Dynamics of faecal contamination in the Waikouaiti estuary and implications for non-commercial harvest

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Abstract
For many New Zealand catchments, rainfall-induced faecal contamination is a primary mechanism for introducing gastro bacterial pathogens into shellfish harvesting environments. Bivalves have the ability to accumulate bacteria through filter feeding, thus putting consumers of shellfish at risk. Commercial shellfish harvesting grounds for human consumption are regulated following quantification assessments of faecal bacteria in both water and the shellfish tissue. However assessments of non-commercial areas are less stringent and only assess faecal concentrations in the water. Additionally, guidelines set by regional councils and national authorities discourage recreational harvesting for several days after a rainfall.

This study assessed the impact of rainfall-induced contamination on Austrovenus stutchburyi (tuaki, cockles or littleneck clams) and Perna canaliculus (kutai, Green Lipped mussels) within the Waikouaiti Estuary, an area that is hypothesised to be impacted by faecal contamination from farming and human sources. To assess the safety of the shellfish harvesting grounds, commercial quality guidelines established by the New Zealand Food Safety Authority (NZFSA) were applied to E. coli data collected for a 13 week monitoring period with the addition of three sampling events during rainfall. Additionally the MfE/MoH (2003) guidelines were used as in recreational settings to determine the safety of the shellfish beds based on assessment of the water only.

NZFSA guidelines stipulate that only 10% of all shellfish samples can exceed 700 E. coli cells per 100 grams of shellfish tissue in order to be deemed safe for human consumption. The shellfish sampling indicated a total of 3/29 (10.3% of samples) breached the NZFSA limits, resulting in borderline acceptance of this guideline. In comparing species, there was only one more contamination event identified in the cockles over the mussels. The MfE/MoH (2003) guidelines for the water specify a 10% allowable limit for exceedances over 43 colony forming units (cfu) per 100 mL and a median measuring below 14 cfu/100 mL. Overall 2/16 (12.5% of samples) of the water samplings exceeded the limit of 43 cfu/100 mL for faecal coliforms therefor surpassing the 10% threshold. On the other hand, the median was 5 cfu/100 mL, thus remaining well within the safe harvesting median limit. These breaches should be further verified with a longer sampling regime containing a larger sampling size.
Both rainfall and river flow correlated with *E. coli* concentrations in the water, but did not provide an accurate indicator of contamination in the shellfish. Correspondingly, there was no correlation between *E. coli* in the water versus concentrations in the shellfish, indicating that other factors should be considered for accumulation. The conditions of the water can affect bivalve filter feeding and should be considered for future work, including: salinity, turbidity, organic content, temperature and the arrival of the contamination to the shellfish site.

Microbial RNA extracts from the collected water samples were interrogated via 16S rRNA sequencing. The resulting sequences were screened for faecal taxa that could be targeted for Microbial Source tracking (MST), and secondly to identify foodborne pathogens associated with the contamination. Samples containing higher *E. coli* abundances and higher river flows increased in faecal and total bacterial diversities. Prospective genera that could be useful for microbial source tracking in the Waikouaiti estuary were *Prevotella, Bacteroides, Enterococcus* and *Bacteroidales*. These have previously been linked to cows, gulls, humans, and pigs as possible sources. The well-documented ruminant genera, *Ruminococcus*, was absent, denoting implications with using this marker for MST, and a negative result for the confirmation of ruminant sources. Sequencing of bacteria enriched on the *E. coli* growth media CM1046 showed an enhanced abundance of foodborne pathogens and faecal-related genera. This media could be useful to enhance the detection of pathogens for water testing protocols.

Current faecal coliform assessments do not indicate contaminant sources, which is important for defining the risk of gastro-pathogens and identifying mitigation strategies. Human sources are of particular concern as this is associated with high-risk pathogens, thus more research using MST is needed to confirm the contaminant sources. This study found that monitoring only the water of shellfish grounds is not an acceptable method for identifying breaches of microbial safety and rainfall-induced contamination in shellfish. We suggest future monitoring of non-commercial shellfish grounds should therefore include monitoring of the shellfish tissue as in commercial regimes.
He mihi - Acknowledgments

To my Nanna and greatest support person throughout this academic journey: Lorna Pāua Bell. I will never forget to ‘Have a good kai and a good laugh everyday, and don’t be one of those that take life so bloody seriously!’ E rere ana tonu ake roimata mōu, kia waimārie tō moe i te korowai i ō tātou aroha. Ānei tītahi taonga mō tō awhi me tō aroha ki ōku haerenga rangahau. Also to our ‘super genius’ Grandfather: Colin Thompson, Nanna Eileen Stone, and Grandad Hina Bell, whom all passed away on this journey. He aroha mutunga kore.

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To our educators: please always tell our rangatahi that ‘they can’, regardless of their background, presentation, attitude, and current abilities - even if they don’t fit your mould of a scientist. All keen and passionate lenses are capable and relevant, and offer new perspectives for combating the environmental challenges we face.

Ki ō ātou Waka Hourua me ngā kaumoana whānui - E’o!
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List of Acronyms

cfu/mL – colony forming units per millilitre
FIO – faecal indicator organisms
HEC – high *E. coli*
HF – high river flow
LEC - low *E. coli*
LF – low river flow
MfE – Ministry for the Environment
MoH – Ministry of Health
MPI – Ministry of Primary Industries
MPN – most probable number
MST – microbial source tracking
NGS – next generation sequencing
NIWA – National Institute of Water and Atmospheric Research
OTU – operational taxonomic unit
PCR – polymerase chain reaction
RNA – ribonucleic acid
rRNA – ribosomal ribonucleic acid
T90 – time it takes for *E. coli* to decrease by 90%
WHO – World Health Organisation
Thesis conventions

The use of the term “shellfish”
Although the term “shellfish” is also used to encompass other phyla (i.e., Crustacea and Echinodermata) for the purpose of this thesis it will only be used in reference to Molluscan bivalves.

Māori language usage
In this thesis all te reo Māori words are italicised, with the exception of New Zealand place names, document names (i.e., legislative acts), trusts and other organisations. This is commonly used when English is the dominant language used in a piece of writing (Rewi 2013), thus italicising is an indication to the reader that the word is not pronounced in an English manner. The use of macrons (i.e., ā, ē, ō, ŭ) is to ensure that the vowel emphasis reflects the correct word or place name referred to.
CHAPTER ONE

Thesis introduction

1.1 General Background
Shellfish harvested from areas containing faecal contaminants may not be safe for human consumption. The mechanism that shellfish use for feeding allows for the accumulation of bacteria including foodborne pathogens (Lees et al. 2010). In order to meet standards for the global food market and suitability for human consumption, shellfish harvested for commercial profit must undergo safety and quality testing (Laing & Spencer 2006). This is based on the concentrations of faecal contamination in the growing waters and contained within the tissue of the shellfish. On the other hand, many non-commercial harvesting areas in New Zealand are restricted to the assessment of the water only, like in the Otago region (Scholes 2009; pers. com. Rachel Ozanne, Water Quality Scientist, Otago Regional Council). This is of concern to non-commercial harvesters because contaminants often accumulate to a much higher concentration within shellfish than that within the overlaying water (Campos et al. 2013).

Legislative regulation differs between commercial and non-commercial harvesting of seafood (Scholes 2009; pers. com. Rachel Ozanne, Water Quality Scientist, ORC). Non-commercial fishing is further broken up into recreational and customary categories. Recreational harvesting is restricted to daily bag limits per person and is enforced by the Ministry of Primary Industries (MPI). Not abiding to bag limits is a punishable offence that can result in the issuing of fines, confiscation of equipment and even jail time for offenders. Under legislation and namely the Treaty of Waitangi (Fisheries Claim Act) 1992, the crown must “recognise and provide for customary food gathering by Māori and the special relationship between tangata whenua and those places which are of customary food gathering importance (including tauranga ika and mahinga mataitai), to the extent that such food gathering is neither commercial in any way nor for pecuniary gain or trade”. Under these provisions, tangata whenua are not restricted by the daily bag limits that are in place for general public. Customary harvesting commences upon the approval of a permit, which is conducted by a tangata kaitiaki (a guardian person), or a person that is appointed by MPI. These rights are commonly exercised to provide seafood for hui (gatherings/meetings) and tangi (funerals).
For humans, environmental contamination can result in social, cultural and health implications. For recreational activities such as swimming and shellfish harvesting, direct contact with contaminated water can impact the health of an individual. Within Te Ao Māori (the Māori world), the degradation of the environment challenges our intrinsic role as kaitiaki (caretakers of the land and waterways). This is a tikanga (a traditional Māori custom) that is cyclic, whereby an environment that is unhealthy and not cared for will also harbour unhealthy inhabitants. Another interpretation to demonstrate this concept is this: if a key food sources are diminished or unhealthy then those that harvest those will either become ill, or lose their food resources all together. Albeit a traditional tikanga, it remains important to our Māori people in contemporary society that the natural environments and its inhabitants are cared for to a sustainable and prosperous future for generations. Thus, the identification of environmental threats and issues are important to Māori and allow for interventions through kaitiakitanga (guardianship, protection, management).

1.2 Human contamination of estuaries
An estuary is defined as a partially enclosed section of the coast that connect rivers to the sea (McLusky 1989). The tidal influence results in the mixing of the saline and fresh waters forming a salinity gradient which decreases proceeding upstream. Both terrestrial and marine waters contribute valuable nutrient inputs that are vital for primary production (Cloern et al. 2014). Estuaries are therefore considered some of the most productive areas on the planet (McLusky & Elliott 2004). The abundance of vegetation and organisms provide ideal habitat for breeding and spawning for fish, as well as feeding and nesting grounds for migratory and local birds (Mcdowall 1976; MPI 2015a).

Centuries of anthropogenic impact have led to the degradation of coastal and fresh water quality throughout the world (McDonald et al. 2016). Estuaries and coastal environments have been fundamental areas for human occupation dating back to 5000 years (Kennett & Kennett 2006). These areas continue to endure the brunt of the human population with 37% of the global population living within a 100 km strip of the coast, that makes up to 20% of the total land area globally (von Glasow et al. 2013). Aside from urbanisation, coastal environments have been heavily developed for industry and agriculture (Ridgway & Shimmield 2002), and result in activities that can discharge wastes into the environment. Centuries of alteration, pollution, and overexploitations of estuarine
ecosystems have led to the worldwide depletion of over 90% of historical animal species (i.e., molluscs, fish, whales, avian), destroyed over 65% of seagrass and wetland habitat, and degraded water quality (Lotze et al. 2006). Agriculture has had the greatest impact in degrading waterways; runoff and sewage inputs are responsible for eutrophication, or a condition of depleted oxygen, resulting in undesirable conditions for a healthy ecosystem and sometimes death for some animals (Royer et al. 2006; USEPA 2006; McLusky 1989; Gray et al. 2002).

Estuarine environments are abundant in species that are highly valued for human consumption. These species are subject to recreational, customary and commercial harvesting. Although they only make up for 0.5% of the world's marine areas, estuaries are among the most productive ecosystems accounting for ~21% of the global catch for fisheries (Houde & Rutherford 1993). Harvested species include fish, crustaceans, molluscs and seaweeds. Harvesting areas are generally at risk of contamination from multiple anthropogenic inputs that are either directly deposited into the estuary or are originating upstream within the wider catchment. Of major concern for human consumption is the potential presence of faecal matter, in particular from human sources because of the abundance of gastrointestinal pathogens (McLellan et al. 2014).

1.2.1 Faecal contamination
The majority of pathogenic outbreaks in humans are associated with mammalian faecal contamination of water (Leclerc et al. 2002). Direct exposure can potentially result in infections of the skin and eyes; septicaemia can result if water is makes contact with an open wound. If the water is consumed then gastroenteritis can result causing mild to severe diarrhoea and vomiting, and in some extreme cases require hospitalization or result in death in the immuno-compromised (Zhao et al. 2016). Well known faecal related gastro pathogens include Salmonella, Campylobacter, Escherichia coli O157 (Avery et al. 2004) and norovirus (Mclellan & Eren 2014). These pathogens are predominantly found within the gut of a variety of animals without posing a health risk, however they have the ability to cause zoonotic infections in humans. Pathogens that are associated with human effluent are considered a higher risk for severity of infection because the human gut is a primary reservoir for human specific enteric-viruses (McLellan et al. 2014).
1.3 Water quality monitoring
The monitoring of water quality is becoming a global interest as awareness and information surrounding the health risks with water contamination increase. Monitoring programs quantify faecal contamination by measuring the concentration of faecal indicator organisms (FIOs). Concentrations are compared to a health risk model to indicate the level of safety for recreational, drinking, bathing, or shellfish harvesting. Suitable FIOs are bacteria that are abundant in the lower intestines of warm-blooded animals such as *E. coli* and *Enterococci*, and are commonly used for risk management assessments of both fresh and marine waters (USEPA 1986). While FIOs are not necessarily pathogenic bacteria themselves they do indicate presence of faecal bacteria and thus are likely associated with gastrointestinal pathogens (Edberg et al. 2000).

Indicative organisms provide a time and cost efficient alternative to screening for a number of different pathogens. It also eliminates the need to isolate a range of pathogens that are otherwise difficult to detect due to low cellular concentrations and specific growth requirements. Epidemiological studies confirm the risk-based approach appropriate for safety assessments, as high concentrations of FIOs are commonly associated with gastrointestinal illness (Balarajan et al. 1991; Cabelli et al. 1979; Haile et al. 1999; Dewailly et al. 1986).

There are currently two key pieces of information currently limiting water quality assessments using FIOs. Firstly, they do not indicate the faecal sources of contamination and secondly, they do not detect the presence of non-faecal pathogens that are in the environment. However, both of these issues could be simultaneously resolved with the use of molecular technologies.

The use of FIOs are not specific to any one particular species of animal and so do not indicate the faecal source. Instead this method only allows for the identification of a spatial location and time (Field & Samadpour 2007). Identifying faecal sources are beneficial for water quality management for the following two reasons; firstly, the severity of potential pathogen presence varies from animal to animal and secondly, source information can support management for mitigation purposes (Cornelisen 2013, Gourmelon et al. 2010). ‘Microbial source tracking’ (MST) is the term used to describe methods that identify a host animal. This is done by targeting components of bacterial microbiota that are specifically associated with the gut of the host animal, or by detecting
human specific chemical markers for waste such as caffeine (Han Tran et al. 2015; Panasiuk et al. 2015). MST is becoming increasingly important for shellfish harvesting areas that aim to manage and minimise pathogenic risks (Gourmelon et al. 2010; Fu et al. 2011).

Faecal pathogens are not the only source of concern in shellfish harvesting grounds, other pathogens such as *Vibrio*, a marine species, are also common vectors of gastrointestinal illness (DePaola et al. 1990; Blackwell & Oliver 2008). It is therefore important to consider a wider scope of microbial hazards when assessing shellfish harvesting grounds for consumer safety. Identifying every pathogen of concern by way of culturing is not a feasible option. Molecular methods have become increasingly popular as they can identify several bacterial signatures simultaneously through PCR. Alternately, microbial profiles of all the bacteria that are in the environment can be generated with microbial sequencing (Mclellan & Eren 2014). These methods can be useful during high-risk of contamination periods such as rainfall events.

1.4 Microbial pollution sources
Faecal pollution sources are categorized as either ‘point’ or ‘diffuse’. Point sources include discharge from a single known source such as outlets from an industrial process, wastewater stations, and sewage overflows (Table 1). Even with treatment wastewater can still contain substantial concentrations of microbial pathogens (Wen et al. 2009), and in some cases contribute up to 90% of the bacterial load within a catchment (Wither et al. 2005a). Sewage overflows may also be common in periods of high sewage system usage; this is particularly common for coastal areas that become popular in the holiday periods (Campos & Cachola 2007). High usage can also strain sewage treatment operations and reduce their efficiency as in the case with UV light disinfection (Kay et al. 2008). Water remediation measures are easier for point sources because these operations can be optimised to reduce biological contaminants before discharge.

On the contrary diffuse pollution does not have a single point of entry into waterways. It is either transported across the land by run-off, or by diffusion into ground water and this causes difficulty in assessing faecal yields. Examples of diffuse sources are waste discharges from boats, urban and rural runoffs, and contamination from wild animals (Table 1). Waterfowl populations can be concentrated around wetlands, coastal lagoons and reservoirs resulting in the accumulation of FIOs. Rural waterways often experience
sporadic peaks of contamination from agricultural activities, such as manure spreading (Collins & Rutherford 2004) and rainfall events (Mallin et al. 2001). Due to the variety and difficulty in tracking source inputs, it is often difficult to control, predict and manage contamination events from diffuse sources (Clements 2013).

For many catchments and estuaries, rainfall-induced contamination is the main cause responsible for the temporal and spatial variability of FIOs. Periods of rainfall can result in increased surface runoff of contaminants associated with agriculture as well as causing sewage systems to overflow into aquatic environments. Variations in FIO concentrations and longevity depend on the magnitude of the rainfall event, land use and topography of the catchment and the riverine networks (Mallin et al. 2000; Baker et al. 2003). When faecal bacteria enter aquatic environments they are exposed to bactericidal effects due to a number of physiological pressures, i.e. osmotic pressures and solar radiation (Anderson et al. 2005; Brash & Haseltine 1982). A decline in cell numbers due to death is observed over an increasing time period. The duration of the rainfall event and other physical parameters such as salinity and temperature will effect the survival of faecal bacteria (Bougeard et al. 2011). These are important factors to consider when assessing the threat of faecal pathogens in shellfish harvesting waters.
### Table 1: Sources of faecal contamination for shellfish harvesting waters. (Table taken from Campos et al. 2013, with modifications for this thesis).

<table>
<thead>
<tr>
<th>Source type</th>
<th>Mechanism of contamination</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diffuse sources</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waste discharges from boats</td>
<td>Associated with the intermittent discharge of raw sewage. Risk of contamination potentially minimised with the provision of pump-out facilities and/or establishment of regulatory measures to restrict discharges.</td>
<td>(Kirby-Smith &amp; White 2006; Mallin et al. 2007)</td>
</tr>
<tr>
<td>Urban runoff</td>
<td>Sewage contamination from human and animal sources which may be detected some distance offshore and a few days after the cessation of the rainfall event.</td>
<td>(Kelsey et al. 2004; Ahn et al. 2005; Mallin et al. 2007; Parker et al. 2010)</td>
</tr>
<tr>
<td>Significant populations of wild animals</td>
<td>Associated with the existence of hydrological connections between dense populations of wild mammals and birds and the receiving water. Lower risk of contamination than that in urban and livestock production areas. Seasonal effects important.</td>
<td>(Whitlock et al. 2002; Wither et al. 2005b; Scott et al. 2004)</td>
</tr>
<tr>
<td>Livestock production areas</td>
<td>Associated with the application of animal manures and sewage sludge to land, especially in the absence of adequate manure treatment technologies and barriers (wetlands, buffer strips) preventing runoff. Higher risk during wet weather.</td>
<td>(Rogers &amp; Haines 2005; Shanks et al. 2006; Fu et al. 2011)</td>
</tr>
<tr>
<td><strong>Point sources</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waste water treatment works</td>
<td>Associated with level of treatment, population equivalent, volume of discharge, plant performance and age and maintenance of sewage transport infrastructure</td>
<td>(Lee &amp; Glover 1998; Webster et al. 2004; Garcia-Armisen &amp; Servais 2007; Kay et al. 2008; Ouattara et al. 2011)</td>
</tr>
<tr>
<td>Industrial discharges</td>
<td>Associated with microbiological content of the effluent.</td>
<td>(Braga et al. 2000; Garcia-Armisen &amp; Servais 2007; Kacar 2011)</td>
</tr>
<tr>
<td>Combined sewer overflows, storm tank overflows</td>
<td>Associated with untreated faecal contamination and potentially high volumes of discharge. Extreme spatial and temporal variability in the patterns of delivery of contaminants.</td>
<td>(Campos &amp; Cachola 2007; Converse et al. 2011; Kay et al. 2008)</td>
</tr>
<tr>
<td>Septic tanks and soak-ways</td>
<td>Usually associated with small volumes of discharge, but significant if not operating properly and potential subsurface transport of contaminated water to surface waters.</td>
<td>(Lipp et al. 2001; Mallin et al. 2007)</td>
</tr>
<tr>
<td>Pig, cattle and poultry units</td>
<td>High density of animals in the proximity of freshwater inputs to the shellfish water.</td>
<td>(Mieszkin et al. 2009)</td>
</tr>
</tbody>
</table>
1.5 Agriculture and water quality
In order to meet global demands for food, a total of 38.4% of global land area is used for agriculture and 26.3% is permanent pasture for farming. Intensive farming can generate large concentrations of animal effluent that is either deposited freely in paddocks, collected and spray irrigated, or treated before being discharged into the water. In dairy farming operations a significant amount of waste is generated in the milking sheds and collected for treatment. Collected effluent goes into an anaerobic settlement pond before flowing into to a second facultative treatment, after this it is discharged into surface water (Bolan et al. 2004) or spray irrigated onto paddocks as fertilizer. This treatment removes a significant amount of suspended solids, but it does not remove the associated nutrients (Hickey et al. 1989; Sukias et al. 2001). Some faecal bacteria withstand these treatment conditions allowing for viable bacteria and pathogens to enter the aquatic environment (Hickey et al. 1989).

Ruminant guts have been previously identified as a reservoir of the virulent *E. coli* 0157:H7 and there have been many examples of this pathogen washing into watercourses (Mainil & Daube 2005). Rainfall induced run-off can also effect ground-water supplies. The Walkerton outbreak in Canada is a famous example where a rainfall event delivered *E. coli* 0157:H7 into drinking water resulting in the infection of thousands and the death of seven individuals (Danon-Schaffer 2001; Auld et al. 2004). In New Zealand, a recent *Campylobacter* outbreak in Havelock North led to an government inquiry. The contamination event resulted in the infection of more than 5,500 people after local bores became contaminated with suspected sheep faeces.

1.6 Water quality in NZ
Contamination of fresh and marine water is widespread in NZ. Nearly half of the total length of streams and rivers throughout New Zealand are contained within developed catchments that are used for farming (43%), forestry (5%) and urban settlement (1%) (MfE 2007). A significantly higher level of contamination is observed in waters found within agricultural and urban areas compared to areas that remain as indigenous forest. The most contaminated waters are those within intensively farmed regions, such as

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1 http://www.fao.org/faostat/en/#data/EL
2 Government Inquiry into Havelock North Drinking Water, June 2017.
Waikato, Southland and Manawatu (Fig. 1). The Auckland region is a densely populated urban area and this is considered the main cause for E. coli contamination (Fig. 1)\(^4\).


Figure 1: Illustration of modelled data for *E. coli* in water across New Zealand as ranked from the highest to the lowest. The dots indicate the location and value of the monitoring data, which was used to train the model. The data used to create the model is based on a 5 year sampling period up to 2012 (figure obtained with permission from MPI\(^5\), with slight modifications for this thesis).

\(^5\) Map and model obtained from the Ministry for the Environment (MfE) personnel, this information and can also be found at [http://www.mfe.govt.nz/environmental-reporting/fresh-water/river-condition-indicator/bacteria.html](http://www.mfe.govt.nz/environmental-reporting/fresh-water/river-condition-indicator/bacteria.html)
In rural New Zealand there is a strong relationship between the quantity of agricultural activity and the degradation of water quality (Quinn & Hickey 1990). Predictably this relationship is also true with a rise in pathogen abundance. In rural NZ, *Campylobacter spp.* is often detected in water within rural catchments (Donnison & Ross 1999; Till et al. 2000) and recreational contact is known to result in outbreaks of illness (Eyles et al. 2003).

### 1.7 Shellfish harvesting

The seafood industry amounts to a significant proportion for the global food market. Reports indicate that an estimated 2.9 billion people regularly consume seafood products which equate up to 20% of the total supply of animal protein (Food & Agriculture Organisation 2012). The New Zealand seafood industry earns $1.71 billion in exports each year\(^6\). Mussel species are dominant in the aquaculture market with the endemic green-lipped mussel (*Perna canaliculus*) representing 73.7% of total aquaculture exports and generating up to $226 million annually (MPI 2012).

Coastal communities throughout the world value recreational seafood resources as a source of sustenance and intrinsic significance (Diegues 1998; Keough & Quinn 2000). In NZ wild shellfish stocks are subjected to frequent customary and recreational harvesting. Shellfish harvesting is an integral part of New Zealand culture and is a highly valued practice for many coastal communities and holidaymakers. Middens found in archaeological *Māori* sites are dominated by shellfish (Worsley & Scott 2000) confirming that they were an important food resource for local *Iwi* (tribe) and used as a trading resource between *Iwi* and *hapū* (subtribe). In contemporary times, seafood remains an important resource that is served at cultural gatherings and represents the second most consumed food at *Marae* (cultural meeting houses) (Rush et al. 2010).

Many New Zealanders are cautious food consumers; top concerns over food safety include: presence of harmful bacteria, unhygienic handling of foods, and chemical usage such as pesticides (Worsley & Scott 2000). However, often shellfish is consumed raw or lightly cooked and are often linked to biological vectors of gastrointestinal illness (Rippey 1994; Potasman et al. 2002; Canesi et al. 2001), this means that consumers are at risk because bacterial pathogens cannot be destroyed without cooking. Furthermore

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\(^6\) [http://www.seafoodnewzealand.org.nz/industry/key-facts/](http://www.seafoodnewzealand.org.nz/industry/key-facts/)
shellfish are filter feeders and can accumulate bacteria to concentrations higher than that in the overlaying water. These factors emphasise the importance of managing safe and healthy shellfish grounds for recreational and customary harvesting.

1.7.1 General information about bivalve shellfish
Bivalves are a class of Mollusca containing ~8500 marine species commonly referred to as shellfish (Potasman et al. 2002). They are comprised of two shells that are hinged together by ligaments that allow the animal to open and close. Two valves are used for filter feeding; water is pumped into the animal through the inhalant siphon, is filtered through the gills, and then exerted from the exhalent valve. Some bivalves such as cockles are sediment burrowers, while others attach themselves to rocks or other solid substrates including oysters and mussels. Filter feeding is an autonomous process that is not controlled at the level of the organism (Jorgensen 1996) but is altered depending on environmental conditions, concentration of food, temperature and turbulence of the water (Jorgensen 1990). Bivalves have the ability to filter large volumes of water on the level of an individual, and thus they are capable of filtering proportionally greater volumes as a collective. In the Pauatahanui inlet in NZ, the estimated cockle biomass (5000 tonnes) is predicted to filter the equivalent of 1.6 million cubic metres of water each tidal cycle (Michael 2008), amounting to one third of the tidal volume. In some cases bivalves are capable of filtering an estuarine system within less then a day (Dame & Prins 1997).

The filtering capacity of shellfish results in the accumulation of matter and pollutants to a degree higher than the overlaying water. Common accumulated pollutants include viruses, bacteria (Jorgensen 1990), and micro-plastics (Browne et al. 2008). The ability for bivalves to accumulate contaminants, and their biochemical and physiological responses make them ideal reporters of water pollution and indicators of ecosystem damage (Nilin et al. 2012; Gupta & Singh 2011).

1.7.2 Shellfish quality
Water quality monitoring in shellfish harvesting areas utilise FIOs to determine the health and safety of shellfish for human consumption. Water quality can directly correlate with land use within the catchment and adjacent to the estuary. For example, Dolah et al (2007) found that estuaries with 50% of urban coverage resulted in 62% of safety

7 http://www.gopi.org.nz/education/cockles/
exceedances in adjacent shellfish growing areas. Aside from bacteria, viral and protozoan vectors are also capable of causing gastrointestinal illnesses through contaminated shellfish (Table 2).

The severity in health and safety risks for shellfish harvesting areas are evaluated based on contributing faecal sources. Usually human sources are considered the highest risk due the association with gastrointestinal pathogens that can readily infect other humans (McLellan et al. 2014). In dry conditions (i.e. low tide) seabirds are a main source of contamination because they defecate directly on top of the shellfish beds (Derolez et al. 2009). This source contains a range of well-known foodborne pathogens such as *Salmonella*, *Shigella*, and *Campylobacter* (Lu et al. 2008).

Rainfall is the most frequently recognised parameter associated with peak abundances of FIOs in shellfish and harvesting waters (Campos et al. 2013). FIOs can be detected in shellfish flesh for up to three to six days after a rainfall event (Coulliette et al. 2009; Kelsey et al. 2004; Lipp et al. 2001). In the UK up to 95% of the total FIO input in coastal waters can be attributed to rainfall (Stapleton et al. 2008). In many instances rainfall is considered an appropriate substitute for faecal indicator assessments and is used to regulate shellfish harvesting (Gourmelon et al. 2010). In Canada and the USA the opening and closing of harvesting from conditionally approved shellfish areas are regulated with rainfall (Campos et al. 2011). Often rainfall has replaced FIOs as a regulating factor because microbiological assessments can take up to 18 - 24 hours to obtain a result.

Due to its association with rainfall, often the use of river flow is considered an additional parameter for identifying periods of potential FIO breaches. In the scientific literature, river flow has been shown to correlate well with faecal pollution, thus is often used in hydrodynamic simulations for predicting influxes of FIOs in shellfish waters and shellfish quality (Bougeard et al. 2011). However for compliance of public health controls in bivalve mollusc fisheries, the use of river flow has been limited (Brock et al. 1985; Fiandrino et al. 2003), although for bathing purposes the hydrographic events of the wider catchment are often considered (Crowther et al. 2002; Kay et al. 2005).
Shellfish risk assessments are based on the quality of the overlying water, however the relationship between the FIOs recovered from the water and its relation to accumulation levels in the shellfish may not be straightforward (Lee & Murray 2010). Many studies have assumed a constant accumulation factor in shellfish across different environments for evaluating shellfish quality, and are based on risk assessments of the faecal microbial content of the water (Riou et al. 2007; Pommepuy et al. 2005). However, often other conditions of the water such as salinity and temperature can impact the feeding of shellfish and therefore the accumulation of FIOs (Derolez et al. 2013).

FIOs are also considered good predictors of other pathogenic organisms in harvesting waters. Regression models for the occurrence of enteroviruses showed that rainfall and temperature were good predictors for viral abundances (Lipp et al. 2001). But FIOs serve as an indicator of pathogens in faecal matter and do not indicate non-faecal sources that can also be present in shellfish harvesting areas (Table 2). Some of these pathogens are part of the natural environment and can be associated with both terrestrial and marine waters (i.e., *Aeromonas spp.* and *Vibrio spp.*).
Table 2: Common pathogens associated with shellfish consumption. (Table taken from Campos et al. 2013, with modifications for this thesis)

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Type</th>
<th>Source</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio vulnificus</em></td>
<td>Bacteria</td>
<td>Environment</td>
<td>Gastroenteritis and septicaemia</td>
<td>(Pommepuy &amp; Le Guyader 1998)</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Bacteria</td>
<td>Environment</td>
<td>Cholera</td>
<td>(Pommepuy &amp; Le Guyader 1998)</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>Bacteria</td>
<td>Environment</td>
<td>Gastroenteritis</td>
<td>(Pommepuy &amp; Le Guyader 1998)</td>
</tr>
<tr>
<td><em>Aeromonas spp.</em></td>
<td>Bacteria</td>
<td>Environment</td>
<td>Gastroenteritis (&quot;Traveller’s diarrhoea&quot;)</td>
<td>(Pommepuy &amp; Le Guyader 1998)</td>
</tr>
<tr>
<td><em>E. coli O157:H7</em></td>
<td>Bacteria</td>
<td>Human and animal excrement</td>
<td>Gastroenteritis</td>
<td>(Chadwick et al. 2008)</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>Bacteria</td>
<td>Human and animal excrement</td>
<td>Gastroenteritis and typhoid fever</td>
<td>(Pommepuy &amp; Le Guyader 1998)</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Bacteria</td>
<td>Human and animal excrement</td>
<td>Listeriosis</td>
<td>(Pommepuy &amp; Le Guyader 1998)</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Bacteria</td>
<td>Human and animal excrement</td>
<td>Gastroenteritis</td>
<td>(Pommepuy &amp; Le Guyader 1998)</td>
</tr>
<tr>
<td><em>Shigella spp.</em></td>
<td>Bacteria</td>
<td>Animal excrement</td>
<td>Gastroenteritis</td>
<td>(Cook 1991)</td>
</tr>
<tr>
<td>Norwalk like virus (Norovirus)</td>
<td>Virus</td>
<td>Human excrement</td>
<td>Gastroenteritis</td>
<td>(Karamoko et al. 2005)</td>
</tr>
<tr>
<td>Human enteric virus</td>
<td>Virus</td>
<td>Human excrement</td>
<td>Gastroenteritis</td>
<td>(Karamoko et al. 2005)</td>
</tr>
<tr>
<td>Small round structured virus</td>
<td>Virus</td>
<td>Human excrement</td>
<td>Gastroenteritis</td>
<td>(Ang 1998)</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Virus</td>
<td>Human excrement</td>
<td>Hepatitis A</td>
<td>(Croci et al. 2003)</td>
</tr>
<tr>
<td>Non B enteral hepatitis</td>
<td>Virus</td>
<td>Human excrement</td>
<td>Hepatitis E</td>
<td>(Swain et al. 2010)</td>
</tr>
<tr>
<td><em>Giardia intestinalis</em></td>
<td>Protozoa</td>
<td>Human and animal excrement</td>
<td>Giardiasis</td>
<td>(Geurden et al. 2010)</td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>Protozoa</td>
<td>Human and animal excrement</td>
<td>Gastroenteritis/Cryptosporidiosis</td>
<td>(Fonseca et al. 2006)</td>
</tr>
<tr>
<td><em>Adenoviruses</em></td>
<td>Virus</td>
<td>Human and animal excrement</td>
<td>Gastroenteritis</td>
<td>(Hundesa et al. 2010)</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>Bacteria</td>
<td>Human and animal excrement</td>
<td>Gastroenteritis</td>
<td>(Roslev et al. 2009)</td>
</tr>
</tbody>
</table>
1.7.3 Shellfish species in NZ

Bivalves that are of customary, commercial and recreational importance in New Zealand include *pipi* (*Paphies australis*), *tuatua* (*Paphies subtriangulata*), cockles (*Austrovenus stutchburyi*), mussels (*Perna canaliculus* and *Mytilus edulis*) and oysters (*Ostrea chilensis*, *Crassostrea gigas*).

The New Zealand bivalve *Austrovenus stutchburyi* (Gray, 1828) is a common occurring species occupying the intertidal zone of estuaries and harbours. *A. stutchburyi* is one of the most common bivalves found within the soft shore sheltered habitats, and are also known as cockles, little neck clams or *tuaki* (MPI 2015b). They burrow within the top 10 cm of sediment (Larcombe 1971) and form beds of 40 to 4,500 animals per m$^2$ (Ministry of Fisheries 2009). The shell is kept tightly closed when they are not submerged in water to prevent the animal from drying out. The cockle is an important recreational and customary resource and has been commercially harvested since the 1980’s (Kainamu 2010). Commercial harvesting still operate in Otago, Golden Bay, Tasman Bay in the South Island, and Whangarei in the North Island (O’Connell-Milne 2015; Williams et al. 2009).

*Perna canaliculus* (Gmelin 1791), *kutai* or the green-lipped Mussel, is an endemic species to New Zealand. The mussel extends byssus threads (small rope like structures) from the shell to anchor itself to hard surfaces such as rocks and wharf piles (Morton & Miller 1973). Green lipped mussels are one of the most common mussel species that are found throughout the intertidal and sub-tidal areas of the NZ coast (Buchanan 1999). It differs from the *Mytilus* species in colour and shape of the shell and shape of the animal inside (Siddall 1980), with the main distinguishing feature being the vibrant green bands along the shell margin. They feed on phytoplankton, zooplankton and detritus through filter feeding (Vakily 1989). Green lipped mussels are commercially grown and harvested in aquaculture throughout the North and the South Island (MPI 2013a).

1.8 Waikouaiti estuary

The Waikouaiti estuary is located within the township of Karitāne. It drains the third largest catchment in the Otago region of the South Island: The Waikouaiti
catchment (Fig. 2). The catchment consists of two branches: the north branch covers 283 km$^2$ of area, and the south branch covers 86 km$^2$. The flood plains and estuary cover 56 km$^2$, equating to 425 km$^2$ in total catchment area (Fig. 2). The two branches converge 8 km upstream from the harbour mouth and the first 5 km of the river is saline. Like other rivers in the Otago region, the Waikouaiti river exceeds safety limits set by the local council for nutrients and faecal bacteria (ORC 2010b; ORC 2014a). The continual development within the catchment has altered water quality and the ecological health of its inhabitants.

The Waikouaiti estuary and Waikouaiti river are important areas for angling, whitebaiting, tourist operations, water sports and shellfish gathering. For local Māori the catchment is of cultural, physical and spiritual importance. As specified by Mules & Prebble (2004) these areas are considered as:

- *Waahi tapu* – a sacred place that holds spiritual value, particularly with regards to the water

- *Kohanga* – an important nursery, breeding or spawning ground for marine/bird species and reseeding ground for shellfish

- *Mahika kai* – a site used as a harvesting resource of food

- *Cultural site* – providing sources of traditional materials such as flax and raupō for weaving

- *Trails* – water bodies that provide traditional and contemporary access ways to different areas throughout the catchment

The saying: *Waikouaiti te awa wairoa o to tātou tini i tupuna, rere tonu, rere tonu*, translates to ‘The Waikouaiti is a river that embodies our health and vitality passed on by our many ancestor, may it continue to flow strong’ (Mules & Prebble 2004). This highlights the intrinsic value of the river and its inhabitants to local Māori and the community.
Figure 2: Branches of the Waikouaiti catchment. The total area for the northern branch is 283 km$^2$ and the southern branch is 86 km$^2$. The lower catchment contains the Waikouaiti estuary as indicated by the red dot.
1.8.1 East Otago Taiāpure

Section 174 of the Māori Fisheries Act permits the establishment of traditional Māori fishing grounds to be recognised as Taiāpure (a coastal patch managed by local people). This enables local communities and tangata whenua to have significant input regarding the fisheries management of these areas. There are currently 10 of these sites throughout New Zealand with several that established within the Kai Tahu (principle tribe of the south island) region, including one within the Otago region. This is managed by the East Otago Taiāpure committee, a group that is made up of representatives from the East Otago Boating Club, Kāti Huirapa Rūnaka ki Puketeraki, Karitāne Commercial Fisherman’s Co-operative, The University of Otago, and River-Estuary Care: Waikouaiti - Karitāne. The expertise within this group utilise both contemporary and traditional Māori knowledge to recommend fisheries regulation and management tools to the Ministry of Primary Industries. Such regulations have altered recreational and commercial harvesting of pāua in aim to replenish an abundant stock that once existed. The East Otago Taiāpure committee collaborate with several University departments to obtain a multidisciplinary understanding of the marine environment to inform regulation decisions (Hepburn et al. 2010; Jackson et al. 2010).

Recently, the safety of shellfish after rainfall-induced contamination has become of concern to the committee. Locals of the Waikouaiti – Karitāne area have particularly raised questions around the safe to harvest standards pertaining to their traditional and local shellfish harvesting sites. This area was of particular interest as customary harvesting is often carried out in this area for the local hapū and marae (pers. comm. Brendan Flack, Chair, East Otago Taiāpure committee). This thesis was developed out of these concerns, with the purpose of addressing the dynamics of rainfall-induced faecal contamination in the shellfish harvesting areas of the Waikouaiti estuary.

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8 Ministry of Fisheries, 1 October 2010
1.9 Thesis Objectives
The primary focus of this research was to identify the microbiological safety of commonly harvested shellfish species in the Waikouaiti estuary after a rainfall. In this study *E. coli* sampling was carried out on cockles and mussels. These concentrations were compared to commercial harvesting standards in order to address the safety for recreational harvesting. Microbial DNA sequencing following contamination events aimed to identify potential faecal sources to address the contamination concerns of the community.

The chapter layout is as follows:

**Chapter One: Thesis introduction**
This section provides background for the thesis and thesis direction.

**Chapter Two: Monitoring of faecal contamination in the Waikouaiti estuary**
This chapter investigates faecal contamination in the Waikouaiti estuary during both baseline and rain conditions. Concentrations of *E. coli* were enumerated for the water and the tissue of cockles and mussels.

**Chapter Three: Next generation sequencing as a tool to detect faecal contamination**
Faecal contamination is further analysed by examining 16S rRNA microbial gene profiles for the water samples collected in chapter two. Profile comparisons were carried out based on *E. coli* abundance, rainfall and river flow. Screenings for faecal related bacteria and potential foodborne pathogens aimed to identify key taxa that could be used for Microbial Source Tracking, and assess other bacterial risks for shellfish consumers. Lastly, the culturing media that was used to enumerate *E. coli* in chapter two was further examined for enriched bacteria.

**Chapter Four: Overall discussion and conclusion**
This chapter links the above chapters and discusses the potential implications for local management, with suggestions for future water quality assessments for recreational and shellfish harvesting areas.
CHAPTER TWO

Monitoring faecal contamination in the Waikouaiti estuary

2.1 Introduction

Like many aquatic environments throughout the globe, the water quality of shellfish harvesting areas is affected by rainfall-induced faecal contamination (Lipp et al. 2001). The Waikouaiti catchment regularly experiences faecal pollution following rainfall, leading to quality issues down stream for recreational and customary shellfish harvesting (ORC 2010a). Water quality monitoring is a legal obligation for both local and regional councils in New Zealand. The results assist with faecal pollution mitigation for regional planning and monitoring for consented waste discharges from local operations. Previous waste interventions in the Waikouaiti catchment have involved re-directing sewage discharge onto land instead of the waterways, and contributions from some local farming operations have seen an improvement in management practices to reduce faecal and nutrient loading in nearby waterways. The combinatory efforts of the community, farmers and council, have substantially lowered E. coli by 12% annually. This accomplishment earned the Waikouaiti River the ‘third most Nationally improved river in the 2013 New Zealand’ at the River Awards (ORC 2014b).

2.1.2 Faecal contamination assessments in shellfish harvesting grounds

*Microbiological media*

Traditional faecal assessments utilised total coliform bacteria to examine and quantify faecal contamination in environmental waters and food products. This was because it was widely accepted that coliforms resembled faecal bacteria from warm-blooded animals due to their ability to utilise lactose. Studies later identified that only a proportion of the coliform group were of faecal origin, and lactose utilising bacteria are abundant in the environment (Leclerc et al. 1996; Rompré et al. 2002; Schraft & Watterworth 2005; Hormann & Hanninen 2006). Thus many environmental coliforms gave a false positive result for these tests, such as *Citrobacter* spp. and *Erwinia* spp. (Wutor et al. 2009; Mclain et al. 2011) which are commonly isolated from, soil, plants and water (Mundy et al. 2003; http://nzriverawards.org.nz/2013-river-awards/)
Kube et al. 2008). Current indicators for faecal bacteria are definitely associated with faeces from warm-blooded animals, commonly used species are *E. coli* and *Enterococci* (Rompré et al. 2002; Schraft & Watterworth 2005). *Enterococci* has an increased survival rate in saline waters and a higher correlation with human effluent, it is therefore commonly used as a faecal indicator in marine environments. *E. coli* is the indicator of choice for assessing freshwater because it is better associated with health outcomes (Prüss 1998).

Bacteria are ubiquitous in seawater and concentrations can reach up to $10^6$ cells per mL (Whitman et al. 1998). Quantifying only one particular group of bacteria requires isolation methods that either excludes the other bacteria from the sample in the analysis, or by targeting an identifiable characteristic that is specific to the group of interest. This is achieved through the use of specialised microbiological broths and agars. Selective media is designed to supply target bacteria with the required nutrients while simultaneously inhibiting non-target groups. Differential media allow for phenotypic identification of specific bacterial species using a colour indicator. Chromogenic media is commonly used for detecting the recognised *E. coli* specific enzyme $\beta$–glucuronidase (Grant 1997; Hallas et al. 2008; Chao et al. 2004).

*Faecal contamination assessments in shellfish*

The current protocol used to safeguard shellfish consumers from faecal contamination worldwide is through the quantification of *E. coli* contained in the tissue of shellfish. In Europe and New Zealand the commonly employed method is the Most Probably Number count (MPN) (MPI 2013b). This is a two-stage method, the first step requires a cell resuscitation phase in a broth followed by a secondary confirmation for *E. coli*. In the first stage a MPN count is carried out whereby a series of tubes are inoculated with varying dilutions of a sample. The tubes are then analysed for the production of gas and a colour change correlating to a presumptive positive result for *E. coli*. These tubes are then subcultured onto a chromogenic agar to visualise Galactosidase production and confirm a positive result. The number of positive tubes is then compared to an index to determine the concentration of cells. Although this is the current method used by Government authorities, there are plating methods that can give a result in 24
hours as opposed to the 48 hours that is required for the MPN method (Clements et al. 2015; 2013). A rapid result is important in ensuring that management actions can respond to immediate health threats.

**Methods for assessing faecal contamination in water**
Two different methods can be used for the analysis of *E. coli* in water: the MPN method (described above) and a plate filtering method (EPA Method 1103.1). The later of the two is a 24-hour single-step method that does not require further sub culturing steps for the confirmation of *E. coli*. The basis of this method requires filtering water samples through an appropriate pore size to trap bacteria of interest. The filters can then be directly plated onto a differential agar, such as m-Endo agar which contains a chromogenic dye with a red indicator when *E. coli* bacteria catabolises glucuronide to glucuronic acid (Dufour et al. 1981).

**2.1.3 NZ biological standards for commercial versus recreational harvesting**
Quality assessments and criteria for shellfish harvesting areas are different for commercial and recreational harvesting. The regulation of harvesting in commercial areas is under the jurisdiction of MPI and are regulated under the Animal Products (Specifications for Bivalve Molluscan Shellfish) Notice 2006 (MAF 2006). The main purpose of the scheme is to identify, monitor, evaluate and manage the risks that are associated with shellfish and shellfish water within harvesting grounds that are intended for human consumption (MPI 2013a).

Under the BMS Notice (MAF 2006) harvesting grounds are given a status based on initial sanitary assessments and regular monitoring results. Generally, the water for approved harvesting areas should meet two main standards throughout each sampling period. Firstly FIO levels should not exceed a mean value of 14 colony forming units (cfu) per 100 mL, and secondly no more than 10% of samples should exceed 43 cfu/100 mL (MPI 2013b). Assessments of shellfish flesh are also carried out in commercial areas. In order to maintain the ‘Standard for approved growing areas affected by point sources’ status these sites must not exceed a median of 230 MPN/100 g and not more than 10% of samples should exceed 700 cfu/100 g. Exceedances of these guidelines can potentially result in the closure of these harvesting areas by an MPI officer.

*Recreational harvesting areas*
Microbiological standards for recreational areas are not as stringently regulated as commercial areas. Additionally, they are not under the jurisdiction of MPI, therefore there are no guidelines that exist for the regulation of non-commercial harvesting grounds (Bay of Plenty Regional Council 2009). Instead local councils perform microbiological surveillance in accordance with ‘Microbiological Water Quality Guidelines for Marine and Freshwater Recreational Areas’ (MfE/MoH\textsuperscript{10} 2003). These guidelines are based on a risk assessment approach that utilises FIO concentrations to stipulate the level of safety for different recreational activities (Table 3). Exceedances of the ‘action mode’ lead to issuing of safety warnings and sometimes beach closures. At the ‘alert mode’, the sampling frequency is increased and a sanitary survey of the wider catchment may follow to identify sources.

The water quality values for recreational grounds are the same for commercial grounds, however there are no requirements for sampling the shellfish flesh. New Zealand Food Safety Authority state that the BMS Notice (MAF 2006) may also be suitable for recreational purposes, but shellfish monitoring is not currently within the councils monitoring scope. Because of its association with pathogens, rainfall is used as a general guideline for warning the public about FIO exceedances in shellfish. Harvesters are discouraged from collecting shellfish 2 days after a rainfall (ORC 2010a), or until the water runs clear for a few days (MPI 2013b).

\textsuperscript{10}`Ministry for the Environment’ (MfE) and ‘Ministry of Health’ (MoH)
Table 3: Guideline values for recreation and shellfish gathering from the Microbiological Water Quality Guidelines for Marine and Freshwater Recreational Areas (MfE MoH 2003). (Table adapted from Mactavish 2013)

<table>
<thead>
<tr>
<th>Guideline</th>
<th>Level/Type</th>
<th>Allowable concentration</th>
</tr>
</thead>
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<td>One single sample between 140 and 280 Enterococci/100 mL</td>
</tr>
<tr>
<td></td>
<td>Action</td>
<td>One single sample over 280 Enterococci/100 mL</td>
</tr>
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<td>Action</td>
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</tr>
<tr>
<td>Recreational shellfish gathering Bacteriological guidance value</td>
<td>The median faecal coliform content of samples taken over a shellfish gathering season shall not exceed MPN of 14/100 mL, and no more that 10% that exceed MPN of 43/100 mL</td>
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</tr>
</tbody>
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2.1.4 Association of nutrients with wastes
Water quality monitoring commonly employs nutrient assessments alongside faecal bacterial assessments (Dolah et al. 2007; Mallin et al. 2007; Templar et al. 2016). This is particularly important for agricultural areas where nutrients are often associated with farm animal wastes and fertilizers (USEPA 2006). Macronutrients are key limiting factors required for the growth and reproduction of primary producers in aquatic environments, however their balance and cycling is vital. Excessive nutrient influxes from anthropogenic activities can significantly burden the ecosystem and lead to eutrophication, plant overgrowth, oxygen depletion, poor water quality and algal blooms (Jarvie et al. 2012). Nitrogen is the limiting factor for marine systems but in freshwater it is often phosphorus (Howarth & Marino 2006; Hecky & Kilham 1988). Concentrations of nitrogen, phosphorus and ammonia can also be associated with animal effluent as well as domestic sewage (Gray et al. 2002; Royer et al. 2006). Farm operations require the use of fertilizers to produce pastures for stock. The nature of its application allow for easy entry into neighbouring waterways (Wilcock et al. 1999).

Local councils monitor nutrients in aquatic environments to manage and regulate waste outputs from industrial and agricultural operations. Concentrations are compared with the guidelines established in ‘Water Quality Guidelines for Fresh and Marine Water’ (ANZECC & ARMCANZ 2000) for both New Zealand and Australian waters. Essentially the guidelines provide ‘trigger levels’ which indicate levels of nutrient enrichment that can potentially cause ecosystem damage in aquatic waters if they are exceeded. Therefore, waste discharges into waters that are well managed should resemble, and be maintained, well below these guideline limits. Unfortunately the ANZECC trigger levels for an estuarine system are derived for low-nutrient (oligotrophic) waters in South-east Australia. NZ waters are considered to be naturally higher in nutrients, often leading to higher number of limit exceedances (Bolton-ritchie & Main 2005; Mills 2014).

2.1.5 Water quality of the Waikouaiti River
The Otago Regional Council (ORC) monitor surface water quality throughout the region as part of the ‘State of the Environment’ (SoE) reporting programme (ORC 2012). Measured parameters include E. coli, Enterococci and nutrient
concentrations in accordance with the MfE/MoE (2003) and ANZECC (2000) guidelines. Under the SoE scheme the Waikouaiti river is sampled monthly 7km up the river at Orbells crossing (see Figure 3 in the materials and methods); additional weekly samplings occur in the summer period and include the Karitāne wharf (1st November to 31st March) (ORC 2005). The sampling frequency is increased for the summer period because recreational activities are considered to be more popular during this season, although this does not account for shellfish harvesting which occurs all year round.

Water quality data for the Waikouaiti River shows that overall the river is currently graded as ‘Very Good’ in the ‘Water Quality Index’. This index measures variables of FIOs and nutrients (ammonia, nitrogen and phosphate), turbidity and dissolved oxygen. Other data indicates that E. coli abundances have substantially decreased from 2001 to 2011, and ammonia concentrations are continuing to improve (ORC 2012).

2.1.6 Potential contamination sources in the Waikouaiti estuary

Agricultural land use within the Waikouaiti catchment

Beef and sheep farming is the primary land use within the Waikouaiti catchment and is particularly dominant in the lower reaches (ORC 2008). Nutrient enrichment of the estuary is reported to be a result of agricultural run-off and FIOs sources are attributed to both agricultural and human sources (ORC 2015). Floodgates are also of concern for water quality because they allow for the accumulation of faecal bacteria, algae and nutrients which can then be delivered into the estuary when the gates are opened (ORC 2010b). Some marshlands extend into stock paddocks that are used for grazing allowing for contaminants to directly enter the associated water (Mules and Prebble 2004).

Waikouaiti Catchment Rainfall

Exceedances in quality guidelines for contact recreation are common in the catchment after a rainfall (ORC 2010b). Average rainfall for the catchment is around 50 mm a month, with the highest averages observed from January to March for the lower branches and December to January for the northern (ORC 2008). An increase in river flow is often observed with rainfall; substantial
rainfalls can be depicted by flow rates measuring above 1.34 cumecs\(^1\). Rainfall-induced contamination is primarily considered to originate from farm run-off, but it is possible that point and diffuse contamination from other operations also contribute (ORC 2015).

**Arm of the estuary**

A previous unpublished report found large amounts of nutrients, *E. coli* and hypoxic sediment in the marshland adjacent to the base of the DCCs oxidation pond (Chand et al. 2015). It was assumed that the observed agricultural activity in this arm is a potential source. Mainland poultry is also located up this arm, it is the largest egg production operation in New Zealand (Ministry of Agriculture and Fisheries 2012). The wastes are transferred into treatments ponds before irrigation onto nearby land. It is expected that some of the irrigated waste could be washed into the coastal environment (ORC 2005).

**Waikouaiti sewage**

The Waikouaiti sewage scheme collects brown waste from both the townships of Waikouaiti and Karitāne. The collected waste is passed through a comminution process (grinds solid wastes) before flowing through to the Waikouaiti Oxidation Ponds and is then spray irrigated onto a pine plantation on a sandy foreshore. The oxidation ponds are situated within 20 metres of the high tide mark adjacent to the Waikouaiti estuary and the pine plantation 300 m to the south of these. Seepage from both of these systems into the coastal environment is possible (ORC 2012). Annual nutrient and invertebrate surveys monitor the Waikouaiti beach and adjacent tidal mudflats, these have so far indicated that there are no diverse effects of the land discharge.

**2.2 Aims**

The objective of this study was to assess the water and shellfish quality in the Waikouaiti estuary after a rainfall. Cockle and mussel harvesting areas were investigated using the faecal indicator *E. coli* as per commercial standards. Nutrient concentrations were also determined to investigate their association with faecal contamination and to compare the concentrations with the ANZECC

\(^1\) http://water.orc.govt.nz/WaterInfo/Site.aspx?s=WaikouaitiConfluence
guidelines. The other aims of this chapter were to examine the (current) method of measuring water quality and comparing these results directly to the concentration of FIOs in the shellfish. A 24-hour method was also used for delivering quantification of faecal contaminants in shellfish.

More specifically, the aims were:

1) To determine the abundance of faecal contamination in the Waikouaiti estuary during periods of rain in the summer to winter months;

2) Identify the potential health risks of *E. coli* contamination for harvesting cockles and mussels in comparison to commercial standards for shellfish harvesting sites;

3) Identify a correlation of rainfall, nutrients and river flow with *E. coli* contamination.
2.3 Materials and methods

2.3.1 Microbiological Media

Tryptic Soy Broth
Tryptic Soy Broth, (211825, BD, France), was made according to manufacturers instructions with autoclaved Milli-Q water. The broth was kept at 4 °C for up to two weeks.

CM1046 Brilliance E. coli coliform selective medium
Brilliance E. coli coliform selective media, (CM1046, Oxoid, Thermo Scientific), chromogenic agar was used for the enumeration of total coliforms and E. coli. Agar plates were prepared according to manufacturers instructions. Total coliform were represented by pink colonies, and purple coloured colonies were indicative of β-glucuronidase activity from E. coli colonies. The plates were then kept at 4 °C for up to two weeks. Plates were incubated at 37 °C (Clements et al. 2013).

Tryptic Soy Agar
Tryptic Soy Agar, (236950, BD, France), was made according to manufacturers instructions. Set plates were kept in a sterile container at 4 °C for up to two weeks.

Butterfields buffered phosphate diluent (Butterfields solution)
This buffer was used as a diluent for the E. coli and shellfish homogenate suspensions. Autoclaved Milli-Q water was mixed with 34 g KH₂PO₄ and the final product was adjusted to a pH of 7.2 using a 5M NaOH solution¹². The final volume was then brought up to a total of 1 litre using Milli-Q water and autoclaved for 15 minutes at 121 °C.

2.3.2 Sampling of the Waikouaiti estuary

Site locations
Sampling sites were located in the coastal settlement of Karitāne 35 km north of Dunedin city centre, New Zealand. All samples were collected on the low outgoing tide. *Austrovenus stutchburiyi* were collected from the Waikouaiti estuary and *Perna canaliculus* from a rock wall along the Harbour mouth (Fig. 3). The site was chosen based on its popularity as a shellfish harvesting area.

Shellfish collection
For each shellfish site a 15 m transect was measured within each channel. Water and shellfish samples were randomly collected from three selected 1 m² plots. For the sampling of *Austrovenus stutchburiyi* each sample consisted of 15 cockles sized between 35-50 mm (Fig. 3). Green lipped mussels (*Perna canaliculus*) were collected along a rock wall of the harbour channel (Fig. 3). A total of 8 edible sized mussels were collected from below the waterline. Samples were kept cool and transported to the Portobello Marine Lab for sampling within 24 hours (in accordance with MPI and MAF protocols).

A plating method for the enumeration of coliforms and *E. coli* was developed from another study that investigated contamination in barnacles and mussels (Clements et al. 2013), with some adaptions. For *E. coli* enumeration, enough shellfish was collected in order to obtain at least 100 g of shellfish contents including both the meat and liquor (collectively referred to as tissue) in accordance with the MPI method (MPI 2013c). All organic matter was removed from the surface of the shellfish using a knife and a sterile pot scrubber under running water. They were then rubbed with ethanol and left to dry before the animals were aseptically shucked. Shellfish tissue was pooled per sample plot and then homogenized in a blender on high for 2 minutes. Dilutions of 1:2 and 1:4 of the homogenates were made in Butterfields solution. Spread plates were made on CM1046 agar plates using 100 uL of each dilution and an undiluted sample. The plates were incubated for 24 hours at 37 °C. Total coliforms and *E. coli* colony counts were displayed as number of colonies per 100 g. Colonies that did not clearly resemble a purple colony were re-streaked onto another plate (of
the same media; CM1046) and incubated (24 hours at 37 °C) for colour confirmation.

**Water sample processing**
Water directly adjacent to the shellfish plots were collected for both the cockle and mussel sites from the respective channel. 500 mL autoclaved bottles were aseptically submerged 6 cm below the waters surface. Collected samples were kept cool during transportation to the lab where they underwent membrane filtration using a developed EPA method 1603 (EPA 2009). For each sample aliquots of 10, 100 and 200 mL were filtered through a 0.45 µm nitrocellulose filter and plated onto CM1046 agar. The plates were analysed for *E. coli* and total coliform colonies after a 24 hour incubation period at 37 °C. Filters with merged colonies, or ones that appeared as a ‘water paint mark’, were labelled as indistinct colonies (IC) and were not counted. Colonies exhibiting an unidentifiable colour were re-streaked onto CM1046 media and incubated (24 hours at 37 °C) for colour confirmation.

**Rainfall measurements**
Weekly rain gauge readings were taken from a gauge deployed at Te Taumata-o-Puaka (Fig. 4). This gauge represented rainfall in the lower catchment of the Waikouaiti. Readings were also taken from another rain gauge within the upper catchment on Mt Misery, this is maintained by the ORC and results are uploaded onto the website. All rainfall readings were taken throughout the sampling period. Data was later collected from a secondary station located in Palmerston (Fig. 4) from the CliFlo National Institute of Water and Atmospheric Research (NIWA) site. The rainfall data was used for statistical tests between rainfall amount and *E. coli* abundances in shellfish homogenate and water.

**River flow measurements**
Flow measurements were obtained from the Otago Regional Council (ORC). Continuous water flow is measured at Orbells (Fig. 3), and is processed and loaded directly to the water section of the ORC website every 10 minutes (http://water.orc.govt.nz/WaterInfo/Default.aspx). Flow rate measurements are made using a Sontek Flow Tracker Acoustic Doppler Velocity current metre, calculations are then made in accordance with the ISO standard ISO748 to
convert the data into a flow rate. All flow data pertaining to the sampling months in this study were obtained from the council. From this the daily maximum flow, minimum flow and daily averages were calculated and used to evaluate the correlation of river flow with bacterial abundances.

Nutrient sampling
Acid-washed syringes and filter housing was fitted with a fresh 1.2 µm filter (Whatman GF/C filter) then rinsed with sample site water three times collecting a 60 mL sample. Samples were collected in triplicate and a fresh acid washed syringe and filter housing are used for each site. Filtered water samples were stored at -20 °C up to the time of analysis. Reagents for nutrient standard curves were prepared a day before the analysis was carried out. Analyses were run on the Lachat Quick Chem 8500 Series FIA Auto Analyser, which contains different channels to simultaneously measure the concentration of nutrients within a sample based on spectrophotometric analyses as a result of chemical reactions.

Phosphate was measured by the reaction of ammonium-molybdate and antimony-potassium-tartate, to antimony-phospho-molybdate, which was quantified by the blue complex formed after the addition of ascorbic acid (measured at 880 nm). The nitrogen species (NOx) was measured by the reduction of nitrate to nitrite by the addition of NEDD (N-(1-napthyl)-ethylenediamine dihydrochloride), and then running the sample through a copper coated cadmium column to measuring the reaction with a pink azo dye which was measured at a wave length of 520 nm. Ammonia was measured based on the Berthelot reaction, where hypochlorite is added to give monochloramine and reacts with nitropusside and phenol to give indephenol blue (measured at 630 nm), which is proportionally equivalent to the amount of the ammonia. These reactions are suitable for samples of brackish, saline and non-saline origin. Concentrations of nitrite/nitrate (NOx), ammonium and phosphate were expressed in mg/ml for comparison with the ANZECC (2000) estuarine trigger values.

Statistical analyses
Statistical analyses used GraphPad Prism 6 version 6.0f for Mac OSX (GraphPad Software, San Diego Califronia USA). Normality of all data was tested using a
D’Agostino-Pearson omnibus test, the data was subjected to parametric or non-parametric tests accordingly.

A nonparametric Spearman’s correlation coefficient (two-tailed significance level; $P \leq 0.05$) was used to test the relationship of *E. coli* abundances in water samples between the mussel and cockle site and identify the similarity in *E. coli* occurrences. A non-parametric T-test was also carried out on this same data set.

One-tailed Spearman’s coefficients ($P \leq 0.025$) were employed to test the correlation of total coliforms and *E. coli* colonies in water samples using combined data from both the cockle and mussel sites. The same test was used for microbiological abundances versus river flow parameters (daily average, maximum and minimum daily flows), and abundances versus nutrients. A two-tailed test was used to analyse the correlation of seven and three day rainfall totals and river flow data with microbial abundances in shellfish.

*Map constructions*

All maps were created using QGIS version 2.20.1 Boston USA. Shape and vector files were sourced online from the LINDZ database website. Waikouaiti catchment maps were provided by Andrew MacKay (ORC, IT/GIS Officer).

*Summary of Background and Rainfall samples collected*

Background monitoring samples were collected consistently once a week for a total of 13 weeks from the 11th of January to the 24th of April 2015, excluding the week 21st February to 1st of March. A total of 13 background samplings were collected and labelled W1 to W13 respectively. Rainfall sampling was carried out after rainfall measuring 9 mm or above was indicated by rain gauges at Te Taumata-o-Puaka and Mt Misery, as well as an increase in river flow that exceeded the median flow (Fig. 4). Three rainfall samplings were carried out in total labelled R1, R2 and R3. A summary of the sampling types carried out and the corresponding dates are outlined in the sampling summary table (Table 4).

The nutrient samples were collected for background samples W12 and W13 and all rainfall samples. All other sample types were carried out for the Rainfall
sample. Specific water samples were also used for DNA sequencing based on the upper and lower limits for *E. coli* (see Chapter Three).
Figure 3: Location of sampling sites, loggers and flow metre in the Waikouaiti river and estuary. The ORC flow metre at Orbells crossing (A) indicates the flow of the river downstream from the confluence in the Waikouaiti catchment. The satellite image (B) indicates the shows the sand bar (green boxes) where cockle sampling was carried out and the rock wall where the mussel site on the rock wall (red boxes).
Figure 4: Rainfall data collection locations. The ORC rainfall data was collected from a logger on Mt Misery, located within the high ranges of the Waikouaiti catchment. A rain gauge measured rainfall at Te Taumata-o-Puaka (in Karitāne) as an indicator of rainfall in the lower reaches of the catchment. Data was also obtained from the NIWA weather station located in Palmerston to analyse the rainfall within the wider region.
**Table 4: Summary of sampling data and type per date.** Sampling data that was collected for each sampling type and date is marked by a ‘Y’, and data that was not available is marked by a ‘-’ symbol.

<table>
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<th>Mussel water</th>
<th>Cockle tissue</th>
<th>Mussel tissue</th>
<th>Nutrients</th>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>Y</td>
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<td>Y</td>
<td>-</td>
</tr>
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<td>Y</td>
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<td>-</td>
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<td>Y</td>
<td>Y</td>
<td>-</td>
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2.4 Results

2.4.1 Background and rainfall sampling summary
Water and shellfish samples were collected for background and rainfall periods to enable comparisons between E. coli abundances during rainfall events to a baseline value, i.e. periods of no rain. Three rainfall events were sampled (R1 - R3) as well as thirteen weeks for background sampling (W1 - W13).

The first samples (W1) were collected after a dry period with little rain (Fig. 5), and a very low river flow of 0.167 m$^3$s$^{-1}$ (Fig. 6). In the week leading up to the W2 sample, 8 mm of rain was observed and the river flow slightly increased to 0.192 m$^3$s$^{-1}$. The river dropped back to flow levels observed during the dry period for W3, and only 3 mm of rain 3 days earlier. W4 was carried out after 18 mm of rain had fallen in 24 hours, raising the river flow to a range of 0.81 to 1.867 m$^3$s$^{-1}$, exceeding the median flow value of 1.34 m$^3$s$^{-1}$.

Two weeks after, W5 was collected; four days prior to collection 10 mm of rain had fallen (this can be seen as a spike in river flow data). Sample W8 had 10 mm of rain three days prior to sampling and river remained low at 0.3 m$^3$s$^{-1}$. Another dry period occurred for the week leading up to the W9 sample although the river flow did shift slightly from 0.261 to 0.293 m$^3$s$^{-1}$. The next week (W10) remained at this same level in flow (0.28 m$^3$s$^{-1}$) and had 5 mm of rain five days prior. A slight shift in river flow occurred before the W11 sample (0.32 m$^3$s$^{-1}$) and remained for the W12 sample, both had 5 mm and 8 mm respectively two and three days prior to sampling.

The first rainfall sample (R1) was collected after a 9 mm rainfall event and the river flow had surpassed the median flow the day prior, reaching 1.5 m$^3$s$^{-1}$. After this rainfall W13 was collected a day after a 10 mm rainfall event and river flow of 0.6 m$^3$s$^{-1}$. The second rainfall sample (R2) was collected a day after the river had increased from 0.4 m$^3$s$^{-1}$ to 1.5 overnight, and 9 mm of rain on the day of collection. The last rainfall sample (R3) was collected 24 hours after a two-day rain event totalling 65 mm, raising the river from 1 to 50 m$^3$s$^{-1}$ in 24 hours. On the day of sampling the river flow averaged at 8 m$^3$s$^{-1}$, well above the median
flow of 1.34 m$^3$ s$^{-1}$. This was the largest rainfall event and river flow observed throughout the entire sampling period.
Figure 5: Rainfall (mm) collected from the Palmerston weather station. Measures rainfall from December 2014 to February 2015 (A) and March to June 2015 (B). Sampling period and type (W1 - W13 = background samples, R1 - R3 = rainfall sampling) is indicated by the purple arrows.

Figure 6: Flow rate data for the Waikouaiti River measured at Orbells. Rate of flow (m$^3$ s$^{-1}$) measurements were taken for December 2014 to February 2015 (A) and March to June 2015 (B). Sampling period and type (W1 - W13 =
background samples, R1 - R3 = rainfall sampling) is indicated by the purple arrows. The annual median flow of the river is indicated by the dotted redline (1.34 m³ s⁻¹).
2.4.2 *E. coli* and total coliform abundances in seawater

An enumeration protocol utilising membrane filtration and chromogenic agar was used to quantify total coliform and *E. coli* in collected water samples. Enumeration was carried out for the background and the rainfall samplings to assess the fitness of the water for shellfish harvesting in accordance with MfE/MoH (2003).

Between the mussel (Fig. 7A) and cockle site (Fig. 7B) there was a similar pattern in the abundances for both *E. coli* and total coliforms, i.e. one increased in abundance with the other and *vice versa*. This relationship was further assessed to determine if other faecal related microbes could be included within the total coliform group (supp. Fig 1). Combined site data for *E. coli* versus total coliforms was analysed using a Spearman’s correlation (non-parametric one-tailed), and showed that there is a positive correlation between total coliforms and *E. coli* (*r* = 0.46, *p* <0.001). Across all samplings, and for both sites, total coliforms were more abundant than *E. coli*.

On some accounts the total coliform colonies could not be counted due to inconstant colonies (IC) (R1, W13 and R3 for the mussel site, and R1 for the cockle site). On the other hand, *E. coli* was countable and was present in all of the samples collected for both sites and sampling types. The background sampling periods that measured above the MfE/MoH (2003) safe harvesting median limit of 14 cfu/100 mL were W2, W4, W7, W9 and W11 for the cockle site, and W9 and W11 for the mussel site. Over all the median of *E. coli* for data from the combined site was 5 cfu/100 mL.

The sample R3 contained the highest *E. coli* exceedances (533 cfu/100 mL) followed by W4 (50 cfu/100 mL). These were the only two samples that exceeded the MfE/MoH (2003) limit of 43 cfu/100 mL for faecal coliforms, of which only 10% of all samples are allowed exceed in order to remain within the bacteriological guidance values for recreational shellfish gathering areas. The remaining rainfall samplings (R1 and R2) did not exceed the either 43 or 14 cfu/100 mL MfE/MoH (2003) unsafe harvesting limits.
The enumerations between the water sampling sites indicated similar abundances of *E. coli* (Supp. Fig 2). A paired t-test data did not show a significant difference between the site data (t = 1.662, p = 0.1405), and showed a strong significant correlation with a Spearman’s correlation analysis (r = 0.8095, p <0.05). These results indicate that the faecal content of the water is the same at both sites.
Figure 7: Mean *E. coli* and total coliform abundances for the mussel and cockle site water samples. Sample type corresponds to background monitoring (W1-W13) or rainfall samplings (R1 - R3). The dotted red line indicates 14 cfu/mL and the solid red line represents 43 cfu/mL, these are the limits specified in the MfE/MoH (2003) guidelines. Samples that were missing are identified with IC (inconsistent colonies) or NS (not sampled). The Y axis is displayed in log scale. Replicates vary per sample, n = 1 to 3. (SEM (± 1)).
2.4.3 No clear association of nutrient concentrations with rainfall in water samples
Concentrations of phosphate, nitrogen and ammonia were analysed for their potential association with rain and faecal contamination. The concentrations were then compared against the estuarine trigger levels outlined in the ANZECC (2000). The results showed variability throughout the sampling period, with ammonia showing the most variability (Fig. 8).

Phosphate values ranged from 0.009 to 0.024 mg/L, with no samples exceeding the 0.03 mg/L limit. Ammonia ranged from 0.015 to 0.063 mg/L and all samples exceeded the trigger level concentration of 0.0156 mg/L. Nitrate (NOx) concentrations ranged from 0.006 to 0.387 mg/L, and exceeded the ANZECC guidelines for samples R2, W13 and R3. Sample R3 measured the highest in nitrate concentration for both the mussel and cockle site (0.4 mg/L).
Figure 8: Nutrient concentrations in the water samples from the rainfall and background sampling. Mean nutrient concentration (in mg/L) for Mussel and Cockle sites (N=3, error bars SEM ± 1). Dotted lines represent estuarine trigger values according to the ANZECC guidelines: nitrate and ammonia = green (0.015 mg/L) and phosphate = red (0.03 mg/L).
2.4.4 *E. coli* and total coliforms in the water significantly correlate with environmental parameters

Associations between the microbial data in the water samples and environmental parameters were examined using the Spearman’s correlation coefficient (one-tailed). The microbial abundances of *E. coli* and total coliforms from both sampling sites were combined and tested with data from rainfall, river flow and nutrients (Table 5).

**Rainfall**

Three day and weekly rainfall totals were collated from the collected data from the Mt. Misery site. A significant correlation was observed for both total coliforms and *E. coli* versus three day and weekly rainfall totals. The three day rainfall total appeared to be more correlated with *E. coli* abundances \( (r = 0.58, p <0.05) \) than the weekly rainfall totals \( (r = 0.47, p <0.05) \). However the opposite were observed for the total coliforms, which correlated more with weekly rainfall totals \( (r = 0.71, p <0.005) \) than with the three day period \( (r = 0.55, p <0.05) \). Correlations were also tested on data collected from the Palmerston and Te Taumata-o-Puaka sites (supp. Table 1), but these did not correlate as well as the Mt Misery site (Table 5). Overall the rainfall data from Mt Misery significantly correlated with microbial abundances in the water samples.

**River flow**

The calculated parameters: daily average, daily maximum, and daily minimum flows were used to assess the relationship of river flow on microbial *E. coli* and total coliforms.

The daily average flow versus total coliforms displayed a significant and strong correlation \( (r = 0.72, p <0.005) \), as well as *E. coli* \( (r = 0.46, p <0.05) \). The maximum daily flow correlations also indicated a positive correlation with total coliforms \( (r = 0.72, p <0.005) \) and *E. coli* \( (r = 0.47, p <0.05) \). The minimum daily flow was also correlated with total coliforms \( (r =0.64, p <0.005) \) and showed the strongest correlation out of all river flow measurements for *E. coli* \( (r = 0.47, p <0.05) \). This demonstrates that when river flow increases so does the abundance of both microbial groups.
**Nutrients**

Ammonia, phosphate and nitrate concentrations were correlated with combined site E. coli data for the water samples. The p-values indicate that the data is inconclusive for E. coli versus ammonia (r = 0.37, p > 0.05) and phosphate (r = 0.29, p > 0.05). However, a significant and strong correlation was found between nitrate and E. coli (r = 0.94, p < 0.0005). This potentially signifies that nitrate concentration increases with the abundance of E. coli in the water.

**Table 5: Spearman’s correlations between microbial abundances measured in the water and environmental variables.** Combined site data for E. coli and total coliform were tested against river flow data (daily average flow, maximum daily flow and minimum daily flow) Mt Misery rainfall data (three day and weekly totals) and nutrient concentrations (ammonia, phosphate and NOx/nitrate). All correlations were carried out using a Spearman’s correlation (one-tailed). For E. coli n = 16 and total coliforms n = 15, *P<0.05, **P<0.01 & ***P<0.001.

<table>
<thead>
<tr>
<th>Total/Type</th>
<th>Total Coliform</th>
<th>E. Coli</th>
<th>Combined E. Coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainfall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>Three day</td>
<td>0.55</td>
<td>0.02*</td>
<td>0.58</td>
</tr>
<tr>
<td>Weekly</td>
<td>0.71</td>
<td>&lt;0.01**</td>
<td>0.47</td>
</tr>
<tr>
<td>Average</td>
<td>0.72</td>
<td>&lt;0.01**</td>
<td>0.46</td>
</tr>
<tr>
<td>River flow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>Max daily</td>
<td>0.72</td>
<td>&lt;0.01**</td>
<td>0.48</td>
</tr>
<tr>
<td>Min daily</td>
<td>0.61</td>
<td>&lt;0.01**</td>
<td>0.47</td>
</tr>
<tr>
<td>Nutrients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>Ammonia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phosphate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NOx</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
2.4.5 *E. coli* and total coliform abundances in cockle and mussel homogenates collected for background and rainfall monitoring

The plate counting method was used to enumerate the *E. coli* abundances in both mussel and cockle shellfish samples throughout the background and rainfall sampling periods (as indicated in Table 4).

Coliforms were found in all shellfish samples except for W3 for the mussels. Total coliforms showed a similar pattern in abundances in both the cockle and mussel homogenates; this was however not the case for the *E. coli* occurrences.

*E. coli* presence in the mussels were identified in samples W4 and W11, amounting to 6,000 cfu/100 g and 500 cfu/100 g respectively. For the cockle samples *E. coli* was found in four of the background sampling periods: W7 (1,000 cfu/100 g), W8 (111.1 cfu/100 g), W9 (1333.3 cfu/100 g) and W13 (333.3 cfu/100 g). *E. coli* was not present in the both the mussel and cockle at the same time throughout the sampling, and interestingly was not found in any of rainfall samples for both species of shellfish.

Overall, two cockle (W7 and W13) and two mussel sample periods (W4 and W11) measured above the 230 cfu/100 g marketable limit for shellfish. A total of three out of all samplings (one mussel and two cockle samples) exceeded the maximum 700 cfu/100 g value for shellfish grounds. The background and rainfall sampling identified that there are unsafe concentrations of *E. coli* in shellfish.
Figure 9: Mean *E. coli* and total coliform abundances for the tissue of mussel and cockle samples. Means for total coliform (grey bar) and *E. coli* (black bar) counts for shellfish samples using the plate counting method (n = 1 to 3, SEM ± 1). Homogenates of (A) mussels and (B) cockles collected from the Waikouaiti sampling site for background monitoring (W1 - W13) or rainfall sampling (R1 - R3). Results displayed as cfu/100 g. Shellfish harvesting standards for *E. coli* according to MPI (or faecal coliforms by MAF) are represented by the blue line for the median limit (230 cfu/100 g) and red for the allowable 10% exceedances (700 cfu/100 g), breach of these limits consider the shellfish as unmarketable.
2.4.6 Environmental parameters do not correlate with accumulated faecal bacteria in shellfish

Associations between the microbial abundances in the mussel and cockle tissue versus rainfall and river flow were calculated using a Spearman’s correlation coefficient (two-tailed). Lastly, a one-tailed correlation was used to investigate the relationship between faecal bacteria present in the water samples and the resulting presence in the shellfish.

Rainfall data from the Mt Misery ORC weather station were collated into weekly and three day rainfall totals, as these have previously found to be the minimal and maximal peak lag times for contamination in shellfish (Lipp et al. 2001; Campos et al. 2011; Kelsey et al. 2004; Coulliette et al. 2009) Rainfall and microbial abundances gave inconclusive results for both rainfall totals (p <0.05), and both microbial groups in cockle and mussel tissue (Table 6). The same test also gave inconclusive results for rainfall data collected from the other sites Palmerston and Te Taumata-o-Puaka (supp. Table 1).

The microbial concentrations from the shellfish was then tested against the river flow data (measured at Orbells). The data was pooled into daily averages, daily maximum and daily minimum flows. The results for the cockle tissue (Table 6) gave inconclusive results (p < 0.05) for both E. coli and total coliforms versus daily average flow (total coliform: r = -0.27, and E. coli: -0.087), maximum daily flow (total coliforms: r = -0.24, and E. coli: r = -0.039), and minimum daily flow (total coliform: r = -0.36, and E. coli: = -0.08). The same analyses were carried out as above for the mussel homogenates (Table 6). The analysis was again inconclusive (p = < 0.05) for all correlations for daily averages (total coliforms: r = 0.046, and E. coli: r = 0.37), maximum daily flow (total coliforms: r = 0.048, and E. coli: r = 0.37), and minimum daily flow (total coliforms: r = 0.01, and E. coli: r = 0.09).

Lastly, a correlation was carried out on microbial abundances of water versus the shellfish. The results indicate that the evidence for the detection of correlations was inconclusive (p = < 0.05) for both mussel (total coliforms: r = 0.11, and E. coli: r = 0.09).
coli: r = 0.33) and cockle abundances (total coliforms: r = 0.51, and *E. coli*: = 0.35).

Overall, there are no conclusive results to suggest that there are any significant relationships between rainfall, river flow, and microbial content in the water, versus abundances of both total coliforms and *E. coli* in the flesh of the shellfish.
Table 6: Spearman’s correlations between microbial abundances measured in shellfish tissue and environmental variables.

*E. coli* and total coliform abundances for cockle and mussel tissue were tested against river flow data (daily average flow, maximum daily flow and minimum daily flow) and Mt Misery rainfall data (three day and weekly totals). All correlations with rainfall and river flow were carried out using a two-tailed correlation, the abundances of measured water flow used a single-tailed. Replicates range from n = 14 to 16.

<table>
<thead>
<tr>
<th>Rainfall</th>
<th>Total/Type</th>
<th>Mussel Total coliform</th>
<th>E. Coli Total coliform</th>
<th>Cockle Total coliform</th>
<th>E. Coli Total coliform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>r  p</td>
<td>r  p</td>
<td>r  p</td>
<td>r  p</td>
</tr>
<tr>
<td>Three day</td>
<td></td>
<td>0.22  0.45</td>
<td>0.30  0.34</td>
<td>-0.01  0.96</td>
<td>0.09  0.26</td>
</tr>
<tr>
<td>Weekly</td>
<td></td>
<td>0.20  0.48</td>
<td>0.41  0.15</td>
<td>0.08  0.76</td>
<td>-0.01  0.17</td>
</tr>
<tr>
<td>Average daily</td>
<td></td>
<td>0.05  0.88</td>
<td>0.37  0.22</td>
<td>-0.27  0.32</td>
<td>-0.08  0.22</td>
</tr>
<tr>
<td>Max daily flow</td>
<td></td>
<td>0.05  0.87</td>
<td>0.37  0.22</td>
<td>-0.24  0.38</td>
<td>-0.04  0.29</td>
</tr>
<tr>
<td>Min daily flow</td>
<td></td>
<td>0.002 &gt;0.99</td>
<td>0.09  0.13</td>
<td>-0.36  0.18</td>
<td>-0.08  0.23</td>
</tr>
<tr>
<td>Water microbial</td>
<td>Total coliform</td>
<td>0.01  0.97</td>
<td>-</td>
<td>0.51  0.06</td>
<td>-</td>
</tr>
<tr>
<td>abundances</td>
<td><em>E. Coli</em></td>
<td>-</td>
<td>0.33  0.25</td>
<td>-</td>
<td>0.35  0.19</td>
</tr>
</tbody>
</table>
2.5 Discussion

The purpose of this chapter was to identify whether rainfall (Fig. 5) and river flow (Fig. 6) increases faecal contamination in the shellfish and shellfish harvesting waters in the Waikouaiti estuary. Nutrients were also measured to assess their association with the presence of faecal indicators. These measurements were compared to guideline limits for an assessment of health and safety on the grounds for faecal waste.

2.5.1 E. coli abundances recovered from water samples

Originally the water from the cockle and the mussel site were examined independently as it was unknown if the additional seawater from the mouth of the inlet contributed a dilution effect on the microbes at the mussel site. The microbiological counts for the water (Fig. 7) and nutrient concentrations (Fig. 8) appeared similar between sites and indicated strong similarity. An analysis of E. coli abundance between sites confirmed a strong correlation (Table 5). Hereafter the water was treated as one for both sites.

Samples R3 (533 cfu/100 mL) and W4 (50 cfu/100 mL) contained the highest abundance of E. coli, and were the only samples that exceeded the 43 cfu/100 mL guideline (MfE/MoH 2003). W4 was the first to contain elevated levels of E. coli, this sample was collected after the first rainfall after a dry period (Fig. 5) and raised river flow above the median flow value (Fig. 6). R1 and R2 were collected after a week of no rain and also surpassed the river flow median (1.34 m$^3$/s$^{-1}$) (Fig. 6), however both these samples did not exceed the MfE/MoH (2003) limits. Although the hydrological conditions were the same, the differences in the abundance could be attributed to the accumulation periods, these are the periods before the rainfall. Faecal contamination from farmland can be sourced from oxidation ponds overflows and stock deposits in the paddocks. These can be flushed out after a rainfall and thus an accumulation period is when the bacteria is allowed time to build up within these reservoirs without rain. These results also indicate that a week without rainfall may not be a long enough accumulation period for a rainfall-induced contamination event.
Across the sampling period the median for *E. coli* did not exceed the 14 cfu/100 mL limit (section 2.4.2), and 10% of samples did not exceed the upper 43 cfu/100 mL guideline. In conclusion water quality is considered safe for shellfish harvesting in accordance with MfE/MoH (2003) guidelines.

Sampling periods containing high abundances of *E. coli* are likely to be associated with other faecal microbes (Silva et al. 2015); these would be represented in a proportion of the total coliform population. A positive correlation of *E. coli* versus total coliforms indicated that the coliforms present could be associated with other faecal microbes and therefore other potential pathogens (Table 5). To further this investigation, molecular techniques need to be employed to identify these other faecal groups present and their sources. MfE/MoH (2003) guidelines state that high faecal contamination counts should be followed up by a sanitary survey, which requires identifying potential contamination sources. An investigation into other faecal microbes and potential sources observed in periods of high and low *E. coli* abundances would be further examined in Chapter Four.

The literature and ORC reports an increase river flow from the median value and rainfall serves as a useful proxy for faecal contamination in the river. This was confirmed in these results where catchment rainfall and river flow (Table 5) correlated with *E. coli* and total coliforms in the water. Out of all of the sites the microbial abundances correlated strongest with rainfall at Mt Misery located in the higher reaches (Table 5) in comparison to the other sites Palmerston and Te Taumata-o-Puaka (supp. Table 1). Interestingly the rainfall data from the lower reaches (Te Taumata-o-Puaka) were inconclusive in determining a correlation with *E. coli* (supp. Table 1). Although this result does not necessarily suggest that there is no correlation at all, the lower reach is largely associated with farmland thus would have been expected to sufficiently correlate with *E. coli* associated with runoff. These findings could also suggest that large rainfalls within the wider catchment (i.e. Mt Misery) contribute to *E. coli* in the water. A larger data set is required to strengthen the evidence.

Previous studies highlight the complexity of environmental parameters that influence faecal contamination events and the resulting bacterial concentrations.
Hay and Roberts (2012) showed that concentration variations of FIOs in the water were not proportional with the amount of rainfall, and furthermore some contamination events can occur independent of rainfall events. For the Motueka river, flow measurements provide an accurate predictor of contamination, but this is not the case for the coastal environment which is influenced by current movement, and other potential bactericidal factors. Lipp et al. (2001) identified concentrations of bacterial and viral faecal indicators in a subtropical estuary were significantly correlated with rainfall and riverflow. These examples demonstrate the differences between catchment systems, thus it is not easy to make specific comparisons between one geographical site and another. Spatial and temporal variations of rainfall induced contamination is dependent on the amount of rainfall, as well as the hydrology, topography, and complexity of the catchment (Mallin et al. 2000, 2001).

2.5.2 Nutrient sampling
Although nutrients do not have a direct adverse affect on shellfish or human health, they often provide indication of pollution associated with farm run off (USEPA 2006). In comparison to the trigger ANZECC trigger levels, all of the ammonia samples exceeded guideline limits and 3/5 sampling periods exceeded limits for nitrate (Fig. 8), but there were no exceedances observed for phosphate. ANZECC exceedances were expected because these guidelines were created for South-East Australian waters, which are not typically as nutrient rich as New Zealand (Cornelisen 2013). As expected, nutrient concentrations were especially higher for the largest rainfall sample (R3) but not for the remaining rainfall samples (R1 and R2); this is also consistent with the E. coli abundances (Fig. 7). Nitrate was the only nutrient to significantly correlate with E. coli abundances in the water suggesting that the faecal matter could be associated with agricultural runoff, at least during strong rainfall events (Table 5).

2.5.3 E. coli abundances in shellfish
According to commercial standards, a shellfish sampling period that measures a median above 230 cfu/100 g for E. coli indicates that those shellfish are not fit for consumption. There were a total of 6 out of the 29 shellfish samples that contained E. coli: 2 mussel samplings and 4 of the cockles (Fig. 9). The median value across all samples for both shellfish types was 5 cfu/100g, thus well within
the aforementioned median limit. However, 10.3% of the samples measured above the 700/cfu 100 g limit, resulting in borderline acceptability with the allowable 10% threshold. Overall, the shellfish in the Waikouaiti estuary could be considered safe to eat according to commercial standards but sporadic contamination events can occur irrespective of rainfall. According to EU standards, any shellfish measuring above 230cfu/100 g would necessitate a two-month depuration step, or, a heat treatment before the shellfish is considered suitable for human consumption.

As expected, there was a difference in the amount of accumulated bacteria measured in cockles (*Austrovenus stutchburyi*) in comparison to (*Perna canaliculus*) (Fig. 9). There was one more accumulation event in the cockles in comparison to the mussels, potentially confirming previous observations showing the former species to be more active feeders (Campos et al. 2013). Interestingly, throughout the monitoring *E. coli* contamination was not found in both shellfish species at any one time, signifying that contamination events could differ between species. It is likely that any differences in accumulation are due to differences in species physiology and not because of spatial differences, because the concentration of *E. coli* in the water were the same for both the mussel and the cockle sites. A larger dataset is required in order to confirm this.

### 2.5.4 Rain as an indicator of *E. coli* in shellfish
The correlations of microbial abundances in shellfish versus rainfall, as well as shellfish versus river flow were inconclusive (Table 6). These environmental parameters did however correlate with the microbial abundances in the water. There was inconclusive evidence that the microbial abundances in the water correlated with abundances present in the shellfish (Table 6). This indicates that the reason for the inconclusive correlation could be due to the lack of data or samples that contained *E. coli*.

Surprisingly, none of the rainfall samples, including the highest water contamination event (R3) did not result in contaminated shellfish. Cornelisen et al. (2011) found that high river flows resulted in increased abundance of *E. coli* in shellfish located 6 km offshore; in some accounts *E. coli* counts of 1,300
MPN/100 g was detected. This indicates that there could be other contributing factors regulating the feeding of shellfish, and potentially shellfish collected further from a river could be more at risk than those in direct proximity.

Filter feeding of bivalves is regulated by physicochemical parameters of the overlaying waters and can impede on the bioaccumulation of faecal bacteria. Temperature and salinity are the main parameters (Campos 2013), of which are altered by rainfall. Fanshawe et al. (1995) showed that the rate of FIO accumulation is faster rate in water with higher salinities. Salinity measurements below 17 parts per thousand (ppt) have been shown to delay filter feeding in the mussel *M. edulis* (Motwani et al. 1956). Warmer periods are also well known to accumulate more faecal contamination in shellfish (Šolić et al. 1999; Sonier et al 2008), and lower temperatures closer to the minimal survival rate can significantly decrease filter feeding (Cusson et al. 2005). Larger rainfall events may decrease salinity and therefore disrupt feeding, this could have been the case for the R3 sample. This indicates that other environmental factors that should be considered for accumulation.

Other conditions that could alter faecal accumulation during a rainfall could be the duration of the rainfall, the areas within the catchment where the rain was received (i.e., lower farm land regions), and travel time for the contaminants to reach and the shellfish beds (Campos et al. 2011). Smaller rainfalls may not induce contamination in shellfish at all as they feed during high tide, thus contamination events could be diluted with the incoming tide.

**2.5.5 Chapter limitations and future improvements to water sampling**

*E. coli* concentrations of the water did not reflect abundances measured in the shellfish. Sampling was carried out during the low tide for access to the shellfish, this poses an issue for obtaining representative abundances of the water that the shellfish feed on during high tide mark. A future amendment would be to sample both the water and shellfish throughout a tidal cycle and rainfall, with particular emphasis on sampling around the high tide. This will give clarity to three main factors; the lag time between rainfall and the appearance of contamination in the water and shellfish; whether abundances change throughout a tidal cycle (i.e. if
contamination peaks in shellfish during high tide); and lastly, the duration of the contamination. The original sampling plan was to take samples before, during, and after the rainfall. However this was not possible due to the distance between the lab and study site, tide times and processing time. Controlled laboratory experiments could also give further insight into the timeframe required for the accumulation and clearance of *E. coli* in the shellfish, thus give recreational harvesters an estimated time required for contamination to clear for the Waikouaiti estuary.

It would have been beneficial to carry out nutrient samplings throughout the entire monitoring period. This is important for understanding the variations of the background levels and creating stronger statistical analyses, and for making correlations between nutrients and *E. coli*.

When using the membrane filtration technique, it is recommended that plates containing 20 - 80 cfu and no more than 200 colonies should be counted (HACH membrane filtration guide) for statistical validity. This study however counted filters containing 1 – 101 cfu per plate, this was because many of the samples had too few colonies. The second issue that restricted counting was the appearance of indistinct colonies (ICs). These colonies generally originated from a suspension of many cells and not one, thus these plates were not counted. Future studies should ensure that the moisture and condensation on the plates are minimised by pre-warming them.

The Specifications for Bivalve Molluscan Shellfish approved methods and sampling regimes were not used in this thesis for measuring faecal contamination (MAF 2006). These use the MPN method to assess *Enterococci* concentrations in the water, as oppose to the membrane filtration technique and *E. coli* as the indicator. The membrane filtration technique was chosen because it is quicker than the MPN method. The recommend a minimum of 30 water samples to be collected over a 12 month period to make an assessment (MAF 2006), where as only 16 were collected over a span of 6 months. Replicates would clarify the variations observed and strengthen statistical tests, the results in this current study can therefore only serve as a snapshot as oppose to a statistically strong analysis.
2.5.6 Suggestions for management

Shellfish sampling indicated that *E. coli* was sporadic and often occurred outside of the measured rainfall events. From the results in this chapter it was clear that rain and river flow and abundances in water were not clear predictors of faecal contamination in shellfish, which is contrary to the discussed literature (Table 6). In other studies salinity has been found to be a good predictor of accumulation of *E. coli* in shellfish and survival of the microbes in the water column. Salinity logging will be carried out in future work to measure changes throughout tidal cycles and rainfall, to better understand the correlation between fresh water and faecal contamination.
2.6 Conclusions

This chapter utilised the commercial guidelines to investigate the safety of shellfish in the Waikouaiti estuary. According to commercial guidelines the total exceedances for the shellfish samples was borderline acceptable. On the contrary, abundances in the water met national standards for shellfish harvesting waters. Sampling confirmed the hypothesis that *E. coli* concentrations are typically higher in shellfish than in the water. Microbial comparisons indicated that contamination in the water was not a certified measure - nor was proportional - to the corresponding abundances in shellfish. This finding proposes that there are other parameters affecting the filter feeding activity of the shellfish, or, that there are inconsistencies in the *E. coli* enumeration methods developed.

Data insufficiency was an issue in this chapter. Future correlations of microbial abundances and the measured hydrological parameters should utilise larger sample sizes. Sampling should occur throughout a tidal period and rainfall event. A salinity logger could also be used in future work to determine feeding of bivalves in response to salinity changes from rainfall. In conclusion faecal contamination is present in the shellfish regardless of rainfall, demonstrating that the relationship between rainfall and contamination events and shellfish quality may not be straightforward.
CHAPTER THREE

Next generation sequencing as a tool to detect faecal contamination in seawater

3.1 Introduction

Current water quality monitoring methods utilising faecal indicator organisms (FIOs) are limited in their ability to provide in depth information regarding specific pathogen presence and faecal contaminant sources. Identifying faecal sources is of particular interest for risk assessments and mitigation programs (Mcellellan & Eren 2014). Another concern with using FIOs is the increasing number of studies demonstrating the ability for *E. coli* to survive and grow in both saline and freshwater environments. In a coastal lagoon in Venice, viable *E. coli* was found adhered to three different types of macroalgae (*Ulva* spp., *Saragassum muticum* and *Undaria pinnatifida*), and genotypic analyses identified some of these strains as pathogenic (Quero et al. 2015). In New Zealand, naturalised clades of bacteria were isolated in freshwater from a creek and wetland water of the Waikato region (Perchec-Merien & Lewis 2012). In these environments, assessments using *E. coli* as a FIO would give a false positive indication of faeces, as a naturalised clade is not associated with a recent contamination event. These findings highlight the importance for developing indicators that are definitely associated with faeces.

Molecular source tracking has become increasingly popular in the exploration of new and more informative water quality monitoring techniques (Han Tran et al. 2015; Ahmed et al. 2014; Mieszkin et al. 2013). Identifying major faecal contamination sources is predominantly useful in local management for mitigation purposes. For example mitigation actions for areas that are predominately impacted by ruminant sources might include improving riparian zones around associated waterways. A succession of earthquakes have damaged sewage infrastructure in Christchurch, resulting in human contaminants making its way into major waterways and potential drinking water sources (Moriarty et al. 2013; Devane et al. 2014). The risk of pathogenic organisms is heightened with human sources because human enteric viruses are primarily sourced from human faeces and sewage (Mcellellan & Eren 2014). It is therefore especially
important to identify human sources within recreationally harvested shellfish beds.

3.1.1 Sequencing
Since the establishment of Sanger sequencing in 1977 molecular microbiology has undergone revolutionary optimisations and are now accessible for a variety of research applications. After three generations of high throughout sequencing this technology is now able to produce up to 100 times the data than its predecessor (Pareek et al. 2011). Sequencing types available are ‘whole’ and ‘partial genome sequencing’, these allow for exploration of whole microbiological communities or the entire genome of a single organism (Davis 2013).

Sequencing in microbial ecology has led to the discovery of new species previously not identified with culturing methods, this is because the conditions required to grow some organisms are unknown or difficult to replicate because they come from extreme environments (i.e. thermal vents). It is now recognised that the microbial diversity is much wider than previously recognised from culturing, for example, in the marine environment it is estimated that up to 99% of bacteria are unculturable (Fergusson 1984).

3.1.2a 16S rRNA gene amplicon sequencing
Alternative techniques to culturing were developed in the 1980s with interests in exploring bacteria in a natural setting through molecular phylogeny (Fuhrman & Hagstrom 2008). 16S rRNA gene sequencing has long been a significant target sequence due to its ability to distinguish between different organisms by partial sequencing (Frierer et al. 2007). This region codes for the 30S small ribosomal sub-unit present in almost every organism (Carter et al. 2000). It contains conserved regions that have undergone slow evolution and so are similar across all prokaryotic hosts. Additionally it contains 1 to 9 hypervariable regions that are species specific and can be targeted for sequencing or other genetic applications (Chakravorty et al. 2007). The homogeneity of the conserved regions allows for the use of universal primers to target multiple bacterial sequences for amplification and sequencing, this allows for simultaneous sequencing and amplification samples containing a diverse range of bacteria (Baker et al. 2003).
Different sequencing depths can be achieved depending on the platform used, i.e., identification level of the genus, phyla or species (Kim et al. 2013).

### 3.1.2b MiSeq Illumina Sequencing platform
The Illumina platform is a high throughput method because of its ability to sequence hundreds of DNA strands simultaneously using the ‘sequencing is by synthesis’ method. A library is firstly prepared by fragmenting the sample DNA and hybridising the strands to a chip containing a lawn of fixed oligonucleotide sequences with complementary adapters (Quail et al. 2012). The fragments are then clonally amplified with each new strand resulting in amplified clusters of each fragment throughout sections of the chip (Pareek et al. 2011; Kircher & Kelso 2010). Sequencing primers are then bound to the strands followed by complementary fluorescent nucleotides. The tags are detected and recorded by a light signal, this occurs across the chip at the same time so all amplified clusters can be read together (Kircher & Kelso 2010). The final sequencing reads are then pooled, paired with their complementary strand reads, and aligned according to homogeneity (Bentley et al. 2008).

The resulting operational taxonomic unit (OTUs) reads are aligned to known taxonomic classification sourced from online databases that contain previously identified microbial sequences, for example the database SILVA (Quast et al. 2013) provides a range of sequences that range from phyla to the species level for organisms isolated from environments such as soil, water, marine and animal guts. Another program is then used to carry out taxonomic assignments and perform analyses such as diversity measures, e.g. Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al. 2012).

### 3.1.3 Microbial Source Tracking
Microbial source tracking (MST) refers to a broad range of techniques and targets with the objective of identifying a faecal contaminant source in an environmental sample. The basis of genotypic MST technology relies on the specific microbiota that inhabit the gut of each animal. Gut bacteria are reflective of the respective diets of animals thus differ from species to species and allow for discrimination between host species (Ley et al., 2008; Shanks et al., 2011). Non-genomic techniques include chemical signals for targets such as caffeine (Field &
There are two main methods for MST, one requires a reference library and the other does not. A library independent method (LIM) is not reliant on a library but instead on a single organism or marker gene that is exclusive to a host source (Roslev & Bukh 2011); for example a variable region of the 16S rRNA gene (Bernhard & Field 2000a). LIMs test contaminated samples utilising markers specific to a known source, while a library dependant method (LDM) compares a dataset of potential targets in a sample to a reference library to extrapolate source origin.

### 3.1.3a Library Independent source tracking methods

The ideal criteria for MST microbial markers are a) anaerobic; b) present in numbers that allow for easy detection; c) have a similar survival rate in the environment as pathogens of interest; d) be associated with pathogens and with faeces of animals concerned and e) have little or no cross over with other animal sources and environmental genera (Mclellan & Eren 2014; Teaf et al. 2011).

Microbes from the order Bacteroidales have been the most explored order of bacteria for MST (McLellan et al. 2014). This is because they are strict anaerobes that cannot survive or divide in aerobic waters, and are genomically distinct between gut species. They make up a large proportion of faeces in warm blooded animals, but is lower in humans (Teaf et al. 2011) and are unlikely to survive in a beach environment (Fiksdal et al. 1985). Available host associated markers for Bacteroidales have been developed for human (Carson et al. 2005; Shanks et al. 2009), chicken (Lu et al. 2008), Canadian geese (Lu et al. 2009), ruminants (Reischer et al. 2006), pigs (Dick et al. 2005) and general faeces from warm blooded animals (Kildare et al. 2007). Other MST applications can target microbial specific genome variants within a species using a simple PCR assay; a good example is the marker Gull-2 that is used to detect seagulls by targeting the microbe *Catellicoccus marimammalium* (Lu et al. 2008).
The first host associated LIM was developed by Bernhard et al (2000a) and utilised the genera *Bacteroides – Prevotella* to distinguish between cow and human faeces. This work has lead to the development of source specific primers that are used in rapid and inexpensive methods for MST on water samples (Bernhard & Field 2000b). Over the years these assays have expanded to include target for human, sewage, ruminants, birds, geese, pigs and other wild animals.

Globally there are many MST assays available, but in order to apply these to new geographical environments they need to be tested for their suitability. The Institute of Environmental Science and Research (ESR) provide those that are currently suitable in New Zealand waters (Table 7). Each marker has been tested for the levels of sensitivity and specificity that should be taken into account with assay selection. For example, the Gull-2 marker requires a heavily polluted sample in order to give a confident reading.
Table 7: Microbial source tracking markers available by ESR. This table was adapted from the markers that are available as presented by the ESR website.

<table>
<thead>
<tr>
<th>Source/Target group/assay abbreviation</th>
<th>Assay type</th>
<th>Sensitivity</th>
<th>Targets from faeces</th>
<th>Assay type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>General -bac marker -GenBac3</td>
<td>PCR</td>
<td>High</td>
<td>Human, Cow, Sheep, Deer, Goat, Pig, Rabbit, Possum, Cat, Dog, Horse, Duck, Swan, Seagull, Geese, Chicken</td>
<td>Bacteroides 16S rRNA</td>
<td>(Siefring et al. 2008)</td>
</tr>
<tr>
<td>Human -BacH</td>
<td>PCR</td>
<td>Medium - 1st preference for human, most sensitive human assay</td>
<td>Human, Cat, Dog, Rabbit, Possum, Chicken, Goat</td>
<td>Bacteroidales spp.</td>
<td>(Reischer et al. 2007)</td>
</tr>
<tr>
<td>Human -BiADO</td>
<td>PCR</td>
<td>Medium - 2nd preferences for human, less sensitive than BacH</td>
<td>Human, Seagulls</td>
<td>Bifidobacterium adolescentis 16S rDNA</td>
<td>(Matsuki et al. 2004)</td>
</tr>
<tr>
<td>Human -HumM3</td>
<td>PCR</td>
<td>Low - requires highly polluted samples</td>
<td>Human, Possum, Rabbit</td>
<td>Cell wall protein</td>
<td>(Shanks et al. 2009)</td>
</tr>
<tr>
<td>Ruminant -BacR</td>
<td>PCR</td>
<td>High - recommended assay</td>
<td>Cow, Sheep, Deer, Goat</td>
<td>Bacteroidales 16S rRNA</td>
<td>(Reischer et al. 2006)</td>
</tr>
<tr>
<td>Bovine -CowM2</td>
<td>PCR</td>
<td>Low - requires highly polluted samples</td>
<td>Cow, Deer</td>
<td>Bovine-specific faecal genetic markers</td>
<td>(Shanks et al. 2008)</td>
</tr>
<tr>
<td>Sheep -Schill</td>
<td>PCR</td>
<td>Medium</td>
<td>Sheep</td>
<td>Cytochrome b</td>
<td>(Schill &amp; Mathes 2008)</td>
</tr>
<tr>
<td>Canine -DogBac</td>
<td>PCR</td>
<td>High</td>
<td>Dog</td>
<td>Bacteroidales spp.</td>
<td>(Dick et al. 2005)</td>
</tr>
<tr>
<td>Avian -GFD</td>
<td>PCR</td>
<td>Medium</td>
<td>Duck, Swan, Seagull, Geese, Chicken</td>
<td>16s rRNA gene</td>
<td>(Green et al. 2012)</td>
</tr>
<tr>
<td>Avian -E2</td>
<td>PCR</td>
<td>Low - not recommended unless samples are highly polluted</td>
<td>Duck</td>
<td>Desulfovibrio spp.</td>
<td>(Devane et al. 2007)</td>
</tr>
<tr>
<td>Seagull -gull2</td>
<td>PCR</td>
<td>Low - requires highly polluted samples</td>
<td>Seagull</td>
<td>16S rRNA gene of Catellicoccus marimamillium</td>
<td>(Ryu et al. 2012)</td>
</tr>
<tr>
<td>Possum</td>
<td>PCR</td>
<td>High</td>
<td>Possum, human, Black Swan, Rabbit</td>
<td>Bacteroidales 16S rRNA</td>
<td>(Devane et al. 2013)</td>
</tr>
</tbody>
</table>
### 3.1.3b Library Dependent methods used in source tracking

Library dependant methods (LDMs) require a collection or library of bacterial sequences for animal sources within the study site (Harwood et al. 2014). The library is then used to compare the sequences obtained from the samples of interest in order to identify the host source. There are few published studies that have utilised community genomics for MST.

The group Jeong et al. (2011) collected sequencing samples from humans, cows and pigs, and used these as templates to compare with sequences water samples. The samples were obtained from a river containing more than 2 million inhabitants of people, pigs and cattle. The Bacteroidales group was the most dominant across all faeces types and accounted for 31–52% of the bacterial sequences, and 0.6% in the river water samples. They also identified host specific genera in the Bacteroidales phyla that were dominant in that particular animal, for example *Veillonella spp* in humans.

Lee et al. (2011) sequenced a range of animal and human faecal sources for identifying source specific genera for MST capabilities in South Korea. Several source specific genera for human and animal samples were found, such as *Yania* for chicken, and *Bifidobacterium* for humans. They found Firmicutes to be the most dominant in clustering analyses, and suggested that these phyla would make good targets for MST markers.

Unno et al. (2010) predicted contaminant sources in watersheds by comparing ratio densities of OTUs in samples from farm animals and humans to contaminated environmental water. A density ratio of the shared OTUs for the faeces and water samples were calculated to identify source. They identified pig and human as potential sources, and also identified the degree of pollution for each sample.

Davis (2013) developed a ‘proof-of-concept’ study to identify the usefulness of 16S rRNA for MST in water samples from NZ. This author tested 16S rRNA techniques on archival water samples that had sources identified by commercial techniques. A total of 6 out of the 10 samples matched commercial findings, with
the remaining 4 samples unable to be identified due to the limited amount of sequencing reads obtained. They identified three major Phyla that dominated all of the faecal samples; these were Bacteroidetes, Firmicutes and Proteobacteria. This suggests that community sequencing capabilities do have potential for use in NZ waters.

Community sequencing technologies for MST are still limited whereby the final result can only hypothesise a source based on phyla ratio comparisons. However, each new analysis broadens the scope of microbial taxa that could be used for MST specific targets in water (Tan et al. 2015), and validates those that already exist, which is of particular importance for establishing global similarities. Another further limitation of LIMs may be that not all contributing sources of contamination may be identified in contaminated waterways and targets for testing could be wasted if not present – the strength of library dependant methods is that all targets can be identified.

3.1.3c Bacteria size

Bacteria typically range between 1 to 100 µm in size (Madigan et al. 2010) with coliform bacteria typically measuring 0.6-1.2 µm in diameter by 2-3 µm in length (Zhang & Farahbakhsh 2007). Standard culturing for faecal bacteria enumeration from water samples require filtration through a 0.45 µm pore size to accumulate bacterial biomass. However recent evidence has shown that some bacteria collected from environmental samples are in fact able to surpass through typical filter sizes. Phyla that are able to pass through the ~0.22 µm filters are coined ultrabacteria, and include phyla without a culturable representative (candidate phyla) such as WWE3, OP11 and OD1 (Luef et al. 2015; Brown et al. 2015). Staley et al. (2013) sequenced river water through two different filter sizes (0.22 and 0.45 µm) and found similar representations of the top abundant taxa, but also found the OTUs of Actinobacteria and Firmicutes were slightly more specific to the 0.22 µm filter than the 0.45 µm filters.

Ultrabacteria bacteria that could be of interest for water quality assessments for shellfish areas include Firmicutes (abundant in animal faeces) and Vibrio (pathogenic genera). Vibrio are known to contain ultrabacteria sized <0.3 µm that
are naturally found in the estuarine and marine environment, and can become dwarfed in nutrient starved conditions (Colwell & Spira 1992). These bacteria could be missed while among larger pore sizes (i.e., 0.45 µm) used for water quality assessments, and therefore could alter what we assume about the population from these tests. The development of genomic techniques for assessing water quality is on going, thus there are currently no standards or consensus for the recommended amount of water and filter size to use (Staley et al. 2013).

3.1.4 Considerations for water quality monitoring for shellfish harvesting areas

3.1.4a Assessing waterborne pathogens associated with shellfish

Campylobacter, Shigella, and Salmonella are commonly found in the gastrointestinal tract of birds and livestock (Stanley & Jones 2003; Patton et al. 2009; Kim & Wells 2016), as well as commonly isolated from estuarine and wastewaters. While these species may show no pathogenicity in the animals they inhabit, they are common waterborne pathogens that cause disease in humans. The strain C. jejuni, is a common cause of foodborne illnesses associated with bird faeces (Malham et al. 2014). Pathogenic strains of Salmonella are known to transmit gastrointestinal infections when harboured in shellfish (Yam et al. 2000). One study showed that the carrying rate of Campylobacter spp, in dairy cows was 70-90% (Stanley et al. 1998) making this pathogen a concern for waters surrounded by dairy farmland.

Faeces are significant sources of waterborne pathogens that have the potential to accumulate in shellfish, however not all pathogens are of faecal origin. There are several marine genus that are also naturally pathogens; Aeromonas contain strains such as A. hydrophila which can cause diarrhoea, and Vibrio strain V. parahaemolyticus, is a well known culprit causing infection when seafood is eaten raw or not properly cooked (Yam et al. 2000). Vibrio spp. is also known for the ability to replicate rapidly in Oysters when they are not refrigerated post harvested (Malham et al. 2014). A comparison with data over the passed 30 years has indicated that the incidence of V. parahaemolyticus is on the rise (Cruz et al. 2015).
Other bacteria are associated with both the environment and animal faeces. *Yersinia* is a bacterial genus containing intracellular parasites to both warm blooded animals and insect hosts. Hong et al. (2013) found these genera are present in soils highly contaminated with pig manure. One strain of particular interest and of faecal origin is *Yersinia enterocolitica* which is associated with human sewage, warm blooded animals and avian species (Li et al. 2015). The diversity in pathogen sources should be taken into account in the case of thorough quality assessments for shellfish grounds.

3.1.4b Adding molecular steps to routine culturing protocols

Traditional culturing techniques remain the golden standard for assessing faecal contamination of water and food. Culturing media have become refined to ensure specific growth and identification of microbes of interest. Media such as CM1046 (Oxoid) is designed for growing lactose utilising coliforms, detecting *E. coli* by β-glucuronidase activity and inhibiting gram-positive bacteria. This media has been used in research for the monitoring of *E. coli* in shellfish (Clements et al. 2013), and used in reports commissioned by the European Union (Andersen et al. 2010). In the US, UK and Australia, chromogenic agars have been accepted as standard methods for water monitoring (Brenner et al. 1996; USEPA 2002; Anon. 2005). Wohlsen (2011) found that CM1046 was a good alternative media for use in the AS/NZS Standard Method. While selective culturing methods for indicator bacteria provide a 24-hour presence/absence indication of contamination, they are still limited in their ability to provide comprehensive and informative information of faecal source and pathogenicity for health and safety risk assessment.

Culture based 16S rRNA libraries have proved useful in identifying some source specific species of bacteria, such as *C. marimammalium* in gull faeces (Lu et al. 2008), and *Lactobacillus sobrius/amylovorus* in pig manure (Unno et al. 2010). Layton et al. (2010) showed that enriching for *Enterococcus* spp. before a PCR protocol could support source identification. Similarly Scott et al. (2005) showed enhanced benefit in the detection of bacterial surface proteins in an assay for *Enterococcus faecium*. Schrank et al. (2001) developed a PCR protocol following *Salmonella* enrichments in the poultry industry to allow for detection of a range of serovars. The addition of molecular steps to routine culturing protocols can
enrich targets for downstream applications that require a substantial amount of DNA, particularly for pathogens that are often found in very low concentrations.

3.2 Aims

Illumina sequencing was employed to identify dominant taxa associated with the presence of *E. coli* in the Waikouaiti Estuary water, as well as assess faecal targets for MST applications of potential genera associated to animal host source. 16S rRNA sequence reads and plate counts for *E. coli* were used in a correlation to examine the relationship of viable cell counts to 16S rRNA copies under different rain/river flow conditions. A comparison was performed of bacterial phyla captured on two different filter pore sizes commonly utilised; 0.22 µm used in microbial ecology, and 0.45 µm used for standard *E. coli* enumeration methods. Of particular interest was to investigate if there was a loss of faecal or pathogenic microbes when using the commonly used larger pore size. Lastly, CM1046 cultured filters were evaluated for enrichment of faecal bacteria, MST targets, and potential pathogens.

Specific questions pertaining to each section:

1) What bacteria are associated with high *E. coli* counts in the Waikouaiti Estuary, and what are their potential animal sources?

2) Are there differences in the recovery of bacterial abundances between filter sizes 0.22 µm and 0.45 µm?

3) Are there differences in the bacteria enumerated between cultured filters on the chromogenic media CM1046 in comparison to filters that are not cultured? Could this method add to an analysis toolkit where cultured filters can then be used for a secondary sequencing step?

This chapter was to address three main hypotheses:

1) Previous results from Chapter Two indicate that out of all the samplings the highest abundances of *E. coli* was detected in the RF3 samples. It is hypothesised that other faecal bacteria might co-occur and therefore more MST targets will be associated with high river flow and high *E. coli* abundances.
2) Previous reports have highlighted the exclusion of some smaller bacteria when filtering water samples using a pore of larger size. From this it is hypothesised that some smaller bacterial groups (i.e. Actinobacteria and Firmicutes) could be excluded with the use of 0.45 µm filters in comparison to 0.22 µm filters;

3) The media CM1046 is used to selectively grow coliforms and faecal coliforms; this leads to the hypothesis that this media could be used to enhance the amount of faecal bacteria for MST targets.
3.3. Materials and Methods

3.3.1 Sample selection and processing
The same water samples collected for monitoring in Chapter Two were used for all experiments assessed in this chapter. Samples selected for sequencing were based on specific criteria such as higher and lower rainfall and river flow (Table 8). High river flows were defined as samples collected during periods of flow rate higher of 1.34 cumecs at Orbells crossing, indicating that a substantial rainfall had occurred in the Waikouaiti catchment (ORC 2009). High *E. coli* counts were sampled that contained more than 14 cfu/mL, and those lower were considered to be low flow; this figure was derived from both the MoH/MfE (2003) guidelines for shellfish harvesting grounds, a sampling median above this figure is considered a health issue for harvesting areas.

Water samples were filtered through nitrocellulose filter membranes using a vacuum pump. A total of 200 mL was filtered for a majority of all the uncultured (not cultured on CM1046) 0.22 µm filters, and only 100 mL was filtered for the RF3 samples for both filter sizes (0.22 µm and 0.45 µm) due to the large amount of biomass clogging up the filters. For the cultured (cultured and incubated on CM1046) filters, the *E. coli* filters enumerated on the CM1046 media in Chapter Two were collected for sequencing. The filters that were not cultured were immediately stored at -20 °C, whereas the cultured filters were stored after a 24 hour incubation.

3.3.1a DNA extraction
DNA was extracted from the thawed filters using the MoBio PowerSoilTM DNA Isolation Kit (MoBio, Solana Beach, CA) according to manufacturer’s instructions, with the exception that the final extracted DNA was eluted in 50 µL instead of 100 µL to concentrate the DNA in the solution. The DNA was quantified using a Nanodrop Spectrophotometer (Thermo Fisher).

3.3.1b Confirmation of amplifiable 16S rRNA
Some DNA concentrations obtained from the extractions were lower than the desired amount of 2 µg/µL. A PCR reaction was carried out on samples ranging in DNA yields, as well as one cultured filter, to ensure that the 16Sr DNA region
was amplifiable. The samples selected and their respective DNA yields were; 21RF3 with 1 ng/µL; 3MW3 with 1.7 ng/µL; 13CW1 with 3.7 ng/µL; 8RF3 with 6.5 ng/µL; 17MW1 with 7.4 ng/µL; and one cultured filter 27MW1 with 345.5 ng/µL.

For the PCR reaction, AmpliTaq DNA polymerase (ABI, ThermoFisher) and forward and reverse primers (Table 9) were combined with the sample DNA as indicated in Table 8. The reaction was carried out in a T100 Thermo Cycler (Bio-Rad) cycle parameters as indicated in Table 10 using the cycle indicated in Table 11. To visualise whether the 180 bp region of the 16S rRNA was amplified, 10 µL of sample amplicon was loaded into the wells of a 1% agarose gel and ran against 2 µL of a 1kp DNA Ladder (DNA plus ladder, ThermoFisher).

After confirmation of amplifiable products (Fig. 10), aliquots of the samples were made for sequencing by diluting the DNA extracts in ddH2O to a concentration of 10 ng/µL. For samples that were under this concentration a total of 100 ng/µL was provided for sequencing.

3.3.2 Sequencing
Samples underwent 16S rRNA gene amplification and amplicon sequencing using the Earth Microbiome Project\textsuperscript{13} barcoded primer set and conditions (Caporaso et al. 2012), the resulting sequences were ran in a Illumina MiSeq 2 x 151 bp for each sample.

3.3.2a Operational Taxonomic Unit assignment
All of the resulting sequence reads were processed in QIIME 1.9 and clustered with 97% similarity. The taxonomy was assigned using the SILVA 119 release reference database (Quast et al. 2013) for reference sequences. The data was normalised to 6,000 reads per sample for further analyses and rarefied a total of 10 times; sequences that did not meet the 6,000 reads were removed (sample 2CW2 did not meet this threshold). The OTU tables were combined and then subjected to R analyses using the Phyloseq package for samples and taxonomic analyses.

\textsuperscript{13} http://www.earthmicrobiome.org/
3.3.2b Phyloseq processing and analyses
Hierarchical clusters (Dendogram) were composed using bootstrap and confidence determination based on Bray-Curtis distancing in order to visualise similar taxonomic compositions between samples in each experiment. For each sample a p-value was calculated along with an approximate unbiased (AU) value and a bootstrap probability (BP). Groups of samples that clustered with 95% confidence were identified and marked with a red box surrounding the group of nodes.

Alpha diversity plots were created in order to determine the level of diversity between two grouped samples pooled by the three different test parameters (river flow, filter size, *E. coli* abundance). For each test, two plots were produced, one displaying the Observed (measure of OTUs unique within each treatment group) and the Shannon index (species richness and evenness index taking into account the percent of occurrence in the treatment).

Relative abundances for phyla and genera were represented as percentages from the relative reads of the corresponding OTUs in relation to the total number of reads in a group of samples. The number of hits for the top OTUs varied per analysis, the specific details of hits obtained are included in each figure legend. The Top 10 significant phyla were defined by their abundance and frequency throughout an experiment in order to identify the most dominant groups throughout all of the sample groups and manually screened for those that contain bacteria from faecal origin (Table 12). Significantly different genera were identified between two treatment groups for those that gave a p-value of less than 0.05 and with fold changes of more than 1.9 and not less than -1.9 were plotted.

To compare dissimilarities between samples within an experiment a NMDS analysis with Bray-Curtis distancing was applied to the sequenced samples. The sample nodes were aesthetically differentiated into treatment types for visualisation of potential effects of the tested parameters on community dissimilarity. These are naturally followed by a monotonic regression (Shepherd plot) to identify whether the dissimilarities within the communities are well preserved.
Uncultured filters were screened against suggested faecal genera with the aim to identify potential faecal markers that can be targeted with MST, as well as potential genera related to faecal sources. This was carried out on the samples selected for the high and low flow, as well as the high and low *E. coli* to identify what conditions offer opportunities for identifying faecal contaminant sources. The genera that were used in this screening were based on previous NGS studies carried out both in and outside of NZ, as well as genera containing established targets for faecal source tracking opportunities (Lee et al. 2011; Davis 2013; Iwamoto et al. 2010; Chidamba & Korsten 2015; Cook 1991; Foster et al. 1995; Finegold et al. 2003; Lu et al. 2008; Lawson et al. 2001; McLellan & Eren 2014). The genera selected for the screening either contained known markers for MST, or were genera that have shown a high abundance in faeces in previous studies.

To determine a relationship between the number of sequence reads and the plate quantification methods, a correlation analysis was carried out on the OTUs of *E. coli – Shigella* versus colony counts observed from the enumeration plates in Chapter Two. This was carried out in order to identify if the OTUs detected were able to translate into cell count quantities and vice versa.
Table 8: Details for the collected water samples subjected to sequencing.

Filters from the water samples processed in Chapter Two were selected based on varying amounts of *E. coli* (cfu/100 mL) and maximum daily river flow for the Waikouaiti river (Max (m$^3$s$^{-1}$)). For each sample week (Week ID) three replicates were randomly processed from either the cockle or the mussel site (Sample ID). Experiment numbers refer to the three comparative analyses carried out based on; 1) river flow analysis; 2) 0.22 µm versus 0.45 µm; and 3) cultured versus uncultured. The filter sizes and culturing varied based on the analyses carried out.

<table>
<thead>
<tr>
<th>Week ID</th>
<th>Sample ID</th>
<th>cfu/100 mL</th>
<th>Filter (µm)</th>
<th>Cultured</th>
<th>Experiment</th>
<th>High/Low flow</th>
<th>Max (m$^3$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W8</td>
<td>1CW1</td>
<td>2.61</td>
<td>0.22</td>
<td>-</td>
<td>1, 3</td>
<td>Low</td>
<td>0.293</td>
</tr>
<tr>
<td>W8</td>
<td>2CW2</td>
<td>1.55</td>
<td>0.22</td>
<td>-</td>
<td>1, 3</td>
<td>Low</td>
<td>0.293</td>
</tr>
<tr>
<td>W8</td>
<td>3MW3</td>
<td>1.55</td>
<td>0.22</td>
<td>-</td>
<td>1, 3</td>
<td>Low</td>
<td>0.293</td>
</tr>
<tr>
<td>W11</td>
<td>4CW2</td>
<td>33.05</td>
<td>0.22</td>
<td>-</td>
<td>1, 3</td>
<td>Low</td>
<td>0.333</td>
</tr>
<tr>
<td>W11</td>
<td>5CW3</td>
<td>33.05</td>
<td>0.22</td>
<td>-</td>
<td>1, 3</td>
<td>Low</td>
<td>0.333</td>
</tr>
<tr>
<td>W11</td>
<td>6MW2</td>
<td>33.05</td>
<td>0.22</td>
<td>-</td>
<td>1, 3</td>
<td>Low</td>
<td>0.333</td>
</tr>
<tr>
<td>RF3</td>
<td>7RF31</td>
<td>53.33</td>
<td>0.22</td>
<td>-</td>
<td>1, 2, 3</td>
<td>High</td>
<td>12.081</td>
</tr>
<tr>
<td>RF3</td>
<td>8RF32</td>
<td>53.33</td>
<td>0.22</td>
<td>-</td>
<td>1, 2, 3</td>
<td>High</td>
<td>12.081</td>
</tr>
<tr>
<td>RF3</td>
<td>9RF33</td>
<td>53.33</td>
<td>0.22</td>
<td>-</td>
<td>1, 2, 3</td>
<td>High</td>
<td>12.081</td>
</tr>
<tr>
<td>W9</td>
<td>10CW1</td>
<td>18.15</td>
<td>0.22</td>
<td>-</td>
<td>1</td>
<td>Low</td>
<td>0.293</td>
</tr>
<tr>
<td>W9</td>
<td>11CW2</td>
<td>18.15</td>
<td>0.22</td>
<td>-</td>
<td>1</td>
<td>Low</td>
<td>0.293</td>
</tr>
<tr>
<td>W9</td>
<td>12MW1</td>
<td>16.50</td>
<td>0.22</td>
<td>-</td>
<td>1</td>
<td>Low</td>
<td>0.293</td>
</tr>
<tr>
<td>W12</td>
<td>13CW1</td>
<td>1.42</td>
<td>0.22</td>
<td>-</td>
<td>1</td>
<td>Low</td>
<td>0.318</td>
</tr>
<tr>
<td>W12</td>
<td>14CW2</td>
<td>1.42</td>
<td>0.22</td>
<td>-</td>
<td>1</td>
<td>Low</td>
<td>0.318</td>
</tr>
<tr>
<td>W12</td>
<td>15CW3</td>
<td>1.42</td>
<td>0.22</td>
<td>-</td>
<td>1</td>
<td>Low</td>
<td>0.318</td>
</tr>
<tr>
<td>W13</td>
<td>16EW2</td>
<td>5.39</td>
<td>0.22</td>
<td>-</td>
<td>1</td>
<td>Low</td>
<td>0.662</td>
</tr>
<tr>
<td>W13</td>
<td>17MW1</td>
<td>11.61</td>
<td>0.22</td>
<td>-</td>
<td>1</td>
<td>Low</td>
<td>0.662</td>
</tr>
<tr>
<td>W13</td>
<td>18MW2</td>
<td>11.61</td>
<td>0.22</td>
<td>-</td>
<td>1</td>
<td>Low</td>
<td>0.662</td>
</tr>
<tr>
<td>RF3</td>
<td>19RF3</td>
<td>53.33</td>
<td>0.45</td>
<td>-</td>
<td>2</td>
<td>High</td>
<td>12.081</td>
</tr>
<tr>
<td>RF3</td>
<td>20RF3</td>
<td>53.33</td>
<td>0.45</td>
<td>-</td>
<td>2</td>
<td>High</td>
<td>12.081</td>
</tr>
<tr>
<td>RF3</td>
<td>21RF3</td>
<td>53.33</td>
<td>0.45</td>
<td>-</td>
<td>2</td>
<td>High</td>
<td>12.081</td>
</tr>
<tr>
<td>W8</td>
<td>22CW2</td>
<td>2.61</td>
<td>0.45</td>
<td>cultured</td>
<td>3</td>
<td>Low</td>
<td>0.293</td>
</tr>
<tr>
<td>W8</td>
<td>23CW3</td>
<td>2.61</td>
<td>0.45</td>
<td>cultured</td>
<td>3</td>
<td>Low</td>
<td>0.293</td>
</tr>
<tr>
<td>W8</td>
<td>24MW3</td>
<td>1.55</td>
<td>0.45</td>
<td>cultured</td>
<td>3</td>
<td>Low</td>
<td>0.293</td>
</tr>
<tr>
<td>W11</td>
<td>25CW1</td>
<td>33.05</td>
<td>0.45</td>
<td>cultured</td>
<td>3</td>
<td>Low</td>
<td>0.333</td>
</tr>
<tr>
<td>W11</td>
<td>26CW3</td>
<td>33.05</td>
<td>0.45</td>
<td>cultured</td>
<td>3</td>
<td>Low</td>
<td>0.333</td>
</tr>
<tr>
<td>W11</td>
<td>27MW1</td>
<td>33.05</td>
<td>0.45</td>
<td>cultured</td>
<td>3</td>
<td>Low</td>
<td>0.333</td>
</tr>
<tr>
<td>RF3</td>
<td>28RF3</td>
<td>53.33</td>
<td>0.45</td>
<td>cultured</td>
<td>3</td>
<td>High</td>
<td>12.081</td>
</tr>
<tr>
<td>RF3</td>
<td>29RF3</td>
<td>53.33</td>
<td>0.45</td>
<td>cultured</td>
<td>3</td>
<td>High</td>
<td>12.081</td>
</tr>
<tr>
<td>RF3</td>
<td>30RF3</td>
<td>53.33</td>
<td>0.45</td>
<td>cultured</td>
<td>3</td>
<td>High</td>
<td>12.081</td>
</tr>
</tbody>
</table>
Table 9: PCR Primers used for confirmation of amplifiable 16S rRNA gene product.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward 16S rRNA</td>
<td>5'-ACTCCTACGGGAGGCAGCAGT-3'</td>
</tr>
<tr>
<td>Reverse 16S rRNA</td>
<td>5'-ATTACCGCGGCTGCTGCG-3'</td>
</tr>
</tbody>
</table>

Table 10: PCR Reaction mixture components in order that they were added.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmpliTaq 360 Master Mix</td>
<td>1X</td>
</tr>
<tr>
<td>DNA template</td>
<td>~5–10 ng</td>
</tr>
<tr>
<td>Primer forward</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Primer reverse</td>
<td>0.5 µM</td>
</tr>
</tbody>
</table>

Table 11: Amplification cycle used to amplify 16S rRNA genes in DNA extractions.

<table>
<thead>
<tr>
<th>Hold stage</th>
<th>1 cycle</th>
<th>95 °C for 10 mins</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR stage</td>
<td>30 cycles</td>
<td>95 °C for 15 secs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65 °C for 20 secs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 °C for 20 secs</td>
</tr>
<tr>
<td>Infinite hold stage</td>
<td>1 cycle</td>
<td>72 °C for 5 mins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cooled to 12 °C</td>
</tr>
</tbody>
</table>
Table 12: Genera of potential faecal markers used in screening. Collected from previous MST papers using NGS. Potential sources and available assays indicated where possible (summarised from Wuertz et al. (2011) and ESR website).

<table>
<thead>
<tr>
<th>Genus</th>
<th>References</th>
<th>Potential Sources</th>
<th>Available assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoogloea</td>
<td>(Davis 2013)</td>
<td>Sewage</td>
<td></td>
</tr>
<tr>
<td>Yersinia</td>
<td>(Lee et al. 2011; McLellan &amp; Eren 2014)</td>
<td>Goose, warm blooded animal, pigs</td>
<td></td>
</tr>
<tr>
<td>Tenacibaculum</td>
<td>(Lee et al. 2011)</td>
<td>Cow</td>
<td></td>
</tr>
<tr>
<td>Sterolibacterium</td>
<td>(Lee et al. 2011)</td>
<td>Cow</td>
<td></td>
</tr>
<tr>
<td>Serratia</td>
<td>(Green et al. 2012)</td>
<td>Pathogens</td>
<td></td>
</tr>
<tr>
<td>Salmonella</td>
<td>(Cook 1991)</td>
<td>Pathogens, birds</td>
<td></td>
</tr>
<tr>
<td>Rhodoplanes</td>
<td>(Lee et al. 2011)</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>Rhodobacter</td>
<td>(Lee et al. 2011)</td>
<td>Human, Gull</td>
<td></td>
</tr>
<tr>
<td>Prevotella</td>
<td>(Davis 2013)</td>
<td>Duck</td>
<td>Distinguishing dog, human, cow (Bernhard &amp; Field 2000a; Bernhard &amp; Field 2000b)</td>
</tr>
<tr>
<td>Owenweeksia</td>
<td>(Lee et al. 2011)</td>
<td>Cow</td>
<td></td>
</tr>
<tr>
<td>Ottowia</td>
<td>(Lee et al. 2011)</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>Oceanimonas</td>
<td>(Lee et al. 2011)</td>
<td>Chicken</td>
<td></td>
</tr>
<tr>
<td>Gemmatimonas</td>
<td>(Lee et al. 2011)</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>E. coli - Shigella</td>
<td>(Iwamoto et al. 2010; Cook 1991)</td>
<td>Pathogens, general faecal marker</td>
<td>E. coli genes for a variety of animals, including cattle, pig, dog, rabbit, bird, bird (as summarised in Wuertz et al. 2011).</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>(Lu et al. 2008)</td>
<td>General faecal marker</td>
<td>Exp gene is used to identify human faeces (McDonald et al. 2006).</td>
</tr>
<tr>
<td>Cellulophaga</td>
<td>(Lee et al. 2011)</td>
<td>Chicken</td>
<td></td>
</tr>
<tr>
<td>Bacteroides</td>
<td>(Davis 2013)</td>
<td>Found in range of faeces</td>
<td>Universal for a range of animals (as summarised in Wuertz et al. 2011).</td>
</tr>
<tr>
<td>Bacillus</td>
<td>(Lee et al. 2011; Lu et al. 2008)</td>
<td>Goose, Gull</td>
<td></td>
</tr>
<tr>
<td>Acidovorax</td>
<td>(Chidamba &amp; Korsten 2015; Davis 2013)</td>
<td>Sewage, Gull faeces</td>
<td></td>
</tr>
<tr>
<td>Agromyces</td>
<td>(Lee et al. 2011)</td>
<td>Goose</td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>(Lee et al. 2011; Davis 2013)</td>
<td>Notable abundances in human, gull, human sewage. A general faecal marker.</td>
<td>Human and seagulls available through ESR</td>
</tr>
<tr>
<td>Bosea</td>
<td>(Lee et al. 2011)</td>
<td>Goose</td>
<td></td>
</tr>
<tr>
<td>Campylobacter</td>
<td>(Cook 1991)</td>
<td>Pathogens</td>
<td></td>
</tr>
<tr>
<td>Cetobacterium</td>
<td>(Davis 2013; Finegold et al. 2003; Foster et al. 1995)</td>
<td>Dog, swan, human, marine animal</td>
<td></td>
</tr>
<tr>
<td>Geobacillus</td>
<td>(Lee et al. 2011)</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>Globericatella</td>
<td>(Lee et al. 2011)</td>
<td>Chicken</td>
<td></td>
</tr>
<tr>
<td>Granulicatella</td>
<td>(Lee et al. 2011)</td>
<td>Chicken</td>
<td></td>
</tr>
<tr>
<td><strong>Helicobacter</strong></td>
<td>(Green et al. 2012)</td>
<td>Gull, geese, chicken, duck</td>
<td>Marker under development for bird, (Green et al. 2012), promising in N</td>
</tr>
<tr>
<td><strong>Ignavigranum</strong></td>
<td>(Lawson et al. 2001; Lee et al. 2011)</td>
<td>Chicken, Human</td>
<td></td>
</tr>
<tr>
<td><strong>Marinospirillum</strong></td>
<td>(Lee et al. 2011)</td>
<td>Cow</td>
<td></td>
</tr>
<tr>
<td><strong>Marinospirillum</strong></td>
<td>(Lee et al. 2011)</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td><strong>Peptostreptococcaceae</strong></td>
<td>(McLellan et al. 2013)</td>
<td>Sewage</td>
<td></td>
</tr>
<tr>
<td><strong>Rhodoblastus</strong></td>
<td>(Lee et al. 2011)</td>
<td>Human/chicken</td>
<td></td>
</tr>
<tr>
<td><strong>Ruminococcus</strong></td>
<td>(Davis 2013; Jeong et al. 2011)</td>
<td>Found in all faeces but notable abundances in sheep</td>
<td></td>
</tr>
<tr>
<td><strong>Sedimentibacter</strong></td>
<td>(Lee et al. 2011)</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td><strong>Selenomonas</strong></td>
<td>(Lee et al. 2011)</td>
<td>Cow</td>
<td></td>
</tr>
<tr>
<td><strong>Sutterella</strong></td>
<td>(Davis 2013)</td>
<td>Chicken, Human</td>
<td></td>
</tr>
<tr>
<td><strong>Tsukamurella</strong></td>
<td>(Lee et al. 2011)</td>
<td>Cow</td>
<td></td>
</tr>
<tr>
<td><strong>Vagococcus</strong></td>
<td>(Lu et al. 2008)</td>
<td>Animal faeces</td>
<td></td>
</tr>
<tr>
<td><strong>Veillonella</strong></td>
<td>(Jeong et al. 2011)</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td><strong>Yania</strong></td>
<td>(Lee et al. 2011)</td>
<td>Chicken</td>
<td></td>
</tr>
</tbody>
</table>
Figure 10: Agarose gel of samples that were amplified for 16S rRNA gene fragments. The negative control is the reaction mixture without any DNA and the positive control is 16Sr RNA sample that had previously been successfully amplified. The amplifiable product and positive control is 180 bp.
3.4 Results

3.4.1a Higher bacterial taxonomic dissimilarity and diversity is more related to high river flow than *E. coli*

Alpha diversity was analysed across all samples as a function of *E. coli* abundance and high or low river flow. High river flow was defined by a value greater than 1.3 cumecs, and high *E. coli* (HEC) was defined by a plate count value greater than 14 cfu/100 mL (obtained in Chapter Two), meaning that the values below this were considered low (LEC).

The RF3 samples diverged from the rest with a Bray-Curtis index of 3.5, showing a 95% confidence that the clusters are significantly different from each other. The highest *E. coli* containing samples (RF3) are the most dissimilar from all the other sample sets, but the remaining HEC samples shared 0.2 - 0.5 dissimilarity with the LEC samples (Fig. 11A).

Higher species richness and evenness were observed for HEC than LEC, with median alpha diversity indices of 4 and 3.7 respectively (Fig. 11B), although the overlap in data suggests that this was not a significant difference. When assessed by river flow the alpha diversity differed from 4 for the low and 6.8 for the high river flow samples (Fig. 11C).
Figure 11: Hierarchical clustering and alpha diversity analysis as a function of *E. coli* amount and high river flow. Hierarchical cluster analysis (A) based on Bray-Curtis dissimilarity index on the Y axis and calculated p-values on the edge of each cluster for each sample period representing BP = Bootstrap Probability p-value, and Approximately Unbiased (AU) p-value. The sample labels correspond to the sampling week that they were collected as in Chapter Two (specific sampling details are summarized in Table 8 of the materials and methods), the blue circles representing samples from the high *E. coli* (HEC), and low *E. coli* (LEC) samples are in grey. The red boxes represent clusters with 95% confidence. Diversity was examined by observing the total OTUs and then calculated the Shannon index for sample groups divided (B) and then for high and low river flow (C). All samples were filtered through a 0.22 µm filter with no further treatment.
3.4.1b Significantly different phyla identified between high and low river flow

Phyla identified in the high and low river flow samples predominantly consisted of Proteobacteria and Bacteroidales (Fig. 12A). There were more phyla detected in the high river flow samples, and a notable increase in the relative abundance of Verrucomicrobia and Bacteroidetes. Firmicutes and Fusobacteria were only present in the high flow and are known to contain faecal bacteria.

Phyla that were significantly different between high and low river flow were analysed (Fig. 12B). Those that were enriched in the high flow are represented in the negative fold change group, and those in the positive fold change group are enriched in the low flow samples. Significantly different phyla with positive fold change were Proteobacteria, followed by Bacteroidetes, Actinobacteria, and Cyanobacteria (Fig. 12B). Of the phyla that exclusively displayed negative fold change were Verrumicrobia, Fibrobacteres, Chlamydiae, Choloflexi, WD272, and Acidobacteria.
Figure 12: Phyla analysis of high and low river flow samples. Relative abundances (percentage of bacterial group compositions) of phyla for high and low flows (A), and significantly different abundant phyla identified between high and low river flow for all samples with high and low E. coli (B). Plotted phyla are those with p-value <0.05, and log fold change (logFC) of more than 1.9 and less than -1.9.
3.4.1c Significantly different genera identified in high and low river flow

Samples grouped by high and low river flow were analysed for significantly different genera and examined for faecal associated bacteria (Fig. 13). Genera that were enriched in the high flow are represented in the negative fold change group, and those in the positive fold change group are enriched in the low flow samples. Of the 80 genera identified 46 had a negative fold change, and 34 had increased, showing that overall there were more genera associated with the high river flow than the low river flow.

*Escherichia* – *Shigella* was identified in the negative fold change (high river flow) group among other potential faecal genera such as *Yersinia, Polynucleobacter, Enterobacter, Novosphingobium, Acinetobacter, Pedobacter, Cytophaga, Aeromonas*, and *Pseudomonas*. Those in the positive fold change group that can be faecal related are *Tenacibaculum*, and *Owenweeksia*, which are also know to be commonly found on the natural marine environment.
Figure 13: Genera trends between high and low river flow samples. Plotted genera are those with p-value <0.05, and log fold change (logFC) of more than 1.9 and less than -1.9.
3.4.1d MST targets and faecal genera more abundant in high than low river flow samples

A selection of pooled samples from experiment 1 (Table 8) were used to screen genera (listed in Table 12) that could be further interrogated for source tracking, and genera that are commonly associated with high *E. coli* abundance. Of the 31 that were screened 19 source tracking related taxa were detected (Fig. 14A). *Owenweeksia* dominated both sample groups (HEC and LEC), with relative abundances of 75% in the HEC samples and 95% in the LEC samples. Both sample groups also contained *Tenacibaculum* (HEC 4.5% and LEC 2%), *E. coli* (HEC 10.5% and LEC <1%), and *Bacillus* (HEC 12.5% and LEC 0.27%), with a higher relative abundance in the HEC samples. The HEC group contained potential MST targets and faecal genera that were not detected in the LEC: *Yersinia* (3%), *Serratia* (3%), *Rhodobacter* (2%), *Acidovorax* (1.5%), and *Rhodoplanes* and *Prevotella* (<1% relative abundance).

The relative abundances for the screened MST targets and faecal genera shows higher abundance and more genera diversity in the analysis by river flow (Fig. 14B) than by *E. coli* (Fig. 14A). Genera shared between both high and low river flow were: *Owenweeksia* (high 2%, low 95%), *E. coli* (high 41% low <1%) and *Bacillus* (high 6% and low <1%). Those only found in the high river flow samples were: *Yersinia* (14%), *Serratia* (10%), *Rhodobacter* (8%), *Acidovorax* (7%), *Gemmatimonas* (6%), *Bacteroides* (3%), *Owenweeksia* (2%), *Rhodoplanes* (<1%) and *Prevotella* (<1%). Genera that were only found in low flow samples were: *Tenacibaculum* (5%) and *Cellulophaga* (<1%).
Figure 14: Source tracking and faecal genera screening as a function of high and low E. coli, and high and low river flow. Relative abundances of genera of interest (Table 12) were analysed for all samples as a function of HEC and LEC (A) and high and low river flow (B).
3.4.1e Observed *Shigella* - *E. coli* sequencing-based abundance did not correlate with *E. coli* plate counts

All *E. coli* plate count positive samples resulted in a positive detection of OTUs for the genera *Shigella* – *E. coli*. The highest abundances of both (i.e., *E. coli* in plates and *Shigella* – *E. coli* sequences) were observed for sampling RF3; however there was not a similar pattern in the abundance of OTUs and plate counts for the remaining samples (Fig. 15A). A Spearman’s correlation between the plate counts and OTUs indicate an inconclusive correlation (Fig. 15B) ($r = 0.80$, $p >0.05$).
Figure 15: Spearman’s correlation of OTU counts versus plate counts. Mean plate counts for *E. coli* from the water (enumerated in Chapter 2) samples were compared with the OTU reads measured for *E. coli - Shigella* (A), for each sample enumeration n = 4 to 6, error bars indicate SEM (± 1). This information was used to run Spearman’s correlations for all samples (B) (n = 6).
3.4.2a Richness and alpha diversity does not differ between 0.22 µm and 0.45 µm filters
This experiment aimed to identify differences in microbial communities between two filter sizes that are commonly used in microbial ecology (0.22 µm) and *E. coli* culture plate enumeration (0.45 µm). Shannon indices show that the median of the pooled samples between the two groups differs by 0.12, and are potentially higher in 0.45 µm filters, but the overlap in the data between the two groups indicate that these differences are not statistical significant (Fig. 16) (ANOVA, F = 0.39, P = 0.57) (Supp. Table 2).
Figure 16: Dissimilarity and richness between 0.45 µm and 0.22 µm filters. Alpha diversity represented by observed species richness (left) and evenness calculated by Shannon index (right) for each treatment type (0.22 µm and 0.45 µm along the x axis).
3.4.2b No significant differences in relative abundance of phyla and genera between different filter sizes

The relative abundances of bacterial phyla and genera were analysed to identify potentially different or missing taxa with the larger filter pore size (Fig. 17A). There appeared to be a shift in the relative abundances of the most dominant phyla (e.g. Bacteroidetes had higher relative abundances in the 0.22 µm filters, and Proteobacteria more in the 0.45 µm filters). However, there were no obvious differences in the abundances of faecal-related phyla: Frimicutes, Fibrobacteres, and Fusobacteria.

Although the relative abundances of genera (Fig. 17B) Erwinia, Mucilaginibacter, and Polynucleobacter appeared possibly more abundant on the 0.45 µm filters than on the 0.22 µm filter, and Psuedomonas, Rickettsiella, and Pedobacter appeared to be more abundant in the 0.22 µm treatment (Fig. 17B), a test for significantly different OTUs found no differences for any OTU.
Figure 17: Relative abundances of phyla and genera between 0.22 µm and 0.45 µm filters. Microbial communities between the two filter treatments were analysed for the relative abundances of phyla (A) and relative abundances of genera (B) (with more than 50 hits only, top 62 OTUs) between the two filter sizes.
3.4.3a Community dissimilarity decreases with culturing on CM1046

Filtered samples cultured and non-cultured on CM1046 were compared to identify whether culturing had altered the bacterial community diversity. A hierarchical cluster analysis was carried out to assess dissimilarity between cultured and uncultured filters (Fig. 18). All cultured groups cluster together with a dissimilarity index of 2.2. The uncultured samples W8 and W11 significantly cluster together with a dissimilarity index of 0.6 and are most dissimilar from all other samples. The remaining samples cluster together, including the uncultured rainfall RF3 samples, which branch with the cultured samples with an index of 2.6.

Figure 18: The effect of culturing on community dissimilarity. Hierarchical cluster analysis was based on the Bray-Curtis dissimilarity index (Y axis) and calculated p-values on the edge of each cluster for each sample period representing: BP = Bootstrap Probability p-value, and AU = Approximately Unbiased. The sample labels correspond to the sampling week that they were collected for as in Chapter Two (sampling details are summarized in Table 4). Samples were colour coded to match their replicates with the uncultured filters (open circles) matching with their cultured pairs (closed circles). Red rectangle marks cluster groups with 95% confidence.
3.4.3b Culturing alters the most abundant genera
The largest rainfall samples (RF3) showed the highest microbial diversity and amount of faecal matter (as measured by *E. coli*); it is for these reasons that they were selected for further examination on the effects of culturing on a diverse microbial community. Among the top 51 OTUs with at least 200 hits, there were 16 faecal-related genera identified in the cultured samples and 26 in the uncultured (Fig. 19). Between the two treatment groups there are six genera shared, these include: an uncultured genera (yellow), *Yersinia, Rahnella, Erwinia, Pseudomonas, Enterobacter,* and *Escherichia-Shigella*. Those that were found in the cultured and not in the uncultured were *Vibrio, Shewanella, Raoutella, Providencia, Photobacterium, Pantoea, Citrobacter, Aeromonas* and *Acinetobacter.*
Figure 19: The effects of culturing on the relative abundance of genera in cultured versus uncultured filters. Analysis was carried out on cultured and uncultured filters from the RF3 samples. Plotting more than 200 hits and the top 51 OTUs.
3.5 Discussion

This chapter assessed the bacterial community in the water samples collected from the Waikouaiti estuary during presence and absence of faecal contamination as measured by *E. coli*. Water samples were analysed using 16S rRNA sequencing to identify: other faecal bacteria associated with *E. coli*, bacteria that could serve as potential MST targets, and other pathogenic genera that might be of concern for shellfish consumers. Community alterations were assessed based on changes in diversity between high and low river flow samples and high and low abundance of *E. coli*. A comparison was carried out on the microbial community collected between 0.22 µm and 0.45 µm pore size filters. Lastly, the affect of culturing with CM1046 media was investigated.

3.5.1 Bacteria associated with *E. coli*

RF3 samples appear to be most dissimilar among all samples. Surprisingly the rest of the ‘high *E. coli*’ (HEC) samples were equally as dissimilar along with the ‘low *E. coli*’ (LEC) samples (Fig. 11A). Alpha diversity was more distinct when samples were separated by high flow (Fig. 11C) than by high/low *E. coli* abundance (Fig. 11B). These results along with an NMDS analysis (Supp. Fig 3) indicate that the RF3 samples were responsible for driving diversity. These observations led to the hypothesis that the ‘high river flow’ (HF) samples contained more faecal microbes than the ‘low river flow’ (LF) samples so were subjected to further analysis for faecal microbes.

The diversity of phyla and genera appeared to be higher in the HF samples than in the low flow samples (LF). Sequencing also revealed the presence of candidate divisions, which are phyla and genera that have no culturable representatives (McDonald et al. 2012). The relative abundances of the faecal phyla Proteobacteria and Bacteroidetes were dominant in both the HF and LF samples (Fig. 12A), and associated genera also appeared to be significantly enriched with both flow types (Fig 12B). Faecal phyla Firmicutes and Fusobacteria were also present in the HF samples but not in the LF (Fig. 12A) and these were not observed to be significantly different between a fold change more than 1.9 and less than -1.9 (Fig. 12B). The phyla Cyanobacteria and Verrumicrobia increased with river flow, these are common natural water and terrestrial isolates (Hedlund
and Gosink 1997; Sun et al. 2014). This finding indicates that a majority of the bacteria found in the HF originate from sources other than faeces and marine environments. Around a dozen detected genera could potentially be linked to faecal contamination (Table 12). The identified genera have previously been linked to cow, gull, human, cat, horse, and pig (Table 12). Three genera containing strains previously responsible for shellfish foodborne outbreaks were found: *Yersinia, Shigella – Escherichia* and *Aeromonas*.

The genera *Shigella – E. coli* encompasses all strains of *Escherichia* and *Shigella*, this is because *Shigella* are treated as a sub-genus of *Escherichia* due to phenotypic and whole-genome homogeneity (Zuo et al. 2013). The faecal-related genus *Shigella - E. coli* was enhanced in the HF samples (Fig. 13) along with other associated faecal-related genera: *Yersinia, Enterobacter, Novosphingobium, Acinetobacter, Pedobacter, Cytophaga, Aeromonas* and *Psuedomonas*. While many of these could be associated with the hypothesised ruminant contamination many are associated with environmental strains linked to terrestrial, fresh water, and the marine environments (Table 13). Faecal-related genera that were found associated with LF (i.e., in the positive fold change group), were *Tenacibaculum* and *Owenweeksia*, and have been previously linked to bovine sources, but again are also largely environmental organisms (Table 13).

Some detected genera contain pathogens that are of concern in shellfish harvesting. An example of this is *Yersinia enterocolitica*, which is a faecal associated bacteria that is associated with sewage, warm blooded animals and avian species (Li et al. 2015). Cells of this taxa are able to persist and remain viable in freshwater for up to 64 weeks (Karapinar & Gonul 1991). *Aeromonas hydrophila* is a known strain to cause illness via contaminated shellfish (Table 13). *Acinetobacter* are ubiquitous soil organisms but can also be associated with cow, gull and human faeces (Table 13). The strain *A. lwoffii* is a potential gastrointestinal pathogen in immunocompromised individuals so it is unlikely that they will cause illness in healthy individuals through the consumption of shellfish (Rathinavelu et al. 2003). *Pseudomonas* strain *P. aeruginosa* have been shown to be associated with the gastrointestinal tract of humans but is also a natural inhabitant of the estuarine environment (Table 13).
Table 12: Details of the genera that were found to be significantly different between high and low river flow (section 4.4.1C). The environments are F = Faecal, M = Marine, S = Soil, FW = Fresh water and O = Other.

<table>
<thead>
<tr>
<th>Genera</th>
<th>Previously isolated sources</th>
<th>Potential Gastrointestinal pathogen present</th>
<th>Faecal host groups</th>
<th>Gastroenteritis strains</th>
<th>Shellfish outbreaks of gastrointestinal illness</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caullobacter</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Grimes 1991)</td>
</tr>
<tr>
<td>Erwinia</td>
<td>O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Starr &amp; Chatterjee 1972)</td>
</tr>
<tr>
<td>Rahnella</td>
<td>S, O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Rozhon et al. 2010)</td>
</tr>
<tr>
<td>Asticcacaulis</td>
<td>FW</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Pointdexter 1964)</td>
</tr>
<tr>
<td>Polynucleobacter</td>
<td>F, FW</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Fernandez-Piquer et al. 2012)</td>
</tr>
<tr>
<td>Aquincola</td>
<td>O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Lechner et al. 2007)</td>
</tr>
<tr>
<td>Oryza sativa Indica Group</td>
<td>O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Ventelon-Debout et al. 2004)</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>F, M, S, FW</td>
<td>Yes</td>
<td>Human, Animal</td>
<td>E. aerogenes</td>
<td>No</td>
<td>(Fergusson &amp; Signoretti 2011; Grimes 1991)</td>
</tr>
<tr>
<td>Luteibacter</td>
<td>S, O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Muangchinda et al. 2013)</td>
</tr>
<tr>
<td>Candidatus Liberibacter</td>
<td>S, O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Liefting et al. 2009)</td>
</tr>
<tr>
<td>Undibacterium</td>
<td>S</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Kämpfer et al. 2007)</td>
</tr>
<tr>
<td>Taibaiella</td>
<td>S, O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Zhang et al. 2013; Tan et al. 2014)</td>
</tr>
<tr>
<td>Achromobacter</td>
<td>FW</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Otta et al. 2014)</td>
</tr>
<tr>
<td>Janthinobacterium</td>
<td>O</td>
<td>No</td>
<td>Cow, Bird</td>
<td>No</td>
<td>No</td>
<td>(Kim &amp; Wells 2016)</td>
</tr>
<tr>
<td>Skermanella</td>
<td>S, FW</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(López-López et al. 2002)</td>
</tr>
<tr>
<td><strong>Marinomonas</strong></td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Satomi &amp; Fujii 2014)</td>
</tr>
<tr>
<td>-----------------</td>
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<td>----</td>
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<td>----</td>
<td>----</td>
<td>----------------------------</td>
</tr>
<tr>
<td><strong>Novosphingobium</strong></td>
<td>F, FW, O</td>
<td>No</td>
<td>Human (gastric contents), Cat,</td>
<td>No</td>
<td>No</td>
<td>(Chidamba &amp; Korsten 2015; Kwon et al. 2011; Delgado et al. 2013; Lubbs et al. 2009)</td>
</tr>
<tr>
<td><strong>uncultured proteobacterium</strong></td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Epilithonimonas</strong></td>
<td>S, M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Ventorino et al. 2016; O’Sullivan et al. 2006)</td>
</tr>
<tr>
<td><strong>Massilia</strong></td>
<td>S, FW</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Cerrone et al. 2011)</td>
</tr>
<tr>
<td><strong>Xylophilus</strong></td>
<td>O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Willems et al. 1991)</td>
</tr>
<tr>
<td><strong>Rhizobium</strong></td>
<td>O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Mendes et al. 2013)</td>
</tr>
<tr>
<td><strong>Escherichia - Shigella</strong></td>
<td>F, O</td>
<td>No</td>
<td>Animal</td>
<td><em>S. sonnei,</em> <em>E. coli O157:H7</em></td>
<td>Yes</td>
<td>(Lan &amp; Reeves 2002)</td>
</tr>
<tr>
<td><strong>Steroidobacter</strong></td>
<td>S</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Wu et al. 2010)</td>
</tr>
<tr>
<td><strong>Simiduia</strong></td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Rua &amp; Thompson 2014)</td>
</tr>
<tr>
<td><strong>Brevundimonas</strong></td>
<td>FW, M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Abraham et al. 1999)</td>
</tr>
<tr>
<td><strong>Acinetobacter</strong></td>
<td>F, M, S, O</td>
<td>Yes</td>
<td>Gull, Cow, Human</td>
<td><em>A. lwaffi</em></td>
<td>No</td>
<td>(Lu et al. 2008; Vandewalle et al. 2012; Jeong et al. 2011; Delgado et al. 2013)</td>
</tr>
<tr>
<td><strong>Cellvibrio</strong></td>
<td>FW</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Chidamba &amp; Korsten 2015)</td>
</tr>
<tr>
<td><strong>Rickettsiella</strong></td>
<td>O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Jasur-Kruh et al. 2013)</td>
</tr>
<tr>
<td><strong>Xanthomonas</strong></td>
<td>M, O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Croci et al. 2006; da Silva et al. 2002)</td>
</tr>
<tr>
<td><strong>Rhizomicrobium</strong></td>
<td>O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Ueki et al. 2010)</td>
</tr>
<tr>
<td><strong>Pedobacter</strong></td>
<td>F, S, M, O</td>
<td>No</td>
<td>Pig</td>
<td>No</td>
<td>No</td>
<td>(Wu et al. 2010; Leung &amp; Topp 2001)</td>
</tr>
<tr>
<td><strong>Cytophaga</strong></td>
<td>F, M, S</td>
<td>No</td>
<td>Horse, Pig</td>
<td>No</td>
<td>No</td>
<td>(Grimes 1991; Leung &amp; Topp 2001; Simpson et al. 2004)</td>
</tr>
<tr>
<td><strong>Table 13: continued</strong></td>
<td></td>
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<tr>
<td>-------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| **Aeromonas** F, M, FW, O | Yes | Chicken | *A. hydrophila*, *A. caviae*, *A. veronii* | Yes | (Grimes 1991; Colakoglu et al. 2006; Kwon et al. 2011; Vandewalle et al. 2012; Wei et al. 2013;)
| **Haliangium** | M | No | No | No | No | (Fudou et al. 2002)
| **Pseudomonas** F, M | Yes | Human, Gull, | *P. aeruginosa* | No | (Sinton et al. 1998; Grimes 1991; Koskey et al. 2014)
| **Flavobacterium** S, FW | No | No | No | No | No | (Grimes 1991; Kacagan et al. 2013)
| **Sulfuricurvum** M, FW, O | No | No | No | No | No | (Tanaka et al. 2012; Roalkvam et al. 2015; Kodama & Watanabe 2004)
| **Chitinophaga** S, FW | No | No | No | No | No | (Kim & Jung 2007; Vaz-Moreira et al. 2011)
| **Sphingomonas** M, S | No | No | No | No | No | (Nogales et al. 2001; Vancanneyt et al. 2001)
| **Mucilaginaibacter** S | No | No | No | No | No | (Pankratov et al. 2007)
| possible genus 04 | / | / | / | / | / | (Woyke et al. 2009)
| marine metagenome | / | / | / | / | / | (Math et al. 2012)
| **Defluviimonas** M, O | No | No | No | No | No | (Lee et al. 2011; Shimizu et al. 2009)
| **Tenacibaculum** F, M | No | Cow | No | No | No | (Holmes et al. 1997)
| uncultured alpha proteobacterium | / | / | / | / | / | (Yang et al. 2015)
| **OM43 clade** M | No | No | No | No | No | (Hwang et al. 2009)
| **Marivita** M | No | No | No | No | No | (Jin et al. 2011)

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<table>
<thead>
<tr>
<th>Table 13: continued</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavobacteriaceae bacterium MEBiC05063</strong></td>
</tr>
<tr>
<td><strong>uncultured marine bacterium</strong></td>
</tr>
<tr>
<td><strong>Prochlorococcus</strong></td>
</tr>
<tr>
<td><strong>Thalassobacter</strong></td>
</tr>
<tr>
<td><strong>uncultured gamma proteobacterium</strong></td>
</tr>
<tr>
<td><strong>Dinoroseobacter</strong></td>
</tr>
<tr>
<td><strong>AEGEAN - 109 marine group</strong></td>
</tr>
<tr>
<td><strong>Litoricola</strong></td>
</tr>
<tr>
<td><strong>SAR92 clade</strong></td>
</tr>
<tr>
<td><strong>OM60(NOR) 5 clade</strong></td>
</tr>
<tr>
<td><strong>NS5 marine group</strong></td>
</tr>
<tr>
<td><strong>Pseudospirillum</strong></td>
</tr>
<tr>
<td><strong>BAL58 marine group</strong></td>
</tr>
<tr>
<td><strong>Marinosciillum</strong></td>
</tr>
<tr>
<td><strong>Lentibacter</strong></td>
</tr>
<tr>
<td><strong>Roseobacter clade NAC11-7 lineage</strong></td>
</tr>
<tr>
<td><strong>Polaribacter</strong></td>
</tr>
<tr>
<td><strong>Oceanicola</strong></td>
</tr>
<tr>
<td><strong>NS3a marine group</strong></td>
</tr>
<tr>
<td><strong>Owenweeksia</strong></td>
</tr>
<tr>
<td><strong>Sulfitobacter</strong></td>
</tr>
<tr>
<td><strong>uncultured</strong></td>
</tr>
<tr>
<td><strong>Glaciecola</strong></td>
</tr>
</tbody>
</table>
### Table 13 continued

<table>
<thead>
<tr>
<th>Starved</th>
<th>Heterotrophs</th>
<th>ACOs</th>
<th>ATP</th>
<th>Chaperones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candidatus Planktomarina (DC5-80-3 lineage)</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><em>Tropicibacter</em></td>
<td>M</td>
<td>No</td>
<td>NA</td>
<td>No</td>
</tr>
<tr>
<td><em>Marinobacterium</em></td>
<td>M</td>
<td>No</td>
<td>NA</td>
<td>No</td>
</tr>
<tr>
<td><em>Nereida</em></td>
<td>M</td>
<td>No</td>
<td>NA</td>
<td>No</td>
</tr>
</tbody>
</table>
3.5.2 Specific genera screening of MST markers suggested faecal genera and foodborne pathogens
The previous sections analysed differences in the whole microbial community, in this section a specific screening was carried out to identify faecal-related genera that were of interest from previous studies. As expected there were more screened targets with higher relative abundances in the HEC and HF samples over LEC and LF (Fig. 15). Of the 41 genera that were screened (Table 12) a total of 19 were detected and have been associated with human sewage, cows, humans, chickens, and goose in previous studies (Fig. 15).

3.5.2a Genera absent in screening
The general faecal marker *Bifidobacterium* was notably absent (Fig. 15). Unfortunately this is an available ESR marker that would have enabled identification between gull and human sources. *Bifidobacterium* is known to have a low persistence in the environment and so are often found in low numbers in polluted waters (Teaf et al. 2011). Another broad marker, *Cetobacterium* was also absent. This is common in marine animals, human and freshwater fish (Davis 2013; Finegold et al. 2003; Foster et al. 1995). This absence could rule out the presence of faeces from swans in the samples as *Cetobacterium* was found to dominate swan faecal samples by Davis (2013). The genera that had previously been identified in chicken faeces *Globicatella, Granulicatella, Ignavigranum, Yania* (Lee et al. 2011) and *Sutterella* (Davis 2013) were not identified. The absence of a combination of these markers could potentially rule out contamination from chicken faeces, and humans. By far the most interesting absence was the genera *Ruminococcus*. This microbe is found in the gut of ruminants in high concentrations, hence is utilised for analysing contamination of water by farming waste in both in New Zealand and overseas studies (Davis 2013). Previous reports have linked the contamination in the Waikouaiti estuary to farming which is most likely (ORC 2010b; ORC 2015), but the absence of *Ruminococcus* suggests that either this is not true, or that the quantities are highly diluted thus limiting its detection threshold.

The absence of the selected screened genera indicate they are not naturally abundant in the Waikouaiti estuary and Waikouaiti catchment environment, as
well as they are not dominant in the faeces that is contributing to the contamination (at least during the time of this study).

Some absences of many of the faecal-related genera could be explained by the fact that many were obtained from overseas studies, thus differences in geographical areas result in variations of animal micoflora. The NZ study carried out by Davis (2013) found that none of the suggested markers from Lee et al. (2010) were found in their faecal samples, but did detect similar markers in comparison to Unno (2010) and Jeong (2011). These last three studies were conducted in Korea, and so faecal-related genera should be treated with caution when applied to new study environments. However, it is also important to rule out those that are applicable to contamination events in new geographical areas for future MST research.

3.5.2b Faecal-related genera identified in the screening
Genera present in high flow but absent in low flow conditions could indicate that they are either a) associated with faecal matter, b) associated with farm run-off (not exclusively faecal), or c) associated with the freshwater inputs. Yersinia is both an environmental genera and is associated with faecal matter from warm-blooded animals. The abundance of this genus increased during periods of high river flow along with Bacillus (an environmental genus that is also dominant in agricultural runoff) and Serratia (an environmental organism also used as a marker for potential pathogens). General faecal indicators Bacteroides and Prevotella were also associated with high flow, and could be associated with a range of animal faeces (human, swan, cows, duck, chicken) (Bernhard & Field 2000a; Kobayashi et al. 2013). Although these overseas studies have shown success in utilising these genera in MST assays they are yet not used in Environment Science and Research Limited (ESR) (Table 7).

Owenweeksia were found to dominate the HEC and LEC samples (Fig. 14A) as well as LF, but not in the HF samples where E. coli was the highest. Other research shows that it is a marine brackish clade (Ameryk et al. 2014), suggesting that its presence might be associated with environmental communities and not faeces. Acidovorax and Zooglea are suggested sewage markers but their use in MST studies should be critically analysed as they are part of Betaproteobacteria
which are generally found through a wide range of terrestrial environments (Davis 2013).

*Tenacibaculum* and *Cellulophaga* did not appear to increase in relation with *E. coli* abundance. *Cellulophaga* has previously been linked to chicken faeces but it is also a marine genus (O’Sullivan et al. 2006). Similarly, *Tenacibaculum* has previously been isolated from seawater (Suzuki et al. 2001; Prabagaran et al. 2007). Those that were detected but were not graphed were *Enterococcus*, *Zoogloea*, *Ottowia*, *Oceanimona*, *Enterococcus* and *Sterolibacterium*. *Sterolibacterium* currently only has one species known to this genus which is responsible for denitrifying and metabolise cholesterol (Tarlera & Denner 2003; Chiang et al. 2008) having previously been found in cow faeces as well in the environment.

*Salmonella* and other bird pathogens (e.g. *Helicobacter*, *Campylobacter*, *Vagococcus*) were detected in low abundances indicating bird faeces were not dominant in any of the samples. This is surprising because the seabird matter was expected to be present with low river flow samples, as birds usually congregate around the harvesting area (Derolez et al. 2009; Wither et al. 2005b). The fact that none of the bird markers were identified suggests that the genera screened might not be sensitive enough, or that they are not appropriate for use in sequencing applications.

Overall the interpretations of the identified genera in the screening should be treated with caution, as many are also natural inhabitants of soil, marine, and fresh water environments and not exclusively faeces related. Both flow conditions contain many terrestrial and freshwater associated genera (Fig. 13).

3.5.3 Quantification of *E. coli* from culturing to sequencing

Another aim of this chapter was to identify a relationship between the OTUs of *E. coli – Shigella* and the corresponding plate counts. The results showed that the correlation is inconclusive (Fig. 15). This could be further explained by two inconsistencies between the methods; firstly the fact that the group *E. coli - Shigella* accounts for both genera OTUs, and secondly the OTUs contributed
from bacteria that are in a ‘culturable but not viable state’ or are dead. The genera *Shigella* are positive for β–glucuronidase (Horakova et al. 2006), so in the plate count these bacteria would have been counted as *E. coli* in the plate enumeration. However some other species in the Enterobacteriaceae family (i.e. *Salmonella, Yersinia, Pantoea, Klebsiella* and *Erwinia*) have also been known to deliver a false positive result on β–glucuronidase based media (Mclain et al. 2011), and false positives are known to commonly occur in brackish and estuarine waters (MoE/MoH 2002). More samples should be collected to obtain more data to strengthen statistical validity for future correlations.

3.5.4 Sequencing of 0.45 µm and 0.22 µm filters show equal representation of microbial communities
In light of recent research regarding ultra-bacteria, an investigation was carried out on the effect of the 0.45 µm filter in comparison to the 0.22 µm in order to identify potential missing genera. It was expected that the 0.45 µm would contain less diversity than the 0.22 µm filters. This hypothesis was however disproven as the results illustrated no statistical differences between species richness (Fig. 16, Supp. Table 2), as well as the relative abundances of phyla (Fig. 17A) and OTUs (Fig. 17B). It was also expected that the 0.45 µm filters would exclude some common ultrabacteria containing phyla previously identified in other studies, such as Actinobacteria and Firmicutes. These studies however used fresh water from rivers as well as ground water (Luef et al. 2015; Brown et al. 2015; Staley et al. 2015) as opposed to estuarine seawater environment.

These findings support that both size filters are equal in capturing the microbial community water samples, and also justifies the use of 0.45 µm for capturing the faecal coliform community using membrane filtration techniques in water quality protocols.

3.5.5 Culturing alters communities
Overall a decrease in bacterial diversity was observed in the CM1046 cultured filters in comparison to samples that were uncultured (Fig. 18 & 19). The bacteria that were enhanced with culturing were those that produced colonies on the media; the OTUs from these bacterial genera would have contributed a majority of DNA therefor diluting and reducing the detection of OTUs from bacteria that
were not enriched by the media (as indicated in Table 8). In particular, the OTUs of marine genera were significantly reduced with culturing (Fig. 20 & Table 14).

Pathogenic genera (both faecal and non-faecal) that have been associated with foodborne outbreaks in shellfish were enhanced by the CM1046 media, including: *E. coli* – *Shigella*, *Yersinia*, *Vibrio*, *Shewanella* and *Aeromonas* (Fig. 20 & Table 14). *Vibrio cholerae* and *Vibrio parahaemolyticus* are naturally found in the estuarine environment (DePaola et al. 1990; Blackwell & Oliver 2008). Gastroenteritis infections caused by strains of *Shewanella* are rare but have been shown to be on the rise in Japan with the high consumption of raw fish per capita (Richards et al. 2008). The genera *Aeromonas* and *Vibrio* are often screened in shellfish products as their association with previous outbreaks have been identified by several of their species (Colakoglu et al. 2006; Athens 1995). The results obtained from this experiment suggest that this media could be useful in combination with sequencing to increase the mass of pathogenic genera. This could be used for genera such as *Vibrio* and *Aeromonas* that are in low abundances without culturing.

Unfortunately species identification was not possible with the level of sequencing used in this study; deeper sequencing methods will need to be employed to address this. Future methods could identify the pathogenic strains that are within the genera to better assess the risk of specific pathogens.

Some foodborne genera detected that have no known cases linked to shellfish poisonings were *Acinetobacter*, *Pantoea*, *Providencia*, and *Photobacterium* (Fig. 19 & Table 14). The species *Raoultella* can be isolated from soil, water and plants; *Raoultella planticola* is the most frequent species found in soils and has been identified in rumen content of cows, this strain along with *Raoultella ornithinolytica* can indirectly cause food related illness via histamine production causing fish poisoning illnesses (Kanki et al. 2002).
Table 13: Details of the relative abundant genera associated with culturing in section 4.4.3c. The sources are F = Faecal, M = Marine, S = Soil, FW = Fresh water and O = Other.

<table>
<thead>
<tr>
<th>Genera</th>
<th>Cultured or uncultured</th>
<th>Previously isolated sources</th>
<th>Gastrointestinal pathogen?</th>
<th>Faecal host groups</th>
<th>Gastroenteritis strains</th>
<th>Shellfish outbreaks of gastrointestinal illness?</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yersinia</strong></td>
<td>Cultured</td>
<td>F, O</td>
<td>Yes</td>
<td>Human, Cow</td>
<td>Y. enterocolitica, Y. pseudotuberculosis</td>
<td>Yes</td>
<td>(Davey et al. 1983; Kishore et al. 2012; Aulisio et al. 1980)</td>
</tr>
<tr>
<td><strong>Vibrio</strong></td>
<td>Cultured</td>
<td>M</td>
<td>Yes</td>
<td>No</td>
<td>V. parahaemolyticus, V. cholera, V. vulnificus, V. fluvialis</td>
<td>Yes</td>
<td>(Cook 1991; Austin 2010)</td>
</tr>
<tr>
<td>Uncultured bacterium</td>
<td>Uncultured</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uncultured bacterium</td>
<td>Both</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Tropicibacter</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>No</td>
<td>(Prabagaran et al. 2007)</td>
</tr>
<tr>
<td><strong>SV1–3</strong></td>
<td>Uncultured</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Sulfobacter</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Park et al. 2007)</td>
</tr>
<tr>
<td><strong>Shewanella</strong></td>
<td>Cultured</td>
<td>M</td>
<td>Yes</td>
<td>No</td>
<td>S. putrefaciens (very rare)</td>
<td>No</td>
<td>(Richards et al. 2008)</td>
</tr>
<tr>
<td><strong>Roseobacter clade NAC11-7 lineage</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Teeling et al. 2016)</td>
</tr>
<tr>
<td><strong>Ralouilletta</strong></td>
<td>Cultured</td>
<td>S, M</td>
<td>Yes - Indirectly via production of histamine</td>
<td>No</td>
<td>R. planticola, R. ornithinolytica, R. terrigena</td>
<td>No</td>
<td>(Kanki et al. 2002)</td>
</tr>
<tr>
<td><strong>Rahnella</strong></td>
<td>Both</td>
<td>S, O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Rozhon et al. 2010)</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Both</td>
<td>F, M</td>
<td>Yes</td>
<td>Human, Gull,</td>
<td>P. aeruginosa</td>
<td>No</td>
<td>(Sinton et al. 1998; Grimes 1991; Koskey et al. 2014)</td>
</tr>
<tr>
<td><strong>Providencia</strong></td>
<td>Both</td>
<td>F, M, S</td>
<td>Yes</td>
<td>Human</td>
<td>P. alcalificiens</td>
<td>No</td>
<td>(O’Hara et al. 2000; Murata et al. 2001)</td>
</tr>
<tr>
<td><strong>Polaribacter</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Teeling et al. 2016)</td>
</tr>
<tr>
<td><strong>Photobacterium</strong></td>
<td>Cultured</td>
<td>M</td>
<td>Yes</td>
<td>No</td>
<td>(Not specified)</td>
<td>No</td>
<td>(Richards et al. 2008)</td>
</tr>
<tr>
<td><strong>Pedobacter</strong></td>
<td>Uncultured</td>
<td>F, S, M, O</td>
<td>No</td>
<td>Monkey, Pig</td>
<td>No</td>
<td>No</td>
<td>(Wu et al. 2010; Leung &amp; Topp 2001)</td>
</tr>
<tr>
<td><strong>Table 14: continued</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td><strong>Pantoaea</strong></td>
<td>Both</td>
<td>S, 0</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Brady et al. 2010)</td>
</tr>
<tr>
<td><strong>Owenweeksia</strong></td>
<td>Uncultured</td>
<td>F, M</td>
<td>No</td>
<td>Cow</td>
<td>No</td>
<td>No</td>
<td>(Lau et al. 2005; Lee et al. 2011)</td>
</tr>
<tr>
<td><strong>Oceanicola</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Cho &amp; Giovannoni 2004)</td>
</tr>
<tr>
<td><strong>NS3a marine group</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Yang et al. 2015)</td>
</tr>
<tr>
<td><strong>Nereida</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>No</td>
<td>(Macián et al. 2005)</td>
</tr>
<tr>
<td><strong>Mucilaginibacter</strong></td>
<td>Uncultured</td>
<td>S</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Pankratov et al. 2007)</td>
</tr>
<tr>
<td><strong>Marinomicrobium</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Yang et al. 2015)</td>
</tr>
<tr>
<td><strong>Marinobacterium</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>No</td>
<td>(Satomi &amp; Fujii 2014)</td>
</tr>
<tr>
<td><strong>Lentibacter</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Li et al. 2012)</td>
</tr>
<tr>
<td><strong>Glaciecola</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>No</td>
<td>(Prabagaran et al. 2007)</td>
</tr>
<tr>
<td><strong>Escherichia - Shigella</strong></td>
<td>Both</td>
<td>F, O</td>
<td>No</td>
<td>Animal</td>
<td>S. sonnei, E. coli O157:H7</td>
<td>Yes</td>
<td>(Lan &amp; Reeves 2002)</td>
</tr>
<tr>
<td><strong>Erwinia</strong></td>
<td>Cultured</td>
<td>O</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Starr &amp; Chatterjee 1972)</td>
</tr>
<tr>
<td><strong>Enterobacter</strong></td>
<td>Both</td>
<td>F, M, S, FW</td>
<td>Yes</td>
<td>Human, Animal</td>
<td>E. aerogenes</td>
<td>No</td>
<td>(Fergusson &amp; Signoretta 2011; Grimes 1991)</td>
</tr>
<tr>
<td><strong>Demequina</strong></td>
<td>Uncultured</td>
<td>S, M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Yi et al. 2007; Finster et al. 2009)</td>
</tr>
<tr>
<td><strong>Citrobacter</strong></td>
<td>Cultured</td>
<td>FW, S</td>
<td>Yes</td>
<td>Human, Gull, Cow</td>
<td>Citrobacter rodentium</td>
<td>No</td>
<td>(Mundy et al. 2003; Kim &amp; Wells 2016; Manja et al. 1982; Patel et al. 2008)</td>
</tr>
<tr>
<td><strong>Candidatus Plankt marina (DC5-80-3 lineage)</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>No</td>
<td>(Buchan et al. 2005)</td>
</tr>
<tr>
<td><strong>BAL58 marine group</strong></td>
<td>Uncultured</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(Simu &amp; Hagström 2004)</td>
</tr>
<tr>
<td><strong>Aeromonas</strong></td>
<td>Cultured</td>
<td>F, M, FW, O</td>
<td>Yes</td>
<td>Chicken</td>
<td>A. hydrophila, A. caviae, A. veronii</td>
<td>Yes</td>
<td>(Grimes 1991; Colakoglu et al. 2006; Kwon et al. 2011; Vandewalle et al. 2012; Wei et al. 2013)</td>
</tr>
<tr>
<td><strong>Acinetobacter</strong></td>
<td>Cultured</td>
<td>F, M, S, O</td>
<td>Yes</td>
<td>Gull, Cow, Human</td>
<td>A. lwoffii</td>
<td>No</td>
<td>(Lu et al. 2008; Vandewalle et al. 2012; Jeong et al. 2011; Delgado et al. 2013)</td>
</tr>
</tbody>
</table>
3.5.6 Limitations and future directions

Assessment of what ‘is’ and what ‘is not’ present in the environment during a high contamination event allows for a broad-spectrum snapshot of what key organisms can be targeted. This is important for selecting the best markers for MST assays, as without prior knowledge a target genera might not even been present in the first place (e.g. *Bifidobacterium*). Source tracking via manual screening of genera is not ideal and can only lead to hypotheses of contamination without statistical validity. Furthermore individual genera are not able to provide source specific information. An LDM application could provide further information but would require sequencing of faeces from all potential hosts that can contribute to the contamination in the Waikouaiti estuary. An alternative could be to obtain reference host sequences from ESR to create such a library for comparison against the collected water samples, as was carried out in Davis (2013).

The overall diversity of potentially faecal microbes was driven by the HF samples from a major rainfall and flood event (samples RF3). It is known from Chapter Two that there was not an accumulation of *E. coli* in shellfish and so the impact of contamination may not be directly associated. The shellfish were possibly not feeding because of stressful environmental parameters such as salinity reduction and increase in organic matter (as previously discussed in Chapter Two). It is therefore important to investigate the faecal contaminants that continue to persist in the estuary after the initial rainfall, and when the animals are feeding normally. Future work should sequence the shellfish itself throughout a major rainfall event to identify risks throughout the rainfall. This would directly assess the accumulated content for pathogens and faecal bacteria that can be source tracked. This will also answer the main question asked by non-commercial shellfish harvesters: ‘is our seafood safe to eat?’.

DNA based techniques will amplify material regardless of cell viability which is an issue for determining the risk of bacteria that are ready to infect people or indicating recent contamination events (Wise & Siragusa 2005). Walters et al. (2009) demonstrated that seawater microcosms inoculated with naked DNA from
Enterococcus faecium can be detected with qPCR after 10 days. In sewage microcosms a culturable detection limit for Enterococcus lasted 5 days, but a qPCR signal could be detected up to 28 days later. In ecological samples a large proportion of bacteria in aquatic environments are in fact dead (Luna et al. 2002). Recent protocols have established differentiation of live/dead bacteria using chemicals to penetrate permeable cells and then bind DNA, and degrade it under the presence of UV light (e.g. Nocker et al. 2007). Live/Dead distinguishing methods are still under optimisation because of the differing concentrations that are required per target. This protocol could be employed in future MST analyses since it offers a quick step to ensure that genomic analyses of dead or naked DNA is inhibited; thus in the context of MST only recently deposited microbes in a contamination event will be detected (Nocker et al. 2007; Bae & Wuertz 2015; Siefring et al. 2008; Roslev & Bukh 2011).
3.6 Conclusions

The key to future water management will be through the utilisation of more informative methods for water quality assessment. The sequencing-based results obtained in this chapter have identified a broad range of bacterial targets that could assist in investigation for source tracking purposes such as genera *Prevotella*, *Bacteroides*, *Enterococci* and phyla Bacteroidales. Optimal conditions that provide MST opportunities were high river flow, which was also found as a main parameter driving microbial diversity in faecal bacteria. The detected faecal-related genera suggest that the sources of contamination could be: cow, gull, human, and pig in the Waikouaiti estuary. Future MST targets should aim at these sources using the aforementioned bacterial targets. Ruminants are considered to be the major contamination source in the Waikouaiti estuary, however the commonly isolated ruminant genus *Ruminoccocus* was absent in this study and therefore could be eliminated as a MST target.

The OTUs recovered using the two studied filter pore sizes (0.22 µm and 0.45 µm) were similar, indicating that standard sampling and enumeration techniques for *E. coli* are adequate for capturing the faecal community. Furthermore CM1046 culturing decreased the relative abundance of marine bacteria but enhanced the growth of terrestrial microbes, faecal-related genera, and gastro pathogens. We suggest that a sequencing step could be used in conjunction with enumeration water quality testing methods, this would allow an improved detection of low-abundant microbial pathogens in shellfish harvesting waters.
CHAPTER FOUR

4.1 Thesis findings

The objective of this thesis was to investigate the dynamics of faecal bacteria in the Waikouaiti estuary after rainfall in order to identify the safety of shellfish for consumption. E. coli was employed as a faecal marker organism and concentrations were enumerated for both shellfish tissue and the overlaying water. These were then compared to safety standards used for commercial harvesting.

A major finding in the results was that there were breaches of safety for shellfish and in the water, but these were not observed simultaneously. Subsequently, correlations of E. coli abundance in the water versus shellfish were inconclusive. This suggested that there are other factors (e.g. salinity, temperature, organic matter load, temporal and scaling effects) that might be important for accumulation of bacteria in shellfish and clearance, or, that more samples are needed.

Throughout the scientific literature periods of no rainfall are considered of little contamination risk because less runoff from pollution sources are expected. However this study showed shellfish were still able to accumulate microbial contaminants to levels that exceed the Category A (>230 cfu/100 g) safe to eat commercial limits during low rain periods. Similar circumstances have previously been identified along the Horowhenua coast (of the North Island) in shellfish beds located downstream of heavily farmed catchments (Newcombe et al. 2014). The inconsistency between rainfall and shellfish quality in these circumstances suggest that quality assessments should focus on the microbial content of the shellfish flesh, and not depend on the quality of the water alone unlike current recreational monitoring protocols.

Farm animals and humans are the hypothesised sources of rainfall-induced faecal contamination for the Waikouaiti River and estuary, however to the best of our knowledge this has not yet been assessed by molecular techniques. Potential sources of contamination identified by 16S rRNA sequencing were: cow, gull,
human, and pig. Sequencing also revealed potentially pathogenic species associated with high measurements of *E. coli*, but not all are necessarily of faecal origin or induced by rainfall.

The identification of contaminant sources is hugely beneficial for mitigation management, while the identification of contamination due to human faeces is particularly important for measuring food safety in shellfish harvesters. MST assays are commonly employed to identify faecal contamination and distinguishing sources. The identified genera, *Prevotella* and *Bacteroides* are currently used as broad-spectrum markers for faecal contamination. Available MST assays using *Enterococci* can be used for distinguishing human sources via the *esp* gene. MST is still a developing technology with the exploration of new assays utilising different genera and the development of new markers. Contaminant source is of great interest to water quality researchers, and thus the identification of genera found in this thesis could be used to re-assess the Waikouaiti Estuary in the future. The continual development of MST assays will also guarantee appropriate markers will arise.

**4.2 Other concerns regarding shellfish safety for consumption**

This study investigated the quality of the Waikouaiti estuary shellfish harvesting beds via the measuring of faecal indicator organism contamination in Chapter Two. Sequencing was also employed to identify other potentially pathogenic bacteria in Chapter Three. However, there are many other types of biological pathogens that can be cause for concern for shellfish health and safety, these include viruses and toxic algae.

Viral foodborne outbreaks in human consumers are considered to be more commonly associated with outbreaks of gastroenteritis than any other pathogen (Lees et al. 2010), with noroviruses accounting for up to 83.7 % of shellfish outbreaks (Bellou et al. 2013). New Zealand is ranked the fourth highest in global norovirus outbreaks associated with seafood, with outbreaks representing 2.7 % of all gastroenteritis cases; in comparison to Australia this is only 1.1 % and Japan represents over half of the outbreaks with 63.6 % (Bellou et al. 2013).
Viral vectors are not represented by measuring microbial FIOs, and are often present in shellfish that are compliant with legal requirements of <230 E. coli per 100 g (Lees et al. 2010). Of further issue is the fact that viruses are more difficult to remove from the bivalve using depuration methods (Johnson et al. 1990; Schwab et al. 1998), and are responsible for the majority of outbreaks associated with depurated shellfish (Lees et al. 2010). Areas that are contaminated by sewage are at greater risk because of the heavy association with viruses such as norovirus (Lodder & Husman 2005). Considering that viruses are among the top leading causes of illness following consumption of shellfish, they should be considered in the context of determining the safety of shellfish for harvesting in the future.

4.3 Source tracking
Molecular microbial analyses are a promising tool for water quality protocols. These can dually identify faecal contamination sources and pathogen presence, therefore allowing for more informative monitoring programs with multidisciplinary tools (Malham et al. 2014). A current research project is being undertaken in Rapaki (Lyttelton Harbour) to assess the safety levels of faecal contamination and identify faecal sources using the ESR toolbox (pers. comm. Lesley Bolton-Ritchie, Environment Canterbury Regional Council). A National review by MPI is currently evaluating the potential inclusion of source tracking technologies to water quality assessments (pers. comm. Chris Tomlinson, Senior Advisor, MPI). These molecular methods could be considered beneficial if applied to the bacteria accumulated within shellfish. Obtaining genetic material from homogenised shellfish can be problematic in that is can often contain inhibitors that can interfere with genomic amplification. A way around this could be to enhance the bacteria by pre-culturing on a selective media such as CM1046, This media which was shown to enrich for both pathogenic genera associated with shellfish outbreaks and genera of faecal bacteria in Chapter Three.

4.4 Considerations for local management
The research presented here aimed to identify these at risks in regards to rainfall, but this was not shown to associate with guaranteed faecal contamination in the water, and to an even lesser degree in the shellfish. This result reflects that although rainfall may introduce faecal contamination the simultaneous decrease
in salinity may prevent the shellfish from regulating feeding. This could indicate that the guidelines for avoiding harvesting after a large rainfall may be misleading, and that it could be smaller rainfall events with smaller changes in salinity that allow for contamination issues in shellfish.

Management of water quality has concentrated on the input of faecal contaminants from diffuse sources such as farmland by inhibiting entry of contaminants into the water in the first place (Chadwick et al. 2008; Kay 2008b). Such concepts for reducing agriculture contamination in waterways include: reducing total farm stock, planting of riparian zones, and removing stock from runoff prone areas during periods of rainfall (Davis-Colley 2013; Collins 2007). The research by Collins et al. (2005) established a large-scale rainfall simulator on a grazed hillside; the author identified that the timing of the last grazing period in relation to rainfall was a significant predictor of \( E. \text{coli} \) abundance in an adjacent stream. It may not be feasible to assess contamination risk by monitoring grazing throughout a whole catchment; an alternative could be to encourage the reduction in animal grazing adjacent to waterways during rainfall (Collins et al. 2005).

4.5 Future research

There were too few exceedances of \( E. \text{coli} \) in the shellfish samples during the monitoring creating difficulty for identifying statistical relationships with river flow, and \( E. \text{coli} \) abundances in water. Solutions to this issue include extending the sampling period, and employ a more sensitive method for enumerating \( E. \text{coli} \) (i.e., the MPN method) to obtain more data points for the lower accumulation limits. However the plate method used should not be rendered impractical, as it could be a suitable method for measuring faecal contamination in areas where higher abundances of contamination are common. Secondly the result time of this method is reduced to 24 hours as oppose to the 48 hours required for the MPN method.

Another objective of this research was to identify a safe harvesting time after rainfall-induced contamination. This is dependent on the survival of faecal pathogens in the water and the time it takes to accumulate and clear from the shellfish (Campos et al. 2013). A series of lab experiments were optimised to
investigate these factors for application to field observations, but are not presented in this thesis due to extreme rainfall restricting lab access and affecting the health of the collected animals. In these experiments, the *E. coli* strain (ATCC 25922) was used to inoculate locally harvested cockles, *A. stutchburyi*, before placing the animals into a flume for depuration. Temporal sampling to determine the clearance rate of *E. coli* was carried out during the expected low tide in order to mimic the period of harvesting in the field and identify a safe harvesting time after the inoculation. These experiments should be carried out in future research, and should also include other shellfish that are harvested in the Waikouaiti estuary, such as *pipi* (*Paphies australis*), *tuatua* (*Paphies subtriangulata*) and mussels (*Perna canaliculus* and *Mytilus edulis*).

### 4.5a Site monitoring

While water and river flow showed to be good parameters for predicting FIO presence in the water, this was not the case for the shellfish. Aside from the lack of statistical quality, this suggests that there could potentially be other factors that are impeding the ability for the shellfish to accumulate contamination. Two major parameters associated with rainfall that disturb filter feeding could be salinity and an increase in sediment loading which can down-regulate filter feeding (Ellis et al. 2002; Jorgensen 1996). Salinity measurements are recommended for future monitoring.

There are numerous cockle beds located throughout the Waikouaiti estuary. Those that were sampled in this study were selected because they are the most popular for recreational shellfish harvesting due to the size of the cockles and the accessibility (adjacent to the carpark). Future studies could include other beds to assess if there is a variation in risk. Unpublished reports on water quality have shown that there may be other contributing water bodies that contain a high concentration of *E. coli*, such as the other arms of the estuary (Chand et al. 2015). It would therefore be valuable to sample cockle beds that are further upstream and adjacent to the different estuary arms where the contamination concentrations could potentially be higher.
To summarise this discussion section, future investigation of water quality assessments of the shellfish in the Waikouaiti estuary should include the following features:

- Identification of contaminant source in water samples collected throughout the river and estuary
- Extend the shellfish survey to include other beds in the estuary
- Measure salinity alongside faecal contaminants in the water
- Include other microbial pathogens such as viruses and toxic algae
4.6 Thesis Conclusions

In terms of answering the question: ‘is there faecal contamination in the estuary’, the data shown in Chapter Two indicates that the levels of faecal contamination do not exceed commercial and recreational standards for water quality. However, the shellfish tissue did not reflect safe levels on several occasions. Hydrological parameters (i.e. river flow and rainfall) correlated with microbial abundances in the water but not for the shellfish. This is an important point to take into consideration for future assessments of shellfish contamination, and the fact that the absence of rain resulted in contamination exceedances in the shellfish. The results found in this thesis indicate that cow, gull, human, and pig, should be further investigated as potential sources for faecal contamination in the Waikouaiti estuary.

Interestingly, contrary to the results in this study, farmed animals are the suggested source of faecal contamination in the Waikouaiti catchment by local tangata whenua and the ORC (pers. comm. Brendan Flack, Chair, East Otago Taiāpūre committee). Despite the importance of sheep farming in the catchment, there was no evidence of faecal contamination from this source using the microbiological marker Ruminococcus. The absence of this marker from samples containing high levels of E. coli suggest that either sheep are not within proximity to contribute substantial contamination, or the marker does not work at all in this setting at all. More work is needed to confirm the absence of sheep farming and the use of this marker as MST marker for ruminants this catchment.

Current FIO enumeration methods for assessments of water quality fail in their ability to identify sources of contamination, which is essential for mitigation efforts. A transition to more informative techniques such as MST methods would allow for effective identification of sources. Another advantage of using these techniques in shellfish harvesting areas is that there could be a simultaneously screening for foodborne pathogens for health and safety assessments. A method that utilises the membrane filtration plate counts with MST tools is suggested, where by source identification is carried out on resulting membrane filters when high plate counts for particular samples are received. Further development of
current water quality methods is important in informing effective and efficient kaitiakitanga, and effective contamination interventions from local bodies.
Supplementary figure 1: Correlation between total coliforms and *E. coli* abundances in the water samples. Abundances of total coliforms and *E. coli* where combined for sampling periods that contained data for both the cockle and mussel sites (A). For each point n = 1 to 6, error bars indicate SEM (± 1). The two groups were also tested for correlation using a non-parametric one-tailed Spearman’s correlation (B) using raw data from (A), n = 82, ****P<0.001
Supplementary figure 2: Correlation of *E. coli* abundances between the mussel and cockle site for the water. The bar graph represents mean *E. coli* abundances (n = 1 to 3, SEM ± 1) for both sites (A). This data was then used for a Spearman’s correlation (B) to detect if the relationship is significant (n = 8, *P*<0.05).
Supplementary figure 3: Ecological distances of HEC and LEC samples 
**based on high and low river flow.** An NMDS plot based on the Bray-Curtis 
 distancing (A) was used to visualise dissimilarity distances. Each point is 
 coloured depending on the amount of *E. coli* (indicated by colour gradient) and 
 indicating high (circle) and low river flow (triangle). A Shepherd plot (B) with 
 monotonic regression ($R^2 = 1$).
Supplementary table 1: Correlations between rainfall and microbial abundances in water. *E. coli* and total coliform data from the cockle and mussel sites were combined and used to test their relationship against 3 day and weekly rainfall totals. Rainfall data collected from both Palmerston and Te Taumata-o-Puaka (TTOP) was tested using a Spearman’s correlation (one-tailed) for both microbial types. For *E. coli*, n = 16 and total coliforms n = 15, *P<0.05 & **P<0.01.

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<td></td>
<td>r</td>
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<td>0.002**</td>
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Supplementary table 2: Table of statistics for filter sizes 0.22 and 0.45 µm used in section 3.4.2a (ANOVA).

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