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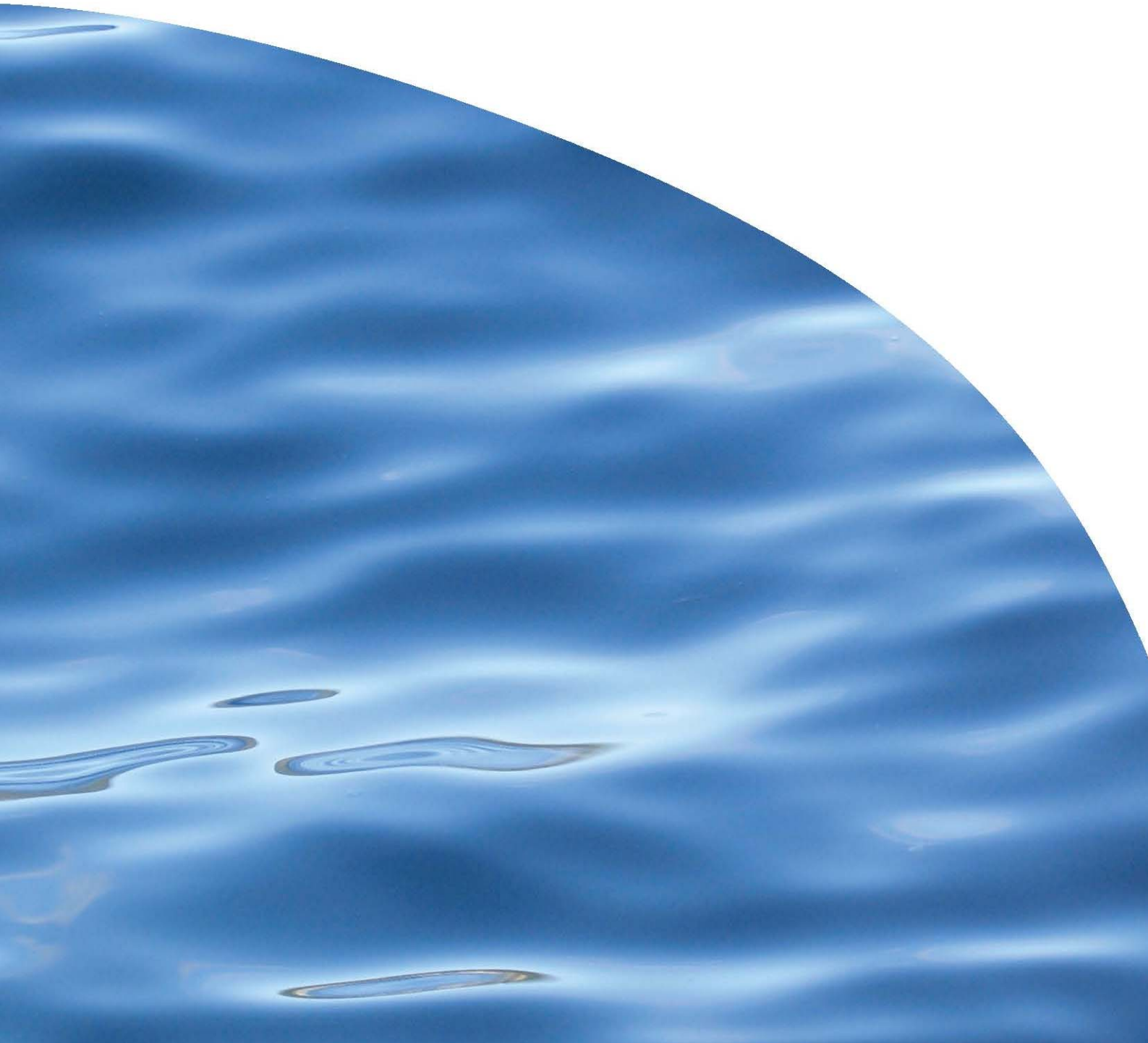
MICROBIAL SOURCE TRACKING (MST) TOOLS FOR WATER QUALITY MONITORING



MICROBIAL SOURCE TRACKING (MST) TOOLS FOR WATER QUALITY MONITORING

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EXECUTIVE SUMMARY

Monitoring of faecal contamination in New Zealand estuaries and coastal waters for the purpose of state of the environment and water quality monitoring programmes is currently limited to periodic measurement of faecal indicator bacteria (FIB) (*e.g.* enterococci) concentrations, which do not provide information on sources of contamination. The primary aim of this project, funded through the Ministry for Science and Innovation Envirolink scheme, was to evaluate the use of quantitative PCR (qPCR)-based microbial source tracking (MST) markers within Regional Council monitoring programmes for identifying the presence and relative contributions of human and ruminant sources of faecal pollution in coastal waterways.

The project involved four steps; (1) selection of existing MST markers most likely to augment water quality monitoring in New Zealand, (2) validation of a range of existing qPCR markers for use in New Zealand, (3) blind tests for evaluating reproducibility of results between two separate laboratories (Cawthron and Environmental Science and Research (ESR)), and (4) a field trial for evaluating the use of the qPCR markers within coastal water quality monitoring programmes across nine regions.

Four bacterial markers targeting the Order Bacteroidales were selected for the project based on their reported abundance and high host-specificity. The four markers included a general Universal Bacteroidales marker (UBac) that serves as an overall measure of faecal contamination but is not host-specific, a Bacteroidales marker specific to humans (HBac), a Bacteroidales marker associated with ruminant animals (RBac) such as sheep and cows, and a bovine Bacteroidales marker (BBac) specific to cows.

Based on the pooled data of both institutes, the universal and ruminant markers are highly sensitive, as they were detected in 97% and 96% of host populations, respectively. The human and bovine markers were less sensitive and were present in 62% and 72% of host population samples. Markers were strongly associated with their target organisms; specificity of human, ruminant and bovine markers was 88%, 81% and 97 % respectively. Some cross-reactivity between markers and non-target organisms, such as possums and rabbits, was present for the human and ruminant markers; however, the probability of contamination from these organisms causing a ‘false positive’ is likely very low in coastal waterways.

Blind tests carried out on samples seeded with varying amounts of municipal wastewater treatment plant influent (human source), and dairy shed slurries (ruminant source) indicated good agreement between estimates derived separately by Cawthron and ESR. Outputs from the blind tests also demonstrated a significant correlation between expected marker concentrations based on the seeded material and detected by the qPCR in the seeded samples. Despite the use of different qPCR instruments and analysis by different scientists, the blind test results demonstrate that the MST analyses can be standardised among laboratories to ensure comparable results across studies and monitoring programmes.

In conjunction with water quality monitoring programmes for nine Regional Councils around New Zealand, a field trial involving collection of water samples on four separate occasions at 53 water quality monitoring sites was carried out between February and May 2011. A total of 206 samples was collected and analysed as part of the field trial. The UBac marker was detected in all but one sample and there was a relatively poor correlation based on the pooled dataset between concentrations of the UBac marker and those of enterococci measured from the same water samples. The poor correlation was most likely due to the coarse spatial and temporal resolution of sampling for the trial and differences in environmental conditions among sampling sites which, in turn, could influence transport and fate characteristics of the two different indicators.

Of the host-specific markers chosen for validation and inclusion in the field trial, the ruminant RBac marker shows the greatest promise as a tool for informing water quality monitoring programmes. The RBac marker was detected in approximately half (51%) of the water samples and at 79% of the sampling sites, indicating pastoral farming is a major driver of faecal contamination in many of the areas sampled. The more specific bovine BBac marker was far less prevalent than the RBac marker and was detected in only 4% of the water samples and at 13% of the sites. Despite its low sensitivity and abundance, the BBac marker is highly specific and would be useful in situations where dairy and/or cattle farming are the dominant land use in upstream catchments. The human HBac marker was detected in only 6% of the samples and at five (9%) of the sampling sites; probably reflecting the efficacy of treatment of human-sourced waste water. However, despite its low prevalence, the human marker was detected on multiple occasions at a number of sites known to be exposed to human waste inputs. There was no indication of contamination source at 17% of the sites based on the suite of source-specific markers used in the trial, which may have been due to the presence of contamination from non-target sources (*e.g*. birds, dogs) or possibly the presence of aged contamination containing persistent populations of enterococci but a low abundance of MST target organisms.

To target sampling and minimise expenditure, it is advised that MST markers be implemented by Councils using a ‘decision tree’ approach. The MST markers validated and tested in this project are one of a suite of tools that can be implemented by Councils. Recommendations for future development of MST and related water quality monitoring tools include the use of multiple human-specific markers to improve the ability to detect human contamination in coastal waterways. Links between laboratories with MST capability should be further encouraged and, where possible, MST and FIB results compiled within a central database in order to evaluate the efficacy of different MST markers at a national level. Development and implementation of MST markers in New Zealand should continue to be aligned with overseas efforts aimed at improving water quality monitoring and standards, such as those being implemented by The United States Environmental Protection Agency and the European Union.

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1. INTRODUCTION

The integrity and health of coastal ecosystems and resources are intimately linked with water quality. Contamination associated with faecal pollution is considered a major threat to water quality worldwide, due largely to its risks to humans through contact recreation and consumption of contaminated shellfish. In New Zealand, poor water quality, identified by high levels of faecal bacteria, often leads to frequent closures of bathing waters and highly valued shellfish harvest areas. Diverse land uses and an associated influx of a number of anthropogenic sources of faecal contamination potentially lead to freshwater and coastal water quality problems in New Zealand (Figure 1). As a result, Councils often have difficulty in determining the contamination source(s), and the relative contribution of each source, in problem situations.

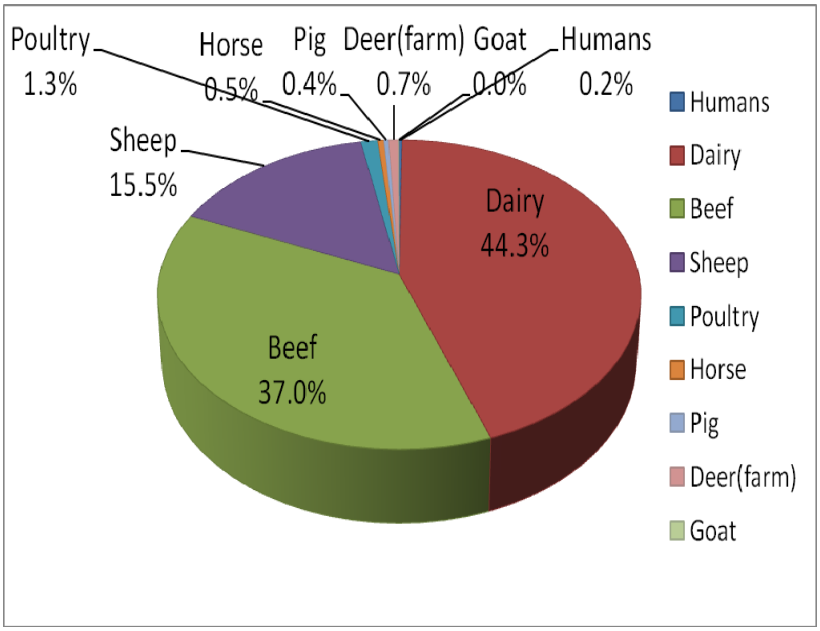


Figure 1 Estimated proportion of faecal matter released in New Zealand by humans and farmed animals.

Monitoring of water quality is a common element of regional authorities' State of the Environment programmes, which act to provide an assessment of environmental problems and illustrate where environmental management has been effective. Current microbiological water quality guidelines set by the Ministry of Health and the Ministry for the Environment, New Zealand (MfE 2003) are based on concentrations of faecal indicator bacteria (FIB; typically *Escherichia coli* in fresh waters and enterococci in marine waters). However, FIB provides no information on the source(s) of contamination, which is critical for prioritising problem areas and enabling effective management and remediation strategies. There is a need for scientifically robust,

practical and cost-effective tools to identify sources of faecal contamination of our near-shore environments that can pose risks to human health and reduce the quality of resources these environments provide.

Microbial source tracking (MST) tools provide a means of identifying contaminant sources, thereby facilitating decision-making and prioritising mitigation measures. There are numerous different types of non-biological and biological tools that can be used to assist in tracking sources of faecal contaminants. Non-biological markers include dye tracers and chemical whitening agents and biological markers include faecal sterols and host-associated microbes. The source of host-associated microbes are identified from a range of Polymerase Chain Reaction (PCR)-based markers that target host-specific (found in only one host species or group) or host-associated (largely confined to one host species or group) indicator organisms (such as viruses, bacteria, and bacteriophages). Researchers worldwide have now published more than 100 assays that could be used for microbial source tracing. A selection of these are presented in Appendix 1

The primary aim of this project, funded through the Ministry for Science and Innovation Envirolink scheme, was to evaluate the usefulness of Quantitative PCR (qPCR)-based MST markers within Regional Council monitoring programmes for identifying the presence and relative contributions of human and ruminant sources of faecal pollution in New Zealand. Outcomes are intended to assist Councils with identifying key problem areas and the sources of faecal contaminants contributing to environmental degradation, which in turn will assist in prioritising and implementing appropriate management actions. National benefits from the project include a country-wide validation of promising qPCR-based MST markers to identify the source, and quantify the relative contribution, of key contamination sources in water samples.

1.1. Approach

The approach used builds on previous and current international research efforts in microbial source tracking. Many of the techniques proposed have already proven their potential in a research context; however, their application in wider monitoring programmes has yet to be realised. The project involved four main steps required to make this transition:

1) Selection of existing MST markers most likely to augment water quality monitoring in New Zealand;

2) Validation of the selected markers against a library of faecal samples representing a range of animals commonly found in New Zealand catchments;

3) Blind tests for evaluating reproducibility of results between two separate laboratories (Cawthron and ESR);

4) A field trial for evaluating the use of the MST markers in coastal and freshwater water quality monitoring programmes across nine Regional Councils.

We can only place confidence in markers that have been investigated for their distribution among hosts present in New Zealand and tested for their sensitivity (likelihood of being detected). Robust validation of markers is required to ensure markers developed overseas are useful in the New Zealand context, *i.e*. that there is limited cross-reactivity between organisms from different hosts. When used for monitoring purposes, there is a need to ensure consistency and to standardise the approach across laboratories that may carry out the analysis. In order to test consistency across laboratories, a blind trial was conducted by Cawthron and ESR scientists. A field trial involving nine Regional Councils was then conducted in order to assess the use of MST tools within routine water quality monitoring programmes.

2. MARKER SELECTION

There are numerous gut bacteria and viruses that can be targeted using MST markers and more than 90 markers have been published to date. Following a review of available bacterial markers we chose to target bacterial species from the Order Bacteroidales, which are reported to be very abundant within the gastrointestinal tracts of warm-blooded animals and can be highly host-specific (Seurinck *et al.* 2005; Mieszkin *et al.* 2010). Representatives of Bacteroidales have been suggested as alternative indicators to faecal indicator bacteria such as *E. coli* since the 1960s (Post *et al.* 1967). Concentrations of Bacteroidales bacteria greatly exceed those of *E. coli* and, more importantly, members of this Order are obligate anaerobes so it is highly unlikely they would find suitable conditions to replicate in the environment. The prevalence of Bacteroidales markers within a water sample can, therefore, provide some indication as to whether contamination is associated with fresh inputs, or alternatively, due to persistent faecal bacteria populations (*e.g. E. coli* is known to persist in the environment once released from a host).

To date, the use of Bacteroidales as an alternative faecal indicator has been hampered by the complicated conditions necessary to cultivate obligate anaerobes. With the advancement of molecular technology, several novel rapid assays have been developed for Bacteroidales markers (Dick & Field, 2004, Converse *et al.* 2009) and the potential of this group as an alternative health risk indicator is being considered for large overseas programmes (*e.g.* recent investigations by the United States Environmental Protection Agency toward refining bathing water quality guidelines).

Based on a review of available Bacteroidales markers and their performance in previous studies, we selected four qPCR-based MST markers for inclusion in the project (see information box below and Table 1). The four markers included a general Bacteroidales marker that serves as an overall measure of faecal contamination but is not host-specific. General Bacteroidales markers used alongside faecal indicators and host-specific markers provide valuable information on the extent and timing (recentness) of contamination events. However, it is acknowledged that some cross-reactivity with environmental material has been indicated in recent literature (Van der Wielen & Medema, 2010). We then selected a Bacteroidales marker specific to humans, since identifying faecal contamination associated with humans is a priority with regard to bathing water quality and health risks (Kinzelmann *et al*. 2011). In addition, we selected a ruminant marker due to the likely important contribution of ruminant sources to diffuse contamination problems in rivers and downstream coastal waters. Several ruminant sources exist in New Zealand catchments; hence, we also trialled a marker specific to Bovines in order to evaluate the potential importance of this specific ruminant source during contamination events.

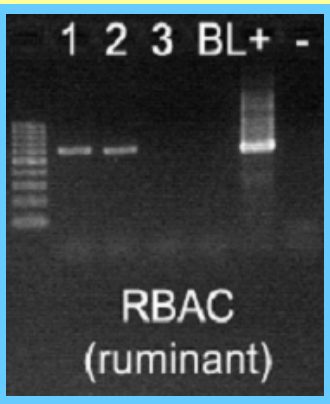
In addition to the general and host-specific markers, we utilised an internal amplification control (IAC) assay to signal any PCR inhibition during analyses. This effectively ensures validity of results and accounts for any inhibition that may be occurring due to other co-occurring compounds (*e.g.* humic acids) within samples. The assay chosen targets a gene within chum salmon, *Oncorhynchus keta* (Haugland *et al.* 2005). This assay was run simultaneously in separate reactions. If any inhibition was detected, samples were diluted to reduce the concentration of inhibiting compounds and re-analysed.

Table 1 Quantitative PCR markers evaluated and/or applied in this study.

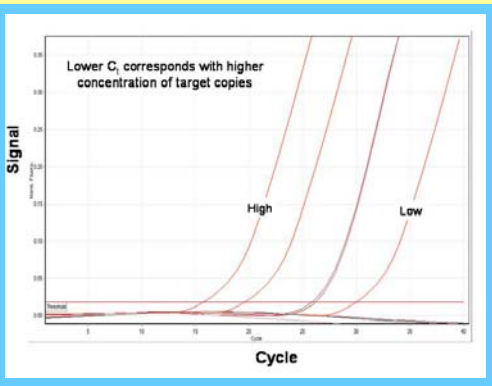
Marker	**Abbreviation**	**Source**	**Reference**
Universal Bacteroidales	UBac	General	(Siefring *et al.* 2008)
Human Bacteroidales	HBac	Human	(Shanks *et al.* 2009)
Ruminant Bacteroidales	RBac	Ruminant	(Reischer *et al*. 2006)
Bovine Bacteroidales	BBac	Bovine	(Shanks *et al.* 2008)

What is Quantitative PCR?

MST markers based on traditional **P**olymerase **C**hain **R**eaction were (and to some extent still are) based on target assays run on an agarose gel, with the "end-point" of the reaction used to assess either the presence or absence of the marker within a sample (see left image). Traditional PCR has advanced from detection at the end-point of the reaction to detection while the reaction is occurring. qPCR detects the accumulation of DNA amplification in "real-time" as the reaction begins and while it is occurring, rather than just at the end-point of the reaction; hence a quantitative measure of the number of copies within a reaction can be obtained by comparing the cycle threshold (C_t) at which the signal/reaction begins (see right image) with the C_t of a suite of calibrated standards.



A ruminant marker present in a positive control (+) and two of three water samples using end-point PCR. BL = a control reaction containing no template.



qPCR output showing the amplification signal of standards prepared with varying amounts of human Bacteroidales markers. The earlier the signal begins, the more abundant the marker is within a sample.

3. MARKER SETUP AND VALIDATION

Prior to validating markers, both Cawthron and ESR developed the qPCR methodologies and ensured high method performance (i.e. expected slope of standard curves) by analysing a number of samples over a range of known marker concentrations. Development also included the testing of negative and positive control samples. Standard solutions were produced from plasmids containing the target genes and concentrations of the indicator organisms in each sample were calculated from the qPCR threshold cycle (Ct) (Figure 2). As part of this process, the following limits of detection for markers were established: <100 gene copies for the bovine marker and <10 gene copies for the other three markers.

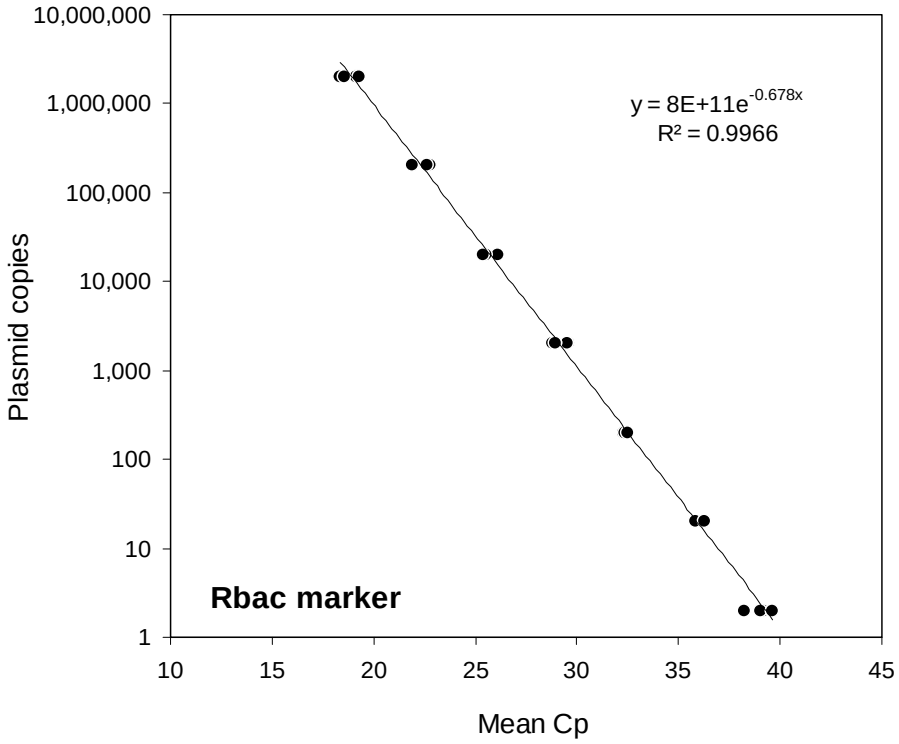


Figure 2 Example of standard calibration curve for the RBac marker. The equation is used to then estimate the number of marker genes within a sample.

When validating MST markers, measures of a marker's *sensitivity* and *specificity* are required in order to establish how commonly a marker is found with a given host species or group and the extent to which a marker is specific to a host, respectively. Both attributes are typically shown as a percentage. For example, if eight out of 10 human faecal samples test positive for the HBac marker, then the sensitivity of the marker is 80%. When nine out of 10 other types of animals do not carry the HBac marker, then the specificity of the marker is 90%. Ideal markers are both highly sensitive and highly specific, which coincides with a greater likelihood of their detection in the environment and reduces the likelihood of a false positive, respectively. Rarely are markers totally specific to a host species. The Bacteroidales markers chosen for this project have high host-specificity, but are known to have limited cross-reactivity with non-target organisms. Validation against a range of organisms in catchments across the geographical range of New Zealand is, therefore, required to understand potential limitations of the markers used.

Robust validation requires screening the markers against as many faecal samples from representative host species and groups as possible. Faecal samples evaluated included those from individuals, and composite samples such as WWTP influent and dairy shed slurries. With the assistance of Regional Councils, a total of 115 faecal samples from 21 different species were collected (Figure 3). Extraction of DNA from faecal samples was carried out by Cawthron, and replicate samples were sent to ESR for validation by both laboratories. In addition, ESR carried out a validation of the markers against an existing faecal library of 100 samples to the 115 collected as part of this project; hence the markers were validated with a total of 215 faecal samples representing 13 groups of organisms (Figure 4). The 100 samples in the ESR library included 16 human samples and four sewage effluent samples,

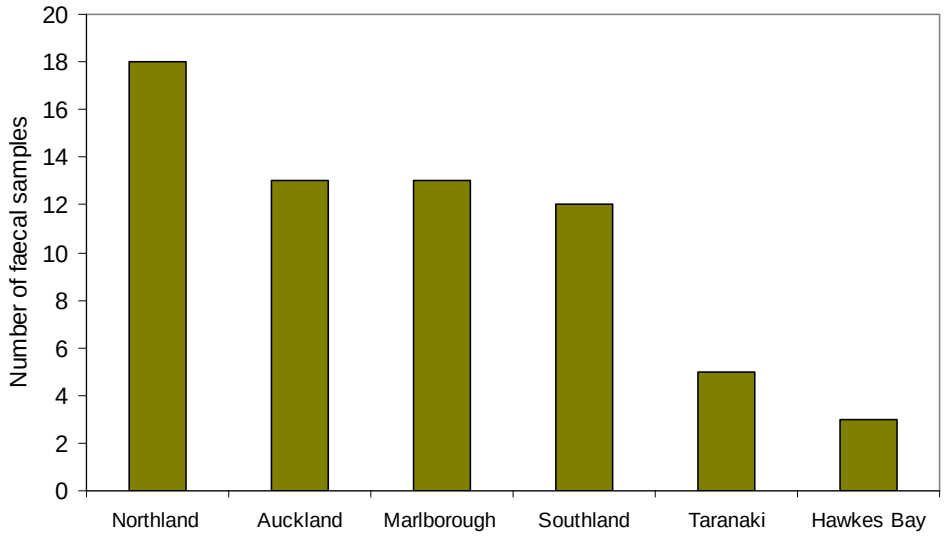


Figure 3 Number of faecal samples collected by Regional Councils.

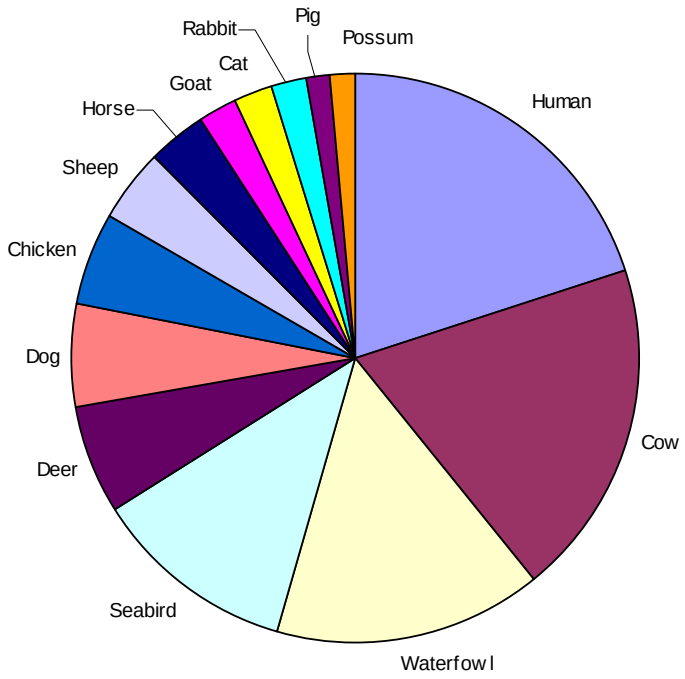


Figure 4 Proportion of faecal samples (n=215) collected for the tools project represented by groups of organisms. Waterfowl included ducks, geese, black swans and pukekos. Seabirds included seagulls and an oyster catcher. Human samples included both discrete samples and composite samples (WWTP influent).

3.1. Validation results

Based on the pooled data of both institutes, the universal Bacteroidales (UBac) marker was detected in 97% of the samples, indicating that this marker is highly sensitive and a good general indicator of faecal contamination. The ruminant (RBac) marker was also highly sensitive (96%). The human (HBac) marker had lower sensitivity, and was detected in 62% and 71% of the human faecal and WWTP samples, respectively (Table 2). The bovine (BBac) marker also has relatively low sensitivity and was detected in 72% of the samples.

The specificity of the HBac assay was 88%, with cross-reactivity primarily associated with possum and rabbit samples. Limited cross-reactivity was also detected in a few samples isolated from dogs, pigs, horses and seagulls. With the exception of possums, marker concentrations in non-target samples were low and would most likely lie below detectable limits in any real-world situations following dilution in the environment. The RBac marker was strongly associated with target organisms with a specificity of 81%; however, there was weak amplification in some samples from non-target organisms such as possums and cats (Table 2). The bovine marker is highly specific; however, the low sensitivity of the assay and the low concentration of the marker within faecal samples likely limit its usefulness to highly contaminated waterways where bovine sources are dominant.

Table 2 Validation results including marker specificity and sensitivity based on the combined dataset (n=215 for each assay)

Assay	Detects	Weakly amplifies	Negative for	Sensitivity	Specificity
General (UBac)	Human, cow, sheep, deer, goat, possum, cat, dog, pig, rabbit, horse, chicken, duck, black swan	Pukeko, seagull	None	97%	NA
Ruminant (Rbac)	Cow, sheep, deer, goat	Possum, cat	Dog, pig, horse, wildfowl, human	96%	81%
Bovine (BBac)	Cow, deer		Possum, cat, sheep, goat dog, pig, horse, wildfowl, human	72%	97%
Human (HBac)	Human and possum	Goat, rabbit	Cow, sheep, deer, dog, pig, horse, wildfowl	62 | 71%[1]	88%

[1] Individual human samples | WWTP sample (composite)

4. BLIND TRIAL

Blind tests carried out on samples prepared in fresh and seawater and seeded with varying amounts of municipal WWTP influent (human source) and dairy shed slurries (ruminant source), indicated good agreement between concentration estimates derived by both institutes (Figure 5). Differences in absolute copy numbers varied between laboratories, which are expected to some extent due to differences in qPCR instruments, cycle thresholds and software calculations. Nonetheless, the relationships between Cawthron and ESR results were linear and, more importantly, the relative differences (ratios) between source-specific markers and the UBac marker were comparable.

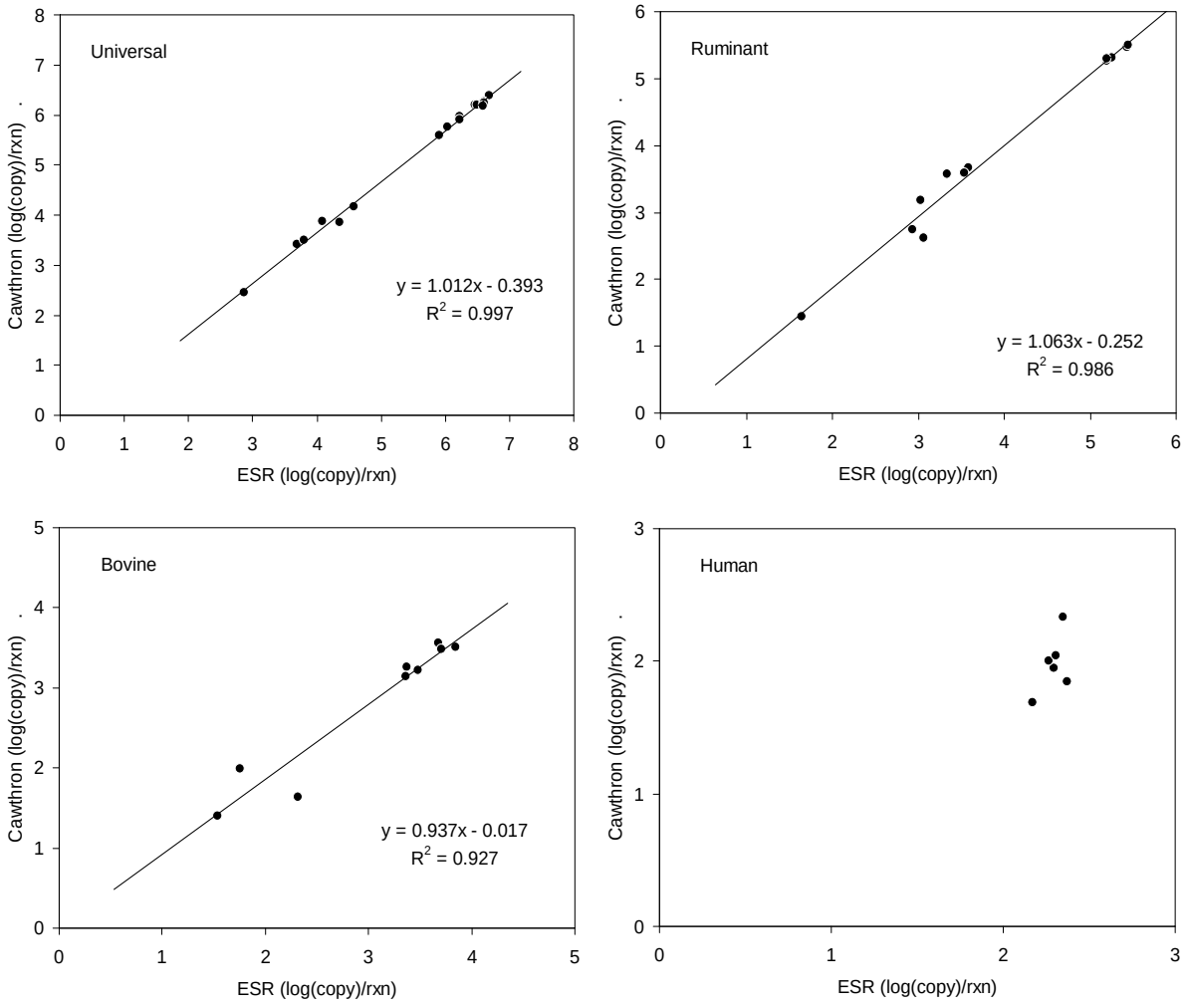


Figure 5 Marker concentrations (copies per reaction) for blind samples analysed separately at Cawthron and ESR.

Results from the blind tests also found a significant correlation between expected marker concentrations based on the seeded material and that detected by the qPCR in the seeded samples (Figure 6). Both laboratories also demonstrated high extraction efficiencies for standards and minimal inhibition, as verified through the use of the internal amplification control assay. Marker performance was the same for samples prepared in freshwater and seawater. Despite the use of different qPCR instruments and analysis by different scientists, the results are in excellent agreement and demonstrate that these tests can be standardised among laboratories to ensure comparable results across studies and monitoring programmes.

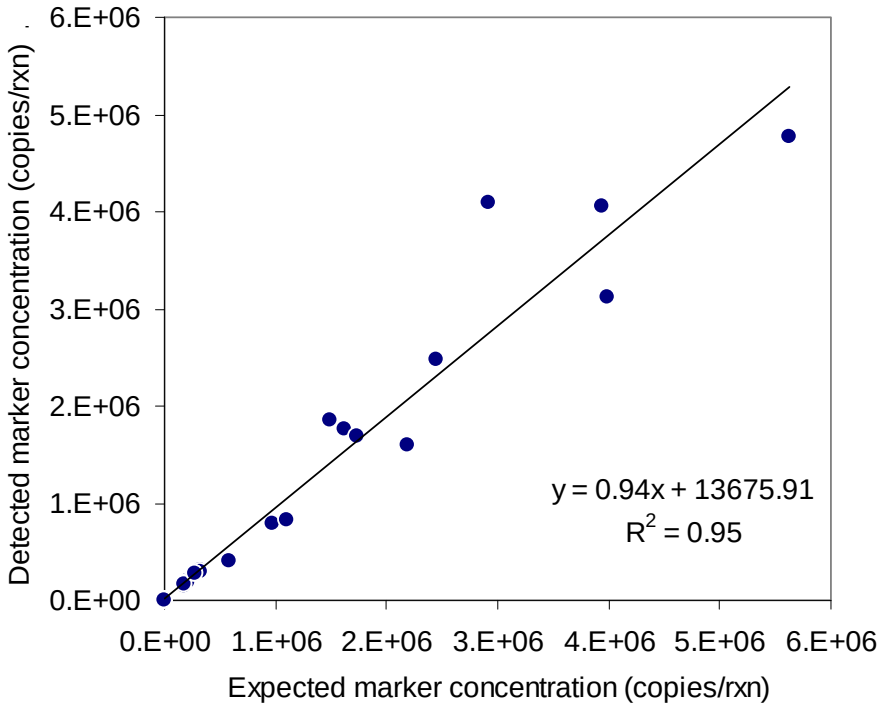


Figure 6 Expected versus detected marker concentrations (as copies per reaction) in seeded water samples (data from both Cawthron and ESR are pooled, n = 45).

5. FIELD TRIAL

In conjunction with water quality monitoring programmes for nine Regional Councils around New Zealand, a field trial involving collection of water samples on one to five separate occasions at 53 monitoring sites was carried out between February and May 2011. Sites selected were generally those identified by Councils as having a high likelihood of contamination, based on previous surveys. The number of samples was split evenly according to Councils and samples were processed and analysed for enterococci concentrations and PCR markers by Cawthron Institute and ESR. Each 500 ml sample was sub-sampled (100 ml) for determination enterococci concentration

(Most Probable Number (MPN)/100 ml) using Enterolert™ (IDEXX Laboratories, Westbrook, ME, USA). Approximately 100 to 200 ml was filtered through a 0.45 µm pore size membrane filter, which was then processed and analysed according to previously-published methods (Table 1).

In most cases, samples for the trial were collected concurrently with routine water quality monitoring samples, which were analysed separately by Councils, as required, for monitoring faecal indicator bacteria (FIB) concentrations (*E. coli* and/or enterococci) and other water quality parameters. Conditions during sampling, including information on rainfall and ancillary data (*e.g.* salinity, turbidity, water temperature) were provided, if available.

5.1. General indicators of faecal contamination

A total of 206 samples were collected and analysed as part of the field trial. The conditions during the first few months of sampling were dry for most of New Zealand. One sampling event across most sites coincided with rainfall and subsequent elevations in faecal indicator bacteria. The range of FIB (enterococci) concentrations varied between near detection limits (<10 MPN/100 ml) to either moderately elevated (100 - 1000 MPN/100 ml) or highly elevated levels (2000 to > 24,000 MPN/100 ml). Only at a few sites, including those with continuous inputs (*e.g.* sites associated with the Christchurch earthquake, and a few sites in Auckland), were enterococci concentrations consistently elevated (see Table 3 for a summary and Appendix 2 for complete results). These results highlight diffuse (non-point source) pollution (usually corresponding with rainfall/runoff) as an important driver of faecal contamination in New Zealand's rivers and coastal waters.

Enterococci concentrations followed a similar trend for duplicate water samples that were collected at the same time and location, but analysed separately. Concentrations were often higher for the samples analysed as part of the trial versus those analysed as part of routine monitoring and in some cases there was a 3-fold difference in values (Figure 7), which highlights the inherent variability in FIB and potential limitations of their use in assessing faecal contamination. Variation in results between laboratories may be associated with actual concentration differences between samples, differences in analysis, or perhaps differences in the duration of time between collection and sample processing.

Table 3 Summary of results for monitoring sites including the range of enterococci and the presence of markers if detected (X) on at least one sampling occasion. On many occasions at most sites, contamination was low and only the UBac marker was detected (often in low concentrations). n = number of samples. Site numbers correspond with locations in Figure 9. Detailed results are provided in Appendix 10.1.

Council	No	Site name	n	Enterococci (MPN/100 ml)	Markers UBac	RBac	BBac	HBac
Auckland	1	Armour Bay	4	<10 - 173	X	X		
	2	Bethells Lagoon	4	97 - 545	X	X		
	3	Cox's Bay	4	20 - 1935	X	X		X
	4	Fosters Bay	4	<10 - 537	X	X		
	5	French Bay	4	10 - 4611	X			
	6	Green Bay	4	<10 - 211	X	X		
	7	Karekare car park	4	145 - 1050	X	X		
	8	Karekare Lagoon	4	341 - 1309	X	X		
	9	Laingholm Beach	4	<10 - 301	X			
	10	Mahurangi town basin	4	84 - 8164	X	X	X	
	11	Piha Lagoon	4	200 - 2187	X			X
	12	Te Atatu Beach	4	<10 - 301	X			
	13	Titirangi Beach	4	10 - 161	X			
	14	Weymouth	3	<10 - 211	X	X		
	15	Wood Bay	4	<10 - 6867	X	X		
Canterbury	16	Ashburton River	5	10 - 265	X	X		
	17	Avon Heathcote Estuary	4	145 - >24,000	X	X		X
	18	Kaiapoi River	5	98 - 657	X	X		
	19	Otukaikino Creek	5	30 - 305	X	X		
	20	Pleasant Point Yacht Club	4	354 - 1008	X	X		X
	21	Tikao Bay	5	<10 - 185	X			
Hawkes Bay	22	Clive River	4	64 - 1700	X	X	X	
	23	Kairakau Lagoon	4	42 - 782	X	X		
	24	Maraetotara Lagoon	4	<10 - 306	X	X		
	25	Porangahau Estuary	4	<10 - 478	X	X		
	26	Puhokio Lagoon	4	306 - 1700	X	X		
	27	Waipatiki Lagoon	4	20 - 364	X	X		
Marlborough	28	Brown River	4	20 - 150	X	X		
	29	Moenui Beach	4	<10 - >2000	X	X		
	30	Momorangi Bay	4	10 - >2000	X			
	31	Rai River; Rai Falls	4	42 - 659	X	X	X	
Northland	32	Bay of Islands at Waitangi	4	<10 - 271	X	X		
	33	Kerikeri at Waipapa	4	<10 - 364	X	X		
	34	Wahiwaka Creek (Kaipara)	4	<10 - 1300	X	X		
	35	Whangarei; Town Basin	4	20 - >2000	X	X		
	36	Whangaroa; culvert	4	31 - 738	X	X	X	
	37	Whangaroa; west of Cape Horn	4	<10 - 697	X	X		
Southland	38	Aparima River	4	10 - 1291	X	X	X	
	39	Jacobs River Estuary	2	0 - 10	X			
	40	Monkey Island	2	10 -20	X	X		
	41	New River estuary	2	20	X	X		
	42	Oreti River	4	10 - 3076	X	X	X	
Taranaki	43	NPDC WWTP sample site	4	<10 - 782	X	X		
	44	Patea River	4	<10 - 99	X	X		
	45	Waitara River town bridge	4	10 - 406	X	X	X	
Waikato	46	Whitianga Harbour	1	<10	X			
Wellington	47	Hutt River at Silverstream Bridge	4	<10 - 164	X	X		
	48	Riversdale Lagoon	4	<10 - 53	X	X		
	49	Ruamahanga River; The Cliffs	4	<10 - 75	X	X		
Wellington (KCDC)	50	Te Horo Beach-Mangaone Stream	4	<10 – 192	X	X		
Wellington (PCC)	51	Plimmerton South Beach	4	<10 - 288	X			X
	52	Porirua Harbour Rowing Club	4	10 - 124	X			
West Coast	53	Orowaiti Lagoon Westport	4	53 - >2000	X	X		

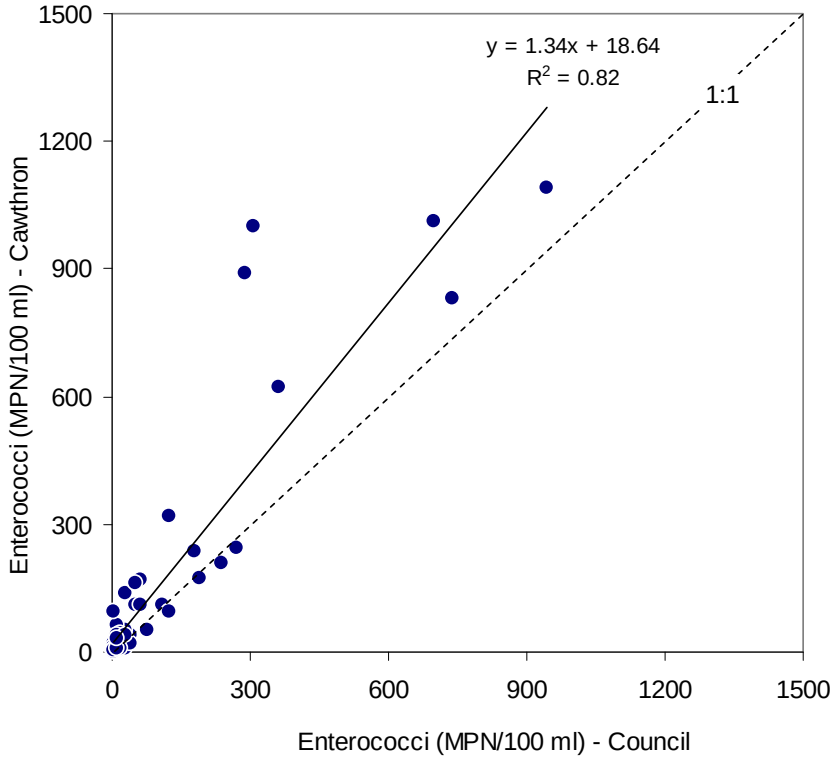


Figure 7 Comparison of enterococci concentrations based on duplicate samples (n = 56 paired samples) collected at the same location and time and analysed separately by Cawthron as part of the trial, and by Councils as part of their routine FIB analysis for water quality monitoring. Samples in which concentrations were given as a greater than value (*e.g.* > 2000 MPN/100 ml) were omitted from the comparison.

Previous studies have demonstrated strong correlations between concentrations of FIB and MST markers, including those targeting Bacteroidales (Reischer *et al*. 2008; Kinzelman *et al*. 2011). Understanding the strength of the correlation between the two is important if quantitative MST markers are to complement FIB results and/or be integrated within the current water quality regulatory framework. Based on the pooled dataset, there was a weak correlation between concentrations of enterococci and the UBac marker (Figure 8). The weak correlation is likely due to a number of factors, including: (1) analytical differences in deriving estimates of FIB versus molecular markers; (2) differences in the population dynamics during transport (*e.g.* die-off) of enterococci compared to the UBac marker that targets an obligate anaerobe (*i.e.* unable to grow and persist in an oxygenated environment); (3) differences in environmental conditions among sampling sites during sampling (*i.e.* tide stage, time of day), which in turn could also influence population dynamics during transport, and (4) the coarse spatial and temporal resolution of sampling for the trial. Studies that have demonstrated strong correlations between FIB and Bacteroidales markers have involved high frequency sampling over the course of single events (*i.e.* floods) and within the same water body (Reischer *et al*. 2006; 2007; Stapleton *et al*. 2009).

Previous studies and the results from the trial indicate that standardised thresholds for assessing water quality using quantitative MST markers (in the way that FIB are currently used) is currently not feasible on a national scale, but they could be implemented within monitoring programmes to better inform FIB monitoring results at the scale of individual catchments or water bodies.

The weak correlation between enterococci MPNs and Bacteroidales concentrations does not reduce the usefulness of MST markers for interpreting water quality data and identifying the likely sources leading to high FIB at a given site. Using both methods simultaneously can reduce the extent of false positive and false negative results from both indicators. For example, FIB such as *E. coli* and enterococci can persist for weeks in river bed sediments and beach sands and can become mobilised when these reservoirs are disturbed (Ishii *et al.* 2006; 2007; Ksoll *et al.* 2007; Brennan *et al.* 2008; Yamahara *et al.* 2007) leading to overestimation of bacterial numbers. By contrast, Bacteroidales does not persist in the environment and can thus assist in assessing the extent to which high levels of FIB may be associated with fresh inputs versus persistent populations in the environment. Additionally, monitoring both FIB MPN and Bacteroidales concentrations can reduce the extent of false positives from MST that can occur when monitoring treated effluent discharges (*i.e.* passed through treatment plant or farm effluent that has been stored) containing nonviable bioindicators.

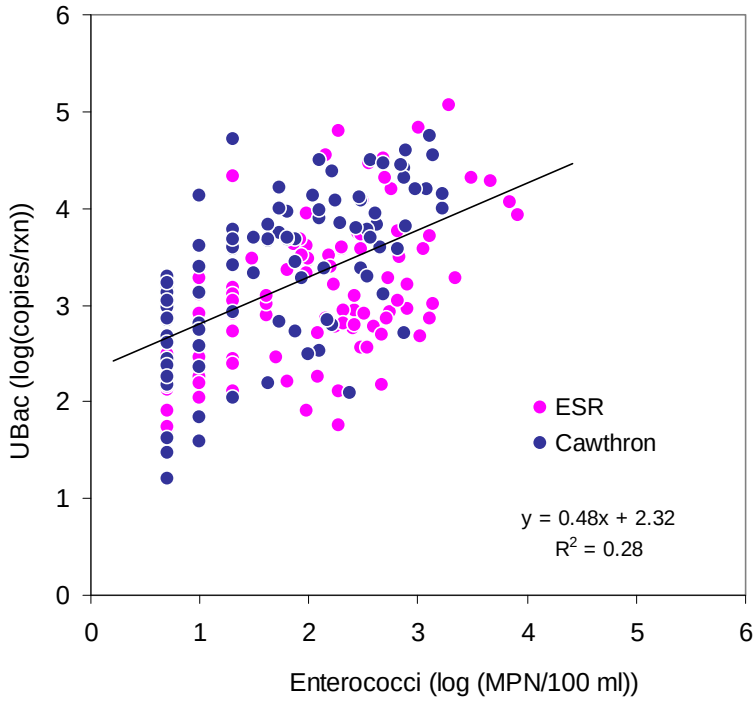


Figure 8 Concentrations of enterococci versus Universal Bacteroidales (UBac) marker. Samples at the detection limit for enterococci (< 10) were assigned a value of 5 and those in which concentrations were estimated as a greater than value (*e.g.* > 2000 MPN/100 ml) were omitted from the comparison (regression is based on the pooled dataset, n = 199).

5.2. Host-specific markers

Of the host-specific markers chosen for validation and inclusion in the field trial, the ruminant Bacteroidales (RBac) marker shows the greatest promise as a tool for informing FIB monitoring results. Due to its high sensitivity and abundance in host organisms, the RBac marker was able to be detected at relatively low levels of contamination (Table 4) and could also be used in a quantitative manner to assess the contribution of ruminants to contamination from mixed sources at a given site (Table 5). The RBac marker was detected in approximately half (51%) of the water samples and at 79% of the sampling sites (Table 3; Figure 9). This frequency of detection and the estimated concentrations of the RBac marker indicate that pastoral farming is likely the primary driver of faecal contamination in many of the areas sampled (Figure 9). Further sampling using the RBac marker alongside FIB data, and over a range of conditions and sites, will further refine the ability to use this marker in a robust, quantitative manner.

Table 4 Thresholds for reporting concentrations of universal Bacteroidales (UBac) marker in trial samples and the likelihood of detecting host-specific markers in Bacteroidales samples from several hosts.

		Likelihood of detection of host-specific markers at different Bacteroidales concentrations		
Bacteroidales Copies/reaction	Universal	Ruminant	Bovine	Human
>10,000	Very strong positive	Detectable if > 0.1% of source	Detectable if abundant	Detectable if abundant
2,000 – 10,000	Strong positive	Detectable if > 1% of source	Possibly detected if dominant source	Will see if abundant
1,000 – 2,000	Positive	Detectable if > 10% of source	Unlikely to be detected	Unlikely to be detected
200 – 1,000	Weak positive	Detectable if the major (up to 100%) source	Unlikely to be detected	Unlikely to be detected
10 - 200	Very weak positive	Unlikely to be detected	Unlikely to be detected	Unlikely to be detected
< 10	Not detected	Unlikely to be detected	Unlikely to be detected	Unlikely to be detected

Table 5 Ranges of the RBac to Ubac ratio and the relative contribution of ruminants to the estimated level of faecal contamination (UBac concentration).

Range of RBac to Ubac ratio	Reported as
10.6 – 22.1	up to 100% (Dominant source)
5.3 – 10.5	up to 50% (Major contributing source)
1.1 – 2.1	Up to 10%
0.11 – 0.22	Less than 1% ruminant
0.01 – 0.02	Less than 0.1% ruminant

The more specific bovine Bacteroidales (BBac) marker was far less prevalent than the RBac marker and was detected in only 4% of the water samples and at 13% of the sites (Table 5). Despite its low sensitivity and abundance, the BBac marker is highly specific and would be useful in situations where dairy farming and/or cattle farming is the dominant land use in upstream catchments. For instance, at some sites in Southland the presence of this marker alongside high RBac concentrations provides evidence that dairy farming is likely the major contributor to faecal contamination in the water bodies sampled (as opposed to sheep farms). The marker could also be used in a quantitative manner to monitor changes in bovine-specific inputs in response to changes to farming practices.

The human Bacteroidales (HBac) marker was detected in only 6% of the samples and at five (9%) of the sampling sites. In cases where it was detected, the concentration of human marker was usually low (close to the detection limit), which probably relates to the efficacy of sewerage treatment and low prevalence of the marker in the host, coupled with further dilution of the marker in coastal waters. This can potentially lead to false negatives (missing human contamination while it is present). Despite its low prevalence, the human marker was detected on multiple occasions at a number of sites known to be exposed to human waste inputs (*e.g.* Cox's Bay Auckland, and the Avon Heathcote Estuary in Christchurch after the earthquake series starting in 2010). Unless human sources are the major contributor to observed faecal contamination (represented by high UBac concentrations), they are unlikely to be detected in downstream river locations or coastal waters where significant dilution occurs (Table 4). The marker was not detected in many samples that were highly contaminated, indicating that human sources are unlikely a major contributor to contamination in these areas.

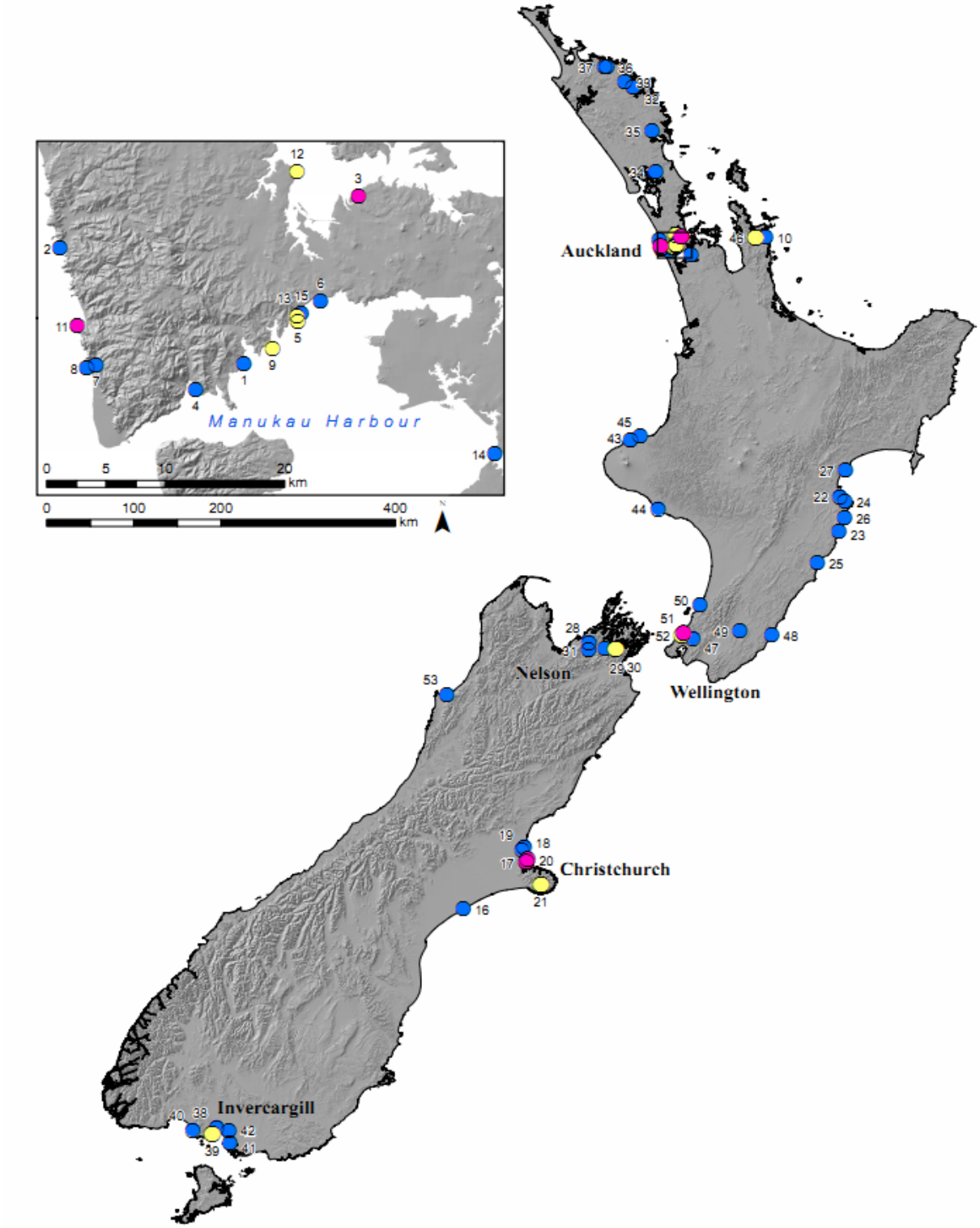


Figure 9 Sampling sites where water samples for the trial were collected by Councils. Numbers correspond with sites listed in Table 3. Colours correspond to sources (● = ruminant, ● = human) contributing to contamination at these sites based on the presence of source-specific markers. In some cases (sites marked ○), faecal indicators (*e.g.* enterococci and UBac marker) were present, but source-specific markers were not detected. See text for further explanation.

The source-specific markers used in the trial were unable to identify the contamination source at 17% of the sites. At some of these sites, faecal contamination levels were simply too low during sampling to detect source-specific markers that are in lower abundance than the general indicators (Table 3). In cases where faecal contamination was clearly present (*e.g.* French Bay in Auckland), it is likely that sources other than ruminant and human were the major contributors to contamination. For example, wildfowl, seagulls and dogs in residential areas and along beaches can represent significant sources of faecal contamination (Wright *et al.* 2009). At some sites, such as Laingholm Beach in Manukau Harbour, enterococci concentrations were elevated despite very low UBac concentrations and an absence of source-specific markers. In this case, it is possible that FIB is associated with persistent populations of FIB in the environment (*i.e.* aged contamination) or possibly treated sources in which FIB are still present, but bacteria targeted by MST markers have died off. In all these cases, further sampling under a range of conditions and possibly using additional markers is needed to confirm sources leading to elevated FIB levels.

6. IMPLEMENTATION OF MST TOOLS

The MST markers trialled and validated as part of this research provide information on the sources of contamination that was not available from FIB monitoring data. Eventually quantitative molecular techniques, such as the MST markers used here, will likely replace traditional FIB in water quality monitoring and regulation. Such a transition will require consistent evidence of the correlations between MST markers and pathogen risk, which in turn must be verified through epidemiological studies. This absence of information does not negate their current usefulness in water quality monitoring programmes, as MST markers can be used to guide land use planning and management. Further implementation of MST markers within monitoring programmes will assist in verifying the utility of current water quality standards and the development of new standards that provide a better indication of health risk, and will assist in monitoring effectiveness of changes to land-use management practices.

In order to minimise expenditure, it is advised that Councils use a ‘decision tree’ approach to applying MST techniques and identifying sites where MST markers will produce the most useful information (go to www.waterquality.org.nz for example). Examples of scenarios encountered during the field trial, and recommendations for further implementation are provided in Table 6. Based on the results from the field trial, source-specific markers are most useful when contamination levels are high. Analysis of MST markers where FIB levels frequently exceed red alert levels (*e.g.* enterococci > 280 MPN 100 ml^{-1}) are more likely to yield useful results.

One option to save on costs is to process and store samples for later MST analysis, which would be contingent on FIB results. Water samples for faecal source tracking analysis need to be analysed promptly for optimal results. Storage of a water sample at 4°C, may result in a 2-log reduction in PCR detectable markers within a week, and continuing degradation over following weeks. A water sample can be frozen, but this results in an immediate 1-log reduction in detectable markers, although the sample is then stable thereafter. Best results occur when a sample is filtered, buffer added and frozen. This is illustrated in Figure 10. Partial processing of samples gives greater flexibility in sampling design, allows samples to be batched, and reduces courier costs. Laboratories interested in validating local partial processing of samples should contact ESR or Cawthron, and we could coordinate a validation between multiple regions at the same time. Appendix 3 outlines the procedure for partial processing of samples. A second step to reducing costs is to use the general UBac marker to identify sites where source-specific markers are likely to yield useful results. For example, the human marker is most likely to be detected when UBac concentrations are above 2000 copies per reaction (Table 4).

Table 6 Example scenarios, interpretation of results and recommendations for further implementation of MST based on the field trial.

Scenario	Example Locations	Interpretation	Recommendation
High levels of UBac marker corresponding with high levels of ruminant marker, particularly following rainfall.	Sites in Northland, Hawkes Bay, Taranaki and Southland	Contamination events driven by diffuse pollution and runoff from agricultural land. Detection of bovine marker confirms that cows/cattle likely a major source.	Consider using the Universal and ruminant (and possibly bovine) markers for monitoring changes over time. Use information to enact changes in land-use practices and monitor their effectiveness.
High levels of UBac marker corresponding with presence of the human marker and an absence or very low concentration of ruminant marker.	Avon-Heathcote Estuary, Cox's Bay in Auckland	Human contamination from damaged or leaking sewerage.	Fix problem, use MST to confirm.
High levels of UBac marker indicating fresh faecal contamination, but no (or very low) signals from host-specific markers.	A number of sites in Auckland and Wellington	Contamination may be associated with sources not screened (birds, dogs, *etc*). Possible differences in transport among target organisms.	Conduct further sampling and consider use of additional markers.
High concentrations of faecal indicator bacteria (FIB) but absence or very low detection of MST markers	Beach sites in Auckland and Wellington	Elevated FIB may be associated with persistent populations of *E. coli* and/or enterococci in the environment (*e.g.* beach sands) or possibly inputs of treated sources that are high in FIB, but low in MST markers.	Identify potential reservoirs for FIB through finer-scale surveys. Conduct further MST analysis under a range of conditions to identify sources.

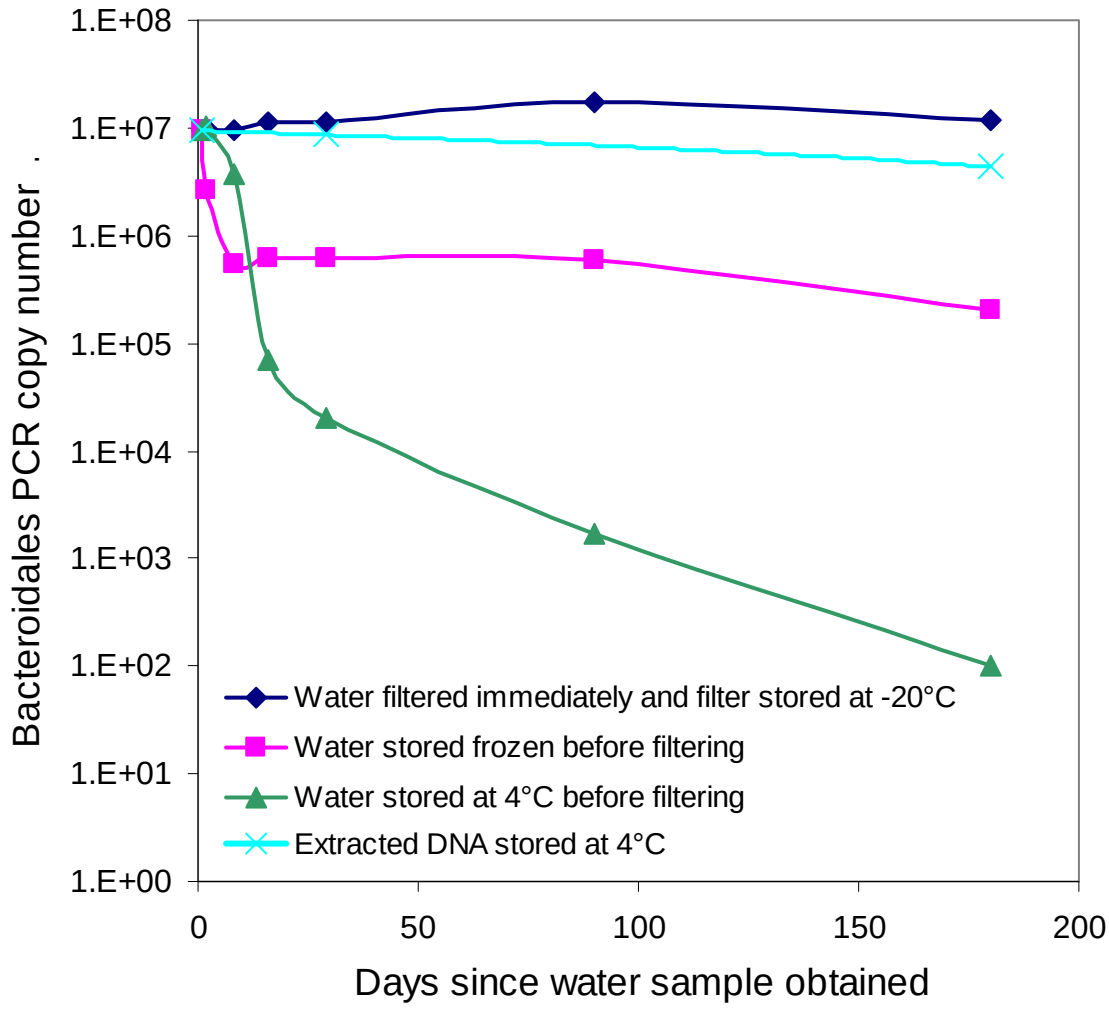


Figure 10 Effects of different processing steps on recovery of Bacteroidetes DNA from water samples spiked with human sewage.

7. FUTURE WORK

Through this Envirolink Tools Project we have developed a protocol for the validation of PCR-based assays in New Zealand and tested this protocol on four Bacteroidales assays including UBac, which is indicative of general faecal pollution (Siefring *et al.* 2008), and assays indicative of humans (HBac; Shanks *et al.,* 2009), ruminants (RBac; Reischer *et al.,* 2006) and bovine (BBac Shanks *et al.* 2008). We have learned much about the use of these assays, having obtained estimates of their specificity and sensitivity (see Table 2).

These assays need to be supported by additional assays for source targets, and also by assays for other potentially important sources in New Zealand including dogs, wildfowl, possums, sheep, horses, *etc.* In order to add assays to the MST toolbox, we need to validate them to determine their specificity and sensitivity against New Zealand sources. The strategy and the library of faecal samples put together in this Envirolink Tools project provide an opportunity to validate additional assays rapidly, so that they can be used in New Zealand with confidence.

Validation of each assay would fit within a Medium Advice Grant ($20,000). We propose the following approach for the validation of each additional marker:

- Screening the PCR marker against the library of samples collected
- Limited collection and screening of additional faecal samples as required
- Screening of a subset of previously-analysed water samples where:
  - Previous analysis indicates significant level of the Ubac marker
  - Target source is a possible source for the sample(s)
- Relating the behaviour of the marker to markers previously validated
- Making recommendations on the appropriate usage of the marker

With support from Councils we would suggest the markers in Table 7 would be useful in New Zealand and should be validated. Additional potential markers that have been published to date are provided in Appendix 1. Although we have already validated a human-associated HBac marker, it was found to have relatively low sensitivity; hence the validation of additional human markers that can be applied in New Zealand would improve confidence in the ability to detect human sources contributing to faecal contamination in coastal waterways.

Table 7 Potentially useful MST markers requiring validation.

Target	Reference
Dog	Dick *et al.* 2005
Duck	Devane *et al.* 2007
Human	Matsuki *et al.* 2004
Human	Reischer *et al.* 2007

8. PROJECT SUMMARY AND RECOMMENDATIONS

Current guidelines around water quality monitoring and compliance are based on faecal indicator bacteria (FIB), and impairments to resource use occur when conservative guidelines are breached. However, in many cases FIB data in isolation do not provide the information required to rectify pollution problems and implement Best Management Practices (BMPs) (Kinzelman *et al*. 2011). Project outcomes demonstrate that the integration of MST markers within routine water quality monitoring programmes will provide the evidence required to address pollution problems by identifying the likely sources of contamination.

Key outcomes of the project included:

- Validation and advancement of MST methods in New Zealand;
- Confirmation that MST methods can be standardised and multiple laboratories can obtain comparable results;
- Knowledge of the strengths and weaknesses of a number of MST markers and their usefulness in water quality monitoring programmes;
- Identification of pastoral farming as a primary source of faecal contamination in New Zealand coastal rivers and near-shore waters;
- Establishment of a platform on which to further expand New Zealand's Faecal Source Tracking toolbox through integration of additional markers and emerging molecular technologies.

Of the MST markers tested, the universal, ruminant and human markers are most useful in the New Zealand context. The ruminant marker is both highly specific and abundant; its application can therefore inform water quality monitoring programmes and provide a measure of the importance of ruminant sources in areas where FIB are historically high. The human marker has limitations due to its low abundance and the ability to obtain robust quantitative results; nonetheless, in combination with additional human markers, it has good potential as a means of screening for human contamination in coastal waterways. Although highly specific, the bovine specific marker was limited in abundance and would likely be most useful in focused source tracking studies or in cases where dairy and cattle farming are the primary upstream land uses.

The MST markers validated and tested in this project are one of a suite of tools that can be implemented by Councils. Based on the outcomes of the project, we recommend the following for future development of MST and related water quality monitoring tools:

- Use multiple human-specific markers to improve the ability to detect human contamination in coastal waterways. There are a number of available markers that could easily be validated against the 'faecal library' and added to the suite of available markers in New Zealand.
- Further encourage links between laboratories with MST capability and, where possible, compile MST and FIB results within a central database in order to evaluate the efficacy of different MST markers at a national level.
- Ensure any further development and implementation of MST markers is aligned with overseas efforts (U.S. EPA and EU) to improve water quality monitoring and standards.
- Integrate the MST markers developed here with other source tracking tools so that a weight-of-evidence approach can be used for water quality monitoring. In problem areas, we recommend the use of spatial sanitation surveys and finer-scale source tracking investigations to obtain the level of information required to appropriately plan and prioritise interventions to improve water quality.

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11. APPENDICES

Appendix 1 A selection of potential PCR assays for faecal source tracking

Target	Assay Name	Target Gene	Target Species	Reference
Bovine	BoBac	16s rRNA genes	Bacteroides Bovine cluster	(Layton *et al.*, 2006)
Bovine	BEV	5'UTR	Bovine enteroviruses	(Fong *et al.*, 2005a)
Bovine	Bovine adenoviruses	protease	Bovine enteroviruses	(Maluquer de Motes *et al.*, 2004)
Bovine	Bovine adenoviruses-nested PCR	protease	Bovine enteroviruses	(Maluquer de Motes *et al.*, 2004)
Bovine	Bovine adenoviruses	Hexon gene	Bovine enteroviruses	(Maluquer de Motes *et al.,* 2004)
Bovine	Bovine adenoviruses-nested PCR	Hexon gene	Bovine enteroviruses	(Maluquer de Motes *et al.*, 2004)
Bovine	BPyV(VP)	vp	Bovine polyomaviruses	(Hundesa *et al.*, 2006)
Bovine	BPyV(VP)-nested	vp	Bovine polyomaviruses	(Hundesa *et al.*, 2006)
Bovine	BPyV(agnoprotein)	agnoprotein	Bovine polyomaviruses	(Hundesa *et al.*, 2006)
Bovine	BPyV(agnoprotein)-nested	agnoprotein	Bovine polyomaviruses	(Hundesa *et al.*, 2006)
Canada goose	Mitochondrial Cytochrome b	Mitochondrial DNA (mtDNA)		(Schill & Mathes, 2008)
Cattle	Duplex Scorpion real-time PCR	16s rDNA	Bacteroides species	(Stricker *et al.*, 2008)
Chicken	Mitochondrial Cytochrome b	Mitochondrial DNA(mtDNA)		(Schill & Mathes, 2008)
Chicken	CP2-9	Metabolism	Bacteroides fragilis	(Lu *et al.*, 2007)
Chicken	CP3-49	Metabolism	C. tetani	(Lu *et al.,* 2007)
Chicken	CB-R2-42	General function gene	Desulfitobacterium hafniense	(Lu *et al.*, 2007)
Cow	Mitochondrial Cytochrome b	Mitochondrial DNA(mtDNA)		(Schill & Mathes, 2008)
Cow	Cow-Bac 1	16s rRNA gene	Bacteroides-Prevotella	(Okabe *et al.*, 2007)
Cow	Cow-Bac 2	16s rRNA gene	Bacteroides-Prevotella	(Okabe *et al.*, 2007)
Cow	Cow-Bac 3	16s rRNA gene	Bacteroides-Prevotella	(Okabe *et al.*, 2007)
Cow	BacCow-UCD	16s rRNA gene	Bacteroidales species	(Kildare *et al.*, 2007)
Cow	15(DQ071632)	Putative helicase	Enterococcus hirae	(Soule *et al.*, 2006)
Cow	10(DQ071647)	Hypothetical Protein	Enterococcus hirae	(Soule *et al.*, 2006)

Target	Assay Name	Target Gene	Target Species	Reference
Dog	Mitochondrial Cytochrome b	Mitochondrial DNA(mtDNA)		(Schill & Mathes, 2008)
Dog	BacCan-UCD	16s rRNA gene	Bacteroidales species	(Kildare *et al*., 2007)
Dog	BacCan-UCD	16s rRNA gene	Bacteroidales species	(Kildare *et al.*, 2007)
Dog	DogBac	16s rRNA gene	Bacteroidales species	(Dick *et al.*, 2005)
Duck	E2	unknown bacterium E2		(Devane *et al.*, 2007)
E. coli	lacZ	lacZ gene	E.coli	(Horakova *et al.*, 2006)
E. coli	uidA	uidA gene	E.coli	(Horakova *et al.*, 2006)
E. coli	cyd	cyd gene	E.coli	(Horakova *et al.,* 2006)
E. coli	sfmD	sfmD gene	E.coli	(Kaclikova *et al.*, 2005)
Enterococcus	Entero1	large subunit rRNA gene of Enterococcus	Enterococcus species	(Siefring *et al.*, 2008)
Horse	HorseBac	16srRNA	Bacteroidales species	(Dick *et al*., 2005)
Human	Mitochondrial Cytochrome b	Mitochondrial DNA(mtDNA)		(Schill & Mathes, 2008)
Human	BtH qPCR	alpha-1,6 mannase	Bacteroides thetaiomicron	(Yampara-Iquise *et al.*, 2008)
Human	HumBac	16s rDNA	Bacteroidales species	(Bernhard & Field, 2000a, Bernhard & Field, 2000b)
Human	E.coli clone	O81 serotype	E.coli	(Clermont *et al.*, 2008)
Human	E.coli clone	B2 clonal subgroup VIII	E.coli	(Clermont *et al.*, 2008)
Human	HF183	16s rRNA gene	Bacteroidales species	(Seurinck *et al.*, 2005)
Human	Bifido adolescentis	16s rRNA gene	Bifidobacterium adolescentis	(Matsuki *et al.*, 2004, Matsuki *et al.*, 1998)
Human	Human-Bac 1	16s rRNA gene	Bacteroides-Prevotella species	(Okabe *et al.,* 2007)
Human	BacHum-UCD	16s rRNA gene	Bacteroidales species	(Kildare *et al.*, 2007)
Human	S.bovis - 43143	16s rRNA gene	Streptococcus bovis	(Whitehead & Cotta, 2000)
Human	esp gene	Enterococcal surface protein	Enterococcus faecium	(Scott *et al.*, 2005)
Human	hum163	bacterial surface proteins	Bacteroides fragilis	(Shanks *et al.*, 2007)
Human	hum366	bacterial surface proteins	Bacteroides thetaiotaomicron	(Shanks *et al.*, 2007)
Human	HuBac	16s rRNA genes	Bacteroides Human cluster	(Layton *et al.*, 2006)
Human	B.theta		Bacteroides thetaiotaomicron	(Carson *et al.*, 2005)
Human	F-specific RNA bacteriophage	GenogroupII	F-specific RNA bacteriophage	(Ogorzaly & Gantzer, 2006)

Target	Assay Name	Target Gene	Target Species	Reference
Human	F-specific RNA bacteriophage	GenogroupIII	F-specific RNA bacteriophage	(Ogorzaly & Gantzer, 2006)
Human	HEV	5' UTR	Human enteroviruses	(Fong *et al.*, 2005b)
Human	HEV-nested	5'UTR	Human enteroviruses	(Fong *et al.*, 2005b)
Human	HAdV	Hexon gene	Human adenoviruses	(Fong *et al.*, 2005b)
Human	HAdV- nested	Hexon gene	Human adenoviruses	(Fong *et al.*, 2005b)
Human	Subgroup II	RNA replicase Beta chain	F+ specific RNA coliphages	(Kirs & Smith, 2007)
Human	Subgroup III	Coat protein	F+ specific RNA coliphages	(Kirs & Smith, 2007)
Human	Duplex Scorpion real-time PCR	16s rDNA	Bacteroides species	(Stricker *et al.*, 2008)
Human	HAd2	Hexon gene	Human adenoviruses	(Maluquer de Motes *et al.*, 2004)
Human	HAd2-nested	Hexon gene	Human adenoviruses	(Maluquer de Motes *et al.*, 2004)
Human	HAdV2	Hexon gene	Human adenoviruses	(Hundesa *et al.*, 2006)
Human	HAdV2-nested	Hexon gene	Human adenoviruses	(Hundesa *et al.*, 2006)
Human	HPyV		Human polyomaviruses	(McQuaig *et al.*, 2006)
Human	66(DQ071640)	Hypothetical Protein	Enterococcus faecalis	(Soule *et al.*, 2006)
Human	67(DQ071641)		Enterococcus hirae	(Soule *et al.*, 2006)
Human	68(DQ071642)	Carbohydrate kinase	Enterococcus faecalis	(Soule *et al.*, 2006)
Human	77(DQ071643)	transcriptional regulator	Enterococcus faecalis	(Soule *et al.*, 2006)
Human	81(DQ071644)	Major tail protein	Enterococcus faecalis	(Soule *et al.*, 2006)
Human	107(DQ071645)	Hypothetical Protein	Enterococcus faecalis	(Soule *et al.*, 2006)
Human	B.adolescentis			(Matsuki *et al.*, 1998)
Human	MtDNA human			(Martellini *et al.*, 2005)
Human	MtDNA human			(Martellini *et al.*, 2005)
Human	HumM2	Bacteroidales-like cell surface associated genes		(Shanks *et al.*, 2009)
Human	HumM3	Bacteroidales-like cell surface associated genes		(Shanks et al., 2009)
Human	MnifH gene	nifH gene	Methanobrevibacter smithii	(Ufnar *et al.*, 2006)
Pig	Mitochondrial Cytochrome b	Mitochondrial DNA(mtDNA)		(Schill & Mathes, 2008)
Pig	Pig-1-Bac	16s rRNA gene	Bacteroidales species	(Mieszkin *et al.*, 2009)

Target	Assay Name	Target Gene	Target Species	Reference
Pig	Pig-2-Bac	16s rRNA gene	Bacteroidales species	(Mieszkin *et al.*, 2009)
Pig	Pig-Bac 1	16s rRNA gene	Bacteroides-Prevotella species	(Okabe *et al.*, 2007)
Pig	Pig-Bac 2	16s rRNA gene	Bacteroides-Prevotella species	(Okabe *et al.*, 2007)
Pig	PTV RNA	RNA	Pig teschovirus	(Jimenez-Clavero *et al.*, 2003)
Pig	PigBac	16srRNA	Bacteroidales species	(Lamendella *et al.*, 2009)
Pig	PAdV qPCR	Hexon gene	Porcine adenovirus genome	(Hundesa *et al.*, 2009)
Pig	Porcine adenoviruses	protease	Porcine adenovirus genome	(Maluquer de Motes *et al.*, 2004)
Pig	Porcine adenoviruses-nested PCR	protease	Porcine adenovirus genome	(Maluquer de Motes *et al.*, 2004)
Pig	Porcine adenoviruses	Hexon gene	Porcine adenovirus genome	(Maluquer de Motes *et al.*, 2004)
Pig	Porcine adenoviruses-nested PCR	Hexon gene	Porcine adenovirus genome	(Maluquer de Motes *et al.*, 2004)
Pig	mcrA	mcrA gene	Methanogen species	(Ufnar *et al.*, 2007) (Lamendella *et al*., 2009)
Possum	Bacteroides		Bacteroides	Devane *et al* submitted
Ruminant	HerbBac	16s rDNA	Bacteroidales species	(Bernhard & Field, 2000a, Bernhard & Field, 2000b)
Ruminant	BacR qPCR	16s rRNA gene	Bacteroidetes	(Reischer *et al.*, 2006)
Ruminant	S.bovis - JB1	16s rRNA gene	Streptococcus bovis	(Whitehead & Cotta, 2000)
Ruminant	Ruminococcus flavefaciens	16s rRNA gene	Ruminococcus flavefaciens	(Tajima *et al.*, 2001)
Ruminant	Rum-2-Bac	16S rRNA gene	Bacteroides	(Mieszkin *et al*., 2009)
Sheep	Mitochondrial Cytochrome b	Mitochondrial DNA(mtDNA)		(Schill & Mathes, 2008)
Universal	GenBac3	small subunit rRNA gene of Bacteroidales	Bacteroidales species	(Siefring *et al.*, 2008)
Universal	Total Bacteroides	16s rDNA	Bacteroides-Prevotella species	(Bernhard & Field, 2000a, Bernhard & Field, 2000b)
Universal	BacPre 1	16s rRNA gene	Bacteroides-Prevotella species	(Okabe & Shimazu, 2007)
Universal	TotalBac	16s rRNA gene	Bacteroidetes	(Dick & Field, 2004)
Universal	AllBac	16s rRNA genes	Bacteroides	(Layton *et al.,* 2006)
Universal	BacUni - UCD	16s rRNA gene	Bacteroidales species	(Kildare *et al.*, 2007)
Universal	BacUni - UCD	16s rRNA gene	Bacteroidales species	(Kildare *et al.*, 2007)

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Appendix 2 Field trial results

ND[1] Not detected: Contamination (as measured by UBac marker) was likely too low.

ND[2] Not detected: Would detect if ruminants a major source

ND[3] Not detected: Would detect if bovine a major source

ND[4] Not detected: Would detect if human a major source

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Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
10/03/2011	Armour Bay	<10	very weak +ve	ND[1]	ND[1]	ND[1]	Low-level contamination present in these samples. Only the fourth sample contained specific markers, where both ruminant and human markers were detected. However, the human marker was at a high level inconsistent with the level of universal marker which casts doubt on the validity of this result. Further sampling at this site under elevated conditions is required.
23/03/2011	Armour Bay	10	very weak +ve	ND[1]	ND[1]	ND[1]	
06/04/2011	Armour Bay	41	weak +ve	ND[2]	ND[1]	ND[1]	
27/04/2011	Armour Bay	173	weak +ve	Present, up to 100% ruminant	ND[1]	Present, although would not expect at this level of Ubac	
10/03/2011	Bethells Lagoon	97	strong +ve	Present, up to 10% ruminant	ND[1]	ND[4]	Relatively low levels of contamination, but ruminant markers detected in three of the four samples suggesting ruminant sources of contamination dominate.

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
23/03/2011	Bethells Lagoon	399	weak +ve	ND, would detect if source was ≥ 0.1% ruminant	ND[1]	ND[1]	
27/04/2011	Bethells Lagoon	545	weak +ve	Present, up to 50% ruminant	ND[1]	ND[1]	
06/04/2011	Bethells Lagoon	156	strong +ve	Present, up to 10% ruminant	ND[1]	ND[4]	
10/03/2011	Cox's Bay	20	very strong +ve	Present, up to 1% ruminant	ND[3]	present	Consistent with human sources, and very low ruminant sources.
23/03/2011	Cox's Bay	1119	strong +ve	ND[2]	ND[1]	ND	
06/04/2011	Cox's Bay	480	very strong +ve	ND[2]	ND[3]	present	
27/04/2011	Cox's Bay	1935	very strong +ve	Present, up to 1% ruminant	ND[3]	present	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
10/03/2011	Fosters Bay	<10	weak +ve	Present, up to 50% ruminant	ND[1]	Present, although would not expect at this level of Ubac	Low-level contamination present of ruminant sources. The detection of human marker is at a very low level, and not consistent with the levels of Ubac.
23/03/2011	Fosters Bay	20	weak +ve	ND[2]	ND[1]	ND[1]	
06/04/2011	Fosters Bay	259	weak +ve	Present, up to 10% ruminant	ND[1]	ND[1]	
27/04/2011	Fosters Bay	537	+ve	Present, up to 10% ruminant	ND[1]	ND[1]	
10/03/2011	French Bay	<10	weak +ve	ND[2]	ND[1]	ND[1]	Low-level contamination of unidentified origin in all but the third sample. Very high levels, but still of unidentified origin in the third sample. At low levels of contamination we would be unlikely to detect either the human or bovine markers and would only detect the ruminant marker if the source was 100% ruminant and fairly recent. For the third sample we would have expected to detect the ruminant marker if it had been present and the human or bovine markers if they were 100% of the source. Thus we cannot attribute the possible source or sources of contaminant in these samples. Potential sources of the universal
23/03/2011	French Bay	1376	weak +ve	ND[2]	ND[1]	ND[1]	
06/04/2011	French Bay	4611	very strong +ve	ND, would detect if source was $\geq$ 1% ruminant	ND[3]	ND[4]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
27/04/2011	French Bay	10	weak +ve	ND[2]	ND[1]	ND[1]	marker are - other faecal sources (*e.g.* wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where differential decay of the specific markers relative to the universal may have occurred.
10/03/2011	Green Bay	<10	very weak +ve	ND[1]	ND[1]	ND[1]	Low-level contamination present of unidentified origin. At this level of contamination we would be unlikely to detect either the human or bovine markers and would only detect the ruminant marker in the third and fourth samples and if the source was 100% ruminant and fairly recent. Thus we cannot attribute the possible source or sources of contaminant in these samples. Potential sources of the universal marker are – low-level human source, low-level or aged ruminant source, other faecal sources (*e.g.* wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where differential decay of the specific markers relative to the universal may have occurred.
23/03/2011	Green Bay	189	very weak +ve	ND[1]	ND[1]	ND[1]	
06/04/2011	Green Bay	211	weak +ve	Present, up to 10% ruminant	ND[1]	ND[1]	
27/04/2011	Green Bay	20	weak +ve	ND[2]	ND[1]	ND[1]	
10/03/2011	Karekare car park	145	weak +ve	ND[2]	ND[1]	ND[1]	Low-level contamination present of unidentified origin. At this level of contamination we would be unlikely to detect either the human or bovine markers and would only detect the ruminant marker if the source was 100% ruminant and fairly recent. Thus we cannot attribute the possible source or sources of contaminant in these samples. Potential sources of the universal marker are – low-level human source, low-level or aged ruminant source, other faecal sources (*e.g.* wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where
23/03/2011	Karekare car park	1050	weak +ve	ND[2]	ND[1]	ND[1]	
06/04/2011	Karekare car park	471	weak +ve	Present, up to 10% ruminant	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
27/04/2011	Karekare car park	298	weak +ve	Present, up to 50% ruminant	ND[1]	ND[1]	differential decay of the specific markers relative to the universal may have occurred.
10/03/2011	Karekare Lagoon	260	+ve	ND, would detect if source was ≥ 10% ruminant	ND[1]	ND[1]	Low-level contamination present of unidentified origin. At this level of contamination we would be unlikely to detect either the human or bovine markers and would only detect the ruminant marker if the source was 100% ruminant and fairly recent. Thus we cannot attribute the possible source or sources of contaminant in these samples. Potential sources of the universal marker are – low-level human source, low-level or aged ruminant source, other faecal sources (*e.g.* wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where differential decay of the specific markers relative to the universal may have occurred.
23/03/2011	Karekare Lagoon	1309	weak +ve	Present, up to 10% ruminant	ND[1]	ND[1]	
06/04/2011	Karekare Lagoon	521	weak +ve	ND[2]	ND[1]	ND[1]	
27/04/2011	Karekare Lagoon	341	weak +ve	ND[2]	ND[1]	ND[1]	
10/03/2011	Laingholm Beach	<10	very weak +ve	ND[1]	ND[1]	ND[1]	Very weak contamination present of unidentified origin. At this level of contamination we would be unlikely to detect any of the specific markers. Thus we cannot attribute the possible source or sources of contaminant in these samples. Potential sources of the universal marker are – low-level human source, low-level ruminant source, other faecal sources (*e.g*. wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where differential decay of the specific markers relative to the universal may have occurred.
23/03/2011	Laingholm Beach	95	very weak +ve	ND[1]	ND[1]	ND[1]	
06/04/2011	Laingholm Beach	327	weak +ve	ND[2]	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
27/04/2011	Laingholm Beach	465	very weak +ve	ND, universal too low to usually detect ruminant marker	ND[1]	ND[1]	
15/03/2011	Mahurangi town basin	84	strong +ve	ND, would detect if source was ≥ 1% ruminant	ND[1]	ND[4]	Contamination of unidentified origin present in first three samples. At this level of contamination we would have expected to detect the ruminant marker if it had been present and the human marker if it was 100% of the source and fairly recent. Thus we cannot attribute the possible source or sources of contaminant in these three samples. Potential sources of the universal marker are – low-level human source, low-level or aged ruminant source, other faecal sources (*e.g.* wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where differential decay of the specific markers relative to the universal may have occurred. Based on previous analysis of faecal samples, the contamination seen in the fourth sample is consistent with100% of the universal marker being derived from a ruminant / bovine source.
05/04/2011	Mahurangi town basin	171	+ve	Present, up to 10% ruminant	ND[1]	ND[1]	
12/04/2011	Mahurangi town basin	98	strong +ve	ND, would detect if source was ≥ 1% ruminant	ND[1]	ND[4]	
26/04/2011	Mahurangi town basin	8164	strong +ve	Present, ≥ 1% of source	present	ND[4]	
10/03/2011	Piha Lagoon	200	strong +ve	ND, would detect if source was ≥ 1% ruminant	ND[1]	present	High level of contamination present in the first sample with decreasing levels in subsequent samples. Based on previous analysis of faecal samples, the detected levels of human marker in the first sample is consistent with100% of the universal marker being derived from a human source. At this level of contamination we would have expected to detect the ruminant marker if it was a contributing source of the contamination.
23/03/2011	Piha Lagoon	2187	+ve	ND, would detect if source was ≥	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
				10% ruminant			We cannot attribute the possible source or sources of contaminant in the subsequent three samples. Potential sources of the universal marker are – low-level human source, low-level or aged ruminant source, other faecal sources (*e.g.* wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where differential decay of the specific markers relative to the universal may have occurred.
06/04/2011	Piha Lagoon	816	weak +ve	Present, up to 10% ruminant	ND[1]	ND[1]	
27/04/2011	Piha Lagoon	253	weak +ve	ND[2]	ND[1]	ND[1]	
10/03/2011	Te Atatu Beach, Waitemata Harbour	<10	+ve	ND, would detect if source was ≥ 10% ruminant	ND[1]	ND[1]	Varying levels of contamination present of unidentified origin. At this level of contamination we would be unlikely to detect either the human or bovine markers but would have expected to detect the ruminant marker if it had been a significant source. Thus we cannot attribute the possible source or sources of contaminant in these samples. Potential sources of the universal marker are – low-level human source, other faecal sources (*e.g.* wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where differential decay of the specific markers relative to the universal may have occurred.
23/03/2011	Te Atatu Beach, Waitemata Harbour	122	very weak +ve	ND[1]	ND[1]	ND[1]	
06/04/2011	Te Atatu Beach, Waitemata Harbour	20	+ve	ND, would detect if source was ≥ 10% ruminant	ND[1]	ND[1]	
27/04/2011	Te Atatu Beach, Waitemata Harbour	301	strong +ve	ND, would detect if source was ≥ 1% ruminant	ND[1]	ND[4]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
10/03/2011	Titirangi Beach, Manukau Harbour	10	very weak +ve	ND[1]	ND[1]	ND[1]	Varying levels of contamination present of unidentified origin. At this level of contamination we would be unlikely to detect any of the specific markers in either of the first two samples and would have only detected the ruminant marker in the fourth sample if the source was 100% ruminant and fairly recent. We would have expected to detect the ruminant marker if it had been present in the third sample and the human marker if it was 100% of the source and fairly recent. Thus, we cannot attribute the possible source or sources of contaminant in these samples. Potential sources of the universal marker are – low-level human source, low-level or aged ruminant source, other faecal sources (*e.g.* wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where differential decay of the specific markers relative to the universal may have occurred.
23/03/2011	Titirangi Beach, Manukau Harbour	63	very weak +ve	ND[1]	ND[1]	ND[1]	
06/04/2011	Titirangi Beach, Manukau Harbour	161	strong +ve	ND, would detect if source was $\geq$ 1% ruminant	ND[1]	ND[4]	
27/04/2011	Titirangi Beach, Manukau Harbour	121	weak +ve	ND[2]	ND[1]	ND[1]	
23/03/2011	Weymouth, Manukau Harbour	51	weak +ve	Present up to 50% ruminant	ND[1]	ND[1]	Low-level contamination present consistent with ruminant pollution.
06/04/2011	Weymouth, Manukau Harbour	211	weak +ve	Present up to 50% ruminant	ND[1]	ND[1]	
13/04/2011	Weymouth, Manukau Harbour	<10	very weak +ve	ND[1]	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
10/03/2011	Wood Bay, Manukau Harbour	<10	very weak +ve	ND[1]	ND[1]	ND[1]	Three of the four samples have very low enterococci and FST markers, of which only the ruminant marker is positive. The sample with high levels of both enterococci and ruminant marker also has ruminant marker present, but consistent with only up to 1% of the contamination. While some ruminant is present, there is likely to other sources present.
23/03/2011	Wood Bay, Manukau Harbour	20	weak +ve	Present, Up to 50% ruminant	ND[1]	Present, (would not expect to see at this level of universal)	
06/04/2011	Wood Bay beach, Manukau Harbour	6867	strong +ve	Present, up to 1% ruminant	ND[1]	ND[4]	
27/04/2011	Wood Bay beach, Manukau Harbour	<10	+ve	ND, would detect if source was ≥ 10% ruminant	ND[1]	ND[1]	

Environment Canterbury

15/02/2011	Otukaikino Creek	305	strong +ve	Present, up to 50% ruminant	ND[3]	ND[4]	Consistent with ruminant source of contamination.
10/03/2011	Otukaikino Creek	74	strong +ve	Present, up to 50% ruminant	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
23/03/2011	Otukaikino Creek	63	strong +ve	Present, up to 50% ruminant	ND[1]	ND[1]	
31/03/2011	Otukaikino Creek	85	strong +ve	Present, up to 50% ruminant	ND[1]	ND[1]	
12/04/2011	Otukaikino Creek	30	strong +ve	Present, up to 10% ruminant	ND[1]	ND[1]	
15/02/2011	Kaiapoi River	313	very strong +ve	Present, up to 5% ruminant	ND[3]	ND[4]	Consistent with ruminant source of contamination.
10/03/2011	Kaiapoi River	279	strong +ve	Present, up to 10% ruminant	ND[1]	ND[4]	
23/03/2011	Kaiapoi River	657	strong +ve	Present, up to 10% ruminant	ND[1]	ND[4]	
31/03/2011	Kaiapoi River	201	strong +ve	Present, up to 50% ruminant	ND[1]	ND[4]	
12/04/2011	Kaiapoi River	98	strong +ve	Present, up to 50% ruminant	ND[1]	ND[4]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
15/02/2011	Tikao Bay	20	very weak +ve	ND[1]	ND[1]	ND[1]	Very low level contamination present. At this level of contamination we would be unlikely to detect any of the specific markers and thus cannot attribute the possible source or sources. Potential sources of the universal marker are - human source too low to detect, other faecal sources (*e.g.* wildfowl), mixed sources, environmental sources and / or aged or partially-treated sources where differential decay of the specific markers relative to the universal may have occurred.
10/03/2011	Tikao Bay	<10	weak +ve	ND[2]	ND[1]	ND[1]	
22/03/2011	Tikao Bay	185	very weak +ve	ND[1]	ND[1]	ND[1]	
31/03/2011	Tikao Bay	<10	very weak +ve	ND[1]	ND[1]	ND[1]	
13/04/2011	Tikao Bay	<10	very weak +ve	ND[1]	ND[1]	ND[1]	
15/02/2011	Ashburton River	41	weak +ve	Present, up to 50% ruminant	ND[1]	ND[1]	Ruminant source of contamination dominates. The human marker detected in one sample was at a high level inconsistent with the level of universal marker which casts doubt on the validity of this result. Further sampling at this site under elevated conditions is required.
09/03/2011	Ashburton River	41	+ve	Present, up to 50% ruminant	ND[1]	ND[1]	
31/03/2011	Ashburton River	97	+ve	Present, up to 50% ruminant	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
06/04/2011	Ashburton River	265	weak +ve	Present, up to 50% ruminant	ND[1]	Present (but wouldn't expect to be able to detect)	
12/04/2011	Ashburton River	10	weak +ve	Present, up to 50% ruminant	ND[1]	ND[1]	
15/02/2011	Pleasant Point Yacht Club	650	weak +ve	Present, up to 10% ruminant	ND[1]	present	Human sources dominant in these samples. May be low levels of ruminant pollution.
24/03/2011	Pleasant Point Yacht Club	573	very strong +ve	ND, would detect if source was > 0.1% ruminant	ND[1]	present	
31/03/2011	Pleasant Point Yacht Club	354	very strong +ve	Present, up to 10% ruminant	ND[3]	present	
14/04/2011	Pleasant Point Yacht Club	1008	very strong +ve	ND, would detect if source was > 0.1% ruminant	ND[3]	present	
15/02/2011	Avon Heathcote Estuary	373	strong +ve	ND, would detect if source was > 1% ruminant	ND[1]	ND[4]	Human sources dominant in these samples. May be very low levels of ruminant pollution.

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
24/03/2011	Avon Heathcote Estuary	>24,000	very strong +ve	ND, would detect if source was > 0.1% ruminant	ND[3]	present	
31/03/2011	Avon Heathcote Estuary	187	very strong +ve	Present, up to 0.1% ruminant	ND[3]	present	
14/04/2011	Avon Heathcote Estuary	145	very strong +ve	Present, up to 0.1% ruminant	ND[3]	present	

Environment Southland

08/02/2011	Oreti River	794	+ve	ruminant present, could be up to 100% of source	ND[1]	ND[1]	Increasing level of faecal contamination during period of sampling - positive increasing to very strong positive. Strong evidence that source is of ruminant origin and that the ruminant source is solely bovine. No evidence of contamination from a human source. However, the level of faecal contamination present in the first two samples is such that we would not have expected to see any human contamination if it had been present and would only have expected to see human contamination in the last two samples if it had
15/02/2011	Oreti River	10	+ve	ruminant present, could be up to 10% of source	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
29/03/2011	Oreti River	677	strong +ve	ruminant present, could be 50-100% of source	ND[1]	ND[4]	been the sole contaminant *i.e.* we would not have seen levels of human contamination <100%.
09/05/2011	Oreti River	3076	very strong +ve	ruminant present, could be up to 100% of source	present	ND[4]	
08/02/2011	Aparima River	1291	strong +ve	ruminant present, could be up to 100% of source	ND[1]	ND[4]	Contamination from ruminants, dominated by bovine sources.
15/02/2011	Aparima River	10	+ve	ruminant present, could be up to 50% of source	ND[1]	ND[1]	
29/03/2011	Aparima River	97	strong +ve	ruminant present, could be up to 100% of source	present	ND[4]	
09/05/2011	Aparima River	504	very strong +ve	ruminant present, could be up to 100% of source	present	ND	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
14/02/2011	New River Estuary	20	weak +ve	ruminant present, could be up to 50% of source	ND[1]	ND[1]	Low-level contamination present from ruminant sources.
28/03/2011	New River Estuary	20	weak +ve	ruminant present, could be up to 50% of source	ND[1]	ND[1]	
14/02/2011	Jacobs River Estuary	0	very weak +ve	ND[1]	ND[1]	ND[1]	Very low level contamination present of unidentified origin. At this level of contamination we would be unlikely to detect either the human, ruminant or bovine markers.
28/03/2011	Jacobs River Estuary	10	very weak +ve	ND[1]	ND[1]	ND[1]	
14/02/2011	Monkey Island	10	very weak +ve	ND[1]	ND[1]	ND[1]	Low-level contamination present from ruminant sources.
28/03/2011	Monkey Island	20	+ve	ruminant present, could be up to 50% of source	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
Greater Wellington Regional Council							
15/02/2011	Hutt River at Silverstream Bridge	20	very weak +ve	ND[1]	ND[1]	ND[1]	Low levels of contamination at this site. Universal Bacteroidales marker concentration very low. Ruminant sources present on the day that enterococci was highest. Detection of the ruminant marker despite low Universal marker indicates ruminants are likely a major contributing source of contamination at this site.
22/02/2011	Hutt River at Silverstream Bridge	<10	+ve	ruminant present, up to 10% of source	ND[1]	ND[1]	
08/03/2011	Hutt River at Silverstream Bridge	164	weak +ve	ruminant present, up to 50% of source	ND[1]	ND[1]	
01/03/2011	Hutt River at Silverstream Bridge	20	weak +ve	ND[2]	ND[1]	ND[1]	
14/02/2011	Riversdale Lagoon	20	strong +ve	ND[2]	ND[3]	ND[4]	Low levels of contamination at this site based on enterococci concentrations. Universal marker concentrations reasonably high with presence of ruminant contamination on one occasion. Additional sampling when contamination is elevated is required to confirm sources.
21/02/2011	Riversdale Lagoon	53	strong +ve	ruminant present, between 1 and 10% of source	ND[1]	ND[4]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
28/02/2011	Riversdale Lagoon	<10	weak +ve	ND[2]	ND[1]	ND[1]	
07/03/2011	Riversdale Lagoon	10	strong +ve	ND, would detect if source was ≥ 10% ruminant	ND[1]	ND[4]	
15/02/2011	Ruamahanga River at The Cliffs	<10	weak +ve	ND[2]	ND[1]	ND[1]	Low levels of contamination at this site. On the last day, contamination slightly elevated as indicated by both enterococci and Universal marker. Ruminants likely a major contributor to contamination on this day. Additional sampling when contamination is elevated is required to confirm sources.
22/02/2011	Ruamahanga River at The Cliffs	<10	weak +ve	ND[1]	ND[1]	ND[1]	
01/03/2011	Ruamahanga River at The Cliffs	<10	+ve	ND, would detect if source was ≥ 10% ruminant	ND[1]	ND[1]	
08/03/2011	Ruamahanga River at The Cliffs	75	strong +ve	ruminant present, up to 50% of source	ND[1]	ND[4]	
16/02/2011	Te Horo Beach at Mangaone Stream	111	very strong +ve	ruminant present, 1 to 10% of source	ND[3]	ND[4]	Low to moderate levels of contamination at this site. Ruminant sources present when contamination elevated. Potentially other sources also contributing to contamination. Additional

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
23/02/2011	Te Horo Beach at Mangaone Stream	192	strong +ve	ruminant present, up to 50% of source	ND[3]	ND[4]	sampling when contamination is elevated is required to confirm sources.
01/03/2011	Te Horo Beach at Mangaone Stream	42	strong +ve	ND[2]	ND[3]	ND[4]	
09/03/2011	Te Horo Beach at Mangaone Stream	<10	very weak +ve	ND[1]	ND[1]	ND[1]	
15/02/2011	Plimmerton South Beach	<10	weak +ve	ND[1]	ND[1]	ND[1]	Low to moderate levels of contamination at this site. Universal signal on last date was high enough that the ruminant marker would normally be detected if ruminants are a major contributing source. Detection of the HBac marker on this occasion indicates potential human source. Further sampling is required to confirm source.
22/02/2011	Plimmerton South Beach	<10	very weak +ve	ND[1]	ND[1]	ND[1]	
01/03/2011	Plimmerton South Beach	75	strong +ve	ND[2]	ND[1]	ND[4]	
08/03/2011	Plimmerton South Beach	288	very strong +ve	ND[2]	ND[3]	Present, although at very low level	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
15/02/2011	Porirua Harbour Rowing Club	124	strong +ve	ND[2]	ND[3]	ND[4]	Low levels of contamination at this site. Universal signal on first sampling day was high enough that the host-specific markers would typically be detected if they were present. Further sampling is required to confirm source(s).
22/02/2011	Porirua Harbour Rowing Club	10	weak +ve	ND[1]	ND[1]	ND[1]	
01/03/2011	Porirua Harbour Rowing Club	20	strong +ve	ND[2]	ND[1]	ND[4]	
08/03/2011	Porirua Harbour Rowing Club	53	weak +ve	ND[1]	ND[1]	ND[1]	

Hawkes Bay Regional Council

14/02/2011	Clive River	64	strong +ve	ND	ND[3]	ND[4]	Moderate to high levels of contamination at this site. Contamination levels corresponded with rainfall. Sources of contamination appear to be dominated by ruminant sources, although other animal sources may be present due to the relatively low level of ruminant marker compared to universal marker. The strong bovine signal on the day corresponding with rainfall identifies cows
01/03/2011	Clive River	164	very strong +ve	Ruminant present, but weak signal	ND[3]	ND[4]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
08/03/2011	Clive River	1700	very strong +ve	ruminant present, up to 50 % of source	Present	ND[4]	as a potential dominant source.
21/02/2011	Clive River	429	strong +ve	ruminant present, up to 10% of source	ND[3]	ND[4]	
14/02/2011	Kairakau Lagoon	42	very weak +ve	ruminant present, 50 to 100% of source	ND[1]	ND[1]	Low to high levels of contamination at this site. Contamination levels (based on the universal marker) corresponded with rainfall. Concentrations of universal marker not always consistent with enterococci, indicating persistent faecal indicator bacteria at this site during dry periods. Fresh sources of contamination are dominated by ruminant sources, which is most evident on last sampling occasion, which corresponded with rainfall.
21/02/2011	Kairakau Lagoon	738	weak +ve	ND	ND[1]	ND[1]	
01/03/2011	Kairakau Lagoon	75	weak +ve	ruminant present, 50 to 100% of source	ND[1]	ND[1]	
08/03/2011	Kairakau Lagoon	782	very strong +ve	ruminant present, 50 to 100% of source	ND[3]	ND[4]	
14/02/2011	Maraetotara Lagoon	124	strong +ve	ruminant present, up to 50 % of source	ND[3]	ND[4]	Low to moderate levels of contamination at this site. Contamination levels (based on the universal marker) corresponded with rainfall. Concentrations of universal marker not always consistent with enterococci, indicating persistent

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
21/02/2011	Maraetotara Lagoon	306	strong +ve	ruminant present, up to 50 % of source	ND[1]	ND[4]	faecal indicator bacteria at this site during dry periods. Fresh sources of contamination are dominated by ruminant sources, which is most evident on last sampling occasion, which corresponded with rainfall.
01/03/2011	Maraetotara Lagoon	<10	+ve	ruminant present, up to 50 % of source	ND[1]	ND[1]	
08/03/2011	Maraetotara Lagoon	64	strong +ve	ruminant present, up to 10 % of source	ND[1]	ND[4]	
14/02/2011	Porangahau Estuary	<10	weak +ve	ruminant present, up to 10% of source	ND[1]	ND[1]	On first three sampling occasions, contamination levels were very low; however, ruminant marker was still detected. Ruminant source also dominant on last sampling occasion, which coincided with rainfall. Ruminants are likely the dominant source of contamination at this site
21/02/2011	Porangahau Estuary	<10	+ve	ruminant present, up to 100% of source	ND[1]	ND[1]	
01/03/2011	Porangahau Estuary	<10	weak +ve	ND[2]	ND[1]	ND[1]	
08/03/2011	Porangahau Estuary	478	+ve	ruminant present, 50 to 100% of source	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
14/02/2011	Puhokio Lagoon	306	very strong +ve	ruminant present, up to 10% of source	ND[3]	ND[4]	Moderate to high levels of contamination at this site. Contamination levels high during both relatively dry and wet conditions. Concentrations of universal marker and ruminant marker highest on the last sampling occasion corresponding with high rainfall. Ruminant contamination likely the dominant source at this site.
21/02/2011	Puhokio Lagoon	1700	very strong +ve	ruminant present, 10 to 50% of source	ND[3]	ND[4]	
01/03/2011	Puhokio Lagoon	344	strong +ve	ruminant present, up to 50% of source	ND[3]	ND[1]	
08/03/2011	Puhokio Lagoon	1200	very strong +ve	ruminant present, up to 100% of source	ND[3]	ND[4]	
13/02/2011	Waipatiki Lagoon	20	strong +ve	ruminant present, between 10 and 50% of source	ND[1]	ND[4]	Low to moderate levels of contamination at this site. Contamination levels (based on the universal marker) corresponded with rainfall. Concentrations of universal marker not always consistent with enterococci, indicating persistent faecal indicator bacteria at this site during drier periods. Fresh sources of contamination are dominated by ruminant sources, which is most evident on last sampling occasion, which corresponded with rainfall.
21/02/2011	Waipatiki Lagoon	344	+ve	ruminant present, up to 10% of source	ND[1]	ND[1]	
01/03/2011	Waipatiki Lagoon	53	strong +ve	ruminant present, up to 1% of source	ND[3]	ND[4]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
08/03/2011	Waipatiki Lagoon	364	very strong +ve	ruminant present, up to 100% of source	ND[3]	ND[4]	
Marlborough District Council							
14/02/2011	Brown River @SH6 Bridge (BRN-2)	150	weak +ve	ruminant present, up to 100% of source	ND[1]	ND[1]	Low to moderate levels of contamination at this site. Contamination levels (based on enterococci) corresponded with rainfall. Ruminant sources are dominant at this site.
21/02/2011	Brown River @SH6 Bridge (BRN-2)	20	very strong +ve	ND[2]	ND[3]	ND[4]	
23/02/2011	Brown River @SH6 Bridge (BRN-2)	137	strong +ve	ruminant present, 50 to 100% of source	ND[1]	ND[4]	
03/03/2011	Brown River @SH6 Bridge (BRN-2)	87	+ve	ruminant present, up to 100% of source	ND[1]	ND[1]	
14/02/2011	Moenui Beach (MOE-1)	10	weak +ve	ND[1]	ND[1]	ND[1]	Low to high levels of contamination at this site. Highest contamination corresponded with the lowest salinity reading (highest freshwater input).

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
21/02/2011	Moenui Beach (MOE-1)	31	ND	ND[1]	ND[1]	ND[1]	Contamination on this occasion was likely driven by ruminant sources.
23/02/2011	Moenui Beach (MOE-1)	<10	weak +ve	ruminant present, 10 to 50% of source	ND[1]	ND[1]	
03/03/2011	Moenui Beach (MOE-1)	>2000	very strong +ve	ruminant present, up to 100% of source	ND[3]	ND[4]	
14/02/2011	Momorangi Bay (MOM-001)	10	weak +ve	ND[1]	ND[1]	ND[1]	Low to high levels of contamination at this site. Highest contamination corresponded with the lowest salinity reading and rainfall. Source of contamination cannot be confirmed.
21/02/2011	Momorangi Bay (MOM-001)	31	strong +ve	ND[2]	ND[1]	ND[1]	
23/02/2011	Momorangi Bay (MOM-001)	>2000	very strong +ve	ND[2]	ND[3]	ND[4]	
03/03/2011	Momorangi Bay (MOM-001)	10	very strong +ve	ruminant present, up to 1% of source	ND[3]	ND[4]	
14/02/2011	Rai River at Rai Falls (RAR-1)	124	strong +ve	ruminant present, 50 to 100% of source	ND[3]	ND[4]	Low to high levels of contamination at this site. Contamination corresponded with rainfall. Ruminants a dominant source of contamination at this site and the detection of the bovine marker indicates cows may be the main ruminant source.

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
21/02/2011	Rai River at Rai Falls (RAR-1)	42	strong +ve	ruminant present, ~ 50% of source	ND[1]	ND[4]	
23/02/2011	Rai River at Rai Falls (RAR-1)	453	strong +ve	ruminant present, 50 to 100% of source	ND[1]	ND[4]	
03/03/2011	Rai River at Rai Falls (RAR-1)	659	strong +ve	ruminant present, up to 100% of source	Present	ND[4]	

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02/03/2011	100211 Whangarei at Town Basin	20	strong +ve	ND, would detect if source was $\geq$ 1% ruminant	ND[1]	ND[4]	Moderate to high levels of contamination at this site. Sources of contamination not consistent but appear to be dominated by ruminant sources, particularly during the last two sampling occasions when salinity was lowest (perhaps associated with rainfall/runoff).
08/03/2011	100211 Whangarei at Town Basin	20	strong +ve	ruminant present, up to 10% of source	ND[1]	ND[4]	
22/03/2011	100211 Whangarei at Town Basin	>2000	very strong +ve	ruminant present, could exceed 50% of source	ND[3]	ND[4]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
26/04/2011	100211 Whangarei at Town Basin	945	very strong +ve	ruminant present, could exceed 50% of source	ND[3]	ND[4]	
28/02/2011	101216 Bay of Island at Waitangi	<10	weak +ve	ND[1]	ND[1]	ND[1]	Contamination at this site was low with the exception of one sampling occasion (coinciding with low salinity) when faecal indicators (enterococci and UBac marker) were elevated. The ruminant marker was detected on two occasions, suggesting ruminants likely a major contributor to contamination.
29/03/2011	101216 Bay of Island at Waitangi	271	strong +ve	Ruminant present, could be 10 to 50% of source	ND[3]	ND[4]	
13/04/2011	101216 Bay of Island at Waitangi	<10	weak +ve	ND[1]	ND[1]	ND[1]	
26/04/2011	101216 Bay of Island at Waitangi	<10	+ve	ruminant present, could be up to 50% of source	ND[1]	ND[1]	
28/02/2011	101526 Kerikeri at Waipapa	<10	+ve	ND[2]	ND[1]	ND[1]	Contamination at this site was low on three of the four sampling occasions. The concentrations of the ruminant marker on two of the occasions suggest that ruminants may contribute to ~ 10 to 50 % of the contamination. Further sampling when contamination is high is required to further confirm sources at this site.
14/03/2011	101526 Kerikeri at Waipapa	<10	+ve	ND[2]	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
13/04/2011	101526 Kerikeri at Waipapa	10	strong +ve	ruminant present, could be about 1 to 10 % of source	ND[1]	ND[4]	
26/04/2011	101526 Kerikeri at Waipapa	364	strong +ve	ruminant present, up to 10 % of source	ND[1]	ND[4]	
28/02/2011	101622 Whangaroa at Culvert	42	strong +ve	ND[2]	ND[1]	ND[4]	Contamination at this site was low to high, with the highest contamination coinciding when salinity was lowest (*i.e.* perhaps following rainfall). This site was an outlier compared to all others with regard to the bovine marker. The bovine marker was present on three out of four occasions, indicating that cows are a dominant source of contamination at this site. However, there is some inconsistency with the overall results of the trial since the bovine marker was detected on more occasions than the ruminant marker, which is more abundant than the bovine marker.
14/03/2011	101622 Whangaroa at Culvert	31	strong +ve	ND[2]	present	ND[4]	
13/04/2011	101622 Whangaroa at Culvert	178	very strong +ve	ND[2]	present	ND[4]	
26/04/2011	101622 Whangaroa at Culvert	738	very strong +ve	ruminant present, up to 10 % of source	present	ND[4]	
28/02/2011	102232 Whangaroa at West of Cape Horn	<10	+ve	ND[2]	ND[1]	ND[1]	Contamination at this site was low to high, with the highest contamination coinciding when salinity was lowest (*i.e.* perhaps following rainfall). The

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
14/03/2011	102232 Whangaroa at West of Cape Horn	<10	weak +ve	Ruminant present with low universal, suggesting ruminants a dominant source	ND[1]	ND[1]	presence of the ruminant marker even when contamination was low, as well as the very strong signal on the day when contamination was high indicates that ruminants are the major contributor to contamination at this site.
13/04/2011	102232 Whangaroa at West of Cape Horn	<10	strong +ve	Ruminant present with low universal, suggesting ruminants a dominant source	ND[1]	ND[1]	
26/04/2011	102232 Whangaroa at West of Cape Horn	697	very strong +ve	Ruminant present, could be up to 100% of source	ND[3]	ND[4]	
02/03/2011	Kaipara at Wahiwaka Creek (109665)	10	very weak +ve	ND[1]	ND[1]	ND[1]	Contamination at this site was high on only one occasion. The very strong signal for the ruminant marker on this day indicates that ruminants are

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
08/03/2011	Kaipara at Wahiwaka Creek (109665)	<10	weak +ve	ND[1]	ND[1]	ND[1]	likely the major contributor to contamination at this site.
04/04/2011	Kaipara at Wahiwaka Creek (109665)	10	very weak +ve	ND[1]	ND[1]	ND[1]	
03/05/2011	109005 Wahiwaka Creek (Kaipara)	1300	very strong +ve	Ruminant present, could be up to 100% of source	ND[3]	ND[4]	
Taranaki Regional Council							
21/02/2011	NPDC WWTP sample site	<10	very weak +ve	ND[1]	ND[1]	ND[1]	Low to high levels of contamination at this site. Highest contamination on the last sampling day coincided with rainfall. Ruminants the dominant source of contamination in last sample.
10/03/2011	NPDC WWTP sample site	238	very weak +ve	ND[1]	ND[1]	ND[1]	
21/03/2011	NPDC WWTP sample site	124	weak +ve	ND[1]	ND[1]	ND[1]	
04/04/2011	NPDC WWTP sample site	782	strong +ve	ruminant present, up to 100% of	ND[3]	ND[4]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
				source			
21/02/2011	Patea River	<10	very weak +ve	ND[1]	ND[1]	ND[1]	Low levels of contamination at this site. Elevated enterococci on the last day that corresponded with rainfall. Ruminants the dominant source of contamination on one sampling occasion.
10/03/2011	Patea River	<10	very weak +ve	ND[1]	ND[1]	ND[1]	
21/03/2011	Patea River	10	weak +ve	ND[1]	ND[1]	ND[1]	
04/04/2011	Patea River	99	weak +ve	ruminant present, up to 100% of source	ND[1]	ND[1]	
21/02/2011	Waitara River town bridge	31	ND	ND[1]	ND[1]	ND[1]	Low to moderate levels of contamination at this site. Highest contamination corresponded with rainfall. Ruminants a dominant source of contamination on one sampling event.
10/03/2011	Waitara River town bridge	64	strong +ve	ruminant present, up to 100% of source	ND[1]	ND[1]	
21/03/2011	Waitara River town bridge	10	+ve	ruminant present, 50 to 100% of	ND[1]	ND[1]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
				source			
04/04/2011	Waitara River town bridge	406	strong +ve	ruminant present, up to 100% of source	ND[3]	ND[4]	

Waikato Regional Council

08/03/2011	Whitianga Harbour	<10	weak +ve	ND[2]	ND[1]	ND[1]	Low-level contamination present of unidentified origin. At this level of contamination we would be unlikely to detect either the human or bovine markers and would only detect the ruminant marker if the source was 100% ruminant and fairly recent.

West Coast Regional Council

17/03/2011	Orowaiti Lagoon Westport at picnic area	1400	very strong +ve	ruminant present, up to 100% of source	ND[3]	ND[4]	Low to high levels of contamination at this site. Universal Bacteroidales marker concentration high. Ruminant sources present and likely a major contributing source.
21/03/2011	Orowaiti Lagoon Westport at picnic area	478	very strong +ve	ruminant present, up to 50% of source	ND[3]	ND[4]	

Date Sampled	Sampling site	Enterococci MPN / 100 ml	Result				Report comment
			universal	ruminant	bovine	human	
29/03/2011	Orowaiti Lagoon Westport at picnic area	53	very strong +ve	ruminant present, 50 to 100% of source	ND[3]	ND[4]	
08/04/2011	Orowaiti Lagoon Westport at picnic area	>2000	very strong +ve	ruminant present, up to 10% of source	ND[3]	ND[4]	

Appendix 3 Partial processing of samples for PCR analysis

1. After collection of water sample, filter 100ml through 0.2µM Supor 200 filter using a vacuum manifold. If filters get blocked too quickly, filter 2x50ml and place both filters in the same 50ml tube (see below).
2. Aseptically remove the filter(s) from the filter holder and place in a 50ml tube. Add 1ml of GITC buffer to the filter(s). When GITC buffer is added use the pipette tip (still attached to pipette) to fold/squash filter(s) so they are fully submerged in the buffer (at the bottom of the tube) and are thoroughly saturated. Vortex, then leave to settle 5min at room temperature. NB: If filter(s) are punctured by the filter tip they are still OK to process.
3. Place the 50ml tubes in -20°C freezer until all samples are ready to be sent.
4. To send place 50ml tubes in a plastic bag, place bag into a chilly bin containing ice packs to keep the filters frozen, and send by courier.
5. Include sample request form(s) and contact details for reporting.

Laboratories should undertake a validation of local processing with Cawthron or ESR before partial processing of samples.