

# Bacterial contamination of seawater and shellfish

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## Executive Summary

The aim of this project was to determine if and how faecal source identification tools could be best applied to determine the source of bacterial contamination of water and shellfish in Marlborough. There were two main components of this work – water sites at Momorangi, and shellfish samples from other sites in the Marlborough Sounds.

### Momorangi

The water sites at Momorangi were analysed using FWA and PCR based assays. FWA levels were below or close to the detection limit for all samples analysed. This indicates that the source of elevated faecal counts does not contain appreciable levels of FWAs. Possible human sources in the area could be tested directly for FWA content – if they contain FWAs then it would suggest they are not a source. If they don't contain FWAs, then it simply means this test is not helpful in source discrimination in this area.

A number of PCR markers were detected in water samples collected at Momorangi. This is a complex environment with multiple faecal inputs. While the current analysis has not resolved the issue, the following observations could be made:

- All but one of the samples analysed contained markers indicative of wildfowl inputs;
- Animal markers were also detected in samples including the coastal bathing site
- Bacteroidetes markers indicative of human inputs and also possum inputs were also detected. As these markers display cross-reactivity with each other, the presence of both could mean either human source, possum source or both.
- MOM-001 sampled on 16/03/2008, contained bacteroidetes human marker and also human indicative *B. adolescentis* marker, with weak possum marker. This is suggestive of human inputs.

Resolving the possum vs human issue would require the use of additional PCR assays, or faecal sterol analysis.

### Shellfish

Examination of shellfish using the PCR based markers was unsuccessful. An alternative technique – Rep-PCR of *E. coli* isolates was demonstrated to be technically feasible. In the preliminary application of this technique, the same genotype of *E. coli* was isolated from Shag poo samples, and from shellfish in the vicinity. While this does provide some support for the suggestion that some of the *E. coli* in these shellfish are from wildfowl, additional work would be required to quantify the actual proportion. This would require analysis of *E. coli* from a larger range of potential sources (including wildfowl, feral animals, human sources), and comparison with shellfish derived isolates

## **Project background**

Some recreational waters in the Marlborough Sounds frequently exceed guidelines levels for safe recreational use. In the past improvements to wastewater treatment systems and public education/information have been undertaken but problems still exist.

The source of bacterial contamination to seawater and shellfish is unclear, thus making it difficult to implement effective management plans to target the issue.

The aim of this project was to determine if and how faecal source identification tools could be best applied to determine the source of bacterial contamination.

The intention of the work was to contribute to more accurately assessing the risk to the public from pathogen contaminated waters/shellfish, and to assess the efficiency and effectiveness of current monitoring programmes, with a view to making changes where appropriate and to communicate this information and the perceived risk to the public.

There were two aspects to this work – water sites at Momorangi and shellfish at other sites in the Marlborough Sounds.

# Momorangi

Momorangi Bay, Grove Arm, Queen Charlotte Sound is 15km from Picton and has a camping ground with 3 basic overnight cabins, 80 power sites, 33 waterfront sites, and 70 tent sites. Facilities include kitchen, laundry, hot showers, and other facilities.



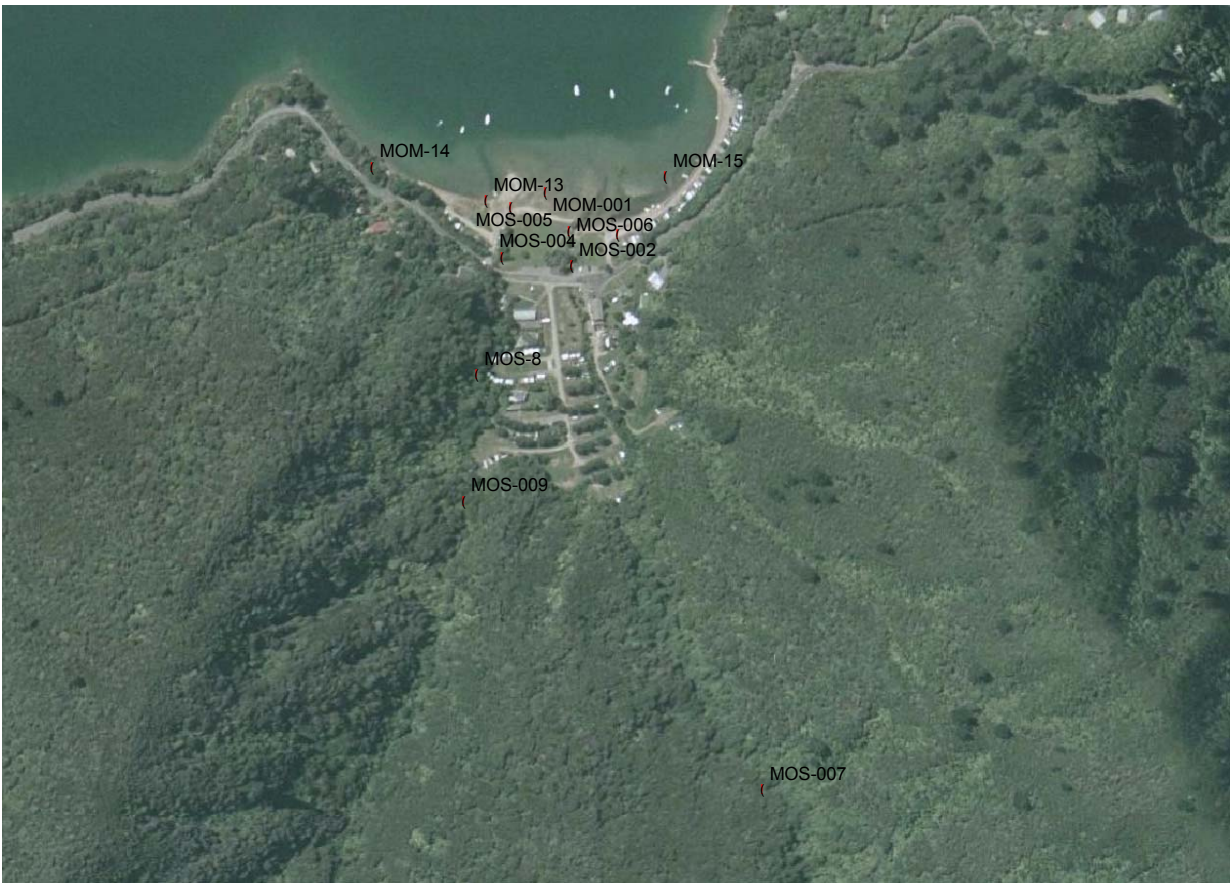
Bathing water is monitored at site MOM-001 which has often exceeded recommended limits. The results from Marlborough Districts Council's bathing water sampling programme for 2006/07 and 2007/08 are compared with the Ministry for the Environments (MfE) bathing water standards in Table 1.

	2006/07 (MPN Enterococci/100mL)	Rainfall (72 hour total)	2007/08 (MPN Enterococci/100mL)	Rainfall (72 hour total)	MfE Alert Guideline	MfE Action Guideline
Week 1			5	9	140	280
Week 2	10	5.0	945	0	140	280
Week 3	111	0.0	40	0	140	280
Week 4	5	4.0	5	0	140	280
Week 5	30	6.5	5	0	140	280
Week 6	20	3.0	384	7.5	140	280
Week 7	75	5.0	20	63	140	280
Week 8	238	4.0	1000	50.5	140	280
Week 9	5	1.0	10	0	140	280
Week 10	111	26.0	164	10	140	280
Week 11	5	4.0	1400	0	140	280
Week 12	429	0.0	87	15	140	280
Week 13	5	18.0	5	0.5	140	280
Week 14	53	0.0	504	5	140	280
Week 15	20	10.0	137	0	140	280
Week 16	560	0.0	150	0	140	280
Week 17	5	8.0	20	4.5	140	280
Week 18	659	0.0	178	13.5	140	280
Week 19	1000	15.5	64	1.5	140	280
Week 20	1700	0.0	20	0	140	280
Week 21	40	1.0	20	0	140	280

**Table 1:** Water quality results for Momorangi Bay for the Bathing Water Seasons (November to March inclusive) 2006/07 and 2007/08.

Sampling has been undertaken at a range of sites (Figure 1). The possible sources of faecal contamination at Momorangi Bay are ducks (particularly Paradise Shelducks and mallards), the wastewater treatment plant at the campground (although thought to be highly unlikely) and possibly poorly performing septic tanks. Domestic and feral animals may also contribute.

The key question addressed here, was “*Are the faecal source tools able to implicate or exonerate a human source of faecal contamination at this site?*”



**Figure 1.** Sampling sites at Momorangi

## **Method**

In addition to normal sampling and analysis, an additional 500ml sample was collected from sites MOM-1, 2, 4, 5 and 6 on the following dates 23 January 2008; 30 January 2008 and the 13 March 2008. These were sent on ice to ESR for analysis using PCR markers, and FWAs.

## **Results**

Results are presented in Table 2 and Table 3.

### **FWA analysis**

FWA analysis was performed on the first two sets of samples. Levels were below the detection limit for all samples on 23/01/2008 sampling, and for east stream and coastal site on the dry condition sampling on 30/1/2008. Levels of 0.03 µg/L were detected at the two west stream sites. Levels of 0.03 µg/L are not consistent with a significant local source of human faecal pollution in the vicinity.

Interpretation: This indicates that the source of elevated faecal counts does not contain appreciable levels of FWAs. Possible human sources in the area could be tested directly for FWA content – if they contain FWAs then it would suggest they are not a source. If they don't contain FWAs, then it simply means this test is not helpful in source discrimination in this area.

Table 2. Sample details and FWA results from Momorangi water samples.

Client Ref No	ESR Ref No	Site	Sample Details	Date sampled	Rainfall (72 hr total)	E.coli	Enterococci	FWA
20080614	CPH0810343	MOS-004	west stream @road bridge	23/01/2008	15mm	1,400	1,100	<0.01
20080612	CPH0810341	MOS-005	west stream@mouth	23/01/2008	15mm	1,400	530	<0.01
20080615	CPH0810344	MOS-002	east stream@road bridge	23/01/2008	15mm	1,500	1,600	<0.01
20080613	CPH0810342	MOS-006	east stream@white footbridge	23/01/2008	15mm	1,100	1,900	<0.01
20080632	CPH0810345	MOM-001	coastal bathing water site	23/01/2008	15mm	75	87	<0.01
20080714	CMB08215	MOS-004	west stream @road bridge	30/01/2008	0.5mm	164	453	0.03
20080712	CMB08213	MOS-005	west stream@mouth	30/01/2008	0.5mm	324	238	0.03
00080715	CMB08216	MOS-002	east stream@road bridge	30/01/2008	0.5mm	222	238	<0.01
20080713	CMB08214	MOS-006	east stream@white footbridge	30/01/2008	0.5mm	271	697	<0.01
20080710	CMB08217	MOM-001	coastal bathing water site	30/01/2008	0.5mm	192	< 10	<0.01
20081696	CMB08282	MOS-004	west stream @road bridge	13/03/2008	1.5mm	111	124	
20081694	CMB08281	MOS-005	west stream@mouth	13/03/2008	1.5mm	560	222	
20081697	CMB08284	MOS-002	east stream@road bridge	13/03/2008	1.5mm	<10	164	
20081695	CMB08283	MOS-006	east stream@white footbridge	13/03/2008	1.5mm	478	782	
20081692	CMB08285	MOM-001	coastal bathing water site	13/03/2008	1.5mm	429	64	

## PCR results

A number of PCR markers were detected in water samples collected at Momorangi (Table 2). This is a complex environment with multiple faecal inputs. While the current analysis has not resolved the issue, the following observations could be made:

- All but one of the samples analysed contained markers indicative of wildfowl inputs;
- Animal markers were also detected in samples including the coastal bathing site
- Bacteroidetes markers indicative of human inputs and also possum inputs were also detected. As these markers display cross-reactivity with each other, the presence of both could mean either human source, possum source or both.
- MOM-001 sampled on 16/03/2008, contained bacteroidetes human marker and also human indicative *B. adolescentis* marker, with weak possum marker. This is suggestive of human inputs.

Resolving the possum vs human issue would require the use of additional PCR assays, or faecal sterol analysis.

Table 3. PCR results from Momorangi water samples

Site	Date sampled	<i>E.coli</i>	Enterococci	TotalBac	Human Bacteroidetes	Human Badolescentis	Wildfowl	Ruminant/ Animal	Possum
MOS-004	23/01/2008	1,400	1,100	positive	positive	faint positive	positive	not detected	positive
MOS-005	23/01/2008	1,400	530	positive	positive	faint positive	positive	positive	positive
MOS-002	23/01/2008	1,500	1,600	positive	positive	not detected	positive	not detected	positive
MOS-006	23/01/2008	1,100	1,900	positive	positive	not detected	positive	not detected	positive
MOM-001	23/01/2008	75	87	positive	not detected	not detected	positive	positive	not detected
MOS-004	30/01/2008	164	453	positive	not detected				
MOS-005	30/01/2008	324	238	positive	not detected				
MOS-002	30/01/2008	222	238	positive	not detected				
MOS-006	30/01/2008	271	697	positive	not detected				
MOM-001	30/01/2008	192	<10	positive	not detected				
MOS-004	13/03/2008	111	124	positive	negative	negative	negative	weak positive	negative
MOS-005	13/03/2008	560	222	positive	negative	negative	positive	weak positive	weak positive
MOS-002	13/03/2008	<10	164	positive	weak positive?	weak positive?	positive	positive	positive
MOS-006	13/03/2008	478	782	positive	negative	negative	positive	negative	negative
MOM-001	13/03/2008	429	64	positive	positive	positive	positive	positive	weak positive

### Faecal sterol analysis

Faecal sterols were analysed in 360ml of sample CMB08285 (MOM-001 from 13/3/2008), and 560ml of a composite sample made from MOS 2, 4, 5 and 6 from (23/1/2008). For comparison a river water sample analysed at the same time known to contain human sewage is included in results.

Low levels of faecal sterols were detected in the two analysed samples. The measured levels do not support human source of contamination. The levels of coprostanol and 24-ethylcoprostanol are too low for ratio analysis to be performed. More conclusive interpretation would require the analysis of larger volume of water – 5 litre or greater volume.

Table 4. Faecal sterol results (Results in PPT)

Site		MOS 2,4,5,6	MOM 1 CMB08285	Human sewage River water
Date		23/1	13/3	
Sterol Name				
coprostanol		1	3	97
24-ethylcoprostanol		3	2	47
epicoprostanol		2	3	16
cholesterol		366	1178	434
cholestanol		24	28	28
24-methylcholesterol		56	17	38
24-ethylepicoprostanol		0	0	4
stigmasterol		140	43	285
24-ethylcholesterol		459	172	348
24-ethylcholestanol		19	8	10
<b>Total</b>		<b>1071</b>	<b>1454</b>	<b>1306</b>
<b>Ratio analysis</b>				
coprostanol/cholestanol	ratio >0.5 indicates human or animal faecal contamination	0.06	0.09	<b>3.45</b>
24-ethylcoprostanol/24-ethylcholestanol	ratio >0.5 indicates human or animal faecal contamination	0.15	0.24	<b>4.84</b>
%Coprostanol/total sterols	> 5.6% suggests human faecal contamination	-	-	<b>7.4%</b>
5 $\beta$ /(5 $\beta$ +5 $\alpha$ stanols)	ratio > 0.7 suggests human faecal contamination	-	-	<b>0.78</b>
coprostanol/24-ethylcoprostanol	ratio > 1.0 suggests human faecal contamination	-	-	<b>2.05</b>
coprostanol:epicoprostanol	ratio > 2.0 suggests fresh/untreated sewage	-	-	6.12
coprostanol/coprostanol+24-ethylcoprostanol	>75% suggests 100% human source	-	-	67.2%
Estimate of % Human sterols		-	-	83.45%

## Overall conclusion

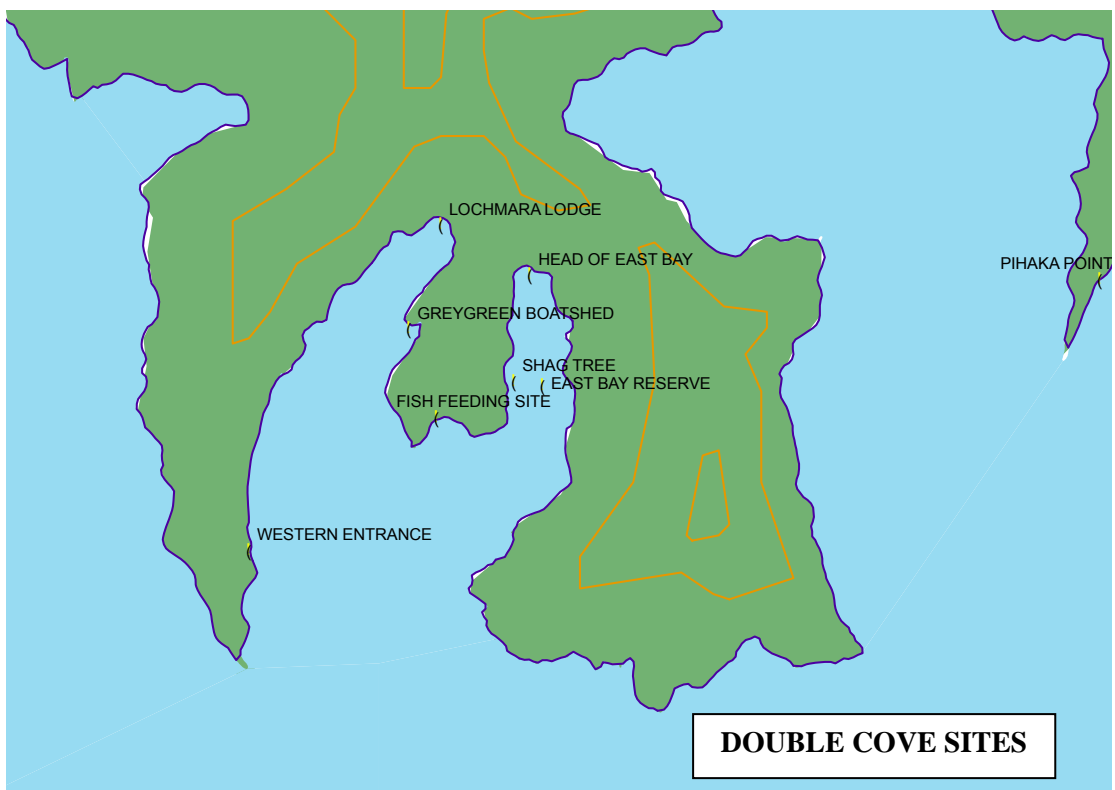
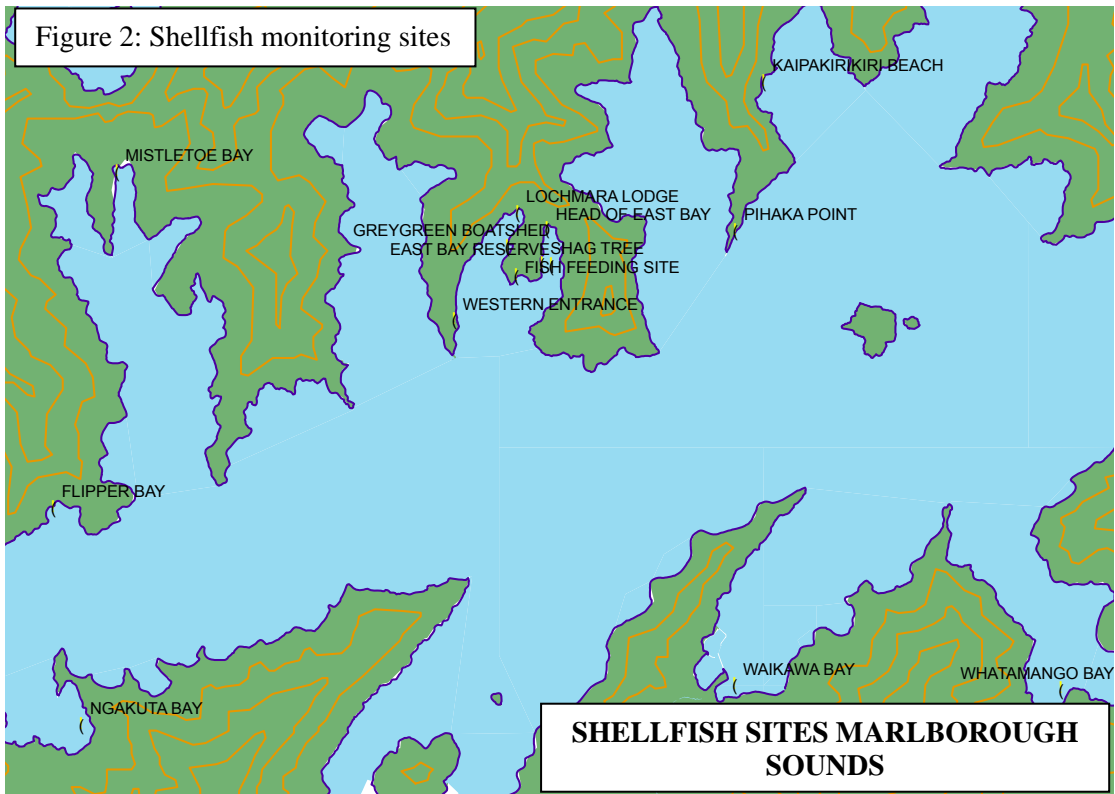
The source of faecal pollution remains unresolved. Although the PCR markers suggested some human inputs, the presence also of possum markers means that the detected signal could be due to either human or possum sources.

The human source is not supported by FWA or faecal sterol analysis. This strength of this support must be tempered by the possibility that potential human sources may not contain FWAs - this could be tested. The faecal sterol levels were low, but higher volume of sample should be analysed to confirm the lack of human input, and perhaps to begin attribution to other sources.

Overall conclusion is that wildfowl inputs are present. There are not significant human inputs to the samples tested. If a human input is involved it will be after movement through soil, or from considerable distance. Certainly not direct inputs at the local scene.

## Shellfish sites, Marlborough Sounds

Shellfish are monitored a number of sites in Marlborough Sounds, the locations of which are indicated below (Figure 2).



Elevated levels of *E. coli* in shellfish collected from these sites have been detected on a number of occasions (Appendix 1). The Ministry of Health and the New Zealand Food Safety Authority recommend that not more than 10% of samples should exceed a level of 230 *E.coli*/100g and no sample should exceed 330 *E.coli*/100g. The possible sources of faecal contamination at these shellfish sites are shags, poorly performing septic tanks and illegal dumping of sewage from boats. Domestic and feral animal contributions are suspected to be low.

Based on previous sampling it was decided to focus on key sites. In this situation, analysis of water samples was unlikely to be useful, as generally very low levels of contamination detected, compared to heavily contaminated shellfish. Two strategies were investigated.

### **Strategy 1 - Direct PCR on shellfish**

Contaminated shellfish were provided, with DNA extractions performed both of the guts and the tissue. Extensive application of PCR assays, and modification of extraction procedures, was unable to extract *Bacteroidetes* spp. or *E. coli* DNA from the shellfish at levels sufficient for these faecal source tracking tools to be used.

Conclusion: Direct PCR on shellfish using *Bacteroidetes* based markers is not currently able to be applied to shellfish. Further development will be required.

### **Strategy 2 - Rep-PCR**

An alternative strategy investigated was genotyping of *E. coli* using Rep-PCR with key question:

*Is genotyping of E. coli from shellfish a useful technique for determination of faecal source?*

Rep-PCR (Appendix 2) is a DNA fingerprinting method which is able to generate a barcode-like fingerprint from *E. coli*. The first step is to recover *E. coli* from shellfish.

Standard analysis of *E. coli* in shellfish uses enrichment techniques, which grow *E. coli* in broth culture and then using MPN format enumerate them. For genotyping, enrichment can't be used as it will alter significantly the types of *E. coli* present, with many being indistinguishable to each other as they are clones. A direct plating method is therefore required. Without enrichment, the detection limit for recovery of *E. coli* will be higher.

### **Shellfish samples - 31 January 2008**

Samples    CMB08220A-D (East Bay)  
              CMB08221A-B (Shag Tree)  
              CMB08222A-B (Fish feeding site)  
              CMB08223A-D (western entrance)

The guts were dissected out from 5 mussels, and pooled together to give one sample (each sample indicated by letter on the end of base number eg. CMB08220A). For East Bay and Western Entrance sites a total of 20 mussels were processed while for the other two sites only 10 were processed. The pooled gut samples were homogenised in an equal weight/vol of buffered

peptone water in whirlpak bags in a stomacher, and 1ml of straight supernatant was plated onto chromocult agar and incubated overnight at 35°C. A shag poo sample was processed at the same time, but with dilutions made.

### **Shellfish samples 28 February 2008**

Sample Number CMB08264- Shag Tree DCO-2

CMB08265- Western Entrance DCO-7

The gut was dissected out from the rest of the mussel flesh. For each of these samples, guts (G) and flesh (F) were processed separately. Guts were approximately 20-30% of the total mussel weight (excluding shell). Dissected guts and remaining flesh were placed into separate whirlpak bags, to which an equal volume of Peptone water was added and then homogenised using a stomacher machine. 1ml aliquots of supernatant were plated directly onto five chromocult plates per sample. Plates were incubated overnight at 35°C, dark blue and turquoise colonies were selected, and rep-PCR performed.

Samples from 13<sup>th</sup> February were analysed only in unsuccessful PCR assay.

### **Results**

A range of Rep-PCR profiles were observed in *E. coli* recovered from the shellfish (Figure 3, Table 4). The shag sample analysed contained two genotypes of *E. coli*, twenty of which were Rep-001, and two of which were Rep-018. The Rep-018 profile was also found in isolates recovered from shellfish at Shag Tree (Figure 4).

We had insufficient isolates from known sources in the area to be able to assign likely sources to the other isolates. Our database does contain isolates from municipal sewage (Christchurch) and dairy cow faeces. These clustered separately to the shag and shellfish profiles, except for the Rep 22, 23 and 24 *E. coli* isolates from Western Entrance Shellfish samples, which clustered among municipal sewage isolates (Figure 5).

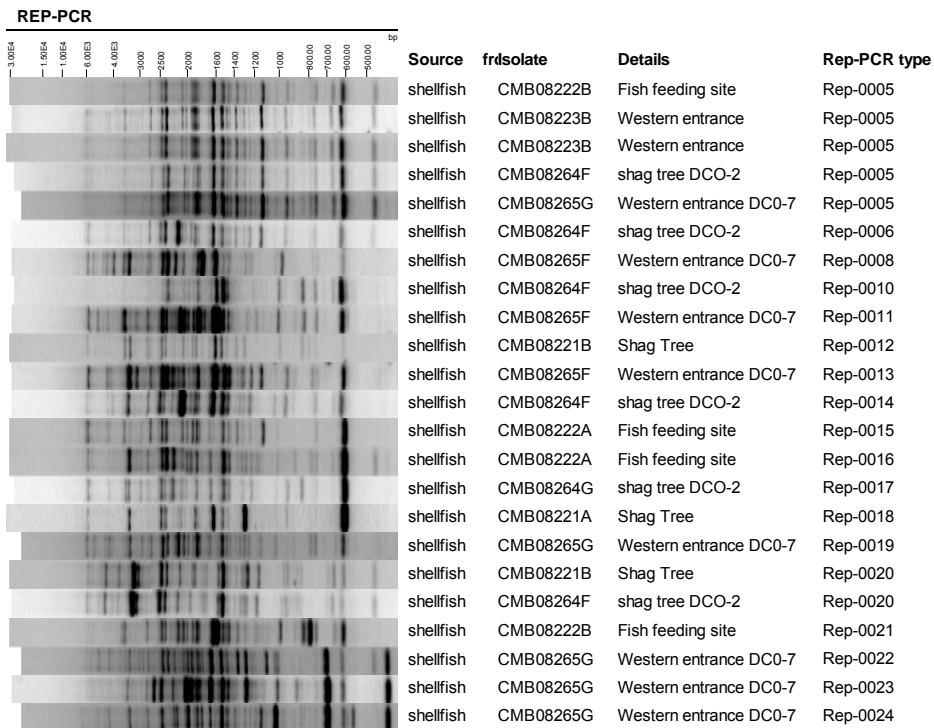


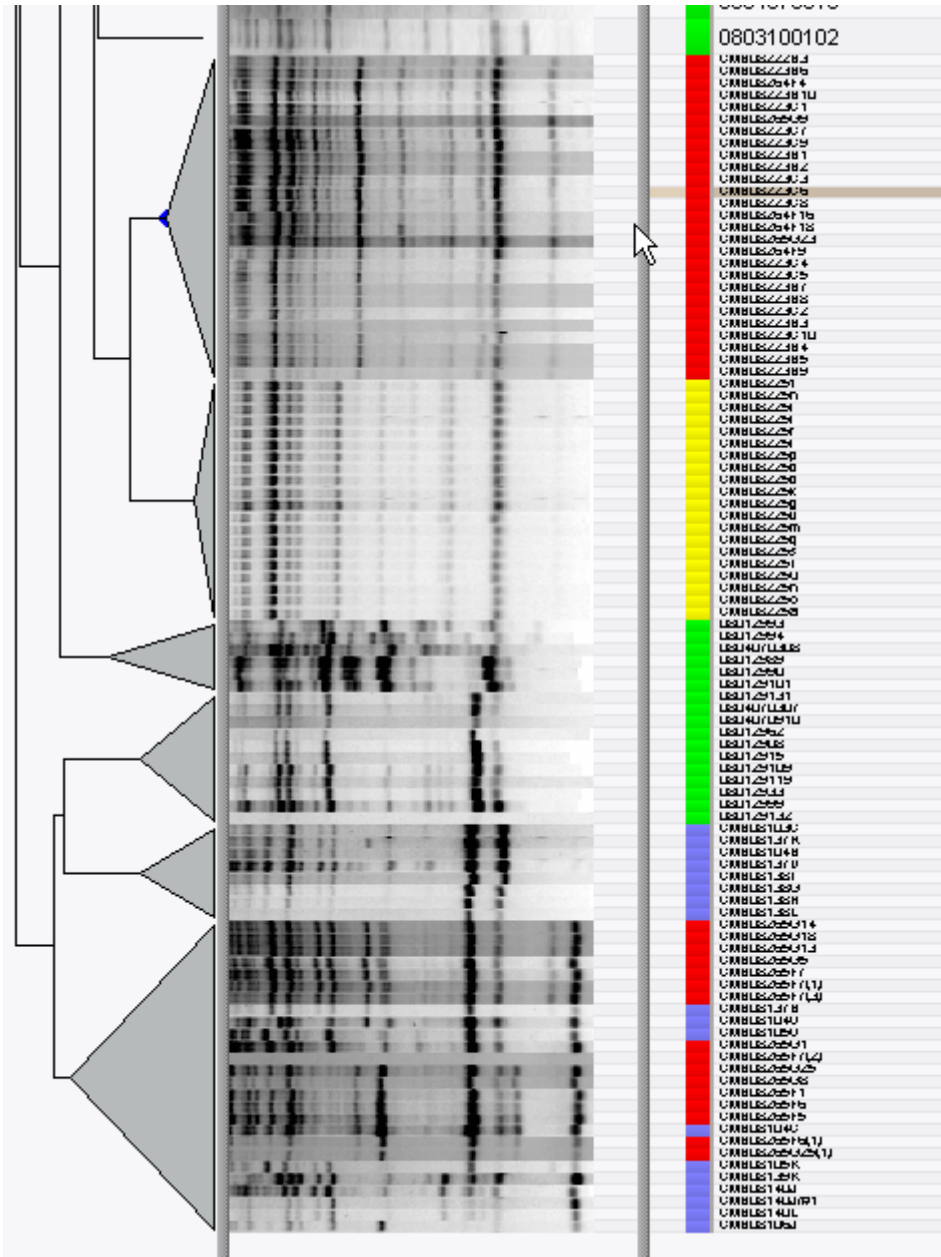
Figure 3. Range of Rep-PCR profiles identified.

Table 5. Rep-PCR profiles identified at each site. Each profile indicates a different Rep-PCR fingerprint or DNA banding pattern. Rep-18 pattern was observed in both the Shag faecal sample and shellfish sampled on the same day.

Sample	Reference	<i>E. coli</i> (MPN/100g)	Rep-PCR profiles (number of isolates with that profile)
31 January 2008			
Shag faecal	CMB08225	not sampled	<b>Rep-18 (2)</b> , Rep-01 (20)
Shag Tree shellfish	CMB08221A	500	<b>Rep-18 (10)</b>
	CMB08221B		Rep-12 (1), Rep-20 (4)
Fish feeding site	CMB08222A	40	Rep-16 (3), Rep-15 (2)
shellfish	CMB08222B		Rep-05 (1), Rep-21 (4)
Western entrance shellfish	CMB08223B	110	Rep-05 (10)
	CMB08223C		Rep-05 (10)
28 February 2008			
Shag Tree shellfish	CMB08264F	1300	Rep-05 (4), Rep-06 (1), Rep-10 (09), Rep-14 (3), Rep-20 (1)
	CMB08264G		Rep-06 (1), Rep-10 (15), Rep-14 (1), Rep-17 (1)
Western entrance shellfish	CMB08265F	1300	Rep-08 (1), Rep-11 (1), Rep-13 (1), Rep-22 (1), Rep-24 (3)
	CMB08265G		Rep-05 (2), Rep-19 (16), Rep-22 (4), Rep-23 (1), Rep-24 (2)

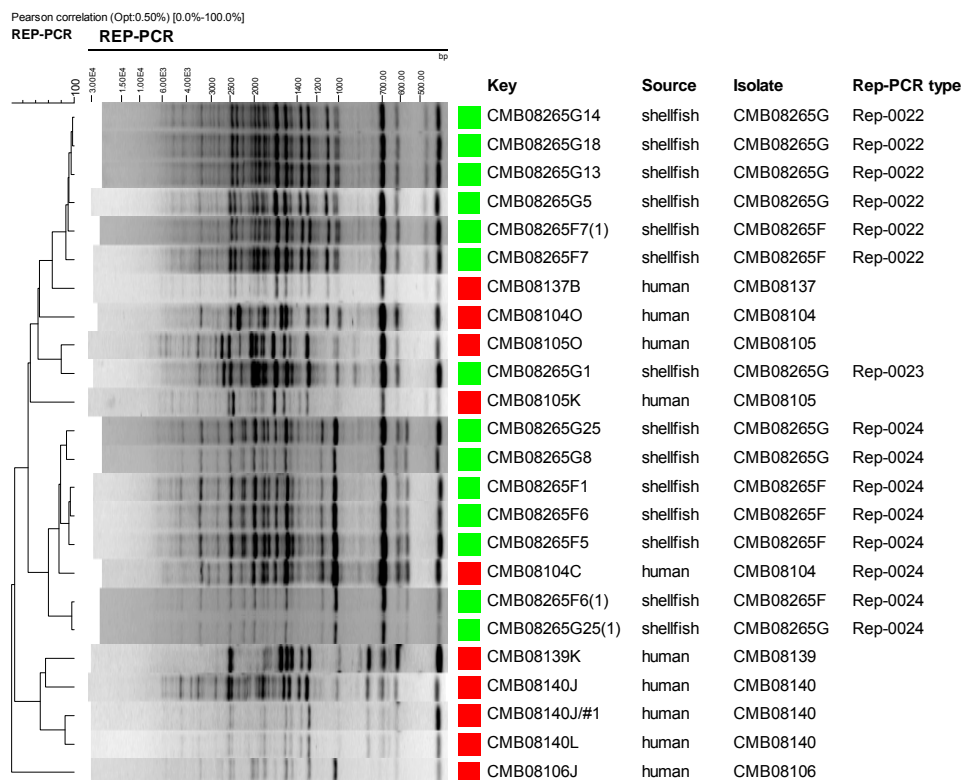


Figure 5. Possible clustering of shellfish and municipal sewage isolates. Shellfish isolates were all from Western Entrance. Software analysis clusters together similar isolates. Shellfish isolates (red) clustered with some human isolates (blue). Shown for comparison are bovine (green), shag (yellow) isolates.



An enlargement of the shellfish and human clustering is shown in Figure 6.

Figure 6 An enlargement of the shellfish and human clustering is shown below (Note shellfish are green and human red)



## Conclusion

Rep-PCR analysis requires analysis of a much larger number of isolates for more conclusive source attributions to be made. This work does however demonstrate the technical feasibility of the analysis. The degree of separation of Rep-PCR genotypes between sources remains to be quantified. However if suspected sources could be sampled concurrently with a contaminated source, it would appear that comparisons of Rep-PCR types could be made, and estimates of contributions made.

Some sites have similar profiles and therefore possibly similar sources.

The finding of the same type (Rep18) in shag poo and shellfish is suggestive that shag poo was a source of contamination of the shellfish.

We compared the isolate patterns from this study, with isolates from municipal sewage (Christchurch) and dairy cow faeces. Our computer software undertakes a statistical comparison of the similarity of the rep-PCR patterns. Patterns that are more similar will cluster together, while those that have greater differences will be positioned separately on the dendrogram. The sewage and cow isolates clustered separately to the shag and shellfish profiles, except for the Rep 22, 23 and 24 *E. coli* isolates from Western Entrance Shellfish samples, which clustered among municipal sewage isolates (Figure 5 and 6).

### Appendix 1 - Previously measured levels of *E. coli* at shellfish sites.

DATE	Rainfall @ Waikawa (mm in 72 hrs)	Shag Tree	East Bay Reserve	Head of East Bay	Western Entrance	Waikawa Bay	Fish Feeding Site	Mistletoe Bay	Lochmara Lodges	Whatamango Bay	Pihaka Point	Grey (Green) Boatshed	Flipper Bay	Tirimoana Terrace	Outward Bound	Ngakuta Bay	Ngakuta Bay	N B
		DCO-2	DCO-3	DCO-1	DCO-7	WKB-8A	DCO-5	OB-2	DCO-4	WMB-1	KAB-002	DCO-6	FLB-1	TIR-5	GRO-1	NGK-1	NGK-3	N B
14-Dec-06	3	5400	1100	90	2400	110	500	310	20	10	-	110	40	70	160	10	20	N B
4-Jan-07	1	2400	90	70	750	70	110	220	20	20	-	10	750	20	70	20	160	N B
18-Jan-07	4	70	1700	160	220	5400	110	1300	18000	40	-	10	10	10	10	310	40	N B
1-Feb-07	18	750	2400	1100	16000	500	750	40	90	40	-	1700	20	50	70	160	10	N B
15-Feb-07	12.5	310	3500	110	18000	20	310	110	70	110		20	3500	20	10	110	70	N B
1-Mar-07	8	3500	2400	1700	200	1300	3500	70	110	90		70	40	40	70	20	70	N B
15-Mar-07	15.5	10	40	750	90	500	310	500	160	310		10	110	1400	310	20	3500	N B
29-Mar-07	1.5	40	500	20	10	70	10	310	40	10		90	20	130	40	20	110	N B
24-Jul-07	0	220	110	1300	40	10	10	10	110	10	-	310	10	-	-	-	-	N B
7-Aug-07	20	1300	10	50	10	20	40	70	20	10	40	20	10	-	-	-	-	N B
21-Aug-07	2.5	310	20	500	10	10	10	110	70	10	40	40	10	-	-	-	-	N B
28-Aug-07	0	220	140	40	40	20	70	20	20	20	110	10	40	-	-	-	-	N B
3-Sep-07	0	430	750	20	200	40	20	20	10	20	600	10	10	-	-	-	-	N B
11-Sep-07	18.5	40	10	10	20	20	40	20	20	10	10	10	70	-	-	-	-	N B
19-Nov-07	0	750	10	40	70	70	250	1100	10	10	10	40	10	-	-	-	-	N B
20-Dec-07	65	9100	310	750	500	310	750	310	220	1300	110	40	500	-	-	-	-	N B
3-Jan-08	0	1300	70		70	70	70	70	310	20	70	200	20	-	-	-	-	N B
17-Jan-08	0	1800	20	110	500	750	110	20	20	160	10	40	40	-	-	-	-	N B
31-Jan-08	0.5	500	20	750	110	40	40	40	220	10	10	18000	40	-	-	-	-	N B
13-Feb-08	20	750	10	10	3500	200	10	40	500	310	40	10	10	-	-	-	-	N B
28-Feb-08	0	1300	10	160	1300	500	40	70	1100	70	20	10	70	-	-	-	-	N B
10-Mar-08	1.5	10	1400	9100	110	200	110	160	310	310	310	90	20	-	-	-	-	N B
1-Apr-08	38	2800	500	5400	70	40	20	90	220	90	40	70	40	-	-	-	-	N B
No. of samples		23	23	22	23	23	23	23	23	23	14	23	23	8	8	8	8	N B
MEDIAN		750	110	135	110	70	70	70	90	20	40	40	40	45	70	20	70	N B
% OF TIME >230/100G		70	43	41	35	30	30	26	22	17	14	13	13	13	13	13	13	N B
HIGHEST		9100	3500	9100	18000	5400	3500	1300	18000	1300	600	18000	3500	1400	310	310	3500	N B



## Appendix 2 - Rep-PCR overview

Repetitive extragenic palindromic-PCR (Rep-PCR), is a type of polymerase chain reaction that targets the repetitive sequences in bacterial genomes using specific primers that are designed complementary to bacterial interspersed repetitive sequences.

### rep-PCR genomic fingerprinting protocol

