp. were detected in Mair Bank and One Tree Point than Waipū River and Ruakākā River, with a higher range of gene copies detected for Mair Bank and One Tree Point.

Temporal variations in gene copy number for *Endozoicomonas* spp. were observed for each population. Gene copies numbers for all four populations showed that for both 2020 and 2021 *Endozoicomonas* spp. was higher in the months of May and August (austral winter) and lower in the months of February and November (austral summer) (Figure 3.10). More gene copies of *Endozoicomonas* spp. were detected in 2021 compared to 2020. A similar pattern of *Endozoicomonas* spp. was observed across the four populations (Figure 3.10). Data from the four populations has a 'bi-modal' data pattern, with a similar detection across the sampling period except for a decline in the summer months (Figure 3.10). Pearson's correlation was used to assess the relationship between salinity and temperature with *Endozoicomonas* spp. gene copies. A weak correlation was detected as temperature increases *Endozoicomonas* spp. intensity decreases ($r = -0.19$, p-value = 1.3e-06). A very weak positive correlation was detected as salinity increases *Endozoicomonas* spp. intensity increases, however, this is not a statistically significant finding ($r = 0.0033$, p-value = 0.93).

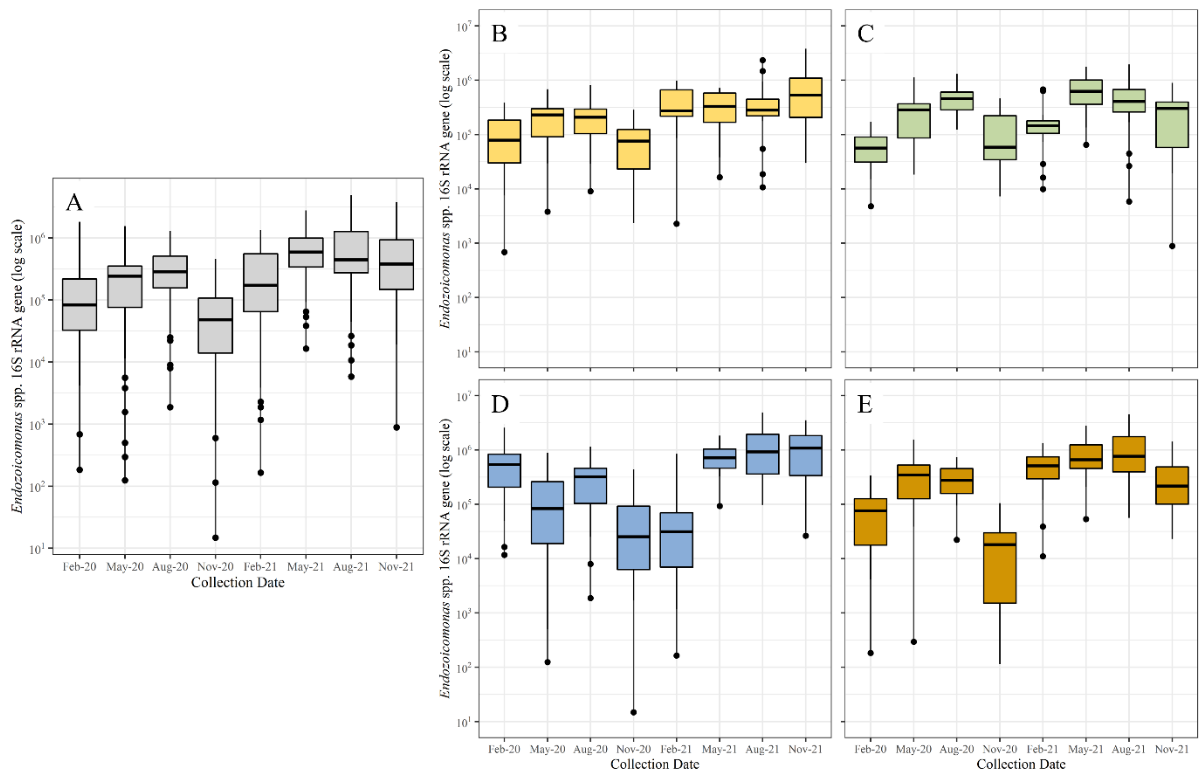

Figure 3.10 Seasonal pattern of *Endozoicomonas* spp. 16S rRNA gene copy number comparison between each collection for the four pipi populations. Line: median; box: interquartile range (IQR); whiskers: minimum and maximum value. A) Combined presence of *Endozoicomonas* spp. present at all four populations, B) *Endozoicomonas* spp. from Waipū River, C) *Endozoicomonas* spp. from Ruakākā River, D) *Endozoicomonas* spp. from Mair Bank, E) *Endozoicomonas* spp. from One Tree Point.

Discussion

Parasites, symbionts, and pathogens

This research provides the foundation for the development of a better understanding of the health of pipi in the Whangārei area. Over the course of two years, four populations of pipi were examined to establish a benchmark of what constitutes 'normal' health in this region. Pipi populations have declined in this area since 2009/2010, with recurrent mass mortality events. One of the reasons that the causes of these events remain unclear is a lack of knowledge of the parasites and pathogens present and their interactions with this native species. Using histology, bacteriology, and qPCR, I have identified common parasites, isolated bacteria, and established the expected levels of *Endozoicomonas* spp. As there are limited data available on the health of wild bivalve populations, this dataset will serve as a reference for future monitoring of pipi not only for this area but throughout New Zealand. It will also enable prompt detection of any deviations from normal health in cases of future mass mortality events. Additionally, because sampling took place over two years, this is the first time there is temporal data on parasites in pipi, which will support any future predictive studies.

As marine environments face mounting pressures, certain species have been disproportionately impacted, with native species being especially vulnerable due to a lack of existing data on their health. Therefore, identifying the cause or causes of health issues during periods of poor health is a challenging task.

As baseline health studies on wild bivalve populations are rare, it is not always clear what features identified under histology are a normal part of health. Histology is a widely used diagnostic method that is commonly used in bivalve disease investigations. From this study we can see that there are several observations that appear to be significant in their presence. However, when in the context of a temporally variable microbiome we can see that they are normal for pipi in this region. Although the observation were not contributing to the declining health of pipi in this region, when observed in isolation these features could be considered harmful, leading to erroneous conclusions about causes of bivalve mortality (Harvell 2019). Therefore, reporting on common parasites, symbionts, and pathogens of bivalve is imperative to aid in researchers attempts to identify causation in declining populations, emerging diseases, or populations undergoing mass mortalities.

The vast majority of histological observations made in this study were expected based on known parasitic and symbiotic fauna of New Zealand bivalves. For example, trematodes (including digenean trematode metacercariae) and hydroids were recorded from all four pipi populations. Bivalves represent intermediate hosts for digenean trematodes and are thus a normal part of their life cycle and, hydroids commonly aggregate within the cavity spaces of bivalves, where they feed on particulate matter around the host gill filaments and within the mantle cavity (Rayyan et al. 2002). Notably, I reported the identification of the hydroid as *Eutima* sp., although observations have been seen under histology in other New Zealand bivalves species identification has previously not been carried out. The identity of the trematode species is unknown at this stage and according to Bennett et al. (2022) no trematodes have been described in pipi previously despite the clear evidence they are frequently infected. Although trematodes and hydroids do not appear to be causing harm to the pipi, it is important to try to determine identification to species level. Identification of species can aid in better understanding their role to host health. An observation of an unidentified putative parasite that requires identification was the donut-shaped cell observed in the haemocytes in pipi from Mair Bank. This was one of the few occasions when a heavy haemocyte response was observed under histology, suggesting it could be important to bivalve health. Unidentifiable parasites are frequently recorded in bivalve (e.g., Lane 2018) and without dedicated effort to their identification, at least to the family level, large gaps will continue to persist in our understanding of parasite biodiversity in New Zealand. Ultimately, this will compromise our ability to identify emerging or invading parasites.

Mucus cells or mucus hyperplasia was a significant observation in the gill tissue of pipi from all four populations. Mucus cell proliferation has been reported in many marine organisms, but the cause of

the proliferation can be hard to determine (Bennion et al. 2022). Mucus hyperplasia in fish and other species has been attributed to a defence mechanism within the animal to protect itself from particulates such as fine sediments, algal blooms, and chemicals or pollutants in the water (Persson et al. 2020). Bennion et al. (2022) reported a higher concentration of mucus cells in an older population of toheroa (*Paphies ventricosa*) and postulated that longer life history of exposure to potential irritants may have been a cause in the higher proliferations. Bivalves that reside in the intertidal zone have to adapt to harsh and varied conditions, with frequent changes of salinity, water temperature, wave action, pollutants, and pathogens (Zhang et al. 2016). It is likely that the mucus proliferation observed in the gills of pipi from this region is an important adaptation that helps them against the fluctuating conditions they experience in the intertidal zone.

The observations reported under histology did not appear to have any seasonal patterns. *Endozoicomonas* spp. in the gills, mantle accumulation and mucus proliferations in the gills were common throughout the two-year study period. Similarly, trematodes, hydroids and *Endozoicomonas* spp. in the digestive gland but at a lower prevalence. In this study I have provided an overview of the presence or absence of histological observation and although this provides an insight into what normal health of these pipi look like, it is hard to quantify the intensity of these observations. Some of the observed parasites may cause greater pathology under high infection intensities. Bennion et al. (2022) provided a solution to the difficulties of quantification of histological observations by providing a semi-quantitative scoring system, enabling consistency across histological analysis and aiding in comparison across studies. Quantification of features also means that data sets, such as the one created here, can be applied in statistical modelling, maximising our knowledge of the meaning of these observations. Although presence/absence data created in the dataset provides us with a baseline understanding of what normal health looks like under histology, it does not provide for the application of semi-quantitative methods of histology which would greatly enhance this study. Indeed, histology could be paired with inherently quantitative test methods such as qPCR (as done in this study with *Endozoicomonas* spp.).

A significant element of this study was the use of bacteriology to identify common or dominant culturable bacteria present within the four surveyed pipi populations. While bacteriology is a commonly used tool in disease diagnosis, when investigating wild bivalve mass mortalities, the results can be difficult to interpret in the absence of a baseline data for comparison (BNZ AHL pers. comms.). Shifts in host microbiota composition have been linked to bivalve mass mortality events (Clerissi et al. 2020). Host-associated bacteria can play an important role in host health, providing services such as nutrient processing and supporting an immune response, and, therefore, when microbiome changes do occur, it could lead to compromised health and increased susceptibility to disease (Lokmer & Wegner 2015). Fluctuations in the amount of bacteria that grew were observed

across this study. There was no significant difference in bacterial growth geographically and the growth of bacteria was similar each month for all four populations. Interestingly, no seasonal patterns could be seen across the two-years, though an overall higher average growth of bacteria was observed in 2020 compared to 2021, with May 2020 having a significantly higher average bacterial growth compared to all other collections. It is unclear what the cause of this could be. One factor could be that water temperatures were warmer in 2020 leading to higher bacterial growth. However, without *in situ* data on environmental conditions, algal blooms, and other aspects of water quality, it is hard to be sure. Higher bacterial diversity was observed in 2020 for all months compared to 2021, except for One Tree Point. Despite observing lower growth and diversity (except One Tree Point) in 2021, it does not mean that the bacteria were not present. Indeed, there are limitations to the methodology used in this study: identification of bacteria was limited to bacteria that were common or dominant across the samples and not every bacteria that grew on the media. Optimising and complementing traditional bacteriology culture with 16S rRNA gene metabarcoding could reveal high levels of diversity not able to be cultured.

Vibrio spp. were the only bacteria common across all four populations. *Vibrio* spp. are a normal component of a bivalve's microbiome and can have a range of complex relationships with the hosts they inhabit (Destoumieux-Garzón et al. 2020). Two species of *Vibrio* were common across the whole collection, *Vibrio artabrorum* (FN668904.1) and *Vibrio splendidus* (KJ423058.1), the latter being more common. *Vibrio* species are a complex group that is highly diverse and not easily identifiable through DNA sequencing of a single gene (Sawabe et al. 2009; Sawabe et al. 2013). Although *Vibrio* spp. were only identified through the atpA gene, this method provides an indication of the diversity of *Vibrio* species present. *Vibrio artabrorum* has been isolated in other clam species but is considered an environmental bacteria (Diéguez et al. 2011; Romalde et al. 2014). *Vibrio splendidus* was isolated from all four pipi populations at most times of the year. There are many species that sit in the Splendidus clade that are known to be a normal part of host microbiome (Romalde et al. 2014), pathogenic to other clam species (Lacoste et al. 2001) and can be opportunistic when the host health is compromised (Beaz-Hidalgo et al. 2010). During this study no signs of pathogenic characteristics could be seen, therefore it is likely that the species isolated in this study is either part of the normal microbiota or alternatively, is opportunistically pathogenic under certain conditions. Without further identification of these isolates, it is hard to postulate to the role in the health of these populations. Interestingly *Vibrio* species were isolated at all four populations at different times of the year. However, *Vibrio* were not isolated at any of the four populations in August of 2020 or 2021. It may be that the *Vibrio* species present are sensitive to cooler temperature and are not present at these times. Studies into this bacteria have shown that during cooler temperature *Vibrio* can enter into a 'viable but non-culturable' state (Johnston & Brown 2002; Li et al. 2014).

Endozoicomonas spp., were observed by histology and detected by qPCR from all four pipi populations. Similarly, to other studies which have reported the presence of *Endozoicomonas* spp., there was no immune response associated with the inclusions under histology (Cano et al. 2020; Bennion et al. 2021). There is much discussion around the role of *Endozoicomonas* spp. in the health of marine organisms. *Endozoicomonas* spp. have been detected in different aquatic organisms, including coral reefs where they are believed to be important symbionts (Bourne et al. 2013; Jensen et al. 2010; Yang et al. 2010; Neave et al. 2016), in cobia (*Rachycentron canadum*) where they are believed to be associated with disease (Mendoza et al. 201), and in shellfish where they have been associated with mass mortalities (Cano et al. 2018; Howells et al. 2021) but also in healthy bivalves such as blue mussels from North America (Schill et al. 2017). It has been postulated previously that *Endozoicomonas* spp. may play a different role in the different host that they inhabit. Howells et al. (2021) detected a higher gene copy number of *Endozoicomonas* spp. in mortality bivalves around New Zealand compared to a healthy population of tuatua (*Paphies subtriangulata*). However, this research was restricted to only looking at healthy tuatua and it may be that *Endozoicomonas* spp. only make up a small portion of the tuatua microbiome making it appear that mortality samples had higher numbers of the bacteria. Howells et al. (2021) reported an *Endozoicomonas* spp. gene copy range of 2-83,400 in healthy tuatua from the lower North Island New Zealand, whereas this study reported a gene copy range of 0-4,889,695 in healthy pipi. Bennion et al. (2021) reported varying gene copy ranges between toheroa (*Paphies ventricosa*) populations from 0-1,688,081. Although belonging to the same genus, these three surf clam species have different levels of *Endozoicomonas* spp. within healthy populations. Therefore, it is likely that the presence of this bacteria varies between host species due to either geographical location, environmental conditions, habitat conditions or the host itself. Interestingly, *Endozoicomonas* spp. had significant negative correlation with water temperature. Clear seasonal pattern can be identified, with higher intensity during the cooler months and lower intensity during the warmer months. The overall temperature was lower in 2021 for all collections and overall, a higher presence of *Endozoicomonas* spp. was detected in 2021 compared to 2020. Cano et al. (2018) postulated that *Endozoicomonas* spp. was linked to warmer temperatures, however, this study shows that there is a correlation with lower temperatures in pipi. Pootakham et al. (2019) saw a similar shift in coral microbiome under heat induced stress causing a marked decline in Gammaproteobacteria of family Endozoicomonadaceae. Biessy et al. (2020) reported other pipi populations in New Zealand with Endozoicomonadaceae present mostly in winter months of July to September, whereas Bennion et al. (2021) did not detect seasonal patterns in three populations of toheroa from New Zealand. Despite the negative correlation, this study only looks at two water quality parameters and a more thorough study would be required to determine drivers of this bacteria. While these data do not elucidate the role of *Endozoicomonas* spp. in the health of pipi, they do provide a benchmark that can be used as a reference point for understanding the role of this bacteria in bivalve health, and in assessing the causes of mass mortality events.

Host health status

None of the pathogens or parasites recorded in this study could be attributed as a cause of pipi mass mortalities or the decline of pipi abundance within the Whangārei area. However, this baseline study does provide some insight into the pipi host health. Host health plays a huge part in disease epidemiology (Lane et al. 2020). Certain features were identified that may be indicative of compromised host health and are discussed below.

Changes in catchment land, construction of marine infrastructures and marine dredging are all known to impact the water quality (Zimmerman & Canuel, 2000; Seitz et al. 2009). Poor water quality management can result in health implications or population shifts to the benthic community (Zhang et al. 2021). Animal condition is important as it provides energy to withstand natural cyclic fluctuations in conditions, whether physical, biological, or chemical. However, when alterations occur that are outside the norm this can impact the animal's condition and compromise their ability to protect themselves (Rainer & Mann 1992). Anoxic sediment was common at Ruakākā River and continually present at One Tree Point. These substrate conditions have previously been attributed to a build-up of sulphides and is largely in response to oxygen availability (Cranford et al. 2017). Not only was black discolouration observed in the substrate at One Tree Point, but black discolouration to the shell and on occasion to the tip of the foot of pipi from this population. These observations likely linked to the substrate condition. The One Tree Point area has undergone extensive development to create an artificial marina and residential estate. This included significant dredging approximately 2 km seaward from One Tree Point pipi population, likely impacting the structure of the surrounding habitat. Marina development and dredging can cause redirection of water, change flow rates, and increase sedimentation, which can alter habitat and water quality (e.g., oxygen availability) of the surrounding areas (Jones et al. 2016). Marine dredging can have direct consequences on benthic communities and has been directly connected to decline of some bivalve species (Baeta et al. 2014). It is possible that these observations and the changes to the surrounding environment may be linked to the increase of mortality events to pipi at One Tree Point. However, research incorporating environmental data and water quality data would be needed to test the hypothesis that these water quality conditions were unfavourable to pipi health.

Data collected on the length of pipi collected from each population tells us that pipi from Mair Bank were larger animals and therefore likely an older cohort. In this study there was a bias to collect pipi ~ 40 mm in length however, there were very few pipi present at Mair Bank between 40-50 mm, the majority being much larger (42-70 mm). Although there are anecdotal stories and archive records of pipi reaching a much larger size of around 80-90 mm (Te Papa records), this is no longer a common occurrence in the Whangārei area (A. Carrington pers. comms). Hooker (1995) postulates that as pipi age they move down the estuarine system towards more open waters and are able to adapt to higher

salinities (~34 ppt). This suggests that Mair Bank could be an older cohort, vulnerable to changes in the environment due to old age. Under histological analysis two observations from Mair Bank that did not appear normal to these populations were granulomas and gonad aggregates. On a number of occasions granulomas were observed within the digestive system. While prevalence of granulomas in pipi was low across the two-year collections, they may be affecting ~ 4% of the population during certain times of the year. Granulomas are a result of inflammation impacting a large area of the body. Granulomas have been observed in cockles, and when observed in high prevalence, have been associated with high mortality (Villalba et al. 2001; Carballal et al. 2001). The cause of the increased inflammation, such as granulomas, is often unknown and may be caused by a number of factors that cause stress on the organisms such as water pollutants, environmental conditions or pathogens (Donaghy et al. 2009). The presence of granulomas in Mair Bank pipi may be an age-related factor and may be that this population is an older cohort, nearing natural mortality and resulting in tissue degeneration. Even if age is a contributing factor to the presence of the granulomas there is still a need to investigate environmental factors and water quality to rule out external stressors as a cause. Histological analysis also revealed the presence of abnormalities within the male pipi gonads observed at Mair Bank. The presence of these abnormalities appeared to be linked to spawning to post spawning reproductive phases and there appears to be clearing that may indicate that it may be a microcell-like organism. It is hard to identify whether the abnormal aggregates are an organism having an impact on male gonads or irregular germ cells that have broken away from the follicle walls with spawning. Alonso et al. (2019) did relate abnormal disruptions to male gonads in farmed *Mytilus galloprovincialis* to the presence of substances in the water, such as tar, which shared similar morphological features as seen in Mair Bank male pipi. The causality of the pathological changes in the male gonads are unknown, though its presentation under histological analysis indicates that this may be something and has also been observed in New Zealand mussels (M. Bestbier pers. comm).

Finally, this study also provides information on the seasonality of reproductive phases of these pipi populations. During spawning to post-spawning-recovery, host are particularly vulnerable (Deudero et al. 2017). Spawning is a high energy activity that requires a large amount of host resources, redirecting the immune systems attention and therefore ability to protect and repair against any other factors during these times (Hong et al. 2014). Several studies documented bivalves experiencing mass mortality events in the spawning to post-spawning-recovery phase (Huvet et al. 2010). It is important to understand the biological processes a host is undergoing as this information may be critical to developing an understanding of host susceptibility to external stressors which may result in degraded health and mass mortality events. From this study we can see the benefit of collecting observational data and host conditions in understanding physiological conditions. Additionally, it would be beneficial to this study and future studies on bivalve health to pair such datum with data on environmental conditions, water quality, information on surrounding anthropogenic activities and the

historical knowledge of the host and the environment. This combined data would help researchers understand the complexity of issues surrounding declining bivalve health and help identify the driving contributing factors.

Conclusion

While this study does not identify the cause of declining pipi health and mass mortality events in the Whangārei area, it does provide the most comprehensive examination to date of pipi baseline health. This data is a reference point for pipi health in this area between 2020 and 2021. Without prior knowledge of host health, diagnosis of mass mortality events can be hindered. Therefore, baseline health data is vital in detecting any deviations from normal health, which can facilitate the identification of the underlying causes. Although this research does not present any new findings regarding pipi health in this region, it establishes a baseline for future reference, which is the first of its kind for native species in New Zealand.

Chapter 4 : Testing baseline health data to investigate pipi (*Paphies australis*) mortality events in Whangārei

Three girls eating pipi/cockle collected from the Petone Beach, 4 January 1979. Alexander Turnbull Library, Wellington, New Zealand (BY CC 4.0).

Introduction

Bivalve molluscs undergo natural cycles of mortality caused by stock density or natural age leading to high numbers of intertidal bivalve being beach-cast (Parada & Molares 2008; Callaway et al. 2013), but the increasing frequency of reports of bivalve mass mortality events suggest they could be unnatural and caused by one or several external factors (Burdon et al. 2014). Bivalves living in the intertidal zone are well-adapted to live in harsh conditions, often experiencing extreme variability in salinity, temperature, wave action (Zhang et al. 2016). Despite the resilience of bivalves to these natural challenges, it appears that increasing human population, and increasing urbanisation of coastal areas is resulting in additional stressors that bivalves are less well adapted to (Kunze et al. 2021). For instance, elevated nutrient levels through increased run-off, seabed disturbances through dredging, harvest pressure, and global stressors, such as warming water temperature from climate change can negatively impact bivalve populations increasing their risk of disease (Tracy et al. 2019; Tricklebank et al. 2020; Balbi et al. 2021).

Data on wild bivalve health globally are rare compared to data on farmed populations. This is probably because wild bivalves occur in rural areas where large mortalities are less likely to be reported and wild populations are generally considered less economically important than farmed species, so people take less notice of their health (Bennion et al. 2022). There are exceptions, however, for instance, when large epizootics occur in commercially caught species, such as the recurrent mortalities of the Aotearoa New Zealand flat oyster (*Ostrea chilensis*) caused by the haplosporidian parasite *Bonamia exitiosa* (Cranfield et al. 2005). Wild bivalves are an important resource for many humans and provide ecosystem services (Smaal et al. 2019). In New Zealand, bivalves are important cultural resource for Māori and support recreational and commercial fisheries (Ross et al. 2017; Guy et al. 2021). Bivalves improve water quality by actively filtering particles from the water column and provide a source of inorganic nutrients and biogenic habitat for other organisms (Smaal et al. 2019).

In New Zealand, there has been an anecdotal increase in observations of wild bivalve mass mortalities and increased reporting of mortality events to Biosecurity New Zealand since 2014 (Bennion 2021; Howells et al. 2021). It is unknown whether there is an emerging cause for the higher number of mortality events or if awareness among the general population is higher leading to them being reported more frequently. Identifying the drivers behind bivalve mortalities is hindered by a lack of baseline health data and the multiple factors that interact with bivalve health (Callaway et al. 2013), such as water quality, host physiological condition, environmental condition and disease (Soon & Ransangan 2019; Soon & Zheng 2019). Baseline health data provides an assessment of current health conditions and a reference point to measure future changes to population health (see Chapter 3 for a health baseline of pipi *Paphies australis* from Whangārei). Monitoring programs required to establish

a health baseline can be expensive, involving high numbers of samples and processing costs, however, an understanding of what constitutes "normal" within wild populations will support deviations for that baseline, enabling drivers behind mortality events to be more easily identified and subsequent management plans to be enacted. Despite the time and expenses in creating baseline data, lack of baseline data can hinder research and can cause years of methodical experimentation to pinpoint causation. As seen with black abalone mortalities in California in mid-1980s, the true cause of the mortalities, Candidatus *Xenohaliotis californiensis*, eluded researchers for many years primarily due to lack of baseline data. Researchers could not identify if microbes they were seeing under pathology were normal in abalone (*Haliotis* spp.) health or was a new threat (Harvell 2019).

In November 2020, May 2021 and July 2022 pipi mortalities from two locations in the Whangārei area were reported to Biosecurity New Zealand to investigate if disease was a contributing factor in the mortalities. Pipi in this area have anecdotally had poor health over the last two decades (A. Carrington pers. comm.), with the population, which is in decline, characterised by periodic mortality events. The cause of decline has never been determined despite several investigations (MPI pers. comms.; Williams et al. 2014). In an attempt to better understand the causes of these mortality events, I investigated the most recent pipi mortality, using the health baseline developed in Chapter 3. Using gross pathology, bacteriology, histology, and a qPCR targeting *Endozoicomonas* spp., I aim to identify if any changes can be observed between health baseline and mortality pipi and to identify the usability of baseline data to interpret causation of mortality events. I discuss these results in light of the health baseline and identify other sources of data that could be acquired to help with ongoing bivalve health monitoring.

Materials & Methods

The same methodology used for sample collection, histology, bacteriology and *Endozoicomonas* spp. qPCR in Chapter 3 was the same as used for establishing the health baseline (see Chapter 3).

Sample collection

Pipi mortalities were notified to Biosecurity New Zealand by the Patuharakeke Te Iwi Trust Board Te Taiao team who aided in the collections of moribund pipi (no longer burying in sand). Moribund pipi were collected at random during a mortality event from Mair Bank in November 2020 (n = 8) and May 2021 (n = 10) (Figure 4.1) (Table 4.1). Moribund pipi were collected at random during a mortality event from Pātaua South in July 2022 by the Patuharakeke Te Iwi Trust Board Te Taiao team (n = 20) (Figure 4.1) (Table 4.1). All pipi were hand collected, placed into zip lock bags transported in chilled (~4°C) seawater to the laboratory in a polystyrene container with ice packs. Water temperature and salinity were recorded during each collection, as well as other notable

environmental conditions. Temperature and salinity measurements were taken using an 837-2_SOL Salinity & Temperature Meter (Gain Express Holding Ltd.).

Table 4.1 Overview of pipi mortality events, including the number of samples tested, and testing performed. Key: H – histology, B – bacteriology, E – *Endozoicomonas* spp. qPCR. *number of samples collected were limited because water conditions made sampling inaccessible and the poor condition of samples upon arrival to the laboratory.

Location	Date	Water temperature °C	Water salinity ppt	*n*	Average length cm (range)	Average weight g (range)	*n*		
							H	B	E
Mair Bank	Nov-20	21.6	34	8*	59 (56-64)	26 (17.1-30.5)	8	7	8
Mair Bank	May-21	18.1	33	10*	62 (52-66)	33 (19.5-44.2)	5	10	0*
Pātaua South	Jul-22	15.8	33.9	20	50.9 (47-55)	15.6 (12.3-18.7)	20	10	20

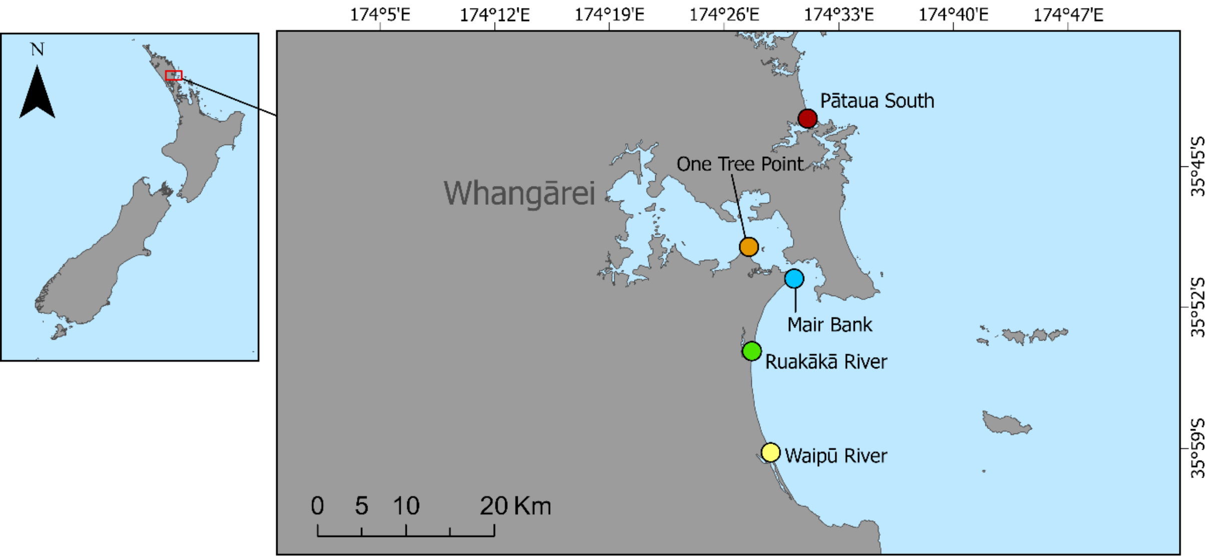

Figure 4.1 Map of New Zealand in the top left corner. Close-up localised map showing the Whangārei area. From north to south, red icon: Pātaua South, orange icon: One Tree Point, blue icon: Mair Bank, green icon: Ruakākā River, yellow icon: Waipū River.

External observation

The external shell surfaces of all collected pipi were assessed for signs of shell damage or unusual growth that may be harmful to the health of the pipi. Shell length and weight (total weight - shell and animal) were measured for each individual from all three mortalities. All samples were measured to the nearest 0.1 mm with vernier callipers and weighed to the nearest 0.1 g using electronic scales.

Histology

Histological sections were made for each pipi collected (Table 4.1). A standard histological section capturing all the major organs was taken from each animal and placed in 10% formalin for a minimum of 24 h. Fixed tissues were processed using standard histological techniques, stained with hematoxylin and eosin (H&E), and cover slipped. Each sample was examined using an Olympus BX51 microscope. Records were made for each individual, including of sex, reproductive phase, haemocyte response (if present), parasites observed and their identification to the lowest possible taxonomic level, presence and intensity of *Endozoicomonas* spp., and any other bacteria.

Bacteriology

Sample processing

Bacteriology was undertaken for seven pipi from the Nov-20 and ten pipi from the May-21 and Jul-22 mortality events (Table 4.1). Using a sterile swab, the adductor muscle was cleaned with 70% ethanol, and a sterile incision was made through the middle of muscle with a new scalpel. A sterile swab was applied into the incision and plated onto tryptic soy agar with 3% sodium chloride (TSA+3%) (Fort Richard) and thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Fort Richard). Swabs were used to inoculate a small area of the selected media and standard streak plating was carried out. The plates were incubated at 22°C aerobically for a total of seven days. Growth was recorded at three days and a final read at seven days.

Bacterial growth on the plate was quantified depending on the quadrant of growth displayed on the plate (Table 4.2). Bacterial growth were grouped into categories: no bacterial growth, mixed bacterial growth (i.e., no common or dominant bacteria), and common or dominant bacterial growth. Common or dominant bacterial growth were characterised by bacterial isolates that were either common across multiple samples or the dominant growth on any singular plate. At both plate readings, any common or dominant isolates were sub-cultured onto the initial growth media and Columbia Sheep's blood agar (BA) to assess for pure growth and identification.

Table 4.2 Categorisation of bacterial growth and the relative scoring associated.

Inoculum area	Relative growth score
No growth	0
Primary inoculum	1
Second quadrant	2
Third quadrant	3
Fourth quadrant	4

Basic biochemical tests were carried out on all common or dominant isolates (Gram stain, indole, oxidase, and catalase) followed by MALDI-TOF MS identification. Isolates that were Gram negative rods and oxidase positive were tested for sensitivity to O/129 (150 µg) (Oxoid Ltd.). Isolates sensitive to O/129 were suspected *Vibrio* spp. and were subjected to PCR and nucleotide sequencing of the atpA gene. Isolates resistant to O/129 (150 µg) were non-suspect *Vibrio* spp. and were subjected to PCR and nucleotide sequencing of the 16S rRNA gene.

DNA sequencing

Nucleic acid was extracted from pure isolates using InstaGene™ Matrix (BIO-RAD). DNA was extracted following the manufacturer's DNA preparation from bacteria protocol. The extracted DNA was quantified (ng/µL) using QubitTM fluorometer (Life Technologies).

The 16S rRNA gene PCR amplified ~1498 bp of the 16S rRNA gene (Lane 1991). The 16S rRNA gene PCR protocol included template DNA (concentration 2.5 ng/µL) added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer 27f and 1525r to a total volume of 25 µL with nuclease free water. Cycling conditions were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 50°C for 15 sec, 72°C for 1 sec. Each PCR had two NTCs and a positive control. For the NTC, molecular-grade water was added to the reagent mix instead of nucleic acid template. For the positive control, *Escherichia coli* (ATCC 25922) DNA was used at 0.1 ng/µL.

The *Vibrio* atpA PCR amplified ~1500 bp of the atpA gene (Thompson et al. 2007). DNA of concentration 1 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer atpA-06F and atpA-04R to a total volume of 25 µL with nuclease free water. Cycling conditions were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 58°C for 15 sec, 72°C for 30 sec. Each PCR had two NTCs and a positive control of *V. anguillarum* (ATCC 19264) at 1 ng/µL.

All PCR amplicons were visualised by electrophoresis in a 1.5% agarose gel and stained with GelRed™ (Biotium). Amplicons of the correct size were purified using a Zymoclean Gel DNA Recovery Kit (Zymogen Research). Purified amplicons were nucleotide sequenced in the forward and reverse direction using the PCR primers for 16S rRNA gene (27f with 1525r) and atpA gene (atpA-06F, atpA-04R, atpA-02F, and atpA-03R). DNA sequencing was carried out on Sanger sequencing platform by Ecogene Landcare Research. DNA sequences generated were imported into Geneious (Biomatters, Auckland), assembled into contigs and manually edited and trimmed. Consensus sequences were queried against the National Centre for BioTechnology Information (NCBI) nucleotide database via BLASTn using default settings.

Statistical analysis

Bacterial growth scores (Table 4.1) were used to compare the relative growth of bacteria for each mortality event. Statistical analyses were performed in R Studio (version 3.6.1, R Core Team 2019). A one-way ANOVA was used to compare the average relative growth scores between each mortality event, and between health baseline pipi and mortality event using Tukey contrasts and p-values adjusted for multiple testing using Benjamini-Hochbergs false discovery rate (FDR) (Benjamini & Hochberg 1995, Hothorn et al. 2008).

Molecular analysis

DNA extraction

DNA was extracted from gill and digestive tissue of eight pipi from the Nov-20 mortality and 20 pipi from the Jul-22 mortality to detect and quantify *Endozoicomonas* spp. (Table 4.1). Approximately 30 mg of gill and digestive tissue was aseptically excised, combined in a tube containing 180 µl tissue lysis buffer (ATL) and 20 µl proteinase K (Qiagen). The tube was incubated at 56°C overnight for tissue lysis. DNA extraction was then carried out using the QIAcube automated extraction robot using the QIAamp HT kit (Qiagen). 18S rRNA gene qPCR (TaqMan Ribosomal RNA Control Reagents VIC™ Probe) was performed on all extracted DNA to check that the DNA was amplifiable. A qPCR cycle threshold between 15 and 25 was considered acceptable.

Endozoicomonas spp. qPCR & statistical analysis

All DNA extracts were tested using a qPCR targeting a 118 bp region of the *Endozoicomonas* spp. 16S rRNA gene as per Howells et al. (2020). Two NTCs and a dilution series of a gblock positive control were included with each qPCR run. A gBlock ® gene fragment was designed from a DNA sequence of *Endozoicomonas elysicola* (Genbank: NR_041264), with dilutions ($1{\times}10^{-4}$ ng μl^{-1}; $1{\times}10^{-5}$ ng μl^{-1}; $1{\times}10^{-6}$ ng μl^{-1}) run in duplicate to create a standard curve to estimate the gene copies per sample.

Gene copy numbers from the qPCR results were used as a proxy to compare infection intensity. Using a GLM, comparisons between the mortality events were tested using Tukey contrasts, and p-values were adjusted for multiple testing using Benjamini-Hochbergs FDR (Benjamini & Hochberg 1995, Hothorn et al. 2008). A p-value <0.05 was considered statistically significant. Statistical analyses were performed in R Studio (version 3.6.1, R Core Team 2019).

Statistical analysis of baseline health data and mortality data

Data from the four health baseline pipi populations studied in Chapter 3 (Waipū River, Ruakākā River, Mair Bank and One Tree Point (Figure 4.1)) were compared against the data collected during the mortality events. A GLM comparison of the *Endozoicomonas* spp. PCR results from the baseline data and mortality data were tested using Tukey contrasts, and p-values were adjusted for multiple testing using Benjamini-Hochbergs FDR (Benjamini & Hochberg 1995, Hothorn et al. 2008). A p-value <0.05 was considered statistically significant. Statistical analyses were performed in R Studio (version 3.6.1, R Core Team 2019).

Results

Field observations & sample collection

There was a recurring mortality event observed at Mair Bank spanning from November 2020 to May 2021but due to associated safety issues, samples could only be obtained in the months of Nov-20 and May-21. Moribund pipi observed during sample collection from Mair Bank were sitting on top of the substrate. Moribund pipi had mantle tissue pulling away from shell interior and the adductor muscles were no longer retracting (Figure 4.2). The mortality event in Pātaua South was first observed on 3rd July 2022, with observations from locals of hundreds of thousands of pipi being washed up in the surf (Cooper 2022). All pipi appeared to be moribund with shell gaping open. All collected samples were measured and weighed (Table 4.1).

Figure 4.2 Moribund pipi from Mair Bank mortality May-20, adductor muscles no longer contracting to close shell.

Histology

Pipi from the Nov-20 mortality were mostly male (87.5%) with a small proportion of female (12.5%). A greater number of female pipi were observed in the May-21 (60%) and Jul-22 (70%) mortality events than the Nov-20 mortality event (Figure 4.3). Mortality pipi were undergoing normal reproductive phases for the time of year they were collected based on the baseline health data (Chapter 3), with the majority of Nov-20 and May-21 undergoing spawning (Figure 4.3). All Jul-22 mortality pipi had spawned (Figure 4.3). Mortality pipi shared common characteristics to the baseline pipi, including the presence of encysted metacercaria, hydroids, mantle accumulations, *Endozoicomonas* spp., and mucus cells present in gill water channel (Figure 4.4). However, several differences were observed in mortality pipi compared to baseline pipi (Figure 4.4). Pipi from the Nov-20 and May-21 mortality events displayed similar pathology, including autolysis of the epithelial cells in the gills and digestive tubules, which was often associated with the presence of rod-shaped bacteria, likely a post-mortem change (Figures 4.5). Post-mortem autolysis within the digestive system was observed in all pipi from the May-21 mortality, presence of rod-shaped bacteria was associated with autolysis (Figure 4.5). Two pipi from the Nov-20 mortality had infiltrations of bacteria, short rod/cocci bacteria, swarming around digestive tubules and swarming into entire tubes (Figure 4.5). No increased haemocytosis was observed between baseline and mortality pipi.

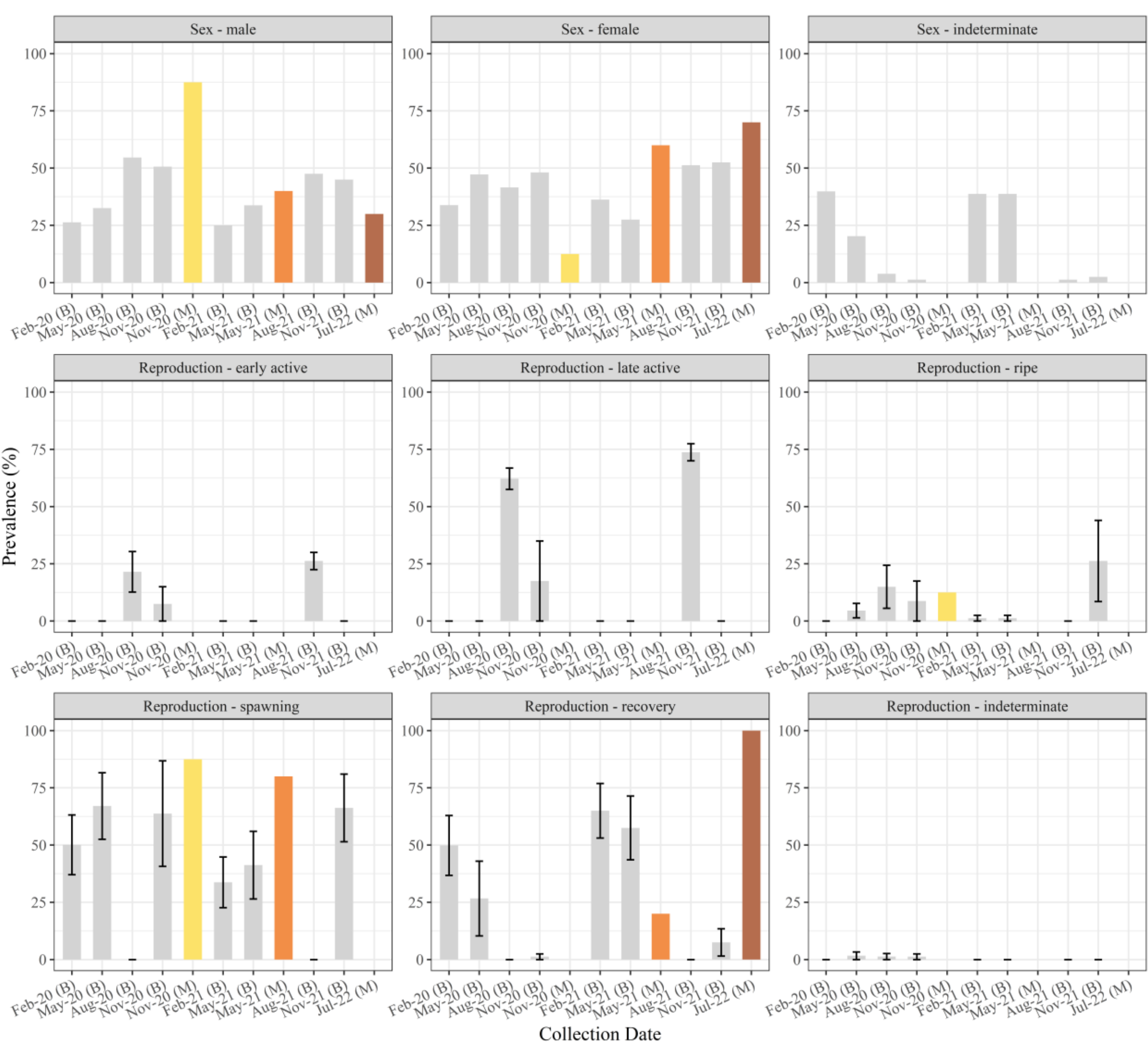

Figure 4.3 Seasonal prevalence of sex and reproductive phase from baseline pipi compared to mortality pipi across the two-year collection, ± standard error of mean. Key: B = baseline health pipi, Nov-20 (M) = Mair Bank mortality 2020, May-21 (M) = Mair Bank 2021 mortality, Jul-22 (M) = Pātaua South mortality 2022 .

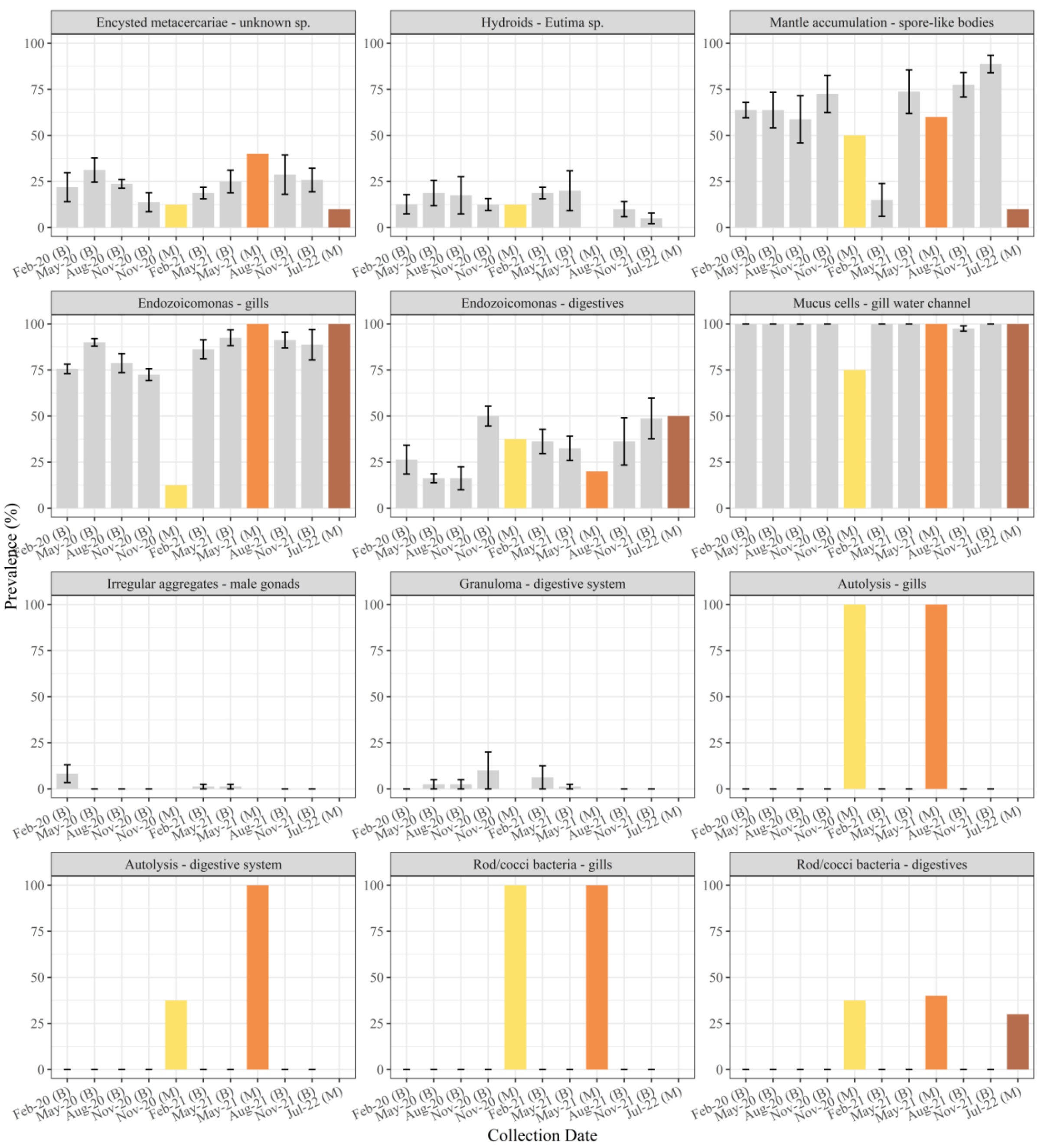

Figure 4.4 Proportion of histological features observed from baseline pipi compared to mortality pipi. Key: B = baseline health pipi, Nov-20 (M) = Mair Bank mortality 2020, May-21 (M) = Mair Bank 2021 mortality, Jul-22 (M) = Pātaua South mortality 2022. Bar chart with standard error of mean

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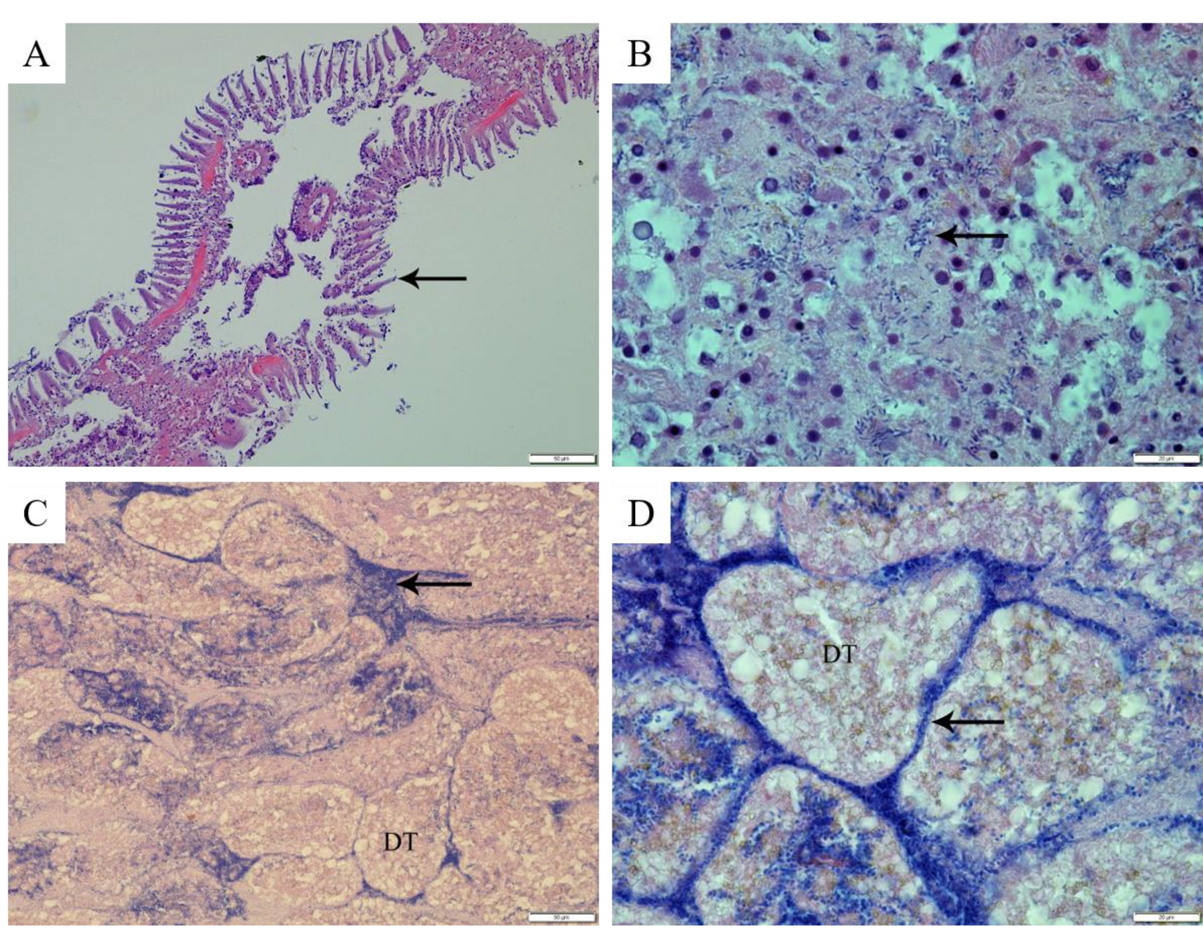


Figure 4.5 H&E photomicrographs from mortality pipi. A) Gill filaments showing necrosis of the epithelial cells, scale bar = 50 µm B) Necrosis in digestive system, rod-shaped bacteria throughout tissue (arrow), scale bar = 20 µm C) Rod/cocci-shaped bacteria (arrow) infiltrating digestive system, DT = digestive tubes, scale bar = 50 µm D) close up photomicrograph of rod/cocci bacteria infiltrating digestive tubules, scale bar = 20 µm.

Bacteriology

The highest average bacterial growth was observed in the Nov-20 mortality, with the lowest growth in Jul-22 mortality (Figure 4.6). A significant difference in the average bacterial growth was detected between the Jul-22 and the Nov-20 and May-21 mortalities. Both Nov-20 and May-21 mortalities had significantly more bacteria isolated from what was expected based on the baseline bacterial growth (except for May-20) (Figure 4.6). Bacterial growth from the Jul-22 mortality was significantly lower than the other mortality events. There was no baseline bacterial growth data for the month July when the Jul-22 mortality occurred, however, significantly different bacterial growth was observed between Jul-22 mortality samples and from May-20 and May-21 baseline growth (Figure 4.6).

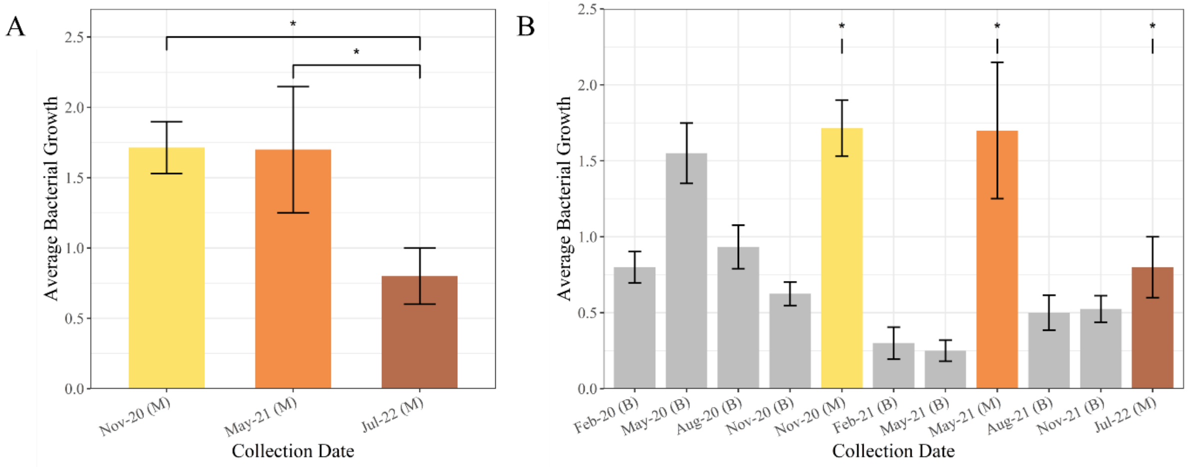


Figure 4.6 A) Comparison of average bacterial growth isolated from each mortality event. B) Average bacterial growth isolated from basline pipi compared to mortality pipi. Bar chart with standard error of mean. * = significant difference measured. Key: B = baseline health pipi, Nov-20 (M) = Mair Bank mortality 2020, May-21 (M) = Mair Bank 2021 mortality, Jul-22 (M) = Pātaua South mortality 2022.

The majority of the bacterial growth isolated had common or dominant growth. Around 70% of the mortality samples had common or dominant growth isolated from each mortality event (Figure 4.7). Baseline samples had fewer than 50% of samples where common or dominant bacteria was isolated (Figure 4.7). Mixed growth was recovered from around 30% of samples from the Nov-20 mortality, which is in line with the baseline samples; no samples from the May-21 or Jul-22 had mixed growth isolated. No bacteria were isolated for 30% of samples from the May-21 and Jul-22 mortality samples (Figure 4.7).

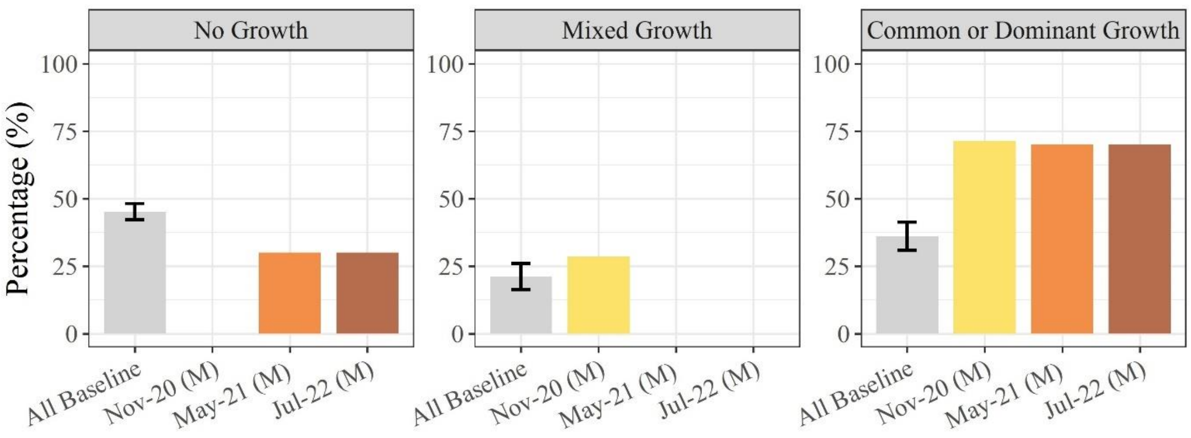


Figure 4.7 A comparison bacterial growth between baseline pipi and mortality pipi. Key: B = baseline health pipi, Nov-20 (M) = Mair Bank mortality 2020, May-21 (M) = Mair Bank 2021 mortality, Jul-22 (M) = Pātaua South mortality 2022.

Nucleotide sequencing of the atpA and 16S genes from common or dominant bacteria revealed 13 different species of bacteria from the three mortalities. The majority of bacterial species identified for

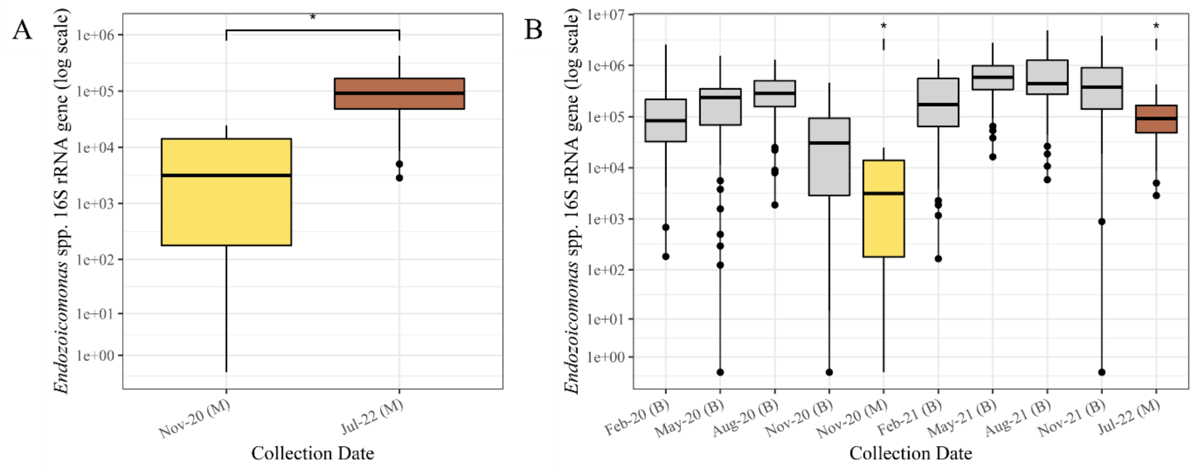
each mortality belonged to the *Vibrio* genus (Table 4.3). Three bacterial species were identified through sequencing of the 16S gene. *Shewanella colwelliana* (KR270290.1) was isolated in the Nov-20 mortality, *Shewanella fidelis* (NR_025195.1) was isolated in the May-21 mortality and *Psychromonas aquimarina* (NR_041604.1) was isolated in the Jul-22 mortality. There was no commonality between the bacteria isolated using the 16S gene in the baseline pipi to the mortality pipi. Although *Shewanella* species were isolated in baseline pipi, they were identified as different species.

Table 4.3 *Vibrio* spp. isolated using atpA gene PCR from the baseline pipi and the three mass mortalities, bacteria recorded as either present (+) or absent (-) for every collection. Identification based on closest BLAST ID (see appendix C, Table C.1 for GenBank reference ID's). *same BLAST ID - geneious alignment confirmed different species. Key: (B) = baseline pipi, (M) = mortality pipi.

Closest level identification	**Feb-20 (B)**	**May-20 (B)**	**Aug-20 (B)**	**Nov-20 (B)**	**Nov-20 (M)**	**Feb-21 (B)**	**May-21 (B)**	**May-21 (M)**	**Aug-21 (B)**	**Nov-21 (B)**	**Jul-22 (B)**
Vibrio atlanticus	-	-	-	-	+	-	-	+	-	-	-
*Vibrio atlanticus**	-	-	-	-	-	-	-	+	-	-	-
Vibrio artabrorum	+	+	-	-	-	-	-	-	-	+	-
Vibrio breoganii	-	-	-	-	-	-	+	-	-	-	-
Vibrio chagasii	-	+	-	-	-	-	-	-	-	-	-
Vibrio cortegadensis	-	-	-	-	-	-	-	-	-	+	-
Vibrio crassostreae	-	-	-	-	-	-	-	-	-	-	+
Vibrio europaeus	-	-	-	-	-	-	-	+	-	-	-
Vibrio genomo	-	-	-	+	+	-	-	+	-	-	-
Vibrio genomo*	-	-	-	-	-	-	+	-	-	-	-
Vibrio genomo *	-	-	-	-	-	-	-	-	-	-	+
Vibrio kanaloae	-	-	-	-	-	-	+	+	-	-	-
Vibrio maritimus	+	-	-	-	-	-	-	-	-	-	-
Vibrio mediterranei	-	-	-	-	-	-	-	+	-	-	-
Vibrio owensii	-	-	-	-	-	+	-	-	-	-	-
Vibrio rumoiensis	+	-	-	-	-	-	-	-	-	-	-
Vibrio sp. Scap24	+	-	-	-	-	-	-	-	-	-	-
Vibrio sp. Scap24*	-	+	-	-	-	-	-	-	-	-	-
Vibrio splendidus	+	+	-	+	+	-	+	+	-	+	-
*Vibrio splendidus**	-	-	-	-	-	-	-	-	-	-	+
Vibrio tetraodonis	+	-	-	-	-	-	-	-	-	-	-
21	6	4	0	2	3	1	4	7	0	3	3

1 *Endozoicomonas* spp. results

2 More gene copies of the *Endozoicomonas* spp. 16S rRNA gene were detected from samples collected
3 from the Jul-22 mortality event than Nov-20 mortality event (Figure 4.8). A significant difference in
4 *Endozoicomonas* spp. gene copies was detected between the Nov-20 mortality and all baseline
5 collections, except Nov-20 (Figure 4.8). A significant difference was detected between the Jul-22
6 mortality and May, Aug of 2020, and May, Aug, Nov of 2021 (Figure 4.8). There were fewer
7 *Endozoicomonas* spp. detected from the Nov-20 mortality pipi than expected at that time of year;
8 these qPCR results are concordant with the fewer number of *Endozoicomonas* spp. inclusions
9 observed under histology (Figure 4.4). There were no data on *Endozoicomonas* spp., from baseline
10 pipi from Pātaua South, which makes comparing the Jul-22 mortality pipi challenging, however, the
11 baseline data from other pipi populations within the Whangārei area can act as a proxy.



12

13 Figure 4.8 A) *Endozoicomonas* spp. 16S rRNA gene copy number of each mortality event. Line: median; box: interquartile
14 range (IQR); whiskers: minimum and maximum value. B) A comparison of *Endozoicomonas* spp. 16S rRNA gene copy
15 number between mortality pipi and baseline pipi. Key: B = baseline health pipi, Nov-20 (M) = Mair Bank mortality 2020,
16 Jul-22 (M) = Pātaua South mortality 2022. * = significant difference measured.

17 Discussion

18 Bivalve mortality events in Aotearoa New Zealand are investigated with little to no data on what
19 constitutes "normal". Bivalve mortality events in New Zealand have included members of the genus
20 *Paphies* and other intertidal bivalves that we do not traditionally have a great understanding of their
21 parasitic fauna compared to more commonly studied bivalve such as flat oysters (*O. chilensis*) or
22 green-lipped mussels (*Perna canaliculus*) (Bennion 2021; Bennett et al. 2022). Baseline health data
23 on populations provides an opportunity to identify potential causes for mortality events by
24 establishing what we can expect to observe in a set of animals at a given time to identify any changes
25 more easily. Baseline health data is useful in biosecurity monitoring but has predominantly been used

on monitoring invasive host species and their interactions with native infection dynamics (Bojko et al. 2013; Frizzera et al. 2021). In New Zealand, baseline surveys have been undertaken for marine pest surveillance to establish what marine pest species are present in a location before monitoring begins to better understand changes in distribution or abundance (Inglis et al. 2006). I employed a similar principle in this study by using baseline health data on pipi from Whangārei (see Chapter 3), to identify differences between mortality and baseline pipi that included the amount of bacterial growth isolated and the intensity of *Endozoicomonas* spp. detected. In aquatic animal disease investigations, histology, bacteriology, and qPCR are routinely used methods to investigate mortality events and therefore, comparing results using these test methods from health baseline and mortality samples provides the best opportunity to improve pathology determinations in disease investigations. I discuss the results from the mortality events in light of the baseline health data and suggest other data to be collected to further enhance disease investigations and identify causes for wild bivalve mortality events.

A significant element of this study was using bacteriology to identify the amount of bacterial growth and identify the common or dominant bacteria present. Significantly higher bacterial growth was isolated from mortality pipi compared to what was expected based on the baseline pipi (Figure 4.4). Around ~70% of the bacterial growth isolated from mortality pipi had common or dominant bacteria, compared to the baseline pipi which had <50% growth with common or dominant bacteria. *Vibrio* species were predominantly isolated from the mortality events but were also commonly isolated from baseline pipi. Pipi collected from the Nov-20 and May-21 mortalities had the same bacterial composition of predominant growth of *Vibrio* spp. with the presence of *Shewenella* sp. The same three *Vibrio* spp. were isolated from pipi collected from the Nov-20 and May-21 mortalities, including *V. atlanticus*, *V. genomo*, and *V. splendidus*. DNA sequencing analysis revealed that the *V. splendidus* were also present in baseline pipi samples, suggesting that *V. splendidus* is environmentally ubiquitous but its role may change under different external stressors. *Vibrio* spp. have a complex role with their hosts that can range from symbiont to pathogen (Destoumieux-Garzón et al. 2021). *Vibrio* spp. can predispose animals to disease or act as opportunistic pathogens affecting immunocompromised hosts. For example, de Lorgeril et al. (2018) demonstrated that OsHV-1 infected Pacific oysters (*Magallana gigas*) were immunologically suppressed and became infected by opportunistic *Vibrio* sp. leading to increased mortality. Moreover, the presence and functioning of marine bacteria, either within the animal or from the surrounding environment, can change under certain conditions (Coffin et al. 2021). Bivalves have developed the ability to gape during anoxic conditions, which helps to enhance oxygen diffusion. Nevertheless, this process exposes the bivalve to anaerobic bacteria that thrive under anoxic conditions (Coffin et al. 2021). Bacteriological culture in this study was purely aerobic, therefore, we could not test for anaerobic bacteria. 16S rRNA metabarcoding may reveal higher abundance of anaerobic Operational Taxonomic Units (OTUs).

Both methods could be useful in future investigations, particularly with increasing rates of eutrophication in the nearshore.

No *Vibrio* species were isolated from baseline pipi during the cooler months (August), whereas pipi were dominated by *Vibrio* spp. growth during the Jul-22 mortality despite these cooler seasonal temperatures. *Vibrio* spp. are known to be particularly sensitive in cooler temperatures and can go into a state of viable but non-culturable (VBNC) condition (Zhang et al. 2020). *Vibrio* sp. was isolated from every pipi that had bacterial growth (~70% of samples) from the Jul-22 mortality. Du et al. (2007) found that in estuarine environments VBNC *Vibrio* spp. could be resurrected by environmental changes, such as a front of warm water, which may well be the reason behind the presence of these bacteria in the cooler months. However, it could also be a significant shift in bacterial community. *V. crassostreae* and *V. splendidus,* which were both present in the Jul-22 mortality, have been associated with bivalve mass mortalities with pathogenic potential (Romalde et al. 2014). We can see from the baseline health data that there has been a deviation from the normal bacterial community in the Jul-22 mortality. Unfortunately, the identification of *Vibrio* in this study was limited to the sequencing of only one gene. Identification of *Vibrio* spp. can be difficult because of interspecific genetic similarities. Ideally multi-locus sequence typing (MLST) or whole genome sequencing should be employed when identifying *Vibrio* species to provide a higher level of identification (Kalia et al. 2015). In all three mortalities we can see that there is elevated bacterial growth and the community of bacteria is comprised of *Vibrio* spp. and may well be worth exploring further to uncover the true nature of these relationships and whether they are pathogenic or opportunistically infected already compromised animals.

Contrastingly to the bacteriology findings, lower levels of *Endozoicomonas* spp. were detected from mortality pipi than expected according to the baseline pipi data. It has been hypothesized that *Endozoicomonas* spp. could be a factor involved in bivalve mass mortalities (Howells et al. 2021). However, such studies have been looking at multiple species, different health statuses, and different geographical locations in New Zealand. In this study, albeit using a small sample size, but from the same species from the same location, a significantly lower number of *Endozoicomonas* spp. were detected from the mortality pipi compared to the baseline pipi from corresponding seasons. *Endozoicomonas* spp. have been suspected as being important to bivalve health, especially for king scallops (*Pecten maxima*) in the United Kingdom (Cano et al. 2018; Hooper et al. 2019), but no studies have demonstrated a direct association between *Endozoicomonas* spp. infection and disease. The lower levels of *Endozoicomonas* spp. detected in this study during a mortality event may represent a symbiotic relationship with pipi, with their abundance correlated to host health. Shifting microbiome is a common occurrence during mass mortality events (Richard et al. 2021), the presence of important bacteria that maintain host health can be superseded by harmful bacteria causing a shift

in overall diversity (Egan & Gardiner 2016). If these bacteria are in fact symbiotic, which they are recognised as in some coral species (Morrow et al. 2015), then the lower presence of *Endozoicomonas* spp. in the event of a mortality may well indicate homeostatic imbalance. In corals, *Endozoicomonas* spp. appeared highly sensitive to environmental shifts and in the presence of stressors a significant reduction was detected. If *Endozoicomonas* spp. have similar roles in pipi or bivalve in general then they could represent a potential candidate monitoring species, akin to a canary in the coal mine for future mortality events; however, this remains to be tested. Moreover, species level identification for *Endozoicomonas* spp., remains elusive. There may be several different species belonging to the genus *Endozoicomonas* that perform different functions. This is an important area of future research to better understand the role of *Endozoicomonas* spp. in bivalve health.

Similar histological observations were made between the baseline pipi and mortality pipi. Histology revealed that the animals from the mortality events were spawning or post-spawning, but this is expected at this time of the year (Figure 4.3). Reproduction is a high-energy process which can be taxing on bivalves and may contribute to a compromised immune system making them more susceptible to external pressures (Brokordt et al. 2019; Hong et al. 2014). If spawning stress alone was the main driver of mortality events, we would expect to see mortalities around the same time each year, which is not the case. Though it is unlikely to be the primary cause of the mortality, in many cases physiological imbalance to the host species can make them particularly vulnerable to unexpected environmental shifts or presence of pathogens and parasites (Samain et al. 2007). However, other than more *Vibrio* spp. isolated and fewer numbers of *Endozoicomonas* spp., the parasitic fauna of pipi were consistent through time, suggesting that environmental factors are likely to be an important factor in mortality events.

From the information gathered from Patuharakeke hapū and observational data collected for the Mair Bank population, there was a recurrent presence of mortality pipi from Nov-20 to May-21 and, therefore, mortality may be from natural die-off from an aging cohort of pipi. Recruitment of bivalves tends to occur in cohorts, with population structures made up of distinguishable age groups (Beukema & Dekker 2005). Hooker (1995b) described how older pipi move downstream and are able to reside in more open ocean areas as they become more tolerant to higher salinities. Based on the length data collected, Mair Bank pipi were a large cohort size and resided in water salinity of around 34 ppt. Presumably the Mair Bank pipi are an older population and natural mortality from old age is likely to be the underlying cause of mortality from Nov-20 and May-21. In contrast, pipi from the Jul-22 mortality were smaller than pipi from Mair Bank mortality (~10 cm shorter in length), indicating that mortality was affecting a different cohort of pipi and therefore a potentially different underlying mortality cause.

From the results, differences in the bacteria growth and intensity of *Endozoicomonas* spp. were observed between the baseline pipi and the mortality pipi, although there is no clear indication in what factors were the primary drivers of the mortality. The histology indicates that the physiological condition of the animals could be compromised due to reproduction, additionally we can see that there is an elevated bacterial growth in mortality pipi compared to baseline health data. Finally, the external observations also show that Mair Bank pipi experiencing mortality were of a larger cohort and may have been age related. To determine if other factors were impacting host health and causing them to be predisposed to the discussed findings, it is necessary to consider and rule out other potential agents. There are several factors that are known to cause host stress and impact the presence of bacterial growth or change the dynamics of pathogens present, such as the environment. Studies on wild abalone (*Haliotis* spp.) in France showed that the host was more susceptible to pathogenic *Vibrio harveyi* when difference of 1°C increase in water temperatures coinciding with host reproductive stress causing higher mortality, representing the emerging risks of disease associated outbreaks with changing climates (Travers et al. 2009). In many mortality events the cause can be multifactorial, initiated from one factor which can lead to secondary infection ultimately resulting in mortality. Environmental and anthropogenic factors need to be taken into consideration as they play an important role in health status.

Conclusion

Similarly to the findings in Chapter 3, baseline data can't be limited to one factor and other data such as water quality and environmental data is required to infer the findings. The results from this study illustrate the importance of gathering baseline data and thereby, being able to carry out direct inference in providing context to the findings. This study provides an insight into one factor, disease, that contributes to bivalve health. Baseline health studies and long-term surveillance of disease, although time consuming and expensive, ensure early detection of pathogens which in turn aid in the implementation of mitigation and management measures. Once baseline health data on disease has been established this data can be easily paired with environmental data and water quality data to start understanding the different factors that interact with bivalve health. Archive environmental data and water quality data is much more readily available than data on disease. These factors will be considered in Chapter 6 in conjunction to bivalve mass mortality events in the Whangārei area.

Chapter 5 : Unravelling the link between environmental factors and *Endozoicomonas* spp.: an experimental study on contributing factors to pipi (*Paphies* australis) mass mortality events in Whangārei

Two women collecting pipi from Oriental Bay, Wellington, 1958. Alexander Turnbull Library, Wellington, New Zealand. (BY CC 4.0).

Introduction

Monitoring the health of marine species in their natural environment is difficult. The array of potential contributing factors, such as salinity, water temperature, and sedimentation, and their complex interactions, makes identifying important drivers of health a challenge (Hewitt et al. 2016). An increase of frequency and severity of local and global stressors as a result of a changing ocean climate will impact nearshore communities, especially benthic communities comprising bivalve molluscs (Lohrer et al. 2016; Bae et al. 2021). Marine species can usually withstand natural fluctuations caused by seasonal and meteorological conditions, however, when fluctuations extend beyond the normal optimal range this can act as a stressor on host health compromising immunity and therefore increasing susceptibility to infection and disease (Sniesko 1974). A better understanding of drivers of marine disease dynamics to help preserve and sustain important marine animal populations is needed.

Salinity, temperature, and sedimentation shape benthic communities in estuarine environments. Water temperature and salinity are frequently studied environmental factors on host health and disease dynamics. Warmer water temperatures can facilitate bacterial growth and increase transmission as demonstrated with withering syndrome in red abalone (*Haliotis rufescens*) and black abalone (*Haliotis cracherodii*) in the USA (Braid et al. 2005). An increase in mortality of European flat oysters (*Ostrea edulis*) in the Netherlands was attributed to warmer water temperatures and increased susceptibility to infection from the haplosporidian parasite *Bonamia ostreae* after prolonged periods of lower-than-normal salinity (Engelsma et al. 2010). Salinity is an important driver in the disease progression of the protozoan marine parasite *Perkinsus marinus* and the American oyster (*Crassostrea virginica*) in the Chesapeake Bay (Burreson & Ragone Calvo 1996). As salinity decreases, infection intensity and prevalence decrease, with the most severe disease events occurring after droughts when high salinity conditions persisted (Burreson & Ragone Calvo 1996). Whilst higher salinity has been known to cause severe disease events, conversely lower salinity can cause the same results. High influxes of fresh water to estuarine environments results in lower salinity causing physiological stress to benthic communities, such as bivalves, weakening the immune system response and causing vulnerability to external factors, such as in bacteria, viruses, or parasites which may thrive or spread more easily in lower salinity environments (Li et al. 2022).

The Impact of sedimentation on marine disease dynamics is not as well understood compared to other stressors such as water temperature or salinity (Lane et al. 2020). Elevated sediment loading in nearshore coastal environments can occur via urban development, forestry, farming, severe weather events, and poor land management practices (Crain et al. 2009). This results in both catastrophic sedimentation events during storms (e.g., cyclone Gabrielle) and ongoing chronic sediment inputs resulting in elevated sedimentation rates and suspended sediment levels in estuarine and coastal waters (Thrush et al. 2004; McMullan 2023). The impact of sedimentation on marine disease

dynamics is not as well understood compared to other stressors such as water temperature or salinity (Lane et al. 2020). Additionally, the interactions of sedimentation with other stressors are also likely to deviate from the normal range under climate change scenarios. Considering the threat of increased sedimentation on the nearshore, especially to bivalve molluscs like pipi, it is important to begin to understand how sedimentation may drive disease dynamics.

Endozoicomonas spp. are intracellular bacteria commonly associated with bivalve mass mortalities in New Zealand but also present in healthy populations indicating they make up a part of the host microbiome (Howells et al. 2021; Bennion et al. 2021). These bacteria have been observed under histopathology in bivalves collected during mass mortality events, submitted for disease investigation to Biosecurity New Zealand's Animal Health Laboratory (BNZ AHL) between 2013-2023 (BNZ AHL, unpubl. Data). Up until 2020, these bacteria were thought to be a *Rickettsia*-like organisms (i.e., RLO) based on morphological features under histology, but through 16S rRNA gene sequencing the bacteria were identified belonging to the genus *Endozoicomonas* (Howells et al. 2021; Bennion et al. 2021; Cano et al. 2020). Internationally there has been an increase in research effort on *Endozoicomonas* spp., leading to an expanding host range and a wider known geographic distribution (Cano et al. 2020; Neave et al. 2017). *Endozoicomonas* spp. have been described in several marine species such as cobia (*Rachycentrum canadum*), coral (*Stylophora pistillata*), and several different bivalve species, including eight from New Zealand (Cano et al. 2020; Neave et al. 2017; Mendoza et al. 2013; Howells et al. 2020). There is still very little knowledge, however, on the role of these bacteria to host health. They have been described as both an important symbiont and opportunistic pathogen under certain conditions. It has been hypothesized that this bacteria becomes opportunistic in king scallops (*Pecten maxima*) when water temperature increases (Hooper et al. 2019).

Identifying which environmental stressors drive marine disease is difficult in the wild. Though laborious in effort, time, and finances, carrying out laboratory simulated trials is often the only way to isolate mechanistic controls to understand drivers of marine diseases and how microorganisms interact with their host (Bernardo et al. 2021). Laboratory experiments are good at elucidating specific mechanisms under controlled conditions. The downside is that these experiments are a simplification of natural processes and physiological responses and may not be representative of a real-world system (Thrush et al. 2000). Despite these limitations, laboratory-based experiments can help clarify mechanisms that underpin real-world processes and should be interpreted within the context of field-based knowledge.

Pipi (*Paphies australis*) in Whangārei have undergone a shift in population dynamics including declining population size and increased reports of mass mortalities (BNZ AHL, unpubl. data; Williams et al. 2017; Pawley et al. 2013). Despite extensive community-based knowledge and efforts, the drivers behind the population shift have not been explained (Williams et al. 2014).

Endozoicomonas spp. have been associated with every pipi mass mortality reported to BNZ since 2010. This raises questions on the role of *Endozoicomonas* spp. on pipi health and its relevance to population decline. I have detected *Endozoicomonas* spp. in wild pipi throughout this thesis (Chapters 3 and 4) and I aim to better understand how they might relate to host health. The aim of this study is to test various environmental stressors that pipi encounter in their natural habitat and examine if unfavourable environmental conditions are linked to increased occurrences of *Endozoicomonas* spp. infection in pipi. By doing so, I aim to determine if this infection contributes to the mass mortality of pipi in Whangārei.

Materials & Methods

Sample collection

Pipi were collected from Mair Bank, Whangārei Harbour (Figure 5.1). Pipi (*n*=600) were collected by hand and placed into an insulated case with icepacks and a protective layer preventing direct contact with the icepacks for transportation. Pipi were gathered with the Patuharakeke iwi under a customary fishery allocation as custodians of pipi in this region.

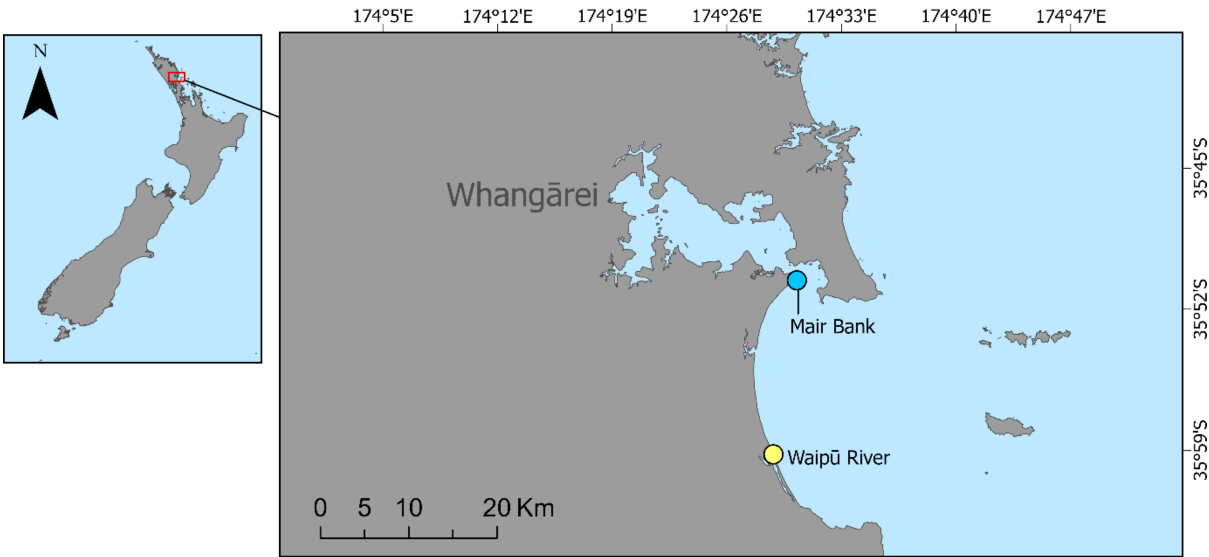


Figure 5.1 Map of New Zealand in the top left corner. Close-up localised map of sample location Mair Bank (blue icon) and Waipū River (yellow icon).

Experiment

Trial experiment

Before conducting the experiment, I trialled the tank layout to test the experimental set-up, including feed type and recirculation system (Figure 5.2). A mortality rate of 10% was deemed acceptable for

this trial. The test experiment lasted for 14 days and involved 20 pipi collected from the Waipū River. Only one pipi died during this period and the experimental layout was deemed acceptable to proceed.

Figure 5.2 Experiment set up showing pipi in a frame supporting them vertically replicating normal position in substrate; each treatment was run in triplicate.

Experimental design

Pipi (n=30) were collected and processed for diagnostic testing immediately on arrival at the laboratory (see test methods in sections below) to serve as a reference point before the experiment started. They were not used in any experimental treatments and classified as ‘field reference’ samples (Table 5.1). Shell length and weight (shell and animal) were taken for each individual before testing (Table 5.1). All samples were measured to the nearest 0.1 mm with vernier callipers and weighed to the nearest 0.1 g using electronic scales.

At the laboratory, 30 pipi were randomly distributed between 18 100L tanks. Shell length and weight were taken for each individual before being placed in the tank (Table 5.1). Pipi were placed on a frame structure to replicate positioning in substrate (Figure 5.2) and acclimatized for three days at 18 °C in 34–35 ppt water. These parameters represented the average water conditions of Mair Bank at the time of collection. Closed system tanks were used with artificial seawater (Aqua One). Water was aerated and filtered through an Aquis 500 cannister (Figure 5.2). Water quality (temperature, salinity, ammonia, nitrate and nitrite) was monitored daily. A 10-50% water change was carried out when water parameters fell outside the acceptable ranges. Pipi were fed with Shellfish Diet® 1800 (Reed Mariculture). Any uneaten food and defecation were removed daily using a siphon. During the study, pipi were checked three times a day, with mortalities removed immediately.

The entire experiment ran for 21 days, excluding acclimatization. Three pipi were removed from each treatment tank every three days at random for testing (i.e., pipi were removed at days 3, 6, 9, 12, 15, 18, and 21). Laboratory testing included *Endozoicomonas* spp. qPCR, histology. Bacteriology was carried out on one pipi from each tank at every interval. The same test methods used in Chapters 3 & 4 were applied here.

Experimental treatments

Experimental treatments were water temperature, salinity and suspended sediments (SS) (Table 5.1). These are common environmental stressors that can influence the presence of marine disease (Braid et al. 2005; Engelsma et al. 2010; Pollock et al. 2016) and simulate environmental stresses that pipi are likely to experience in their natural habitat. The control conditions and experimental treatments were based on water quality data from Mair Bank collected by the Northland Regional Council between 2010-2020 (Griffiths 2021). Control conditions were the average conditions experienced and the treatments represented extreme conditions identified (Table 5.1). All treatments were run in triplicate, including the control. Tank conditions were maintained daily, the water temperature for all conditions were maintained at ± 1°C of the set temperature and salinity was maintained at ± 1 ppt of the set salinities.

The ambient room temperature was set at 18°C and temperature treatment tanks (21°C and 24°C) were created using individual tank heaters. Temperature was checked three times a day to ensure they remained within ± 1°C of the set temperature. For salinity tank treatments (17 ppt and 24 ppt), artificial seawater was diluted from 34 ppt to the required salinity with fresh water in a holding tank before being distributed. Salinity was checked three times a day and was maintained at ± 1 ppt of the set salinities. For the treatment of SS, marine sand collected from Whangārei Harbour was used. The sand was weighed and suspended in the water column using a circulation pump (Fluval ®) constantly at a rate of at 750 mg/l-1. Before use, sand was dried and sieved to ≤ 250µm using woven wire accredited sieves (Endecotts Ltd), placed into a glass beaker and autoclaved at 121°C for 20 minutes three times (Otto et al. 2018). Autoclave runs were validated by taking 20 g of autoclaved sand, placing it in sterile water, vortexed (3 min at 10,000 rpm) and plating 100 µL of the solution onto tryptic soy agar with 3% sodium chloride (TSA+3%) (Fort Richard), and Columbia Sheep's blood agar (BA) (Fort Richard) in triplicate and incubated at 22°C. Plates were checked for bacterial growth at days 3 and 7. Acceptable criteria range for successful sterilisation was no bacterial growth observed after 7 days.

Table 5.1 Summary table of experimental treatments and metadata of pipi used in each tank. SS –suspended sediments.

Treatment	***n***	**Temp. (°C)**	**Salinity (ppt)**	**SS**	**Average length (mm)**	**Length range (mm)**	**Average weight (g)**	**Weight range (g)**
Field reference	30	NA	NA	NA	62.39	41.5-70.46	30.27	14.3-43.9
Control	90	18	34	NA	58.46	19.50-70.50	27.12	6.00-48.20
21°C	90	21	34	NA	59.46	44.00-68.50	26.37	9.50-47.30
24°C	90	24	34	NA	58.31	44.00-67.00	25.41	7.50-38.10
SS	90	18	34	≤ 250μm	56.90	44.00-65.50	23.66	8.90-33.70
24ppt	90	18	24	NA	61.92	47.00-69.50	30.11	11.10-53.50
17ppt	90	18	17	NA	62.59	56.50-71.50	29.29	19.60-42.00

Endozoicomonas spp. qPCR

The intensity of *Endozoicomonas* spp. in response to changing environmental conditions were assessed using qPCR. DNA was extracted from gill and digestive tissue separately to detect and quantify *Endozoicomonas* spp. from every pipi. Approximately 30 mg of gill and digestive tissue was aseptically excised and placed separately into tubes containing 180 µl tissue lysis buffer (ATL) and 20 µl proteinase K (Qiagen). The tube was incubated at 56°C overnight for tissue lysis. DNA extraction was carried out using the QIAcube automated extraction robot using the QIAamp HT kit (Qiagen). 18S rRNA gene qPCR (TaqMan Ribosomal RNA Control Reagents VIC™ Probe) was performed on all extracted DNA to check that the DNA was amplifiable. A qPCR cycle threshold between 15 and 25 was considered acceptable for extracted DNA.

All DNA extracts were tested using a qPCR targeting a 118 bp region of the *Endozoicomonas* spp. 16S rRNA gene as per Howells et al. (2020). Two no template controls (NTCs) and a dilution series of a gblock positive control were included with each qPCR run. A gBlock ® gene fragment was designed from a DNA sequence of *Endozoicomonas elysicola* (Genbank: NR_041264), with dilutions (1×10^{-4} ng μl^{-1}; 1×10^{-5} ng μl^{-1}; 1×10^{-6} ng μl^{-1}) run in duplicate to create a standard curve to estimate the gene copies per sample.

Statistical analysis

Gene copy numbers from the qPCR results were used as a proxy to compare infection intensity between the treatments. Using a GLM, comparisons between the different tank treatments were tested using Tukey contrasts, and p-values were adjusted for multiple testing using Benjamini-Hochbergs false discovery rate (FDR) (Benjamini & Hochberg 1995, Hothorn et al. 2008). An initial comparison was carried out of *Endozoicomonas* spp. between each tank treatments and the two different tissue types to understand the presence of *Endozoicomonas* spp. between the two organs. A secondary

comparison was carried out separately for each tissue type between each tank. A p-value <0.05 was considered statistically significant. Statistical analyses were performed in R Studio (version 4.1.3, R Core Team 2022).

Histology

All samples underwent histological analysis to observe any pathology and changes in *Endozoicomonas* spp. infection intensity in response to varying environmental treatments. A standard histological section was taken from each animal and placed in 10% formalin for a minimum of 24 h to fix. A standard histological section included a cross section that captured all major organs, including gill, mantle, digestive gland, gonad, connective tissue, and foot muscle (Howard 2004). Fixed tissues were processed using standard histological techniques, stained with hematoxylin and eosin (H&E), and cover slipped. Each sample was examined for general health status using an Olympus BX51 microscope. Additional records were made for each individual, including sex, reproductive phase, haemocyte response (if present), parasites observed and their identification to the lowest possible taxonomic level, *Endozoicomonas* spp. presence and intensity, and bacteria observed.

Bacteriology

Bacteriology testing was performed on pipi from each tank treatment to understand differing bacteria isolated from each treatment. Bacteriology has been performed throughout the thesis (Chapters 2, 3, 4) informing a good understanding of what is expected in healthy pipi. One pipi from each tank at every testing interval was tested for bacteriology. After opening the pipi, the adductor muscle was cleaned with 70% ethanol using a sterile swab. Using a new scalpel, an incision was made through the middle of the muscle. A sterile swab was plunged into the incision and plated onto growth media directly. Swabs were plated onto TSA+3% and thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Fort Richard) in the laboratory. Swabs were used to inoculate a small area of the selected media and standard streak plating was carried out. The plates were incubated at 22°C for a total of seven days. Growth was recorded at three days and a final read at seven days.

Bacterial growth on the plate was recorded as a score depending on the quadrant of growth displayed on the plate (Table 5.2). Isolated bacteria were grouped into categories: no bacterial growth, mixed bacterial growth (no common or dominant bacteria), and common or dominant bacterial growth. Common or dominant bacterial growth was the main group of interest and were characterised by bacterial isolates that were either common across multiple individuals or were the dominant growth on any singular plate. All common and dominant isolates were sub-cultured onto the initial growth media and BA to assess purity and for identification.

Table 5.2 Categorisation of bacterial growth and the relative scoring associated.

Inoculum area	Relative growth score
No growth	0
Primary inoculum	1
Second quadrant	2
Third quadrant	3
Fourth quadrant	4

Basic biochemical tests were carried out on all common or dominant isolates (Gram stain, indole, oxidase, and catalase) followed by identification with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Isolates that were Gram negative rods and oxidase positive were tested for sensitivity to O/129 (150 µg) (Oxoid Ltd.). Isolates sensitive to O/129 were suspected *Vibrio* spp. and were subjected to PCR and nucleotide sequencing of the atpA gene. Isolates resistant to O/129 (150 µg) were non-suspect *Vibrio* spp. and were subjected to PCR and nucleotide sequencing of the 16S rRNA gene.

DNA sequencing

DNA was extracted from separate pure isolates using InstaGene™ Matrix (BIO-RAD). Briefly for each isolate, five individual colonies were scraped off the agar and homogenised in 200 µL vortexed InstaGene™ Matrix in a centrifuge tube. The tube was then heated at 56°C for 30 mins, vortexed, and heated at 72°C for 10 mins before being vortexed again then centrifuged at 3,000 g for 3 minutes. The resulting DNA was quantified (ng/µL) using QubitTM fluorometer (Life Technologies).

Isolates not suspected to be *Vibrio* spp. were subjected to a bacterial species PCR targeting ~1498 bp of the 16S rRNA gene (Lane 1991). Template DNA concentration 2.5 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer 27f and 1525r to a total volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 50°C for 15 sec, 72°C for 1 sec. For every PCR performed, two NTCs and a positive control were used. For the NTC, molecular-grade water was added to the reagent mix instead of nucleic acid template. For the positive control, *Escherichia coli* (ATCC 25922) DNA was used at 0.1 ng/µL.

Suspect *Vibrio* spp. were subjected to a PCR targeting 1500 bp of the atpA gene (Thompson et al. 2007). DNA of concentration 1 ng/µL was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer atpA-06F and atpA-04R to a total volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 58°C for 15 sec, 72°C for 30 sec. Each PCR had two NTC and a positive

control of *Vibrio anguillarum* (ATCC 19264) at 1 ng/µL. One suspect *Vibrio* species did not amplify from the atpA gene PCR so was run on the 16S rRNA gene PCR to obtain an identification.

All PCR amplicons were visualised by electrophoresis in a 1.5% agarose gel and stained with GelRed™ (Biotium). Amplicons of the correct size were purified using a Zymoclean Gel DNA Recovery Kit (Zymogen Research). Purified amplicons were nucleotide sequenced in the forward and reverse direction using the PCR primers for 16S rRNA gene (27f with 1525r, and PA-GSF with PA-GSR) and atpA gene (atpA-06F, atpA-04R, atpA-02F, and atpA-03R). DNA sequencing was carried out on Sanger sequencing platform by Ecogene Landcare Research. DNA sequences generated were imported into Geneious (Biomatters, Auckland), assembled into contigs and manually edited and trimmed. Consensus sequences were queried against the National Centre for BioTechnology Information (NCBI) nucleotide database via BLASTn using default settings.

Statistical analysis

Bacterial growth scores were used to compare the relative growth of bacteria between the five treatments and the control. Statistical analyses were performed in R Studio (version 4.1.3, R Core Team 2022). A one-way ANOVA was used, using Tukey contrasts and p-values adjusted for multiple testing using Benjamini-Hochbergs false discovery rate (FDR) (Benjamini & Hochberg 1995, Hothorn et al. 2008).

Mortality statistical analysis

Mortality of pipi was observed in each of the treatment tanks across the experiment. Diagnostics of *Endozoicomonas* spp. qPCR, histology and bacteriology were carried out on fresh mortality pipi where possible given the condition of the sample and time restrictions (e.g., if the animal had died overnight and tissue autolysis had commenced). Test results from the mortality samples were compared to pipi sampled when alive

A primary analysis looked at the survival probability between the six treatments to understand which treatment caused the most stress on the pipi health. Survival probability rates from each of the tank treatments were compared to identify which tank had the highest influence on mortality using Cox proportional hazard model. As quantitative data was collected for *Endozoicomonas* spp. qPCR and bacteriology in both health and mortality pipi, the results from these two methods could be easily compared against each tank treatment. The same statistical analysis as described above were carried out to compare the difference between healthy and mortality pipi from the same tank treatment.

Results

Endozoicomonas spp. qPCR

A significant difference (p-value < 0.05) in gene copy numbers of *Endozoicomonas* spp. was detected between the gills and digestive tissue between each treatment. Gene copies detected in the gills ranged from 1,845-8,640,754 and 5-153,874 from the digestive tissues. Because of the difference gill and digestive tissue were not compared against each other and tank treatments were only compared against the same tissue type.

Field reference samples had the lowest median number of *Endozoicomonas* spp. gene copies and was significantly different from the control and 21°C treatment (p-value < 0.05) (Figure 5.3). Significantly more gene copies were detected in the 21°C treatment than 24°C (Figure 5.3). There was no significant difference in gene copies of *Endozoicomonas* spp. between any other treatment. Gill tissue from treatment of 24°C had the lowest median gene copies and the lowest minimum and maximum range of gene copies. Tank treatment of SS had the highest median gene copies followed by 21°C which had the highest maximum number of gene copies present. A separate comparison between the tank treatments with digestive tissue was carried out (Figure 5.4). Interestingly, the results from the digestive tissue were the opposite to gene copies measured in gill tissue with 24°C having the highest *Endozoicomonas* spp. gene copies detected. A significant difference in gene copies was detected between tank treatment of 24°C and all other tank treatments (p-value <0.05). *Endozoicomonas* spp. gene copies were lowest at 24 ppt and were significantly lower than gene copies measured in the field reference sampling.

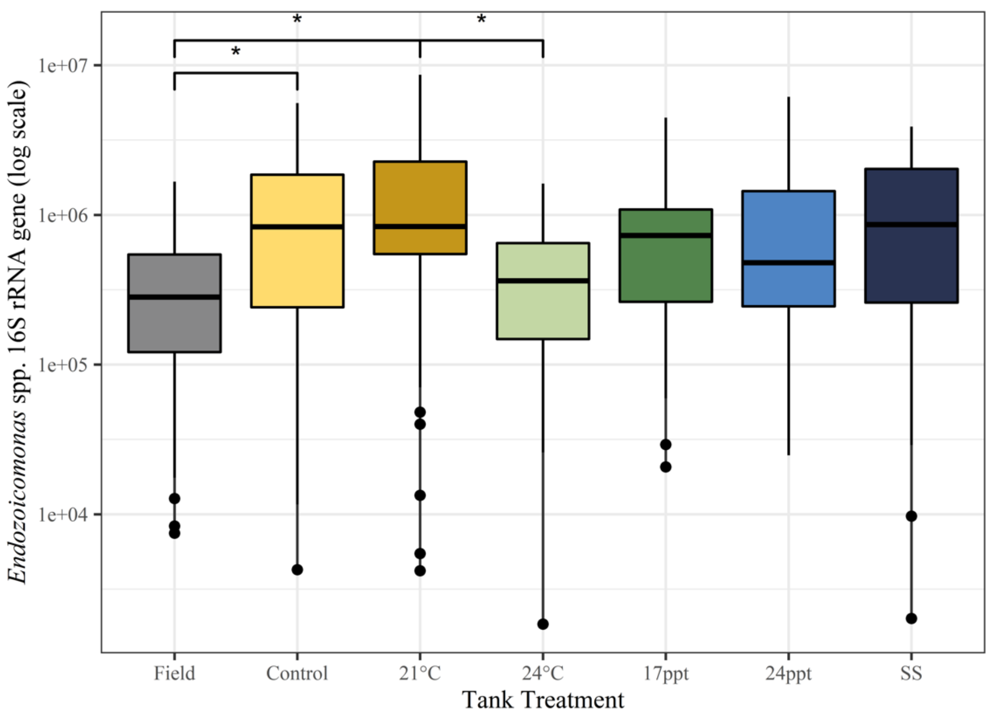


Figure 5.3 *Endozoicomonas* spp. 16S rRNA gene copy number comparison between tank treatments and field reference pipi from gill tissue. SS –suspended sediments. Line: median; box: interquartile range (IQR); whiskers: minimum and maximum values. * represents significant difference measured (p-value < 0.05).

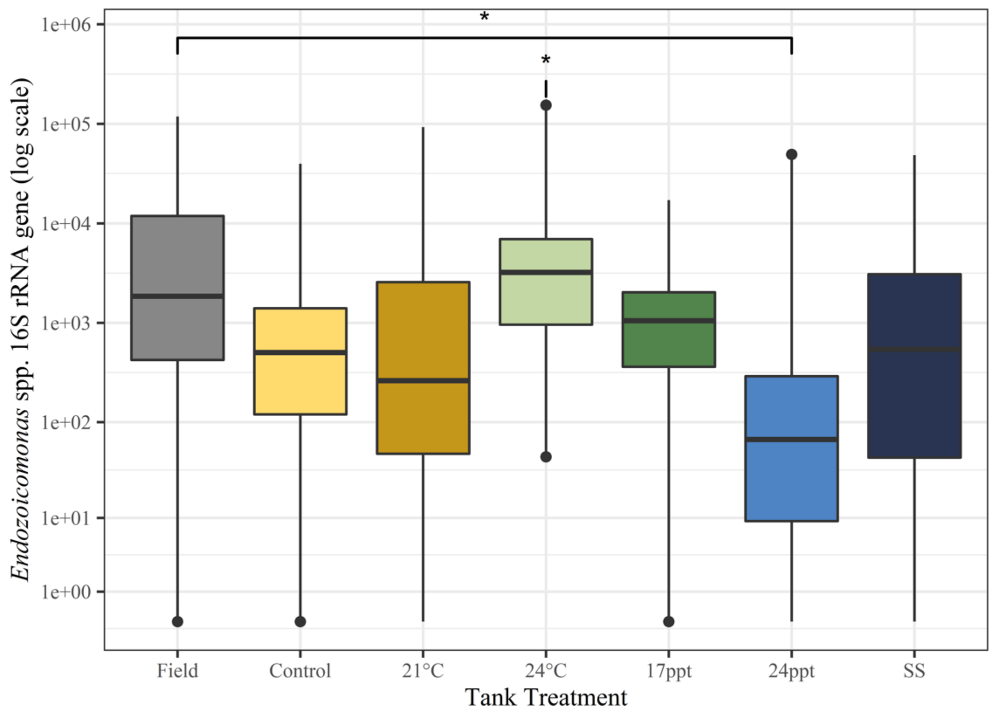


Figure 5.4 *Endozoicomonas* spp. 16S rRNA gene copy number comparison between tank treatments and field reference pipi from digestive tissue. SS –suspended sediments. Line: median; box: interquartile range (IQR); whiskers: minimum and maximum values. * represents significant difference measured (p-value < 0.05).

Histology

Endozoicomonas spp. was more commonly observed in gill epithelium then the digestive epithelium (Figure 5.5). *Endozoicomonas* spp. was observed at a similar prevalence in different tissue types between the treatments. No haemocyte response was associated with the presence of *Endozoicomonas* spp. under any treatment.

An even ratio of male and female pipi was used across all treatments (Figure 5.5). Field reference pipi were ripe and spawning, whereas the pipi that were placed into tanks were mostly spawning with some post-spawning (Figure 5.5). Pipi examined shared several characteristics between treatments and the field reference samples. These included the presence of *Endozoicomonas* spp., dilated digestive tubules, mucus cells present in gill water channels, encysted metacercariae and hydroids (Figure 5.5). Several differences were observed, however, in experimental pipi compared to field reference pipi (Figure 5.5). All pipi, except those exposed to 24 ppt, had the presence of rod/cocci bacteria and tissue autolysis in both the gill and digestive system (Figure 5.5). An increased haemocyte response was observed in the connective tissue around the digestive tubules of pipi from control, 24°C, 24 ppt and SS tanks (Figure 5.5).

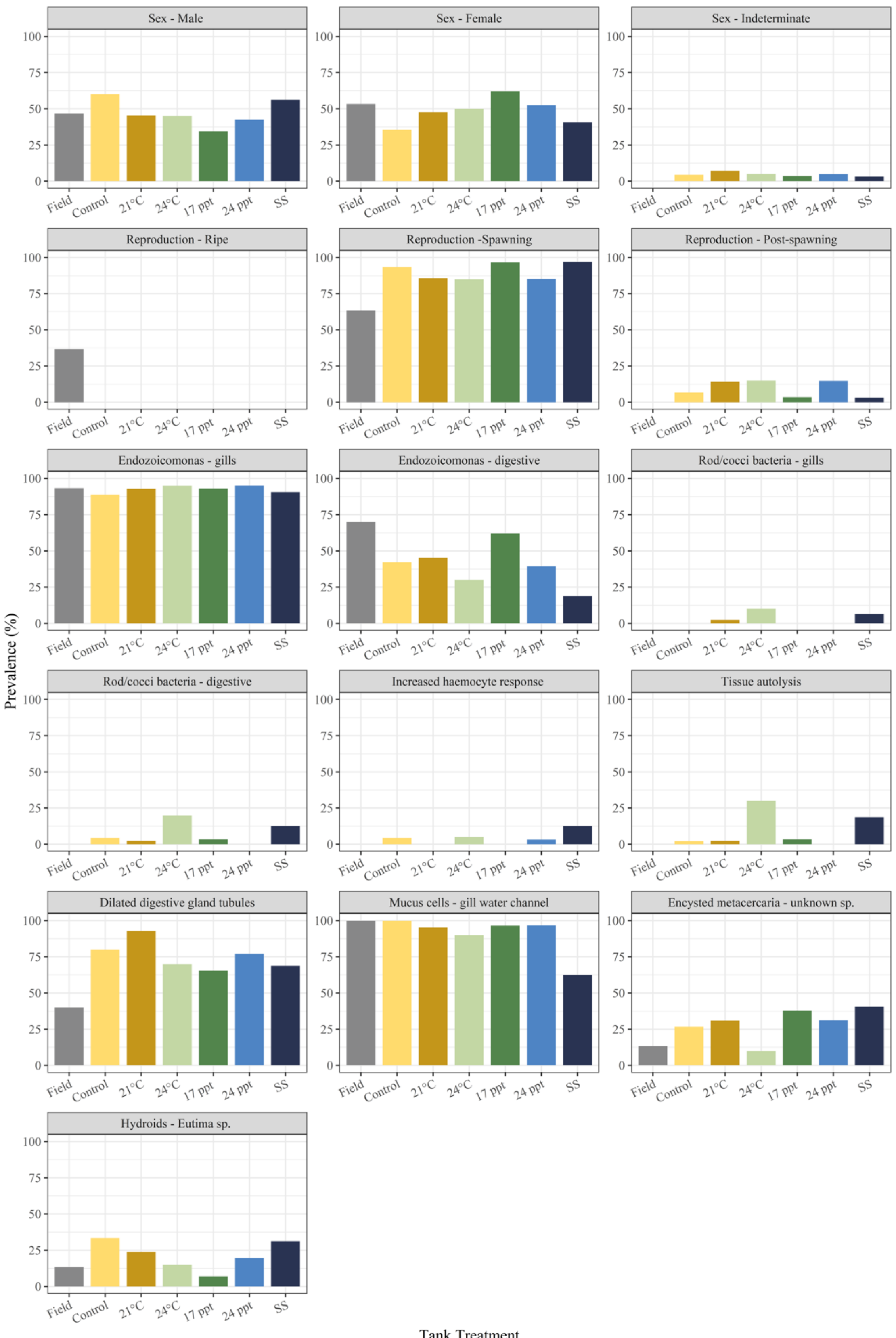


Figure 5.5 Bar chart showing the percentage of individuals with certain characteristics under histology between each treatment and field reference pipi. SS –suspended sediments.

Bacteriology

There was no significant difference in the average bacterial growth observed between the five treatments and the control (Figure 5.6). Three genera were classified as common or dominant bacteria across all treatments: *Hydrogenophaga, Shewanella and Vibrio* (Table 5.3). The number of genera were lowest in treatment 24°C and the highest in pipi from the control tank and 17 ppt, although the diversity between these two tanks did differ (Table 5.3).

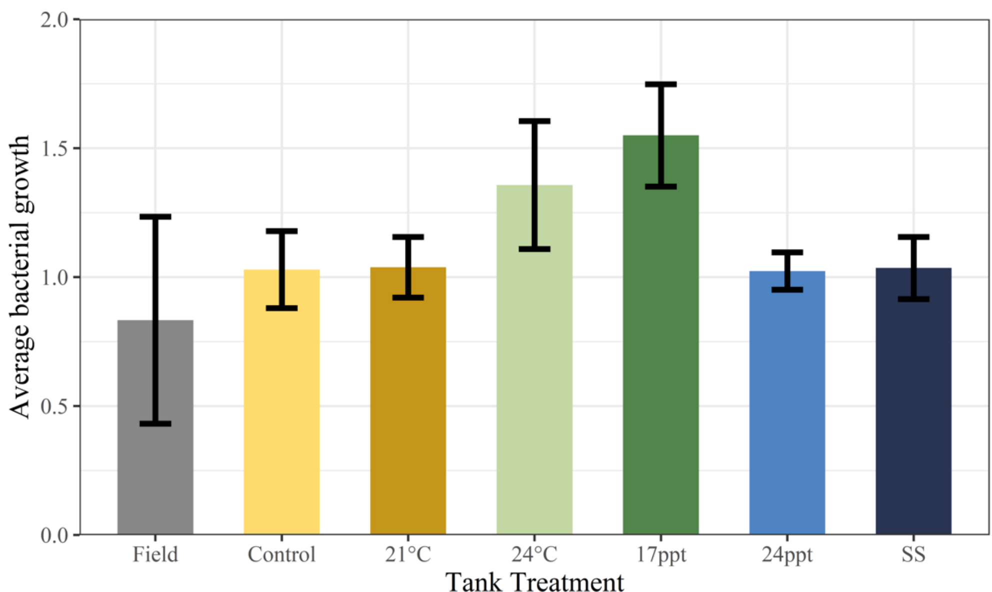


Figure 5.6 Average bacterial growth measured for each tank treatment and field reference pipi. SS –suspended sediments. Bar chart with standard error of mean.

Table 5.3 Summary of bacterial genera isolated (Gram negative & Gram positive) from all five treatments, the control and field reference pipi. SS –suspended sediments.

Gram	**Genus**	**Field**	**Control**	**21°C**	**24°C**	**17ppt**	**24ppt**	**SS**
Positive	*Dietzia*	+	+	+	-	+	+	+
	Microbacterium	+	+	-	-	+	+	-
	Rhodococcus	-	-	-	-	+	+	-
Negative	*Ferrimonas*	-	-	-	+	-	-	-
	Flavobacterium	+	-	-	-	-	-	-
	Hydrogenophaga	-	+	+	+	+	+	+
	Kangiella	-	+	-	-	-	-	-
	Marinobacterium	-	+	-	-	+	-	-
	Photobacterium	-	-	-	-	-	-	+
	Shewanella	+	+	+	+	+	+	+
	uncultured bacterium	-	+	+	-	+	+	+
	Vibrio	+	+	+	+	+	+	+
Total *n* genera present:		5	8	5	4	8	7	6

Mortality pipi

During the experiment some level of mortality of pipi was seen under all treatments, including the control. Significantly higher mortality was seen in the treatments 24°C and SS, with a total cumulative frequency of ~80% and ~65%, respectively. The 24 ppt treatment had the lowest cumulative mortality (Figure 5.7).

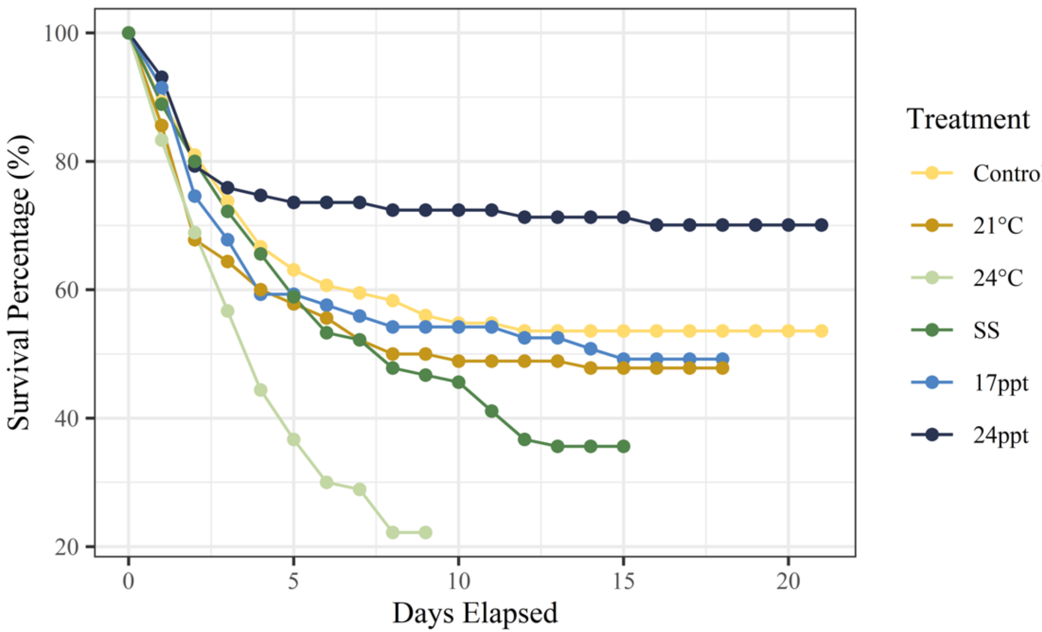


Figure 5.7 Survival percentage of pipi from each treatment in this study. SS –suspended sediments.

Mortality Endozoicomonas spp. qPCR

Endozoicomonas spp. results showed there was a significant difference in *Endozoicomonas* spp. gene copies in the gills from live and mortality pipi. Mortality pipi had a significantly lower median copy number ($p < 0.05$) (Figure 5.8). Significantly lower gene copies of *Endozoicomonas* spp. were detected in the gill tissue of mortality pipi compared to live pipi (p-value < 0.05) (Figure 5.8). Median gene copy numbers of *Endozoicomonas* spp. in the digestive tissue were lower for mortality pipi in control, 21°C, 24°C and 17 ppt (Figure 5.9). Higher gene copy numbers of *Endozoicomonas* spp. were detected at 24 ppt and SS for mortality pipi than live pipi, with a significant difference detected at 24 ppt (p-value < 0.05) (Figure 5.9).

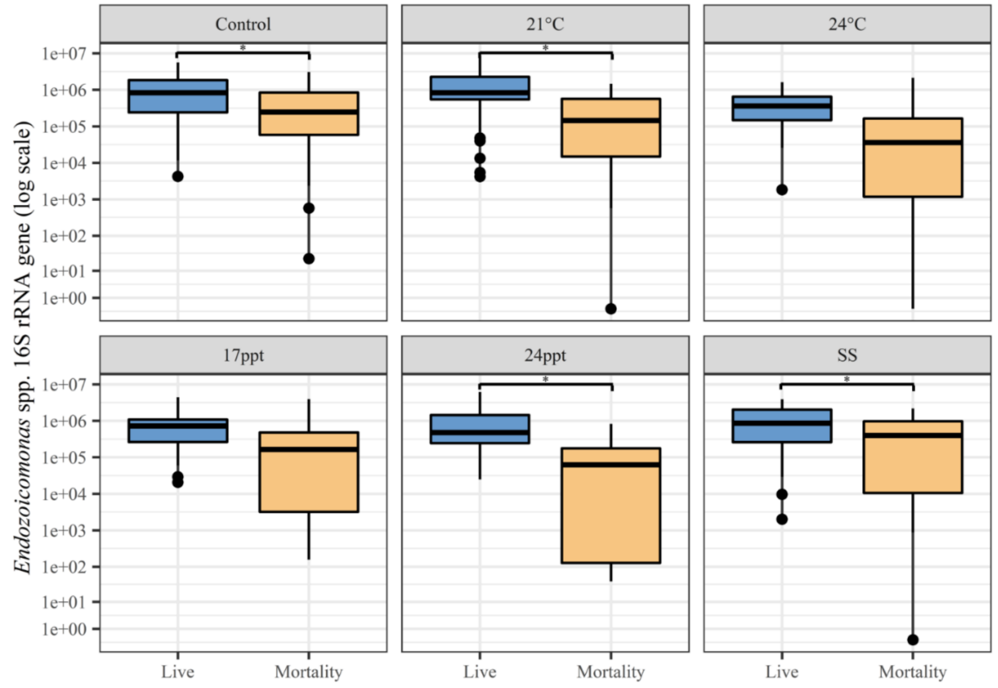


Figure 5.8 *Endozoicomonas* spp. 16S rRNA gene copy number com parison between live and mortality for gill tissue between each tank treatment. SS –suspended sediments. Line: median; box: interquartile range (IQR); whiskers: minimum and maximum values. * represents signficant difference measured (p-value < 0.05).

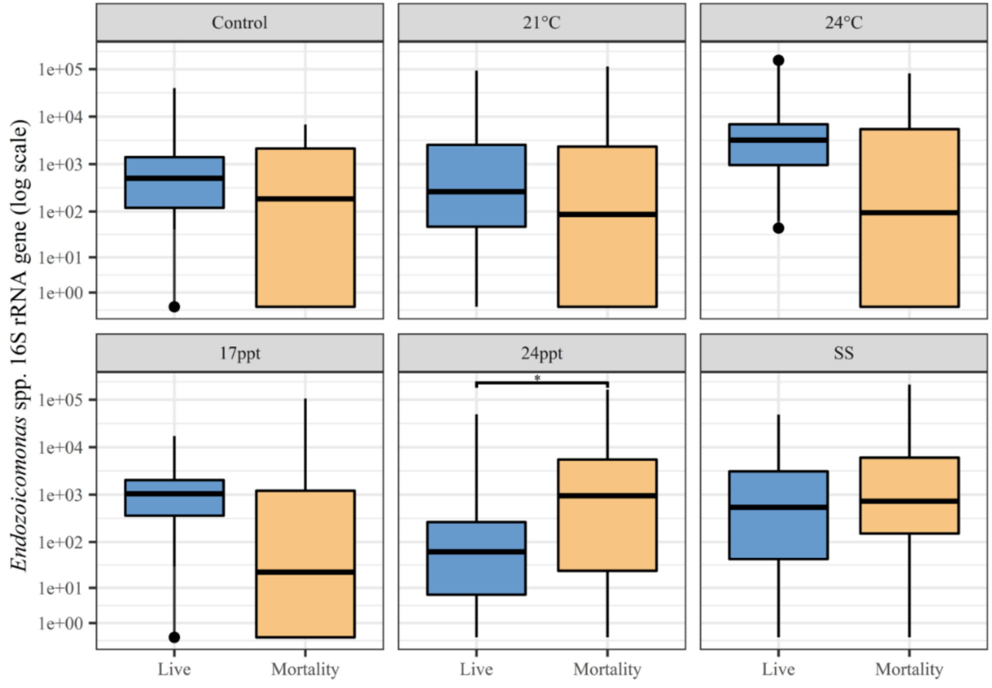


Figure 5.9 *Endozoicomonas* spp. 16S rRNA gene copy number com parison between live and mortality for digestive tissue between each tank treatment. SS –suspended sediments. Line: median; box: interquartile range (IQR); whiskers: minimum and maximum values. * represents significant difference measured (p-value < 0.05).

Mortality histology

Similar observations were made between the live and mortality pipi by histology (Figure 5.10). The main differences observed were a higher proportion of autolysis and a higher prevalence of rod/cocci bacteria in the gills and digestive system in mortality pipi. Mortality pipi also had a lower prevalence of *Endozoicomonas* spp. in the digestive tissues, encysted metacercariae and hydroids than live pipi. Feeding could not be determined due to the level of autolysis in the digestive system.

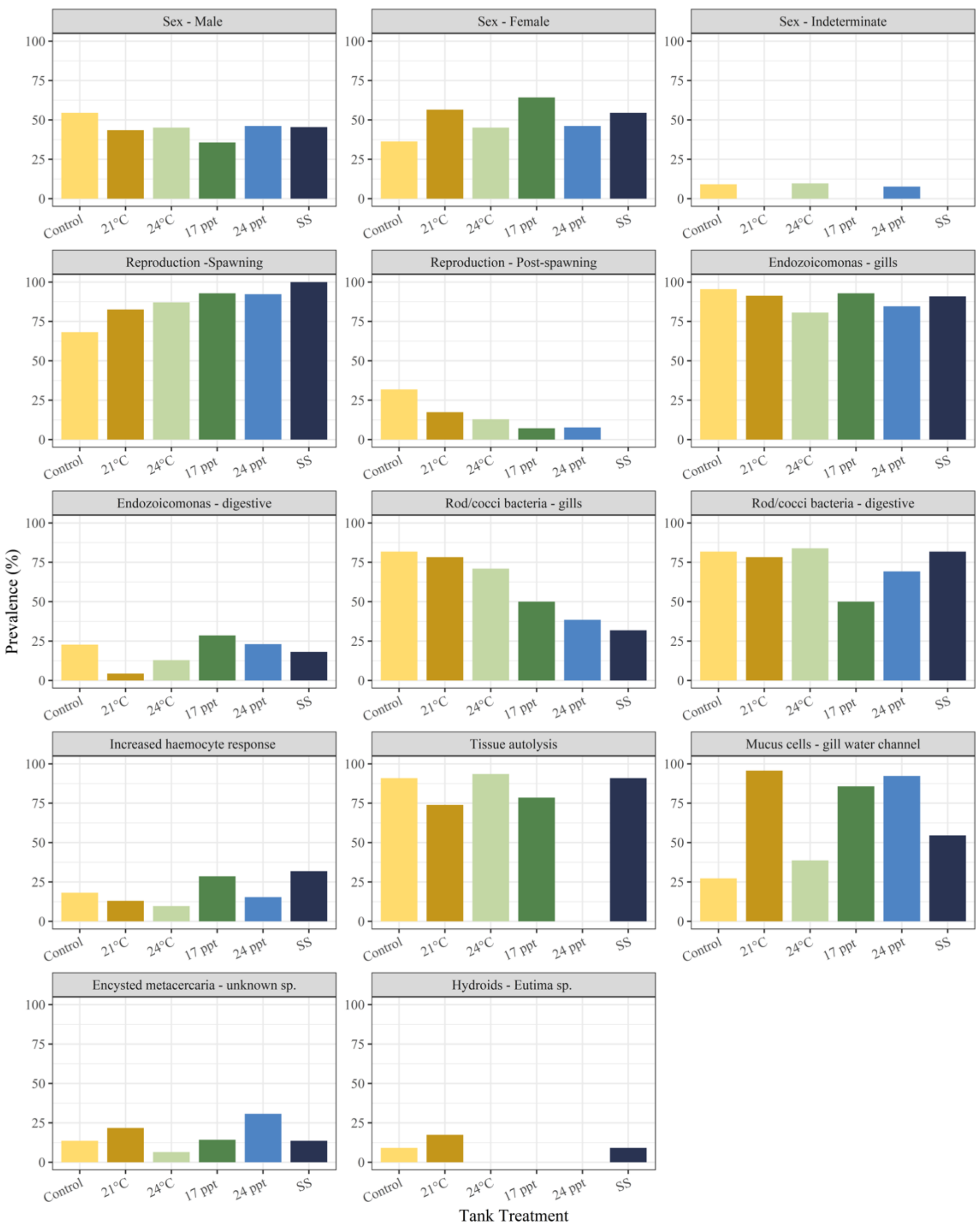


Figure 5.10 Bar chart showing the percentage of individuals with certain characteristics under histology between each treatment for mortality pipi. SS –suspended sediments.

Mortality bacteriology

Significantly more bacteria were grown from mortality pipi than live pipi in all treatments except 17 ppt (p-value < 0.05) (Figure 5.11). *Vibrio* was the only genus that was common across all treatments in mortality pipi. A much lower number of genera was present in mortality pipi in comparison to live pipi (Table 5.4 & Table 5.3).

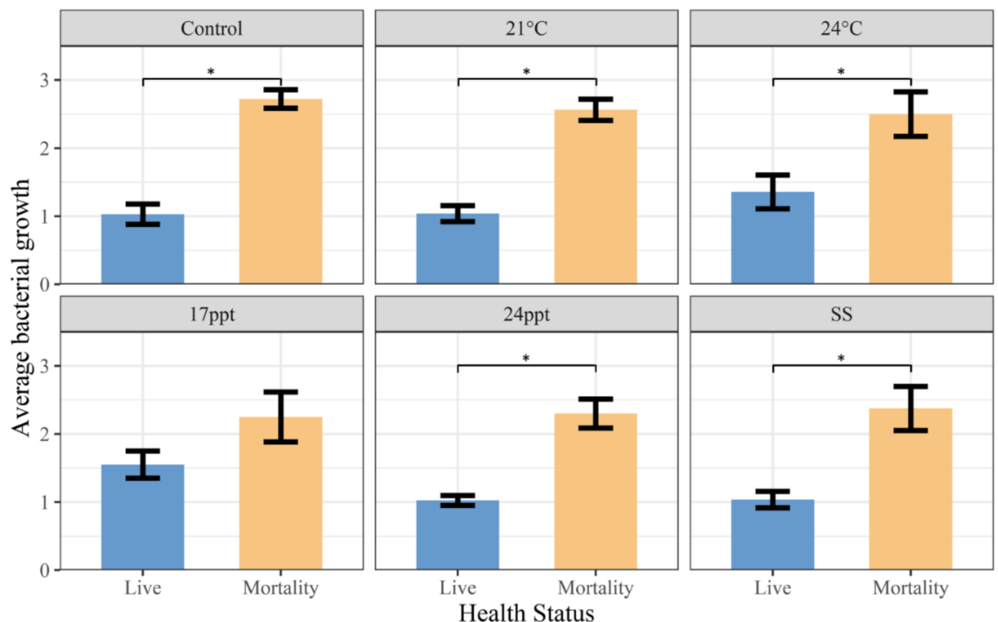


Figure 5.11 Average bacterial growth measured between live and mortality pipi under each treatment. SS –suspended sediments. Bar chart with standard error of mean.* represents signficant difference measured (p-value < 0.05).

Table 5.4 Summary of bacterial genera isolated (Gram negative & Gram positive) from all 6 treatments in moribund pipi. SS –suspended sediments.

Gram	**Genus**	**Control**	**21°C**	**24°C**	**17 ppt**	**24 ppt**	**SS**
Positive	*Dietzia*	+	-	-	-	+	+
	Microbacterium	-	+	+	+	+	+
Negative	*Hydrogenophaga*	+	+	+	-	+	+
	Shewanella	+	+	+	+	-	+
	Vibrio	+	+	+	+	+	+
Total *n* genera present:		4	4	4	3	4	5

Discussion

The aim of this study is to test various environmental stressors that pipi encounter in their natural habitat and examine if unfavourable environmental conditions are linked to increased prevalence and intensity of infection from *Endozoicomonas* spp. By doing so, the study seeks to determine if

Endozoicomonas spp. contribute to mass mortality events of pipi in the Whangārei area. I found that the overall presence of *Endozoicomonas* spp. was highest at a temperature of 21°C and lowest at 24°C. No significant difference in the presence of the bacteria was observed between the other treatment tanks, suggesting that extreme conditions did not affect the presence or intensity of infection. A direct comparison between live and mortality pipi showed a significantly lower presence of *Endozoicomonas* spp. in mortality pipi. Histology and bacteriology allowed for a further exploration into other potential factors contributing to the decline of pipi in Whangārei considering the *Endozoicomonas* spp. results.

Endozoicomonas spp. intensity varied significantly between host gill and digestive tissue. A significantly higher infection was measured in the gills compared to the digestive tissue. An overall average of 1,067,379 gene copies were detected in the gills compared to 5,579 in the digestive system. Differences in bacterial quantity and diversity is common. Higher bacterial diversity was reported from siphons of pipi compared to the digestive tissue of pipi from ten populations around New Zealand (Biessy et al. 2020). Like siphons, gills are exposed to a greater diversity of microorganisms from the surrounding water. Gills serve as the primary organs for both respiration and the capturing and transportation of food particles (Carroll & Catapane 2007), whereas the digestive system is a more isolated organ that is commonly dominated by a lower diversity of bacteria that are associated with specific organ functioning (Lokmer et al. 2016).

Endozoicomonas spp. qPCR results indicated that temperature influenced the infection of *Endozoicomonas* spp. in pipi, with very little difference between the control tank (18°C) and 21°C and then a significantly lower presence of *Endozoicomonas* spp. at 24°C. The results are comparable to those presented in Chapter 3 regarding the pipi baseline, where the mean water temperature during the sampling period was 19°C and a maximum of 26°C. A negative correlation was observed between warmer temperatures and intensity of *Endozoicomonas* spp. The laboratory and field results show that temperature has a negative impact on *Endozoicomonas* spp. intensity. No significant difference in the presence of *Endozoicomonas* spp. was observed in the other treatments tested, suggesting that the bacteria remain relatively stable under changes to salinity and increased amount of suspended sediments.

Increased mortality of pipi in the tank trials allowed us to look at the direct interaction of this bacteria between live and moribund pipi. Assuming that the physiological condition of the pipi was compromised by stressors, such as transportation, tank treatment, change in food source, alterations to exposure of light or other unknown factors, allowed me to determine whether *Endozoicomonas* spp. is an opportunistic pathogen while the host health was compromised. Interestingly, significantly lower intensity of *Endozoicomonas* spp. was observed in the gills of mortality samples compared to live pipi. Host tissue autolysis was common among mortality pipi, indicating that *Endozoicomonas* spp.

that were present in the gills were released and free within the tank water. This could explain why a lower intensity was detected in mortality pipi. Tank water was not collected or tested during this study to test this. However, given that no increase in infection intensity or prevalence was observed across treatments, the results suggest *Endozoicomonas* spp. detected were not opportunistically infecting pipi and therefore unlikely to be a contributing factor in the declining population of pipi in Whangārei.

The decline in *Endozoicomonas* spp. in mortality samples could be indicative of a change in host biome. Anthropogenic stressors on coral species (*Pocillopora verrucosa* and *Acropora hemprichii*) lead to an imbalance in microbiome community and resulted in a decrease in the dominant microbes belonging to Endozoicomonaceae (Ziegler et al. 2016). Research into the sponge (*Arenosclera brasiliensis*) microbiome has shown that *Endozoicomonas* spp. produce a range of bioactive compounds, and it is likely that this bacteria produces antibiotic compounds in sponges which may play a role in protecting their host organisms from pathogens and environmental stressors (Rua et al. 2014). If *Endozoicomonas* spp. has a similar role to that in coral and sponges as in bivalves the decline in the presence of this bacteria under stressful conditions may be indicating an overall decline in the microbiome health of the host. The role of these bacteria in bivalve health can only be hypothesized based on the findings in the study and research on these bacteria in other host organisms. The next steps to better understanding the relationship between *Endozoicomonas* spp. and bivalves would be to culture this bacteria and perform whole genome sequence to assess its transcriptomic and proteomic response in the host tissue as has been done in coral and sponges.

Interestingly, despite its much lower intensity, the presence of *Endozoicomonas* spp. in the digestive system did the opposite of that in the gill in the live pipi. At 24°C *Endozoicomonas* spp. was significantly more abundant than all other tank treatments (Figure 5.4). In assuming that at 24°C the pipi becomes stressed it could be hypothesized that if these bacteria are in fact mutualistic. When the pipi becomes stressed it may impact respiration and nutrient uptake in the gills and proliferation of this bacteria may be compromised. Whereas the digestive system is an internal organ and may maintain a source of nutrients which may allow this bacteria to thrive whilst other organs of the bivalve are stressed. It is therefore hypothesised that the gill tissue may well be a more accurate organ to test the abundance of this bacteria due to its stability in the microbiome and as an indicator to a stressed host.

Although wild pipi are subjected to a multitude of internal and external stressors, this controlled experiment provides an insight to some of the host, pathogen, environment interactions that may be at play other than the interactions of the bacteria *Endozoicomonas*. Histology and bacteriology diagnostic testing provided an additional snapshot of the physiological condition and presence of aerobic bacteria present in pipi. Similar observations were made by histology and similar bacteria were isolated between treatments. Although not statistically significant, a higher rate of bacterial

growth was observed in conditions of higher temperature and lower salinity. This suggests that with increasing climate change interactions, a negative effect of these two stressors may intensify, potentially impacting the health of these species. A significant difference was observed when pipi died during experimentation. Bacterial growth in every tank treatment between live and mortality had a significant difference with the growth in mortality being significantly higher. Additionally, the community of bacteria present was less diverse in mortality pipi then live pipi. During mortality events in oysters (*Crassostrea* sp.), the bacterial community in oysters can shift towards lower diversity, with a higher abundance of certain opportunistic pathogenic bacteria (Lokmer et al. 2015). This shift in bacterial community can be caused by physiological stress that can affect their immune system functioning affecting the microbial environment within the animal (Richard et al. 2021. *Vibrio* was constant across all mortality pipi. *Vibrio* spp. are complex bacteria that are known to have opportunistic pathogenic characteristics however identification of this bacteria is complicated. The genus *Vibrio* contains over 100 identified species that are closely related and difficult to distinguish (Jones 2017). If *Vibrio* spp. were a causative agent in these mortalities, it was likely an opportunistic species that likely thrived under either the tank treatments or the physiological stress of the animals.

Mortality observed in the tank trials could well be a physiological weakness in these cohort of pipi due to their age. From the length data collected, we can see that these pipi are an older cohort (maximum length 71 mm). Mair Bank pipi have had increasing reports of declining populations size and increased presence of mortality in this area and physiological weakness due to old age may be a factor (Williams et al. 2017). Significantly higher mortality was experienced in treatments of 24°C and suspended sediments which are likely to be more stressful on the hosts than the other treatments used. Another observation of note that could have impacted the physiological condition is spawning in these pipi. Histology revealed that all pipi, male and female, were undergoing spawning to post-spawning reproductive phases. The cooccurrence of warm temperatures and spawning has been linked to summer mortalities of other bivalve species (Hong et al. 2014). However, even after spawning, surviving individuals may experience physiological stress caused by post-spawning energy deficiency. Bivalves with altered physiological states are unlikely to be able to defend themselves against secondary stressors, such as modifications to environmental conditions (Hong et al. 2014). This may well be what has caused the high mortality observed in these tank trials. Although acclimatisation conditions were adopted based on the environment they came from, laboratory-based work cannot replicate every detail and the pipi would have had some element of stress to the system from both the transportation and the change in environmental conditions. I could see in histology from the field reference pipi, that a number of the pipi were in ripe/full reproductive phase and it may be that transportation of these animals unfortunately coincided with a spawning event. Interestingly from the cumulative mortality we can see that pipi held at 24°C had the highest cumulative mortality, similar to that of cooccurrence of spawning and warm temperatures causing summer mortalities in

Pacific oysters (Huvet et al. 2010). The combination of an older cohort, spawning and a secondary stressor of transportation and changing environment is likely to cause significant stress to physiological condition of these animals.

Interestingly pipi held at 24 ppt have a relatively low mortality rate compared to the other treatments including the control which was based on the average water conditions these pipi usually experience at Mair Bank, Whangārei Harbour. It is believed that as pipi mature into adult life stage, they develop a greater tolerance for higher salinity water, which enables them to relocate from estuarine habitats to more open water environments with higher salinities (Hooker 1995). This is likely an adaptive behaviour to avoid other environmental stressors (Cummings & Thrush 2004), as it has sometimes been observed as an adaptation for the success of spawning or feeding (Tettelbach et al. 2017). It is intriguing to note that, despite their potential preference to lower salinities, as seen in this study, if pipi are found in the harbour mouth in higher salinity water it may be a survival technique to evade other stressors. This suggests that they may be relocating from a stressful environment to another environment that is comparatively less stressful, that may still cause physiological weakness in this population.

The knowledge gained from this experiment should not be considered as stand-alone findings but should be paired with field-based experiments and observations, such as was collated in Chapters 3 and 4 of this thesis. *Endozoicomonas* spp. is not responsible for the decline of pipi in the Whangārei region based on data collected during field surveys and laboratory experimentation. A comparison between live and mortality pipi under different environmental treatments revealed a lower intensity of infection, indicating that *Endozoicomonas* spp. could be an important bacteria to bivalve microbiome similar to other hosts it has been identified in. Environmental conditions that pipi were exposed to during this experiment led to mortality. Warm water (24°C) and sedimentation had a significantly higher mortality rate than the other treatments. It is likely these environmental conditions will be unfavourable to pipi health in natural settings. It is unknown if these changes were to occur over an extended period whether pipi would be able to adapt to these conditions, though it does suggest that if there was a sudden shift to these extremes it is unlikely that pipi would be able to cope. This study only looked at a singular stressor per tank, the impacts of multiple stressors is an ecological reality that cannot be replicated under laboratory settings. Bivalves commonly experience multiple stressors within the environment, for example the stressors of spawning coinciding with storm events, which can alter salinity levels, increasing sedimentation is not an uncommon occurrence. Although this study looked at singular characteristics it can still provide some knowledge on the simplified interactions between some of the stressors that bivalves may be influenced by to aid in the interpretation of field-based observations.

Chapter 6 : A retrospective analysis of mortality events of culturally significant bivalve pipi (*Paphies australis*) in Whangārei

1949 Ruakākā River and Whangārei Harbour. Alexander Turnbull Library, Wellington, New Zealand (BY CC 4.0).

Introduction

The occurrence of mass mortalities in marine organisms is commonly attributed to a combination of factors rather than a single cause (Petton et al. 2021). The combination of factors, such as pathogens and pollutants, collectively put pressure on the host, resulting in stress and reduced resilience to environmental extremes or infection (Soon & Ransangan 2019; Esposito et al. 2022). For cultured bivalves, there is a considerable amount of information on viruses, bacteria, fungi, protozoans and helminths (Gosling 2015). This is often not the case for wild remote bivalve species, which are less studied from a disease perspective than commercially important farmed populations (Bennion et al. 2022; Lane et al. 2020). It is normal to observe bacteria, fungi, protozoans and helminths when examining wild shellfish health, but their presence doesn't necessarily result in disease. They could make up the normal host parasite community and form part of the normal host microbiome (Zannella et al. 2017; Bennion et al. 2022). Some parasites, however, may be opportunistic leading to greater pathogenicity when conditions are favourable for the agent or stressful for the host, compromising its immune system (Destoumieux-Garzón et al. 2020). Howells et al. (2021) highlighted the scarcity of baseline health data on wild bivalves, which poses challenges when attempting to determine whether a particular agent is part of the host microbiome or a potential cause of a mass mortality event. This issue becomes even more pronounced when studying lesser-studied species with little data on parasites and pathogens (Lane et al. 2020; but see Bennion et al. 2021). Baseline health data can provide valuable insights by establishing expected prevalence and infection intensity in specific hosts at specific times (Chapter 3). Understanding how external factors may influence prevalence and intensity is the next step in uncovering drivers of mass mortality events (Chapter 4).

The Whangārei area has a rich diversity of bivalve species, including pipi (*Paphies australis*), tuatua (*Paphies subtriangulata*), cockles (*Austrovenus stutchburyi*), green-lipped mussels (*Perna canaliculus*) and scallops (*Pecten novaezelandiae*) (Chetham 2014). Since 2009, there have been several documented mass mortalities of pipi, cockles, and tuatua in the region that have been submitted to Biosecurity New Zealand (BNZ) for disease investigation (Bingham 2013; BNZ unpubl. data). No exotic disease was identified during these investigations, although several bacteria were isolated and detected but not identified as the causative agent (Howells et al. 2021). Pipi, in particular, in the region have had increasing anecdotal reports of declining health which coincides with a substantial decrease in pipi biomass (Pawley et al. 2013; Williams et al. 2017). Unfortunately, despite the combined efforts from a number of institutes looking into the health of pipi in this region, there has been no definitive answer to the causes of decline (Williams et al. 2014).

Wild bivalve mass mortalities are not commonly reported in scientific literature and assigning causation is very difficult (Soon & Ransangan 2019). Wild bivalve populations represent a significant part of the ecosystem, supporting fisheries, habitat, and cultural and social services (Kainamu-

Murchie et al. 2018; Smaal et al. 2019). There is a need to review what information is available on factors that can influence bivalve health to understand drivers of mass mortality events and stock declines. Bennion et al. (2022) provided a comprehensive study on the culturally significant surf clam toheroa (*Paphies ventricosa*) in New Zealand that has undergone dramatic declines in health with no signs of recovery. In that study, disease wasn't identified as a driver in the declining health of toheroa, however, if it were, it would have been identified too late and effective management wouldn't have been put in place in time. Bennion et al. (2022) highlighted the need for such work to be undertaken before stock of these wild populations reach a point of no return to ensure that sustainable conservation and management efforts can be enacted in a timely manner. Conducting surveillance on all wild bivalve species around New Zealand in a "timely" manner is not always possible and therefore other avenues of resources need to be explored to aid in early detection of declining health, whether the cause is an emerging disease or a shift in water quality.

Often data remains underutilised due to a lack of awareness about its existence and accessibility. Biosecurity New Zealand (BNZ) plays a crucial role in identifying and preventing the entry of unwanted diseases into the country and managing endemic diseases (Lane et al. 2020). Annually, BNZ investigates anywhere between 10 and 30 bivalve mortality events (Williams 2021; Williams 2022; Taylor 2022). Investigations of suspected exotic disease use various diagnostic testing methods including histology, bacteriology, and PCRs for different marine pathogens. As a result, BNZ possesses information on the presence and absence of a range of organisms including bacteria, fungi, helminths and protozoans that are incidentally observed during the testing process. Essentially, this information serves as a baseline reference for the occurrence of agents in wild bivalve mass mortality events throughout the country. To date, this data set remains relatively unexplored as a source of baseline data in monitoring long-term trends.

This chapter examines archival data from pipi mortalities submitted to BNZ from the Whangārei area, compiling observations and incidence of organisms made during disease investigations. It compares these findings with observations from healthy pipi in the same area (Chapter 3) to offer insights for future diagnostic assessments. Additionally, the study aims to analyse the abundance of *Endozoicomonas* spp. and correlate them with water quality data to examine the relationship of this bacteria and potential factors driving the occurrence of these mortalities.

Materials & Methods

Mortality pipi

The BNZ Laboratory Information Management System (LIMS) was searched to identify archived pipi mortality cases in the Whangārei area. Ten separate pipi mortality events occurred between 2013 and 2022 from five different locations around the Whangārei area. Data could not be retrieved any further

back than 2010 due to retrieval problems with non-digitised records. The mortality events occurred at Marsden Bank, Mair Bank, Munroe Bay, One Tree Point and Pātaua South (Figure 6.1) (total pipi n = 134). Refer to Table 6.1 for sample summary. It is important to note that not all information from the archive investigation was available. In many cases, weight and length data were missing as this was not routinely recorded, and bacteriology and molecular work were not always conducted likely due to poor sample quality. Mortality samples from the Mair Bank Nov-20 and May-21, and Pātaua South Jul-22 were used in Chapter 4.

Healthy pipi

Healthy pipi used for analysis in this chapter were pipi collected in Chapter 3. Healthy pipi were collected from four populations in the Whangārei area, including Waipū River, Ruakākā River, Mair Bank and One Tree Point between 2020 and 2021 (total n = 640) (Table 6.1) (Figure 6.1).

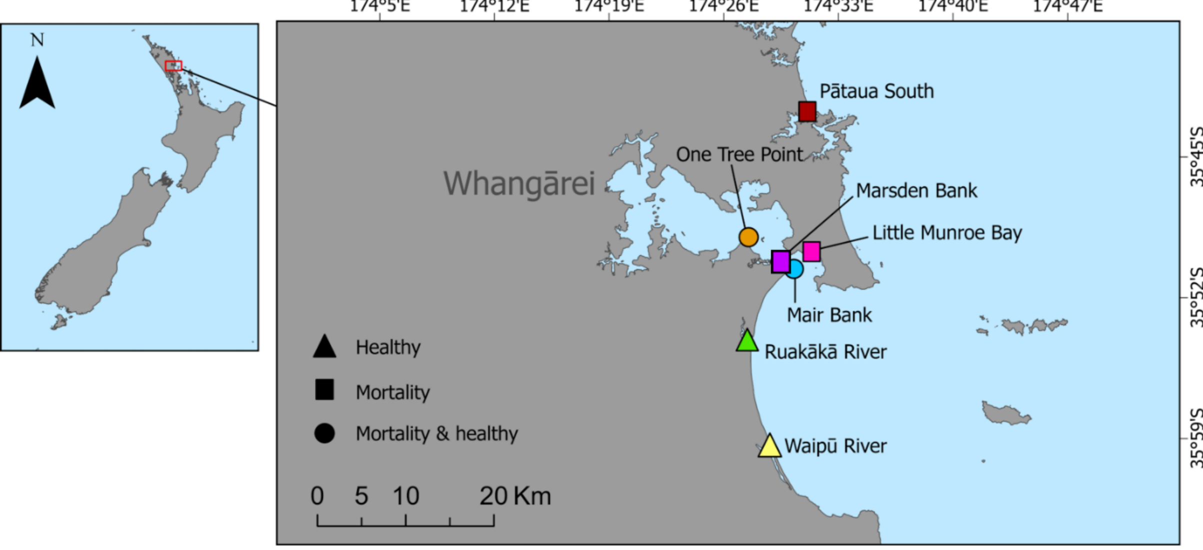


Figure 6.1 Map of New Zealand in the top left corner. Close-up localised map showing Whangārei and locations of sample collections. Key: triangle: healthy samples, square: mortality samples, circle: both health and mortality sample collections.

Table 6.1 Summary of mortality and healthy pipi events in the Whangārei area used in this study. With details on locations, health status, average length and weight, total number of animals collected and used in Histology (Histo.), Bacteriology (Bacto.) and *Endozoicomonas* spp. qPCR (qPCR).

Collection Population	Habitat type	Date	Health status	*n*	Avg. Length (mm)	Avg. Weight (g)	*n* Histo.	*n* Bacto	*n* qPCR
Mair Bank	Lower estuary	Apr-13	Mortality	10	NA	NA	100	0	0
Mair Bank	Lower estuary	Nov-20	Mortality	12	59	25.8	8	7	8
Mair Bank	Lower estuary	Dec-20	Mortality	18	58	25	10	10	18
Mair Bank	Lower estuary	May-21	Mortality	10	62.1	33.4	5	10	0
Mair Bank	Lower estuary	Feb-20	Healthy	25	54.7	NA	20	10	20
Mair Bank	Lower estuary	May-20	Healthy	20	59.9	NA	20	5	20
Mair Bank	Lower estuary	Aug-20	Healthy	20	56.6	NA	20	5	20
Mair Bank	Lower estuary	Nov-20	Healthy	25	59.9	25.6	20	10	20
Mair Bank	Lower estuary	Feb-21	Healthy	20	53	17.9	20	5	20
Mair Bank	Lower estuary	May-21	Healthy	25	56.9	22.8	20	5	20
Mair Bank	Lower estuary	Aug-21	Healthy	20	52.4	19.9	20	5	20
Mair Bank	Lower estuary	Nov-21	Healthy	20	61.1	30.2	20	5	20
Marsden Bank	Lower estuary	May-13	Mortality	10	NA	NA	10	0	0
Marsden Bank	Lower estuary	Feb-18	Mortality	15	NA	NA	15	15	13
Pātaua South	Lower estuary	Jul-22	Mortality	10	51.2	16.63	10	5	5
Munroe Bay	Estuary	Jan-19	Mortality	9	NA	NA	5	0	9
One Tree Point	Estuary	Feb-19	Mortality	20	NA	NA	20	0	20
One Tree Point	Estuary	Mar-19	Mortality	20	NA	NA	20	0	20
One Tree Point	Estuary	Feb-20	Healthy	25	39.9	NA	20	10	20
One Tree Point	Estuary	May-20	Healthy	20	45.9	NA	20	5	20
One Tree Point	Estuary	Aug-20	Healthy	20	44.4	NA	20	5	20
One Tree Point	Estuary	Nov-20	Healthy	25	47	13.9	20	10	20
One Tree Point	Estuary	Feb-21	Healthy	20	45.3	11.8	20	5	20
One Tree Point	Estuary	May-21	Healthy	25	43.7	10.4	20	5	20
One Tree Point	Estuary	Aug-21	Healthy	20	45.6	12.2	20	5	20
One Tree Point	Estuary	Nov-21	Healthy	25	46	12.8	20	5	20
Ruakākā River	River	Feb-20	Healthy	25	35.7	NA	20	10	20
Ruakākā River	River	May-20	Healthy	25	40.4	NA	20	5	20
Ruakākā River	River	Aug-20	Healthy	25	38.7	NA	20	10	20
Ruakākā River	River	Nov-20	Healthy	25	41.7	6.2	20	10	20
Ruakākā River	River	Feb-21	Healthy	20	39.9	5.6	20	5	20
Ruakākā River	River	May-21	Healthy	25	38.6	5.4	20	5	20
Ruakākā River	River	Aug-21	Healthy	20	40.5	6.8	20	5	20
Ruakākā River	River	Nov-21	Healthy	25	41.5	6.7	20	5	20
Waipū River	River	Feb-20	Healthy	25	42.5	NA	20	10	20
Waipū River	River	May-20	Healthy	20	42.9	NA	20	5	20
Waipū River	River	Aug-20	Healthy	25	45.9	NA	20	10	20
Waipū River	River	Nov-20	Healthy	25	43.6	8.6	20	10	20
Waipū River	River	Feb-21	Healthy	20	43.9	8.9	20	5	20
Waipū River	River	May-21	Healthy	25	41.6	7.8	20	5	20
Waipū River	River	Aug-21	Healthy	20	44.1	10.4	20	5	20
Waipū River	River	Nov-21	Healthy	25	45.7	10.9	20	5	20

Archived test results

Laboratory results of healthy pipi were the same as presented in Chapter 3. Laboratory results of mortality pipi from Mair Bank Nov-20 and May-21, and Pātaua South Jul-22 were the same as Chapter 4.

Diagnostic tools used for archived cases (sites Mair Bank, Marden Bank, Munroe Bay and One Tree Point for years 2013-2019, and additionally Mair Bank in Dec-20) were similar to those used in Chapter 3 and Chapter 4, however, specific methodology might vary between cases. We reviewed all case work and test data held by the BNZ Animal Health Laboratory. Observations and results from each of the mortalities and baseline pipi cases were extracted and used in the analysis. We extracted data on histology, bacteriology and qPCR results for *Endozoicomonas* spp.

For histology, all archive mortality histology slides were reanalysed for this study. Each sample was examined for general health status using an Olympus BX51 microscope. Histology was analysed based on the presence or absence of pathological observations rather than the intensity of these observations.

For bacteriology, case reports were reviewed and bacteria isolated and notes of growth were extracted for the purpose of this study. All bacterial isolates that were described as common or dominant in each mortality were considered significant to this study.

Quantification of *Endozoicomonas* spp. was generated from archived qPCR BioRad CFX Manager files, a standard curve was used to convert cycle threshold (Ct) values into quantitative values based on known concentration of target gene from the positive controls used. Quantitative values were extracted for the purpose of this study.

Environmental data

Environmental data were sourced from the Northland Regional Council (NRC). NRC routinely collects water quality samples from 44 sites in the Northland region (refer to Figure 6.1 for location of Northland). From 2010-2017, samples were collected bi-monthly and monthly from 2017 onwards. For this study, water temperature, salinity, total suspended solids, and dissolved oxygen were selected for analysis because these are known variables to impact host and organism conditions (Table 6.2). Environmental data were not always available from the specific sample site or date of collection for mortality events. I selected the closest water quality site to the mortality event and the closest date preceding the mortality event (Appendix D, Table D.1). For healthy pipi, water quality sample sites were collected from all four populations (Waipū River, Ruakākā River, Mair Bank and One Tree Point) and from the closest date prior to collection (Appendix D, Table D.1).

Table 6.2 Water quality parameters used in this study and NRC sampling procedure used (Griffiths 2021).

Parameter & unit measured	NRC sampling procedure
Temperature (°C)	In situ field measurement handheld YSI meter
Salinity (ppt)	In situ field measurement handheld YSI meter
Total suspended solids (mg/L)	Total suspended solids by gravimetry APHA 2540 D
Dissolved oxygen (mg/L)	In situ field measurement handheld YSI meter

Statistical analysis

Endozoicomonas spp. qPCR

Gene copy numbers from the qPCR results were used as a proxy to compare infection intensity between each of the mortality cases and each healthy collection (healthy samples from Chapter 3 were pooled). Using a general linear model (GLM), comparisons between healthy and mortality pipi were tested using Tukey contrasts, and p-values were adjusted for multiple testing using Benjamini-Hochbergs FDR (Benjamini & Hochberg 1995, Hothorn et al. 2008). A p-value <0.05 was considered statistically significant. Statistical analyses were performed in R Studio (version 4.1.3, R Core Team 2022).

Endozoicomonas spp. qPCR & environmental data

Pearson's correlation was carried out between *Endozoicomonas* spp. gene copies and each of the four environmental variables (Appendix D, Table D.1) separately for healthy and mortality pipi. A correlation matrix was performed between the four environmental variables: temperature, salinity, total suspended solids and dissolved oxygen to understand the colinear relationship in these variables to aid in interpretation of data with the diagnostic results. Statistical analyses were performed in R Studio (version 4.1.3, R Core Team 2022).

Results

Histology & bacteriology

Histological observation provided information on the host, comprising of sex and reproductive phases pipi were undergoing and pathological observations seen in the different organs of both healthy and mortality pipi (Figure 6.2). It was common to see light focal haemocyte response in all healthy and mortality pipi around the digestive structures and gills tissue. Inflammation was only recorded when it exceeded a low level of what is considered to be common. The haemocyte response was observed to be either diffused with a significant increase in abundance, extensively targeted to one organ, or observed throughout (Figure 6.2).

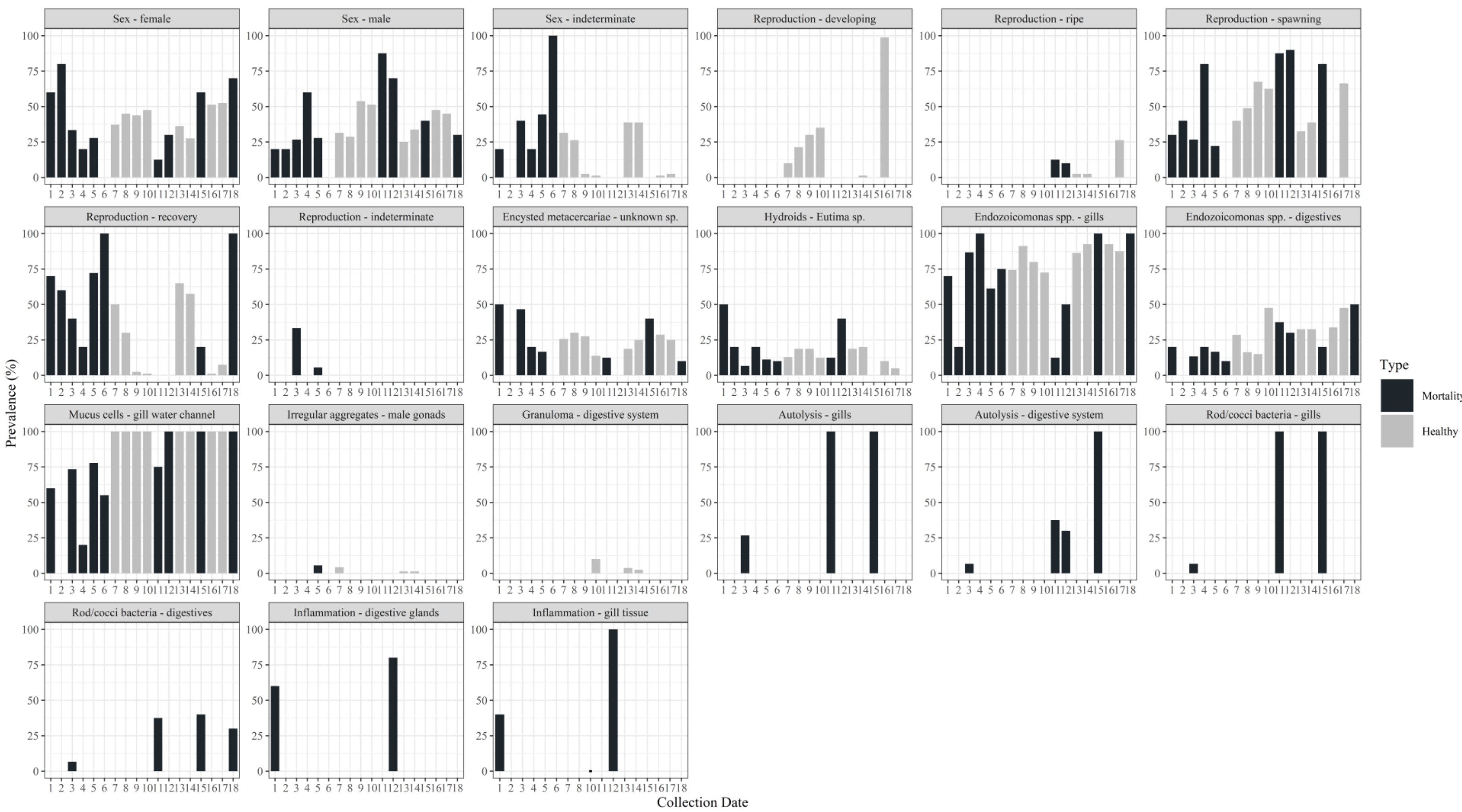


Figure 6.2 Proportion of observations in histopathology from each of the collection of pipi from Whangārei for both mortality (black) an d healthy (grey). Key for the x axis: 1. Apr-13, 2. May-13, 3. Feb-18, 4. Jan-19, 5. Feb-19, 6. Mar-19, 7. Feb-20, 8. May-20, 9. Aug-20, 10. Nov-20, 11. Nov-20, 12. Dec-20, 13. Feb-21, 14. May-21, 15. May-21, 16. Aug-21, 17. Nov-21, 18. Jul-22.

Categorisation of the growth of bacteria between healthy and mortality showed that healthy pipi had a higher percentage of samples that exhibited no growth and mortality pipi had a higher percentage of samples with common or dominant bacteria growth (Figure 6.3). Of the common or dominant bacterial growth from the healthy and mortality pipi cases, isolated bacteria were identified and a comparison of the occurrence of these isolates was carried out (Table 6.3). Of the bacteria present three genera were common in both healthy and mortality pipi: *Photobacterium* spp., *Shewanella* spp. and *Vibrio* spp. (Table 6.3).

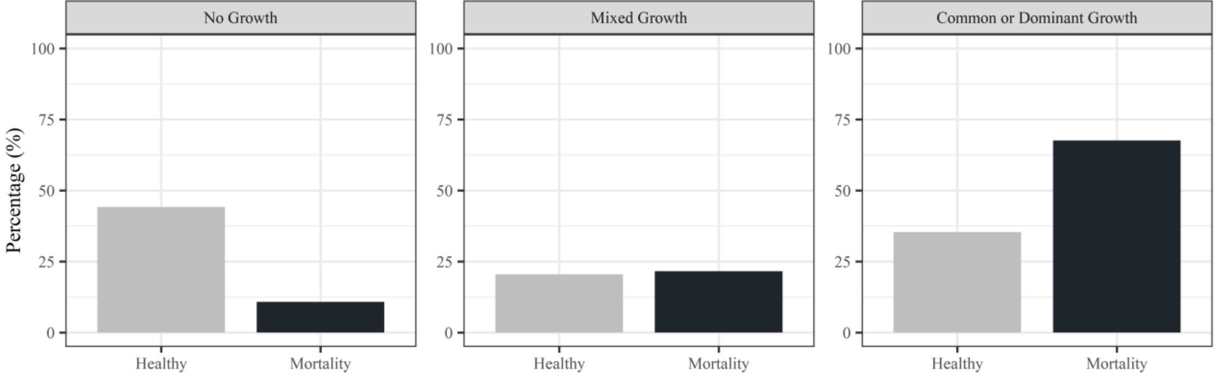


Figure 6.3 Percentages of the type of bacterial growth for all samples collected are shown between healthy and mortality pipi collected.

Table 6.3 Isolated genera of bacteria from each collection where bacteriology was performed, bacteria recorded as either present (+) or absent (-) for every collection. Key: MDB = Marsden Bank, MB = Mair Bank, PS = Pātaua South.

		Mortality					**Healthy**							
Gram	**Genus**	**Feb-18 MDB**	**Nov- 20 MB**	**Dec-20 MB**	**May-21 MB**	**Jul-22 PS**	**Feb-20**	**May-20**	**Aug-20**	**Nov-20**	**Feb-21**	**May-21**	**Aug-21**	**Nov-21**
Positive	*Staphylococcus*	-	-	-	-	-	-	-	-	+	-	-	-	-
Negative	*Kistimonas*	-	-	-	-	-	+	-	-	-	+	+	+	+
	Marinomonas	-	-	-	-	-	-	-	+	-	-	-	-	-
	Pectobacterium	-	-	-	-	-	-	-	-	-	-	-	-	+
	Photobacterium	+	-	-	-	-	+	-	-	-	-	-	-	-
	Pseudomonas	-	-	-	-	-	-	-	+	-	-	-	-	-
	Psychromonas	-	-	-	-	+	-	-	-	-	-	-	-	-
	Shewanella	-	+	-	+	-	-	-	+	+	-	+	-	+
	Uncultured bacterium	-	-	-	-	-	-	-	+	-	-	-	-	-
	Vibrio	+	+	+	+	+	+	+	-	+	+	+	-	+
Total *n* genera present:		2	2	1	2	2	3	1	4	3	2	3	1	4

Endozoicomonas spp. qPCR and environmental data

A significant difference was detected between the overall abundance of *Endozoicomonas* spp. for mortality pipi and healthy pipi. Healthy pipi had a significantly higher number of gene copies present. Significantly higher *Endozoicomonas* spp. gene copy numbers were detected between healthy pipi collected in May-21, Aug-21 and Nov-21 and all mortality pipi (Figure 6.4). For healthy pipi, a weak negative correlation between *Endozoicomonas* spp. gene copy number and temperature was observed, whereas a weak positive correlation was observed between *Endozoicomonas* spp. and dissolved oxygen (Figure 6.5 & 6.6). No correlation was detected between any environmental variable and *Endozoicomonas* spp. abundance for mortality pipi (Figure 6.5 & 6.6).

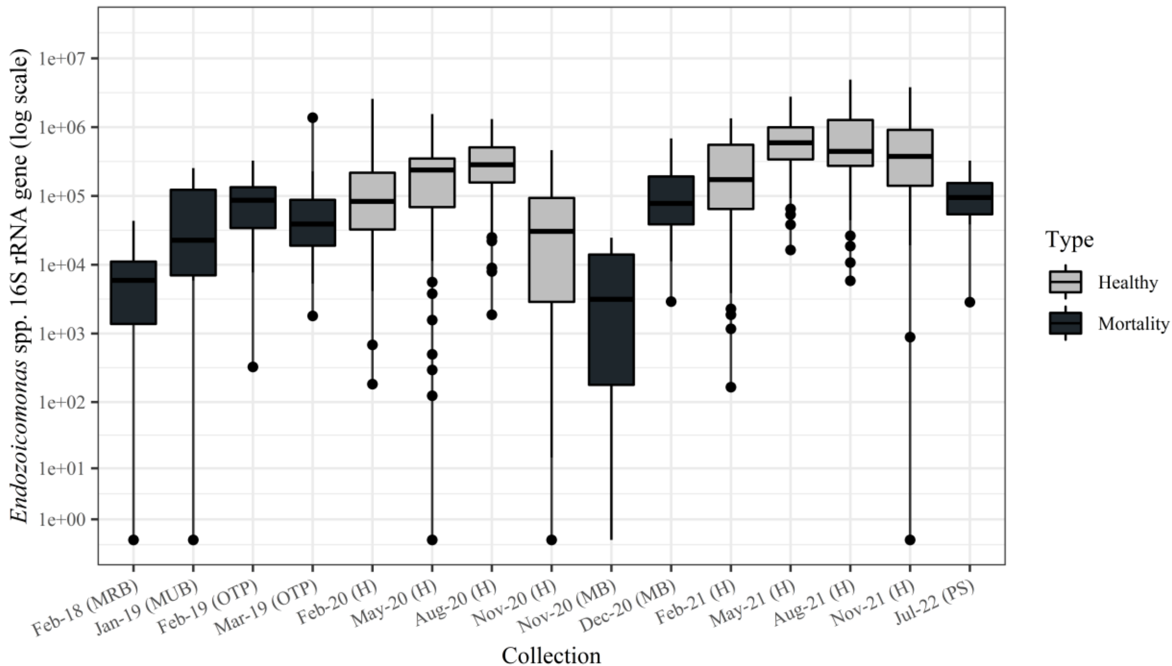


Figure 6.4 *Endozoicomonas* spp. 16S rRNA gene copy number for each collection both healthy and mortality. Line: median; box: interquartile range (IQR); whiskers: minimum and maximum values. Key: MRB = Marsden Bank, MUB = Munroe Bay, OTP = One Tree Point, H = all healthy pipi, MB = Mair Bank, PS = Pataua South.

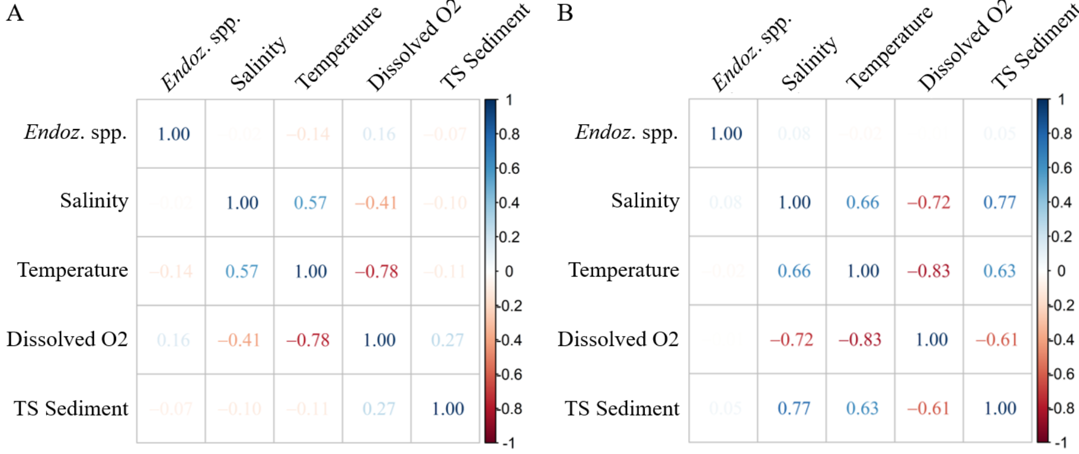


Figure 6.5 Correlation matrix between the environmental variables and *Endozoicomonas* spp. abundance showing correlation coefficient, 1 representing a strong positive correlation, 0 representing no correlation, and -1 representing a strong negative correlation. A) healthy pipi B) mortality pipi. Key: *Endoz.* spp. = *Endozoicomonas* spp. gene copies., Salinity = salinity (ppt), Temperature = Temperature (°C), Dissolved O2 = Dissolved oxygen (mg/L), TS Sediment = Total suspended sediments (mg/L).

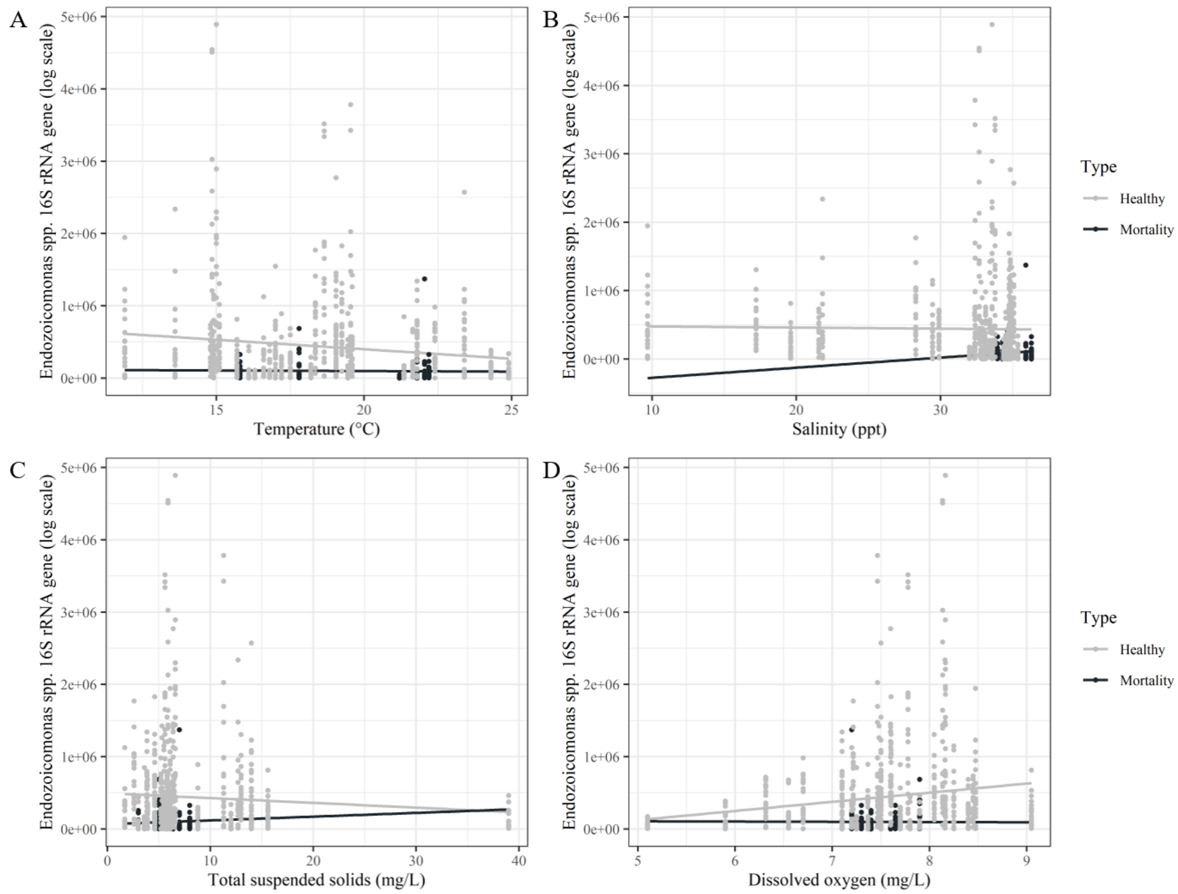


Figure 6.6 Scatter plot of *Endozoicomonas* spp. abundance from both healthy and mortality pipi and four different environmental variables. A) temperature (°C), B) salinity (ppt), C) total suspended sediments (mg/L), D) dissolved oxygen (mg/L).

Discussion

The aim of this study was to compile archived diagnostic results from pipi mass mortality events in the Whangārei area and collate with diagnostic results from healthy and mortality pipi generated in this thesis (Chapters 3 and 4) to add to health baseline of pipi from the area. Additionally, I analysed *Endozoicomonas* spp. abundance data from the above data set to determine if water quality factors were a driver in abundance and could be a factor in mortalities. Examining archival test results from histology, bacteriology and *Endozoicomonas* spp. qPCR supports what has been demonstrated throughout this thesis. This included: (1) *Endozoicomonas* spp. were significantly less abundant in mortality pipi then healthy pipi (Figure 6.4); (2) all mortality events happened when pipi were spawning to post-spawning/recovery reproductive phases (Figure 6.2); and (3) *Photobacterium* spp., *Shewanella* spp. and *Vibrio* spp. were commonly isolated bacteria between morality and healthy pipi with *Vibrio* spp. isolated in every case except for two (Table 6.3). The inclusion of archival mortality events spanning from 2013 to 2020 provides an additional layer of information and data regarding the health of pipi in the Whangārei area. This enriches our understanding of this culturally significant species and enhances the existing knowledge base. Different parasites and host pathology were consistent across healthy and mortality pipi for the period 2013-2022. The collation of both data sets enables assessment of any changes in prevalence and intensity of parasites commonly observed or identify an incursion of a new parasite. This highlights the enduring relevance of the curated baseline data in this thesis and underscores its value in assessing the health of pipi in this region.

Historical data

Historical data is vitally important to understand changes over time and help determine the drivers behind observation of poor health or mass mortalities in the marine environment (Schwerdtner Máñez & Poulsen 2016). Historical data enables anomalies and trends to be identified to support effective health monitoring (Friedman et al. 2014). Natural patterns and trends possibly driven by seasons or host age can be monitored that were previously hard to identify on a case-by-case basis. For example, I have incidentally collected data on the reproductive phases of pipi through this thesis. Unexplored data holds great potential for valuable information that could have significant benefits. In an analogous example, there is an abundance of information from data companies, corporate business and the financial market that have demonstrated that the vast amount of data collected has the potential to contribute to revenue increase but are often left unexplored (Nissim 2022). By utilising the disease investigation data collected by BNZ, emerging patterns and trends may be uncovered which could have critical implications for marine health, particularly in the face of growing marine pressures resulting from climate change (Tracy et al. 2019). Exploring and leveraging this unexplored

data can lead to a better understanding of the marine environment and support informed decision-making processes aimed at preserving and managing marine ecosystems effectively.

An interesting observation from this study was the absence of reports from the Whangārei area sent to BNZ between 2013 and 2018. This could be attributed to various factors. It is possible that there were no instances of mortality or ill health in pipi during that time. Additionally, some people within the community may not have been aware of whom to report such events to, as they may not have recognised it as an ongoing issue. Further, the remote locations of these wild populations make it challenging to firstly detect a mortality event and therefore report them (Howells & Brosnahan 2022). Alternatively, sometimes people lose faith in the available reporting lines and fail to report them either through disengagement or instances where no solution or response could be obtained. Community engagement and reporting is an essential part of an effective passive surveillance system. There is a need for general public and community-based engagement whether biosecurity is managed on a central government scale or by local councils (Payne et al. 2023). Communities are typically more invested and aware of the general overall health of their "backyard" and are therefore more likely to identify when something doesn't look quite right (Payne et al. 2023). Acquiring consistent data on bivalve mass mortalities requires communication and engagement. People are more likely to report another mortality event if they are aware that the data is going to be utilised to improve surveillance monitoring purposes.

Environmental correlations

In mortality pipi no correlation was detected between *Endozoicomonas* spp. abundance and environmental variables, but a weak positive correlation was detected between abundance and dissolved oxygen in healthy pipi. When examining the distribution of the data, however, it is unlikely to be a true correlation and it suggests instead that an optimal environment for *Endozoicomonas* spp. exists at approximately 7-8.5 mg/L of dissolved oxygen. In this range, the conditions may be more favourable for the growth and proliferation of *Endozoicomonas* spp. Similarly, although a weak negative correlation was detected between water temperature and abundance of *Endozoicomonas* spp., the highest abundance was detected between 15-20°C. In the Northland region between 2018-2020 the median coastal water temperature recorded from 44 sites was between 16.6°C and 18.4°C (Griffiths 2021). Water temperatures above 20°C are therefore likely to be unfavourable to pipi in this region, that in turn will reduce the abundance of *Endozoicomonas* spp. if the bacteria are indeed symbiotic. This trend was detected during tank trials (Chapter 5), when significantly lower abundance of *Endozoicomonas* spp. was detected at a temperature of 24°C and had significant impact on host survival probability. The distribution of the salinity data was influenced by sampling location. For example, lower salinity data was available for pipi collected from a river compared to higher salinity at the harbour mouth. This creates data stratification. As a result, it becomes more challenging to

identify and assess the correlation between salinity and *Endozoicomonas* spp. Likewise with total suspended sediments, it was hard to identify the true correlation, because the majority of data is distributed between 0-15 mg/l, with only one sampling event occurring at ~40 mg/L and therefore difficult to discern the correlation between *Endozoicomonas* spp. and suspended sediments. If *Endozoicomonas* spp. are symbiotic or mutualistic then increased sedimentation would compromise host health and lead to lower abundance of *Endozoicomonas* spp. (Norkko et al. 2006).

Unfortunately, correlations with environmental data could not be carried out on histology and bacteriology data. Unlike *Endozoicomonas* spp. data, only presence and absence was recorded for bacteriology and histology. Presence-absence data alone does not provide insights into variations within specific spatial or temporal contexts. For instance, if one animal showed the presence of one hydroid through histology, while another animal at a different time of the year exhibits 20 hydroids, presence-absence data cannot differentiate between prevalence and load. Therefore, identifying the drivers behind this occurrence cannot be inferred. When analysing bacteriology and histology considerations need to be made into making semiquantitative and quantitative approaches to enhance the data that is collected. By incorporating such methods, a more comprehensive understanding can be gained regarding the abundance and distribution of microorganisms, aiding in the identification of potential drivers behind observed patterns.

When assessing the impacts of environmental pressures on the health of both the host and an organism, it is crucial to consider certain factors, such as which component of the environment is the driving force or is the environment impacting host immunity or the prevalence of the organism present (Zgouridou et al. 2021). In this study, water quality measurements were obtained from the NRC from the surrounding area prior to collection. However, it is important to note that environmental factors may have cumulative effects and there may be a delay until the consequences of these pressures become evident (Thrush et al. 2021). Thus, it becomes challenging to predict which elements have the greatest impact when relying on a single data point. To address these challenges, it is necessary to gather long-term water quality data to identify any cumulative factors. The environmental data used in this study were collected monthly. This can result in missing valuable information about environmental factors. Gaps in both spatial and temporal coverage also make statistical analysis more difficult. It is important to acknowledge that this data is not specifically collected for the purpose of this study but rather to monitor the overall health of the surrounding water quality in the region. Nonetheless, it does provide a comprehensive resource. However, when aiming to detect small-scale changes in real-time, it is essential to conduct long-term data collection, which would be better suited for the application of this study (Danovaro et al. 2016). Utilising automated or satellite methods would be particularly beneficial in capturing the desired level of detail and accuracy.

Conclusion

In this study, the combined archival and baseline study results indicate that mortality events in pipi coincided with the spawning to post-spawning periods. Additionally, there was an observed increase in the amount of bacteria isolated in mortality samples, although the types of bacteria were common among mortality and healthy pipi. It is likely, therefore, that the mortality events are multifactorial and not all factors have been elucidated in this study. This chapter does show that simple comparisons of archive data can aid in teasing out components that are relevant in these events. Unfortunately, without long-term real-time data the environmental variable that may have been triggered in these events cannot be elucidated. However, what the result of this study does tell us is that during spawning and recovery periods pipi are likely more vulnerable and therefore more susceptible to mass mortality. It would be worth looking at other geographically different pipi mortality events, and other wild bivalve species, to see if similar patterns can be deduced as this may be a significant component in the causation of wild bivalve mass mortalities.

Chapter 7 : General Discussion

Pipi gathering at Paihia, Bay of Islands, 1950s, Islands, Bay of, by Eric Lee-Johnson. Purchased 1997 with New Zealand Lottery Grants Board funds. © Te Papa. CC BY-NC-ND 4.0. Te Papa (O.009045).

The implications that will be felt by the loss of wild bivalve populations are hard to calculate. Biosecurity New Zealand (BNZ) investigates reported incidents of mass mortality events of wild bivalve populations from around the country with results often being inconclusive or multifactorial (Williams 2014; Williams 2022). An exotic disease has never been detected during investigations into bivalve mortality events belonging to the genus *Paphies*. Nevertheless, the identification of causes for recurring mortality events remains elusive, yet it is crucial for the future productivity of wild bivalve populations. Therefore, additional research is required to elucidate the potential factors that could be contributing to mortality events and declining biomass. This was the motivation of this thesis research. Marine bivalves are taonga to indigenous communities and have been an integral part of the lifestyle for generations of New Zealanders, principally through recreational and commercial harvesting (Cisneros-Montemayor et al. 2016). The pattern of loss has been seen before with toheroa (*Paphies ventricosa*). This native surf clam is culturally significant to Māori but populations of them are threatened. Significant declines in population abundance over the last 40-50 years to a level where they are unlikely to be restored to levels they once were (Williams et al. 2013). Recent study efforts have attempted to address causes of the population decline of toheroa in New Zealand, in particular the role of disease (Ross et al. 2017; Bennion 2021; Taikato 2021). This prompts the question, however, of whether it is a case of too little too late. If awareness of the implications of loss were raised earlier and research efforts had been initiated sooner, there might have been an earlier implementation of measures to understand their health and develop effective conservation tools.

The drivers of mass mortality events in bivalves will vary for each event, involving different primary drivers and combinations of drivers. For an example with a gastropod mollusc, the disease Withering-Syndrome Rickettsia-Like Organism (WS-RLO) caused a massive change in the population structure of abalone (*Haliotis* spp.) by causing high mortalities. Although the primary factor in these mortality events was the organism, a number of other factors exacerbated the effects and were identified as key drivers. The genetics of the host was a key factor, with susceptibility varying among the *Haliotis* genus identifying this . When the agent was finally identified as WS-RLO, a bacteriophage caused inhibition to WS-RLO (Friedman et al. 2014; Crosson & Friedman 2018). WS-RLO was still present within the abalone but no longer resulting in mass mortalities (Friedman et al. 2014). If a driver can be pinpointed and could be managed effectively through human action this is a vital step in future sustainable conservation efforts. It is crucial to establish baseline data on the health and mortality of host organisms to detect deviations from norm or patterns of illness that may help identify the primary drivers behind mass mortality events. This requires an examination of not only pathogens but also the interplay of factors such as environment, host health, and the pathogen itself, as demonstrated in the chapters of this thesis. This moves away from the singular discipline focus that commonly occurs in scientific research.

In this final chapter I address key considerations for wild bivalve health in New Zealand, with a focus on pipi (*Paphies australis*) health in Whangārei and other bivalve species in New Zealand. I finally discuss the application of diagnostic tools in assessing health and future considerations for modifying the techniques that were applied in this study.

Assessing the baseline health of pipi

Surveillance aids in the identification of emerging diseases, changes in the prevalence and intensity of endemic pathogens, shifts in geographical locations, and changes in host ranges (Groner et al. 2016). Surveillance of all species is not possible and is normally isolated to wild species of significance, normally species with high economic value such as the New Zealand flat oyster (*Ostrea chilensis*) in the Foveaux Strait (Michael et al. 2022). Baseline health data serves as an alternative approach that can offer significant insights into potential factors contributing to mortality events. It can also provide valuable insights by helping to identify factors that are not contributing mortality events, although they may look like it e.g., *Endozoicomonas* spp. as demonstrated in this research (Ward & Lafferty 2004). By excluding non-causative factors, it helps to refine the understanding of the complex dynamics surrounding mortality events and enables more targeted efforts in identifying and addressing the actual drivers of these events.

Baseline health data can be used to start answering some important questions in terms of factors contributing to population decline. For instance, with baseline health data I was able to investigate whether *Endozoicomonas* spp. is responsible for the decline in pipi health and identify at what life stage and what time of year pipi mortality events are more likely to occur. The creation of baseline data achieved in this thesis (Chapter 3) showed that despite its existence that causation can't be fully elucidated yet and additional data is needed to complement the work presented in this thesis research. Chapter 3 and 4 show some important findings on the presence of *Endozoicomonas* spp. and other interesting bacteria and pathological observations under healthy and mortality pipi. The prevalence and intensity of *Endozoicomonas* spp. in healthy and mortality pipi show that *Endozoicomonas* spp. is not an opportunistic bacteria and not the driver behind the declining health of pipi in this region. This was further confirmed when trying to identify whether unfavourable environmental variables to pipi were a potential driver in abundance in Chapter 5. It was found that different environmental conditions did not play a significant role in the intensity of this bacteria in pipi. Performing genomics on *Endozoicomonas* spp. will be critical to understanding the role of this bacteria in bivalve health. (Schreiber et al. 2016; Pogoreutz &Voolstra 2018).

This study has collected a lot of information on common bacteria isolated from pipi and different observations in histopathology. This information is not only valuable for disease diagnosis, but some of the data obtained in this study should also be considered in conservation efforts, particularly if

stock assessments indicate that the pipi population in this region is stabilising. For example, understanding the reproductive behaviour and recruitment success of pipi in this area are crucial factors when considering sustainable conservation strategies for the species (Bargione et al. 2021). If the pipi biomass at Mair Bank remains consistently abundant and any associated catch-bans and rāhui are lifted, insights from this study could be used in management efforts such as implementing seasonal catch closures during periods when pipi are more vulnerable. Although pipi have a lengthy spawning period, spanning from November to May across different populations in Whangārei, fishing restrictions could be imposed during these times to ensure the protection of pipi during their spawning season. This is a common technique used in other marine organisms in an effort to ensure stable population biomass stocks (Clarke et al. 2015). In addition, implementing community monitoring programs for pipi mortalities during known spawning periods can be valuable. Such programs would ensure that if a mortality event occurs, relevant samples can be collected for testing to investigate potential causes and comprehend patterns and drivers behind these events.

The baseline health study indicates that disease is not a contributing factor to the declining biomass of pipi in Whangārei. There is no evidence of any pathological changes in the health of pipi that indicate a bacterial agent as the cause in the decline of biomass, however, the possibility of a bacterial agent, such as *Vibrio*, as an opportunistic element in mortality events cannot be ruled out and requires further exploration. While disease was not identified as a factor, it is important to recognise that the risks associated with disease still exist. Despite not being directly implicated in the observed mortality events, the potential presence of pathogens or other disease-causing agents cannot be completely ruled out in future events. It is predicted that the threat of disease will escalate under the pressures of climate change (Lane et al. 2022; Hutson et al. 2023). Therefore, this baseline data will serve as a valuable resource for future investigations into mass mortality events of pipi in the Whangārei area.

The interface of effective engagement and collaboration in science

The mortalities that are occurring or that are being reported to BNZ appear random, with no common linkage between them. An important outcome of this thesis is the demonstration that the close partnership with the Patuharakeke hapū provided information and insights into issues within the hapū that would otherwise not have been known. Community engagement is essential for biosecurity systems to identify risks. This could be with local public residents or industry stakeholders (Payne et al. 2023). The system alone cannot work effectively without this knowledge. There are many community run activities around New Zealand that focus on the health environment of their own monitoring programmes and generally have better awareness of their own “backyard” than science institutes or government bodies do (Peters & Hamilton 2016; Williams et al. 2017). Collaboration across the “silos” is essential for progression. This thesis demonstrates that causation is not attributable to just one factor, as highlighted within the existing text, and there are likely a multitude

of reasons for mass mortality events. However, the Patuharakeke hapū alone cannot find the answer and has been working on many research projects to try to elucidate the causations. This study demonstrates that when performing disease diagnosis, a suite of other findings can be identified incidentally. It is therefore recommended that there be more open transparency in sharing the information that is gathered, fostering a more open-source approach. The information that research institutes or other bodies gather can include important findings regarding a hapū's taonga (treasure), and thus, this information shouldn't be solely owned by that organisation. Instead, all the gathered information should be handed back as a record for the hapū to keep as guardians. It is also important to note that within research organisations and government bodies who are working to aid in elucidating causation, turn-over of staff can be high and the information or knowledge can be lost over time. However, mātauranga Māori (Māori knowledge) shows that knowledge on the species within their rohe and efforts that have previously been carried out has stood the test of time.

The last chapter of this thesis demonstrates that baseline data is relevant and can aid in identifying patterns looking at historical data. Performing similar analysis would be beneficial but also requires communicating these results out to the community. It is common for results in bivalve mortalities to be "multifactorial". By combining all the results from mortalities, patterns can emerge that indicate what could be a factor, and this needs to be communicated out in that way to ensure that communities and other potential stakeholders are not dejected in their efforts when they receive an answer of "multifactorial". Communication needs to see the bigger picture and social science is essential in communicating the right message on a community scale.

Surveillance of wild bivalve mass mortality events

Pipi are not the only wild bivalve species that has experienced repetition of mass mortality events in New Zealand (Howells et al. 2021; Chapter 1, Figure 1.4). The risk of incursion from exotic and emerging diseases is growing with increasing pressures from climate change (Hutson et al. 2023). Wild populations remain vulnerable to the impacts of disease and there is no "barrier" to safeguard them. Early detection is essential in minimising the impacts and spread of disease and therefore baseline data has a place in all wild bivalve populations. However, the creation of baseline health data like the one carried out in Chapter 3 cannot be carried out for every bivalve species in every different habitat type in New Zealand. It would be logistically impossible and prohibitively expensive. Moreover, some of the species from these wild populations are taonga, so working with community groups to understand the fragility of these populations and the cultural significance is important to understand upfront. Although the results show that the baseline data was relevant for interpreting the health of pipi in the Whangārei region, this data hasn't been tested on pipi in other geographical regions within New Zealand. To do this you would have to look at healthy pipi in a similar habitat type from the ones created in the Whangārei baseline, e.g., river, lower estuary and estuary, for a

similar time of year and see whether they shared similar observations before you could assume that the baseline data created in this study could be applied to the species at large. That is only one species. Similar studies as the one carried out here have been created on other wild bivalves. Bennion (2021) carried out one of the most comprehensive studies on toheroa health and would be worth utilising that data in comparison to archive toheroa mortalities that have been observed to make the most of this resource. However, that still poses a problem for surveillance on other species where no baseline health data has been created. Attending scientific conferences and staying updated on current research, as well as conducting thorough scans of various university portals to explore ongoing master's and PhD projects, offers oversight of the crossovers in research that are being carried out across the institutes. Similarly to what was presented above, if institutes had a more open-source way of working, baseline data or surveillance work could be carried out on the back of research that is been carried out elsewhere. Furthermore, it is important to consider the impact on these animals when they are taken from their natural environment, emphasizing the need for careful handling and responsible utilisation. Sharing efforts in this regard will contribute to the respect and conservation of these environments and species, rather than exploiting them for individual purposes.

Archived data are an important resource for surveillance. Similar to what was done in this thesis, where archival data from BNZ was analysed to examine changes in pipi health over time in Whangārei, the same approach could be applied to investigate other significant wild bivalve species. A scan of Ministry for Primary Industries surveillance magazine series shows that historical research on wild and farmed bivalves have been carried out (see Surveillance magazine, MPI). In 2000, a health survey was carried out on 1,200 greenshell mussels (*Perna canaliculus*), 1,050 Pacific oysters (*Crassostrea gigas*), 300 rock oysters (*Saccostrea glomerata*), 252 scallops (*Pecten novaezelandiae*) and 150 clams (*Austrovenus stutchburyi*) as part of active surveillance required under WOAH guidelines (Hine 2002). While the primary objective of surveying these species was to gain a deeper understanding of the status of WOAH-notifiable diseases in these commercially significant organisms, the collected data could be utilised as a baseline reference for assessing the overall health of these species. Additionally, it can provide valuable insights into the prevalence of specific pathogens, if present, and other observations that may serve as essential references for future health analysis. Secondary research can look for patterns across data spanning several years and identify trends or use it to verify early hypothesis statements and establish whether it’s worth continuing research into a prospective area.

Future diagnosis of bivalve mass mortalities

The tests that are performed and the way data are analysed is essential for disease diagnosis (Helman et al. 2020). Histology and bacteriology are commonly used disease diagnostic and research tools (Aranguren & Figueras 2016; Howells & Brosnahan 2022) and were used in this study so that the data

collected could be used and compared in other disease diagnosis events. The tools used in this study are classics for a reason. However, modifying or adapting these traditional diagnostic methods could open up new avenues of data collection or provide additional insights. Histopathology and bacteriology can be presence/absence. Chapter 6 shows the limitations of combining these types of data with information on water quality and other environment measurements. Whereas the qPCR results were easier to integrate and explore the drivers behind the abundance of *Endozoicomonas* spp. Chapter 2 demonstrates that there are methods that can be adapted to integrate information in making it semi-quantitative and quantitative and applicable to the question being asked in research. Bennion et al. (2022) shows that grading scales of histological observation provides quantitative measures that allow for interpretation of the potential environmental driver behind certain observations. However, when diagnosing an animal under histology the tool must be practical in terms of its uses. For example, BNZ has a core service, it is not a research institute and time is of the essence when ruling out disease as a factor. It is recommended that at least semi-quantitative methods be applied when time is limited. Grading methods such as mild, medium, and severe would at least provide some quantitative value to compare intensity of observations. However, this can lead to ambiguity within scoring systems between researchers and institutes. Creating objective definitions with criteria or by figures, along with an associated scoring system in a standard operating procedure, would be essential to enhance the grading value.

A secondary difficulty that is a running theme throughout this thesis is the lack of baseline data and general knowledge on wild bivalve species. Although exotic pathogens have not been identified in any of these bivalve mortalities, endemic pathogens pose as much a risk to marine organisms (Hutson et al. 2023). Shifts within the natural environment can have huge consequences on the host microbiome (Masanja et al. 2023). Within the microbiome is a plethora of bacteria, some potentially pathogenic (Shuping et al. 2023). The results from each chapter show that there is continuous presence of *Vibrio* species isolated from both healthy and mortality pipi, however the tools available were not able to fully elucidate its role in health of pipi. This indicates a need to improve diagnostic capabilities in the space. Commonly in wild bivalve mass mortalities the potential causative agent is unknown and therefore a variety of generalised diagnostic tools are employed such as histology and bacteriology to look at the overall health of the whole animal. This can be time consuming, and often the causative agent isn't identified. Looking at archive cases provides evidence base for traits that are common and traits that are not. This can inform where research efforts should be focussed around and what diagnostic capabilities may need additional resourcing to aid in diagnosis of wild bivalve mass mortality events.

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Appendix A: Bacteriology & Bivalves

A guide to using transport media swabs in the field on bivalves

What you will need per bivalve:

- A utensil to open the bivalve e.g., scalpel or shucking knife
- One sterile swab
- Ethanol ≥ 70%
- Sterile scalpel or sterilised sharp knife
- Transport media swab (e.g., Amies agar gel without charcoal)

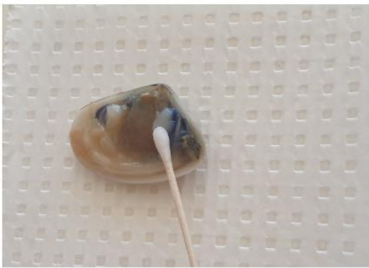
1. Collect bivalves

This can be applied to live or dead bivalves.

2. Open bivalve

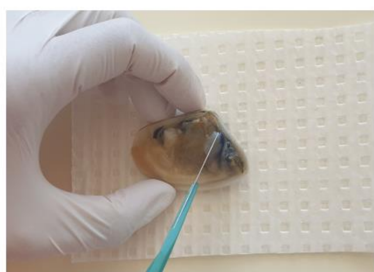
Open bivalve carefully. Once open identify the adductor muscles.

3. Clean adductor muscle



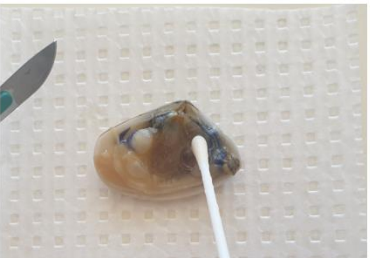
Using a sterile swab clean one of the adductor muscles with ethanol.

4. Open adductor muscle



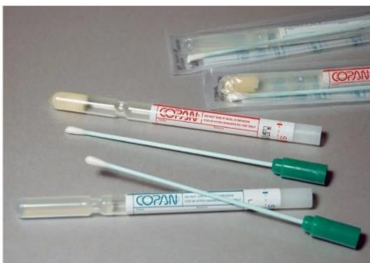
Using a sterile scalpel make a small incision in the adductor muscle that you cleaned.

5. Collect a bacteria sample



Place transport swab in the incision and "soak" up the bivalves haemolymph aka. blood.

6. Send swab for testing



Send transport media swab for testing. Keep chilled where possible, don't freeze.

Figure A.1 Field swabbing method used in Chapter 2.

Table A.1 GenBank reference IDs for bacterial isolates in Chapter 2. *Same GenBank reference ID as other isolate but confirmed different species when sequence aligned in Geneious, DNA sequences were analysed using a % identity matrix in Geneious to assess similarity.

Associated Chapter	**Associated Table**	**Closest level identification**	**GenBank reference**
Chapter 2	Table 2.4	*Marine bacterium*	KJ469398.1
		Pseudoalteromonas agarivorans	CP033065.1
		Sphingobium naphthae	NR_157779.1
		Tenacibaculum sp.	MH150835.1
		Vibrio sp.	MG388155.1
		Vibrio sp.*	MG388155.1
		Vibrio splendidus	KJ423058.1
		Vibrio tetraodonis	MT741837.1
		Vibrio artabrorum	FN668904.1
		Vibrio kanaloae	CP065150.1
		Vibrio genomo sp.	GU378413.1
		Staphylococcus warneri	MT328647.1
		Staphylococcus pasteuri	MT539733.1
		*Staphylococcus pasteuri**	MT539733.1
		*Staphylococcus warneri**	MT328647.1
		Staphylococcus epidermidis	MT613456.1
		Marinomonas profundimaris	NR_148262.1
		Marinomonas pontica	NR_042965.1
		Kistimonas sp.	KF444399.1
		Pseudomonas sp.	MT539780.1
		Pseudomonas lurida	MT505164.1
		Pseudomonas poae	MT631989.1
		*Shewenella colwelliana**	KR270290.1
		*Shewenella colwelliana**	KR270290.1
		Shewenella hafniensis	KX271692.1
		Shewenella baltica	MT516278.1
		uncultured bacterium	LR650284.1

Appendix B: Baseline health of pipi

Table B.1 Collection and location information at the time of each collection. Key: WR = Waipū River, RR = Ruakākā River, MB = Mair Bank , OTP =One Tree Point (Chapter 3).

Collection Date	Moon phase	Tidal range	Weather conditions	Observations
10/02/2020	Full moon	Spring tides	Clear with some clouds 21/19°C, SSW 19km/h winds, humidity 54%, barometer 1021 mbar.	WR: Pipi found in fine sand in fast flow of river. RR: Pipi found in sandy substrate, discolouration, and eggy smells to substrate layers. MB: Densely packed shell layers make up substrate surrounding pipi population. OTP: Pipi found in sandy/muddy substrate. Substrate blackened and smelly.
18/05/2020	First Quarter Moon	Neap tides	Clear with some clouds 16°C, ESE 34km/h winds, humidity 70%, barometer 1024 mbar.	WR: No new observation, same as previous collection. RR: No new observation, same as previous collection. MB: No new observation, same as previous collection. OTP: No new observation, same as previous collection.
4/08/2020	Full Moon	Spring tides	Mild wind, surface waves. ~15km/hr ESE winds.	WR: Discolouration to water – yellow tinge and eggy smell. RR: Recent flooding over the edge of the channel 2 weeks prior to collection. Eggy smell when substrate disturbed. MB: No new observation, same as previous collection. OTP: Mangroves seeding in area. Seagrass in sand, more cockles present then previous collections. Red algae present. Sediment is fine and takes a while to settle down.
16/11/2020	New Moon	Spring tides	Clear weather with some clouds, 11km/hr westerly winds.	WR: Heavy rain one week prior. RR: Heavy rain one week prior. MB: Heavy rain one week prior. OTP: Heavy rain one week prior.
22/02/2021	Waxing Gibbous	Neap tides	Clear with some clouds 20/21°C - ENE 27km winds.	WR: Heavy rain one week prior. Lots of red algae in water, *Spyridia filamentosa* (NIWA 2018). RR: Heavy rain one week prior. MB: Heavy rain one week prior. OTP: Heavy rain one week prior.
4/05/2021	Last Quarter Moon	Neap tides	Clear with some clouds. SW 10km/h, 18/15°C, humidity 52%, Barometer 1027 mbar.	WR: No new observation, same as first collection. RR: Removal of mangroves in surrounding area. New seawall being built in close proximity to pipi population. MB: No new observation, same as first collection.

				OTP: No new observation, same as first collection.
4/08/2021	Waning crescent	Neap tides	Clear with some clouds. SW 13km/h, 14°C, humidity 67%, barometer 1015 mbar.	WR: New sand bank at river mouth. RR: New sea wall got washed into river with storm whilst under construction. MB: Bank substrate very sandy in comparison to previous collections. Present of much smaller pipi then is usually seen. OTP: Sea wall going in around the corner of Pile Road West. Ongoing construction of marina, 750 houses been built on edge of marina.
29/11/2021	Waning crescent	Neap tides	Clear with some clouds. ENE 2km/h, 24°C, humidity 63%, barometer 1023 mbar.	WR: No new observation, same as first collection. RR: No new observation, same as first collection. MB: Increased observations of cockles across collection site. OTP: Increased observations of cockles across collection site.

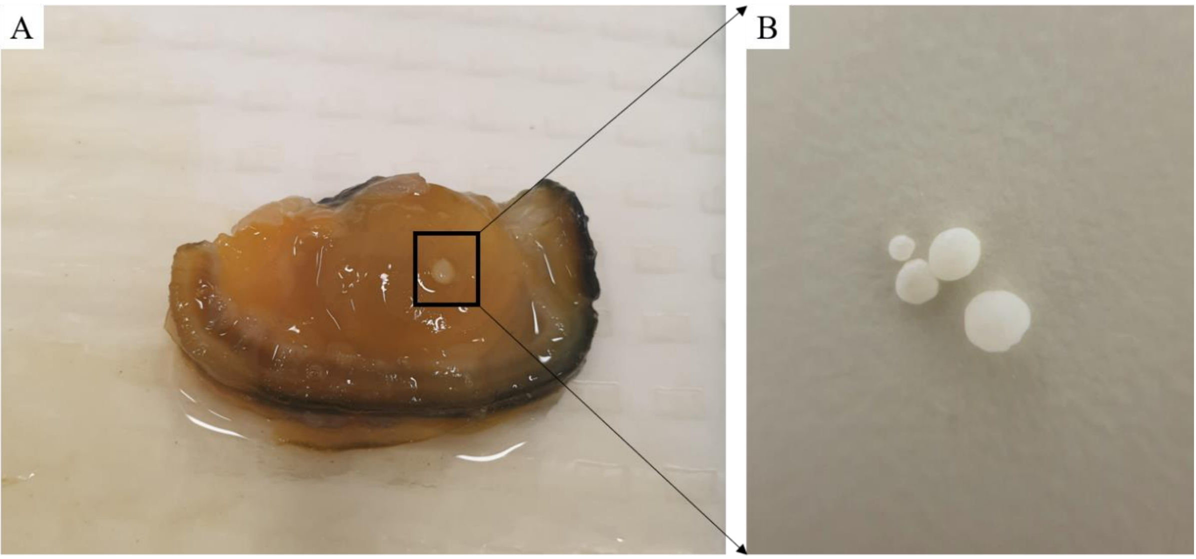


Figure B.1 Observations of a pearl under the mantle of a pipi from Mair Bank. A) external observation of pearl under mantle layer, B) different pearl sizes removed from a number of pipi at Mair Bank (Chapter3).

Table B.2 GenBank reference IDs for bacterial isolates in Chapter 3. *Same GenBank reference ID as other isolate but confirmed different species when sequence aligned in Geneious, DNA sequences were analysed using a % identity matrix in Geneious to assess similarity.

Associated Chapter	Associated Table	Closest level identification	GenBank reference
Chapter 3	Table 3.5	*Kistimonas* sp.	KF444399.1
		Kistimonas sp. *	KF444399.1
		Kistimonas sp. *	KF444399.1
		Kistimonas sp. *	KF444399.1
		Marinomonas pontica	NR_042965.1
		Pectobacterium brasiliense	CP024780.1
		Pseudomonas lurida	MT093468.1
		Pseudomonas orientalis	MN197609.1
		Pseudomonas sp.	MT539780.1
		Shewanella baltica	CP028355.1
		Shewanella colwelliana	KR270290.1
		Shewanella colwelliana *	KR270290.1
		Shewanella colwelliana *	NR_043074.1
		Shewanella hafniensis	KX271692.1
		Staphylococcus pasteuri	MT539733.1
		Staphylococcus warneri	MT328647.1
		uncultured bacterium	LR650284.1
Chapter 3	Table 3.6	*Vibrio artabrorum*	FN668904.1
		Vibrio breoganii	CP016177.1
		Vibrio chagasii	KJ423057.1
		Vibrio cortegadensis	HF955043.1
		Vibrio genomo	GU378413.1
		Vibrio genomo*	GU378413.1
		Vibrio kanaloae	CP065150.1
		Vibrio maritimus	CP090438.1
		Vibrio owensii	CP045859.1
		Vibrio rumoiensis	AP018685.1
		Vibrio sp. Scap24	CP041332.1
		Vibrio sp. Scap24*	CP041332.1
		Vibrio splendidus	KJ423058.1
		Vibrio tetraodonis	MT741837.1

Appendix C: Testing baseline health data

Table C.1 GenBank reference IDs for bacterial isolates in Chapter 4. *Same GenBank reference ID as other isolate but confirmed different species when sequence aligned in Geneious, DNA sequences were analysed using a % identity matrix in Geneious to assess similarity.

Associated Chapter	Associated Table	Closest level identification	GenBank reference
Chapter 4	Table 4.3	*Vibrio atlanticus*	FM954972.2
		*Vibrio atlanticus**	FM954972.2
		Vibrio artabrorum	FN668904.1
		Vibrio breoganii	CP016177.1
		Vibrio chagasii	KJ423057.1
		Vibrio cortegadensis	HF955043.1
		Vibrio crassostreae	AP025476.1
		Vibrio europaeus	CP053541.1
		Vibrio genomo	GU378413.1
		Vibrio genomo*	GU378413.1
		Vibrio genomo*	GU378413.1
		Vibrio kanaloae	CP065150.1
		Vibrio maritimus	CP090438.1
		Vibrio mediterranei	CP033577.1
		Vibrio owensii	CP045859.1
		Vibrio rumoiensis	AP018685.1
		Vibrio sp. Scap24	CP041332.1
		Vibrio sp. Scap24*	CP041332.1
		Vibrio splendidus	KJ423058.1
		*Vibrio splendidus**	CP089205.1
		Vibrio tetraodonis	MT741837.1

Appendix D: Retrospective analysis

Table D.1 Summary of environmental variables for each of the collections, sorted by location and habitat type. Key: TSS = total suspended solids, DO = Dissolved oxygen (Chapter 6).

Location	Population habitat	Type	Date	Temperature (°C)	Salinity (ppt)	TSS (g/m3)	DO (mg/L)
Mair Bank	Lower estuary	Mortality	Apr-13	21.6	34.1	-	7.47
Mair Bank	Lower estuary	Mortality	Nov-20	16.1	34.64	3	7.8
Mair Bank	Lower estuary	Mortality	Dec-20	17.8	34.64	5	7.9
Mair Bank	Lower estuary	Mortality	May-21	19.25	34.765	4.6	7.605
Mair Bank	Lower estuary	Healthy	Feb-20	23.4	35.07	14	7.5
Mair Bank	Lower estuary	Healthy	May-20	17.2	34.82	8.8	7.7
Mair Bank	Lower estuary	Healthy	Aug-20	14.9	29.435	5.5	8.25
Mair Bank	Lower estuary	Healthy	Nov-20	16.1	34.64	3	7.8
Mair Bank	Lower estuary	Healthy	Feb-21	21.35	34.835	3.6	7.25
Mair Bank	Lower estuary	Healthy	May-21	19.25	34.765	4.6	7.605
Mair Bank	Lower estuary	Healthy	Aug-21	15	33.575	6.6	8.165
Mair Bank	Lower estuary	Healthy	Nov-21	18.65	33.78	5.6	7.78
Marsden Bank	Lower estuary	Mortality	May-13	19.65	35.4	-	7.5
Marsden Bank	Lower estuary	Mortality	Feb-18	21.2	34.3	6.4	7.6
Pātaua South	Lower estuary	Mortality	Jul-22	15.8	33.94	5	7.65
Munroe Bay	Estuary	Mortality	Jan-19	21.8	34.3	3	7.4
One Tree Point	Estuary	Mortality	Feb-19	22.2	36.3	8	7.3
One Tree Point	Estuary	Mortality	Mar-19	22.05	35.9	7	7.2
One Tree Point	Estuary	Healthy	Feb-20	24.9	35.35	12	7.1
One Tree Point	Estuary	Healthy	May-20	17	34.74	5.8	7.5
One Tree Point	Estuary	Healthy	Aug-20	14.8	21.565	6.5	8.45
One Tree Point	Estuary	Healthy	Nov-20	16.3	34.6	4.6	7.7
One Tree Point	Estuary	Healthy	Feb-21	21.8	34.92	3.85	7.1
One Tree Point	Estuary	Healthy	May-21	19.05	34.83	6.4	7.6
One Tree Point	Estuary	Healthy	Aug-21	14.85	32.67	5.9	8.135
One Tree Point	Estuary	Healthy	Nov-21	19.6	33.42	5.3	7.48
Ruakākā River	River	Healthy	Feb-20	24.3	32.01	3.6	5.1
Ruakākā River	River	Healthy	May-20	16.6	32.79	1.7	7.2
Ruakākā River	River	Healthy	Aug-20	15.1	17.215	13	8.05
Ruakākā River	River	Healthy	Nov-20	16.8	33.59	39	7.9
Ruakākā River	River	Healthy	Feb-21	21.65	32.355	4.6	6.55
Ruakākā River	River	Healthy	May-21	18.35	28.285	2.6	7.215
Ruakākā River	River	Healthy	Aug-21	11.9	9.695	6.1	8.475
Ruakākā River	River	Healthy	Nov-21	19.45	32.88	6.1	7.365
Waipū river	River	Healthy	Feb-20	24.3	33.07	5.6	5.9
Waipū River	River	Healthy	May-20	17.5	34.52	3.9	8.4
Waipū River	River	Healthy	Aug-20	15.7	19.61	15.6	9.05
Waipū River	River	Healthy	Nov-20	18.2	34.38	14	7.7
Waipū River	River	Healthy	Feb-21	22.4	33.125	5.2	6.7
Waipū River	River	Healthy	May-21	19.05	29.89	5.8	6.315
Waipū River	River	Healthy	Aug-21	13.6	21.81	12.7	8.16

Waipū River	River	Healthy	Nov-21	19.55	32.395	11.3	7.465