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Advanced Indicators for the Identification of Faecal Pollution Sources

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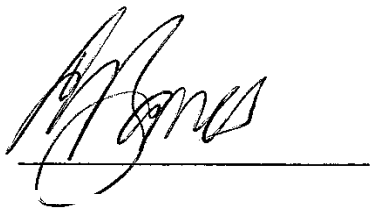

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Advanced indicators for the identification of faecal pollution sources

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Prepared for
Auckland Regional Council

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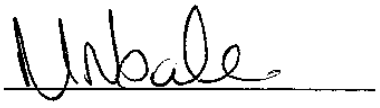
Dr Martin Neale  28 March 2008

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1 Executive Summary

This report describes each of the currently available tools for faecal source discrimination.

The most basic level of discrimination is the ability to detect human faecal pollution, usually in a background of non-human pollution. Fluorescent whitening agents (FWAs) are the primary tool recommended for this purpose. FWAs are components of most washing powders and in both septic tanks and community effluent, become associated with human faeces. As such, FWAs are a useful indicator of human effluent. FWAs are relatively easy to assay and in a number of studies have proven their usefulness. As chemicals however they may have different movement and survival characteristics to microbial pathogens that are usually principal concern. As such, FWAs should be supported by additional source-specific indicators.

There are a number of micro-organisms such as *Bifidobacterium adolescentis*, and some strains of *Bacteroides* which appear specific to humans. Other organisms such as *Rhodococcus coprophilus* appear restricted to herbivores. Traditional culturing and identification is not practical for many of these organisms. The polymerase chain reaction (PCR) makes the specific detection of these and other source specific organisms possible. Total DNA can be extracted from a water sample, and screened with a suite of PCR assays specific to different organisms. ESR is currently evaluating and developing this methodology which should not only allow the confirmation of human effluent, but the identification of non-human components.

Faecal sterol analysis is also useful, particularly for the identification of human effluent. While less distinctive, animal faecal pollution can also be identified to a limited extent. Faecal sterol analysis is however time consuming and expensive, and therefore best suited to very targeted sample evaluation.

Practical examples of the use of some of these indicators in New Zealand situations is presented, together with some general conclusions.

A number of other faecal source indicators are also described in this report including:

- phenotypic and genotypic subtyping of E. coli and enterococci by antibiotic resistance, ribotyping and rep-PCR,
- detection of caffeine, bile salts, and LABs
- detection of bacteriophages and viruses

- faecal coliform:faecal streptococci ratios

In their current state of development, these other indicators are not recommended, because they do not offer significant benefits to faecal source discrimination in New Zealand. Faecal source discrimination is an area of science that, due to increasing interest worldwide, is developing rapidly.

2 Recommendations

The traditional microbial indicators remain the best tools for routine evaluation of microbial water quality. When elevated levels of faecal indicators are detected, and identification or confirmation of the source is required, the following general strategy is recommended.

1. Undertake a thorough site examination around the areas of concern. Utilise local knowledge from residents, farmers, local regulators and others to design a sampling strategy to cover temporal and spatial variations. Further sampling for traditional indicators to confirm the severity and consistency of the contamination is recommended. Identification of sampling locations by GPS, and with digital photographs is useful.

2. Undertake a preliminary collection of samples, and analyse for E. *coli* or enterococci, molecular markers, and FWAs.

3. Where the most significant contamination is identified, resample with a more comprehensive and targeted strategy. Repeat analysis for indicators in step 2, and collect, and filter water samples (4 litres or more) for later faecal sterol analysis if required.

4. Where sewage pipe leakage is suspected, additional evidence could be gathered through the use of television camera inspections of sewage pipes, and rhodamine WT tracers to follow exfiltration. In addition, determination of flow rates in pipes could also assist with evaluation of exfiltration.

5. To confirm suspected sources, additional source discrimination tools may be useful to clarify or exclude potential sources. For example *Clostridium perfringens* in comparison with faecal coliforms may indicate canine pollution, while specific bile salts are specific to pigs.

3 Introduction

The public has become increasingly aware of the potential health hazards of faecally contaminated water. This heightened awareness is resulting in an increased frequency of water quality monitoring for the traditional microbial indicators, faecal coliforms, *E. coli* and enterococci. There is also an expectation that when these indicators are detected, corrective action will be taken to eliminate these faecal indicators - and by inference the faecal pollution - from the water. While these traditional indicators are usually a good indication of microbial quality, and therefore the health risk posed, they provide little guidance as to the source of the faecal pollution. Faecal coliforms and other traditional indicators are present in the faeces of humans, cows, sheep, dogs, ducks, seagulls and a wide range of other animals. Identifying the source of faecal pollution can be crucial for effective water management, particularly when for example, a single seagull can excrete per day as many as 3×10^8 faecal coliforms and 3×10^9 enterococci (Wood and Trust 1972).

Faecal source discrimination is a developing area of research worldwide. The 2003 American Society for Microbiology Annual Conference had 48 presentations covering the development or use of faecal source discrimination tools, compared with only three in 2000. In Europe an EU funded group from Spain, The Netherlands, United Kingdom, Sweden, and Cyprus are collaborating to evaluate a number of approaches, including the use of faecal sterols, antibiotic resistance profiles and bifidobacteria.

This report describes the current state of the most promising and popular approaches to faecal source discrimination, and for each draws a conclusion on its applicability to New Zealand. The Foundation for Research Science and Technology have funded a 6 year research project evaluating many of these tools, and together with the Auckland Regional Council has allowed these to be applied to real life situations. The results of these studies are also presented. Finally a recommended approach to faecal source discrimination is presented along with proposed research directions.

4 Microbial indicators

4.1 Total coliforms, faecal coliforms and *E. coli*

The coliform group of bacteria consists of gram negative, non spore forming, oxidase negative, rod-shaped facultative anaerobes that are defined by their ability to ferment lactose (using the enzyme galactosidase) with the production of acid and gas within 48 hours at 36 ± 2°C. The bacterial genera included in the coliform group are *Escherichia, Citrobacter, Klebsiella and Enterobacter*. These total coliforms can be of both faecal origin (human and animal waste from septic systems, sewage, animal dropping etc) and/or vegetative origin (from soil, vegetation, sediment, insects etc.).

A subset of total coliforms are the faecal coliforms (FC), which are those coliforms that ferment lactose & produce gas at 44.5 +/-0.20°C within 24 +/- 2 hours. This group includes *Escherichia, Klebsiella* and *Citrobacter*. Faecal coliforms are usually associated with human or animal waste. The enteric bacterium ***Escherichia coli***, is the member of this subgroup most commonly cited as an exclusive indicator of faecal contamination.

Thus total coliforms, faecal coliforms and *E. coli* are all indicators of water quality, and are associated with bacteriologically polluted water. All may be associated with disease causing organisms. The presence of total coliforms may not indicate any pollution derived from faeces. Faecal coliforms and more specifically *E. coli* are stronger evidence of faecal pollution, but not whether it is from human sources, or from animal or bird sources.

The Ministry for the Environment and the Ministry of Health guidelines for drinking water stipulate less than 1 *E. coli*/100mL, and for fresh water bathing less than 126 *E. coli*/100mL. For fresh water bathing, counts greater than 273 *E. coli*/100mL require a sanitary survey to report on sources of contamination.

Conclusion: Faecal coliforms and *E. coli*, while generally good indicators of faecal contamination of water, are unable to discriminate between sources of faecal pollution.

4.2 Ratio of faecal coliforms to faecal streptococci

The group comprising the faecal streptococci (FS) includes the enterococci *Entercoccus faecium, E. faecalis, E. durans, E. avium, E. gallinarum* and two non-enterococci, *Streptococcus bovis* and *S. equinus* (Sinton *et al.*, 1998). The interest in the FS group as indicators of faecal pollution stems from the fact that they are present in human faeces

at concentrations of approximately 10^6 per gram, which is significantly less (by a factor of 10-100) than the concentration of faecal coliforms in human faecal material. In comparison, their concentration in domestic animal faeces such as sheep faeces (10^7/g) and cow faeces (10^6/g) is higher than that of faecal coliforms in the same animals.

This lead to the establishment of a **faecal coliform:faecal streptococci (FC:FS) ratio** to indicate the likelihood of contamination sources. In human faeces the FC:FS ratio is reported to be >4 in contrast to a ratio of <0.7 in animal faeces. This ratio was widely used as an approach to identify sources of pollution, but has fallen out of favour due to the number of variables encountered when interpreting results, the most significant of which is differences in survival characteristics between the two groups of bacteria. Studies have indicated that faecal streptococci are more persistent in aquatic environments than faecal coliforms (Sinton *et al.*, 1998). There is also the potential for growth of faecal coliforms in soils in tropical areas, and this may also apply to temperate climates (McLellan *et al.*, 2001). In addition, when there are fewer than 100 FS/100 ml, there are often difficulties in differentiating natural populations of faecal streptococci in soil and aquatic ecosystems from contamination sources. The biggest advantage of the FC:FS ratio is the relatively low cost of setting up the testing procedure.

Conclusion: The different survival rates of faecal bacteria limits the methodology to applications where faecal contamination is of a recent origin (within 24 hours), and not mixed with significant existing faecal indicators.

4.2.1 Ratio Shift of FC:FS

A variation of the FC:FS ratio capitalises on the differential survival rates of the FC and FS groups to look for trends in the shift of the ratio as a means to trace the source of pollution (Feachem, 1975). A sample is assayed, then stored and re-assayed. Important to this shift concept is the fact that human faeces are dominated by the enterococci group, whereas the streptococci group dominates in animal populations. It has been proposed that because the enterococci are more persistent in the environment than the faecal coliforms, contamination by a human source should exhibit an initially high FC:FS ratio, which will subsequently fall as the FC die off. In contrast, if the pollution source is animal in origin it could be expected that the initially low FC:FS ratio would rise as the streptococci are less persistent in the environment compared with the faecal coliforms.

Conclusion: FC:FS ratio shift approach is only reliable with fresh faecal inputs that are not mixed with existing or alternative sources. The assay is also time consuming. As such this ratio has limited practical application and should not be relied on.

4.3 *Clostridium perfringens*

C. perfringens is an anaerobic organism which is valued as a faecal indicator due to its long survival characteristics, which *may* better mimic pathogens such as Cryptosporidium. McBride *et al.* (2002) recorded a maximum of 120 cfu/100 mL in New Zealand surface water samples, with median of less than 1. High levels of *C. perfringens* have been observed in the faeces of dogs – equivalent in number to faecal coliforms (Table 1) (Leeming *et al.*, 1996). High relative levels of *C. perfringens* may therefore be an indicator of dog faecal pollution.

Table 1. Faecal coliform and Clostrium perfringens counts in faeces from selected animals

		Human	Dog	Bird	Sheep
Faecal Coliforms	Cfu/g	107	108	108	107
Clostridium perfringens	Cfu/g	104	108	102	104

Conclusion: There has been very little research to substantiate the validity of use of *C. perfingens* in conjunction with other indicators to implicate fresh canine pollution. While appealing, further investigation is clearly required.

4.4 *Rhodococcus coprophilus*

Rhodococcus coprophilus is a natural inhabitant of the faeces of herbivores. It is a nocardioform actinomycete, forming a fungus-like mycelium, which breaks up into Gram-positive, aerobic, bacterial cells (Sneath *et al.*, 1986). These bacteria contaminate nearby grass and hay, are eaten by herbivores, survive passage through their digestive systems, and thus again infect the voided dung (Al-Diwany & Cross, 1978).

The potential use of *R. coprophilus* as an indicator of herbivorous faecal pollution in water was first recognised by Rowbotham and Cross (1977), who noted its presence in the faeces of domesticated herbivores, pasture run-off and associated receiving waters and sediments, but its absence from human faecal wastes. It has since been frequently isolated from the dung of herbivores such as cows, donkeys, goats, horses and sheep, from poultry reared in proximity to farm animals, and from fresh waters and wastewaters polluted with animal faecal material (Mara & Oragui, 1981; Mara & Oragui, 1983). Savill *et al.* (2001) isolated from cow faecal samples an average of 1.3×10^6 *R. coprophilus* CFU/g of faecal material. Sheep and horse faecal samples contained 2.7×10^5 CFU g^{-1}, while the count in deer was lower, at 5.5×10^3 CFU g^{-1}. All other animals tested, (possum, duck, pig, rabbit) and the five human samples, were negative.

Al-Diwany and Cross (1978) found that *R. coprophilus* counts were reasonably well correlated with faecal streptococci, suggesting a faecal origin. In Africa, Jagals *et al.*

(1995) found *R.. coprophilus* to be more strongly associated with animal, rather than human, faecal pollution of water.

A major impediment to the use of *R. coprophilus* as an indicator of animal faecal pollution is the cumbersome and time-consuming methodology associated with culturing this organism (Rowbotham & Cross, 1977; Mara & Oragui, 1981; Sneath *et al.*, 1986). Conventional culturing takes up to 21 days to establish a result, which severely limits its application. Species identification by biochemical means is also difficult (Bell *et al.*, 1999).

There are two additional problems associated with the use of *R. coprophilus* as an animal faecal indicator. First, the organism appears to be more persistent in the environment than traditional faecal indicators such as *E. coli* and faecal streptococci (Mara & Oragui, 1983). Thus, it may need to be used in conjunction with more short-lived animal indicators. Second, the species has been found in activated sludge scums (Sezgin *et al.*, 1988), which can be composed of both human and animal waste and its reported presence in river foams probably still originated from animal sources (Al-Diwany & Cross, 1978).

Conclusion: Difficulties and the time required to culture *R. coprophilus*, mean that detection of *R. coprophilus* by culturing is not a practical tool for faecal source discrimination.

4.5 Bifidobacteria

Bifidobacteria are anaerobic, gram positive bacteria, which are present in the faeces of humans and animals. Their usefulness as indicators of human faecal contents stems from the high numbers (up to 10^{10} CFU/g) present in the human gut (Kaneko & Kurihara, 1997). This density is typically 10-100 times greater than those of the coliform bacteria (Carrillo *et al.*, 1985).

There are currently 31 known species of bifidobacteria (Miyake *et al.*, 1998). Of these, species such as *Bifidobacterium adolescentis* are associated with human faeces, whereas other species such as *B. thermophilum* appear to be exclusive to animals (Gavini *et al.,* 1991). The human bifid sorbitol agar (HBSA) was developed by Mara & Oragui (1983) to specifically culture human bifidobacteria based on their unique ability to ferment sorbitol. Sorbitol-fermenting bifidobacteria are not reported from other animals with the exception of isolates of bifidobacteria from pigs, which are unable to grow on HBSA (Mara & Oragui, 1983). In the same study *B. adolescentis* was found to be the predominant *Bifidobacterium* species in human faeces but was not isolated from pig

faeces, although previous studies have reported a low frequency of isolation (8%) from pig faeces (Resnick & Levin, 1981).

Rhodes and Kator (1999) have suggested that due to the low survival rates of bifidobacteria in aquatic environments and competition from non-bifidobacteria the HBSA culture method is neither selective nor sensitive enough to detect human faecal contamination in waters receiving diffuse pollution sources. This method requires anaerobic incubation for at least 2 days followed by colony identification to confirm the presence of *B. adolescentis*. This is a labour intensive procedure and biochemical differentiation is sometimes unreliable (Matsuki *et al.*, 1998).

Lynch *et al.* (2002) identified BFM media (Nebra & Blanch, 1999) as the best media for the selective growth of *B. adolescentis* from sewage. From the municipal effluent sampled, a mean of 5×10^3 colonies, all of which were of similar morphology, grew on BFM plates. Hybridization of these colonies with a *B. adolescentis* specific probe identified 90-100% of the colonies as *B. adolescentis*. The other media (YN-17, RB, HBSA, modified HBSA) were less selective, with up to a hundred-fold more colonies isolated from the sewage samples analysed. On these media a wide mixture of colony types were observed, of which only a maximum of 10% hybridised with the *B. adolescentis* probe. Serial dilutions of pure cultures of *B. adolescentis* and *B. thermophilum* grew an equivalent number of colonies on all five media tested, suggesting that maximum recovery of *B. adolescentis* was achieved on BFM.

The growth of species of *Bifidobacterium* and other genera, were evaluated on BFM agar plates. Pure cultures of the *Bifidobacterium* species tested grew well on BFM agar, except for *B. coryneforme* which did not grow at all, and *B. asteroides*, *B. boum*, *B. magnum*, and *B. ruminantium* which only very small colonies grew. Most of the non-bifidobacteria species did not grow on BFM, except for *Rhodococcus equi, Salmonella menston, Staphylococcus* spp. and the *Streptococcus faecalis* strains, which produced very small colonies.

Once deposited into the environment, bifidobacteria are unlikely to reproduce in aquatic environments, due to their requirement for anaerobic growth conditions and poor growth at less than 30°C. This makes them useful as an indicator of recent faecal contamination (Scott *et al.*, 2002a). However there are conflicting reports in the literature about their survival characteristics in aquatic environments. Wheater *et al.* (1980) noted a large decrease in total bifidobacterial numbers (from 10^9 to 10^4) during the initial transformation of faeces into sewage. However this figure had stabilised to 85% of the initial bifidobacteria count at 5°C, and 64% at 18°C. Some researchers have found similar survival between bifidobacteria and coliforms in natural waters (Gyllenberg *et al.*, 1960). The findings of Carillo *et al.* (1985) have suggested that bifidobacteria do not survive as well as *E. coli* in tropical freshwaters. Rhodes and Kator (1999) performed

in vitro studies of *B. adolescentis* survival in filtered estuarine water of varying salinities and temperatures. They reported enhanced survival of *B. adolescentis* at lower temperatures (4 weeks at 10°C) compared with a non-recoverability at temperatures of 23°C and 30°C after 5-9 days. Their in vitro observations correlated with their field studies suggesting that differences in seasonal persistence of *B. adolescentis* could limit its usefulness as a faecal source indicator. This study also reported the negative prevalence of *Bifidobacteria* in sediments from estuarine environments.

Conclusions: Growth of Bifidiobacteria on BFM is indicative of human faecal pollution, although colonies observed may not exclusively be of human origin. Further genetic, biochemical or microbial identification of *B. adolescentis* colonies is technically possible, but probably not practical on a routine basis. There is also a lack of data relating to survival characteristics of Bifidobacteria particularly in comparison to coliform survival. Some studies have suggested that there is an initially high die-off rate of Bifidobacteria, but that this rate slows down and is reduced at lower water temperatures.

4.6 F-specific phages

Bacteriophages (or "phages") are viruses that infect and replicate in bacterial cells. The term coliphage refers to the subgroup of bacteriophages that infects coliforms, including *E. coli* and possibly other Enterobacteriaceae, which are found in the intestinal tracts of all warm-blooded animals. The two major groups of bacteriophages believed to be the most useful as indicators are somatic coliphages and F-specific phages.

The F-specific phages are comprised of two families - F-RNA phages and F-DNA phages - both of which consist of single-stranded genetic material, and do not contain tails (Leclerc *et al.*, 2000). Both types infect host bacteria via the F+ pilus. F-DNA phages infect through the tip of the pilus whereas F-RNA phages infect through its sides (Duckworth, 1987). The F+ pilus is encoded for by the F-plasmid classically found in *E. coli* K-12, but it has also been introduced into other hosts in the laboratory to eliminate detection of somatic coliphages (IAWPRC, 1991).

Serological classification of F-RNA phages yields four general RNA phage groups that may provide some insight into the origin of faecal contamination. Serotypes II and III are mainly isolated from human faeces, while serotypes I and IV are usually found in animal faeces (Furuse, 1987). Molecular probes specific for each serotype have been designed, and allow direct plaque hybridisation to study the subgroups present in a water sample. Subgroups II and III predominate in water contaminated with human pollution, while subgroups I and IV are found more often in animal impacted water (Hsu *et al.*, 1995).

A potential limitation of F-RNA phage however is the different apparent survival rates of the four subgroups (Schaper *et al.*, 2002). Subgroup I appears the most resistant to all inactivating treatments and conditions, while subgroup IV the least resistant.

A second limitation is the apparent low levels of F-RNA phage found in New Zealand sewage and waters. McBride *et al.* (2002) found less than 2 pfu/100 mL in 75% of the water samples assayed. Comparative *E. coli* levels were 100-fold higher, and somatic phage 25-fold higher.

F-DNA phage and somatic coliphages have had little investigation as to their potential for faecal source discrimination.

Conclusions: While the groups of F-RNA phage may shown some source specificity, variability in survival rates, and more importantly the apparent low levels present in polluted water, make F-RNA phage an experimental rather than practical tool at this stage.

4.7 *Bacteroides fragilis* phages

Bacteroides fragilis is one of about 11 species, which are loosely placed together in the'*B. fragilis*' group. They are gram-negative, anaerobic, rod shaped bacteria. Tartera and Jofre (1987) tested twelve strains of different *Bacteriodes*-species and found that one *B. fragilis* strain, HSP40, was detected in 10% of 40 human faecal samples and was not detected in faeces of other animal species. They suggested that the detection of bacteriophages by strain HSP40 of *B. fragilis* could be used to distinguish between faecal pollution of human and animal origin. Grabow *et al.* (1995) found *B. fragilis* phages in 13% of 90 human stool samples but not in any animal or birds feces. Many researchers have investigated the detection of bacteriophages infecting strain HSP40 of *B. fragilis*. Tartera *et al.* (1989) reported that phage infecting *B. fragilis* HSP40 have the same origin as human viruses and were able to multiply under anaerobic conditions, but did not replicate significantly in the environment. Jofre *et al.* (1989) found a significant correlation between the numbers of *B. fragilis* phages and human enteric viruses. Jagals *et al.* (1995) investigated a stream and river exposed to predominately faecal pollution of domestic animal origin and to run-off. *B. fragilis* HSP40 phages were not detected by direct plaque assays in any of their samples. They concluded that more sensitive detection methods were required for the phages. Bradley *et al.* (1999) reported that the numbers of *B. fragilis* bacteriophages, were higher than the other bacteriophages, including F+ bacteriophages in their sampling site but they failed to isolate *B. fragilis* HSP40. They pointed to a lack of these bacteriophages in sewage in their study area and a need to concentrate the samples before assay as reasons for their failure to isolate *B. fragilis*.

Conclusions: The use of bacteriophages of *B. fragilis* HSP40 has the advantage of high specificity for human faecal pollution. In some areas strain HSP40 detects up to 10^5 phage per 100ml of urban sewage and polluted water. However other studies both internationally (Jagals *et al.*, 1995; Bradley *et al.*, 1999; Puig *et al.*, 1999) and in New Zealand (Sinton *et al.,* 1998), have found them in low or zero concentrations both in sewage and in natural polluted water Therefore the use of phages of *B. fragilis* HSP40 may be limited, and currently is not recommended.

4.8 Enteroviruses and other human enteric viruses

When detected enteroviruses, adenoviruses, noroviruses and other human enteric viruses are good indicators of human faecal pollution, due to their apparent host specificity. They are however difficult and often expensive to assay, with appropriate methodology for concentration of large volumes of water the key limitation (Sinton *et al.,* 1998).

Conclusions: Detection of human enteric viruses is a good indication of human faeces. However due to intermittent presence in faeces the absence of these indicators does not exclude human faecal pollution.

4.9 Conclusions

Many of the potential faecal source indicators discussed above have been evaluated and tested at various stages over the last forty years. None have been proven to provide a satisfactory level of discrimination. They may however yet prove useful in conjunction with some of the more recent approaches discussed in the following sections.

5 Subtyping methods

While *E. coli* and enterococci are not source specific, considerable attention has been paid to the possibility that certain strains or subtypes of *E. coli* or enterococci are specific to particular human or animal sources. *E. coli* and enterococci are the most intensively studied indicators, are reasonably cheap and easy to assay, are specified in existing legislation, and in most cases the presence of high levels of these indicators are the reason that sources of faecal pollution are being investigated.

A number of phenotypic and genotypic subtyping methods have been developed, which try to differentiate between the host sources of either *E. coli* or enterococci. A brief description of the major genotypic subtyping methods is included in Table 2. This review however focuses on just three subtyping methods – ribotyping, Rep-PCR and antibiotic resistance analysis.

Table 2. Genotypic Subtyping Systems

Subtyping Method	Target	Brief Description
Pulsed Field Gel Electrophoresis (PFGE)	Entire Genome	Restriction enzyme (RE) cleavage followed by DNA separation on agarose gel. Different DNA cleavage patterns are indicative of strain variation.
Denaturing Gradient Gel Electrophoresis (DGGE)	Entire genome or specific gene e.g. flagellin	RE Cleavage of DNA is followed by denaturing gradient gel electrophoresis which detects differences in the melting behaviour of small DNA fragments (200-700 bp) that differ by as little as a single base substitution
Restriction Fragment Length Polymorphism (RFLP) Amplified Ribosomal DNA Restriction Analysis (ARDRA)	Gene(s) specific Ribosomal DNA	PCR amplification of a specific gene(s) followed by RE cleavage and separation by electrophoresis. Different DNA patterns are indicative of strain variation.
Random Amplified Polymorphic DNA (RAPD)	Entire genome	PCR amplification using short random (non-specific) primers which amplify regions of the genome. The number and location of these sites varies for different strains of a bacterial species. Separation of the PCR products by electrophoresis generates different patterns, which are indicative of strain variation.
Amplified Fragment Length Polymorphism (AFLP)	Entire genome	Restriction digestion of genomic DNA by two REs. PCR of the fragments by two primers based on the two RE sequences amplifies only those fragments flanked by both RE sites. One of the primers contains a fluorescent or radioactive label and PCR products are analysed on denaturing polyacrylamide gels. 80 – 100 bands are generated by this technique.
Multi Locus Sequence Typing (MLST)	Entire genome	Double stranded DNA sequencing of at least 7 conserved genes in an organism. Comparison of the allelic differences within each gene is indicative of strain variation.
Ribotyping/riboprinting	Multiple copies of ribosomal RNA gene(rRNA)	Cleaved genomic DNA is electrophoresed followed by Southern blot hybridisation with a probe specific for rRNA genes.

5.1 Ribotyping

E. coli and other micro-organisms contain multiple copies of the ribosomal RNA (rRNA) gene loci within their genome. The presence of multiple copies of the rRNA and its highly conserved nature make it a useful target for subtyping by the technique termed ribotyping. This technique involves cleavage of the genomic DNA by restriction enzymes (R.E.) and electrophoresis of the resulting DNA fragments on an agarose gel, which separates the DNA into distinct bands. The DNA bands containing fragments of rRNA genes are detected by Southern blot hybridisation with a probe specific for ribosomal genes (Scott *et al.*, 2002a). The resulting DNA pattern (fingerprint) produces a high level of discrimination between *E. coli* subtypes. The number of DNA bands generated depends on the R.E., for example, *Hind*III for *E. coli* produces between four to 12 bands over a size range of 0.7 to 20 kilobases (kb) (Parveen *et al.*, 1999).

Ribotyping was the subtyping tool applied to determine the sources of faecal contamination of four beaches in the San Diego, USA (CSDDEH, 1999). The study obtained 489 isolates of *E. coli* from water samples and was able to match 353 of these isolates to 12 source groups. These groups came under the broad headings of domestic animals (dogs and cats), humans, avian species and indigenous mammals, including rodents and racoons. The number of unmatched isolates was 179, which represents 37% of isolates having no known source of contamination. The human isolates provided the predominant matches during dry weather episodes. In contrast, dogs (>30%) and avian isolates (~24%) were dominant in samples collected during wet weather. The results of this study provided direction for development of performance measures for best management practices, including implementing management strategies to reduce faecal loading attributed to dog contamination.

A study of *E. coli* isolates from the Appalachiola Estuarine system in Florida, USA used discriminant analysis of ribotypes to distinguish human (HS) and non-human sources (NHS) of faecal contamination (Parveen *et al.*, 1999). The study concluded that 97 % of NHS and 67% of HS isolates were correctly classified, with an average rate of correct classification of 82%. Analysis of *E. coli* isolates obtained directly from human and animal faeces showed that ribotyping correctly classified 67% of human and 100% of animal faeces. This represented an average rate of correct classification of 84%.

Carson *et al.* (2001) applied the same method of discriminant analysis to ribotypes of *E. coli* faecal isolates derived from human and seven animal/bird hosts. The study showed that 95% of the human ribotypes and 99.2% of the collated non-human ribotypes were correctly classified. When all of the eight individual ribotype sources were analysed the overall average rate of correct classification (ARCC) was 73.6%. This classification rate could be improved when the number of groups analysed was limited to three. For

example, a comparison of RTs derived from human, dog and horse faeces revealed an ARCC of 94.2%.

Conclusions: For each organism a large database of ribotypes from known sources is required to enable comparison with the ribotypes of unknown isolates. Database libraries need to contain isolates from a wide geographical range of environments or be exclusively designed for the specific study of a watershed's potential for faecal contamination. The method is expensive and labour intensive. However, automated ribotyping is available for high through-put applications and 16S and 23S rRNA probes are commercially available (Parveen *et al.*, 1999).

5.2 Repetitive element PCR (Rep-PCR)

Repetitive portions of DNA sequence are located throughout the genome of prokaryotes. Primers can be designed to specifically amplify these repetitive elements to generate DNA fingerprints that are specific for species and strains of bacteria (Scott *et al.*, 2002a). Three methods have been developed which target different families of these repetitive elements, including repetitive extragenic palindromic sequence PCR (REP-PCR); enterobacterial repetitive intergenic consensus sequence PCR (ERIC) and BOX PCR, which targets extragenic repeating elements. The number of amplification products (visualised as bands on the agarose gel) generated by REP-PCR is between 13 and 22 and the amplicons range in size from 300 bp to 6 kb. ERIC primers generate 7 to 13 amplicons and are of a similar size range (McLellan *et al.*, 2003). Box primers generate between 18 and 30 amplicons, which increases the discriminatory power of this method in comparison to the other two rep-PCR techniques (Carson *et al.*, 2003). As with other library based subtyping techniques, Rep-PCR requires a large database of DNA fingerprints to which unknown isolate DNA patterns can be compared.

Dombek *et al.* (2000) used BOX and REP primers to generate DNA fingerprints from 154 *E. coli* strains obtained from human and animal faeces. They reported that the BOX primers were more effective in their grouping of the *E. coli* strains into different faecal sources. One hundred percent of chicken and cow isolates were assigned to the correct source, compared with 83% for humans and between 90 and 78% for geese, ducks, sheep and pig isolates. However subsequent work by this group as they have analysed larger number of samples, has identified a huge variety of fingerprint types (M. Sadowsky, personal communication). From almost 2,466 fingerprints generated, 65% of them were unique (Table 3). Therefore to comprehensively represent all types in a catchment, a very large library of several thousand isolates is required.

Table 3. Rep-PCR fingerprints from animal sources.

Animal Source	Individual Animals Sampled	*E. coli* Fingerprints Obtained	Unique Fingerprints
Cat	37	108	53
Chicken	86	231	158
Cow	115	299	203
Duck	42	122	75
Horse	44	114	86
Human	197	307	226
Sheep	37	101	67
Total	**982**	**2466**	**1616**

McLellan *et al.* (2003) conducted a comparison study of the ability of REP-PCR to differentiate between different sources of faecal contamination when compared with ERIC PCR and pulsed field gel electrophoresis (PFGE). DNA fingerprints of *E. coli* isolates generated from multiple samples were collected from sewage (n = 180), gulls (n = 133) and dairy cows (n = 121). The results of the subtyping of a single isolate from the multiple samples revealed a high diversity of subtypes within each sample type by each of the three subtyping methods. Within a host group, pairwise comparison of similarity indices of the REP-PCR fingerprints ranged widely from 98% to 15%. The fingerprints did not cluster into specific host groups, but consisted of subclusters of closely related subtypes. Of these subclusters the majority, which produced a similarity index of 80 % or higher, belonged to the same host group. The sewage isolates were the predominant sample type with subclusters of 80% and higher. Overall the three subtyping techniques were comparable, although PFGE was more discriminatory between closely related subtypes than the two PCR techniques.

In this study McLellan *et al.,* (2003) also performed analyses to determine the reproducibility of the REP PCR and ERIC PCR methods. They concluded that duplicate analysis of the DNA fingerprint of each isolate generated by these two PCR systems was consistent and therefore the techniques were reproducible. The study analysed the diversity of multiple *E. coli* isolates from a single host animal (human/dog/gull) by the REP PCR method. In most samples there was either a single or predominant subtype isolated. The other subtypes were isolated at a much lower frequency. The researchers caution using multiple isolates from the same sample as their results suggested that each sample contains a predominant subtype, which would inflate the frequency of a particular subtype when incorporated into a database.

The high diversity of *E. coli* subtypes (one-third of subtypes were <65% similar to other subtypes) reported in this study is consistent with the findings of Carson *et al.* (2001) and Dombek *et al.* (2000) and confirms that an extensive database for reliable faecal source determination is required.

A comparison study between the ability of rep-PCR and ribotyping to distinguish between *E. coli* faecal isolates from humans and seven non human sources (cattle, pig, horse, dog, chicken, turkey and goose) was performed by Carson *et al.* (2003). The rep-PCR was based on the BOX primer system, which generates the most banding patterns of the three rep-PCR methods and therefore has the higher discriminatory power between isolates. The researchers found that overall rep-PCR using the BOX primer system was significantly superior to ribotyping. The ARCC for ribotyping was 73% in comparison with an ARCC of 88% for rep-PCR for isolates from all faecal sources. Individual examples of rates of correct classification (RCC) for sources that were statistically significant included human isolates with an RCC of 89% by ribotyping and 97% by rep-PCR and dog isolates RCC of 76% compared with 98% (respectively). Only one non-human faecal source had an RCC for ribotyping that was higher compared with the RCC for rep-PCR, however the difference was not statistically significant. The better performance of rep-PCR in comparison with the ribotyping technique was attributed to the higher number of bands (18 to 30) generated per isolate by the rep-PCR. Ribotyping patterns typically consisted of 6 to 12 DNA bands.

Carson *et al.* (2003) also commented on the efficiency of the two methods, reporting that they found rep-PCR to be highly reproducible generating high-quality patterns 95% of the time. In contrast, ribotyping generated high-quality patterns 85% of the time and manual ribotyping required 10 to 12 days for completion of analysis from initial sampling to computer analysis of DNA fingerprints, in comparison to 7 to 8 days for rep-PCR. Ribotyping requires more individual steps during analysis, including a more technically demanding hybridization step.

Conclusions: Rep-PCR is more rapid and cost effective than PFGE and ribotyping. It does not require isolation and purification of DNA, because it is able to utilise whole cell suspensions (Dombek *et al.*, 2000). However it does requires a large database of DNA fingerprints and sophisticated computer software for data analysis.

5.3 Antibiotic Resistance

The antibiotic resistance of isolates from water samples has been used to identify sources of faecal pollution. Either *E. coli* or enterococci isolates are analysed against a panel of antibiotics to generate a multiple antibiotic resistance (MAR) profile.

For example Bahirathan *et al.* (1998) performed antibiotic susceptibility tests with 10 therapeutic antibiotics on yellow-pigmented enterococci. In three antibiotics, cephalothin, erythromycin, and vancomycin, there was a significant ($p =< 0.05$) association between susceptibility and source. Vancomycin resistance was significantly ($p =< 0.001$) higher in isolates from wild sources compared with that in isolates from other sources. Results suggested that vancomycin-susceptibility testing of yellow-

pigmented enterococci may have potential value in the identification of sources of faecal pollution, especially when combined with traditional quantitative methods.

Guan *et al.* (2002) examined resistance to 14 different antibiotics of 319 *E. coli* isolates from the faeces of cattle, poultry, swine, deer, goose, and moose, as well as from human sewage, and clinical samples. Using discriminant analysis 46% of the livestock isolates, 95% of the wildlife isolates, and 55% of the human isolates were assigned to the correct source groups by the MAR method. They found however that amplified fragment length polymorphism (AFLP) analysis was more discriminatory.

Harwood *et al.* (2000) examined the antibiotic resistance patterns of faecal streptococci and faecal coliforms isolated from domestic wastewater and animal faeces to nine antibiotics at four concentrations each. Antibiotic resistance patterns of faecal streptococci and faecal coliforms from known sources were grouped into two separate databases, and discriminant analysis of these patterns was used to establish the relationship between the antibiotic resistance patterns and the bacterial source. The average rate of correct classification for the faecal streptococcus database was 62.3%, and that for the faecal coliform database was 63.9%.

Hagedorn *et al.* (1999) established a database of antibiotic resistance patterns from 7,058 faecal streptococcus isolates from known human, livestock, and wildlife sources in Montgomery County, Virginia. Correct faecal streptococcus source identification averaged 87% for the entire database and ranged from 84% for deer isolates to 93% for human isolates. They isolated faecal streptococci from three highly contaminated sites, and MAR analysis classified almost 80% of isolates as being from cattle, with small proportions from waterfowl, deer, and unidentified sources (approximately 7% each). Based on these results, cattle access to the stream was restricted by installation of fencing and in-pasture watering stations. Faecal coliforms were reduced at the three sites by an average of 94%, from prefencing average populations of 15,900 per 100 ml to postfencing average populations of 960 per 100 ml. After fencing, <45% of faecal streptococcus isolates were classified as being from cattle.

Conclusions: While the MAR method for differentiating between faecal sources can work this method is time intensive for the field and laboratory work and its laboratory procedure is complicated and costly. Parveen *et al.* (1999) noted that the antibiotic resistance patterns of bacteria are influenced by selective pressure and thus maybe different in other geographical areas and may vary over time.

5.4 Limitations of library based methods

There are two potentially serious limitations to library based subtyping of isolates for faecal source discrimination. Firstly the size of the library, and secondly the number of isolates required for analysis.

Library based methods require isolates from known sources which are then subtyped and used to compare unknowns against. Ideally a library should contain all possible subtypes and these should not be represented in more than one group.

Initial publications by Dombek *et al.* (2000) on the use of Rep-PCR were very promising. However as they have analysed larger number of samples, the huge variety of fingerprint types is becoming evident (M. Sadowsky, personal communication). From almost 2,466 fingerprints generated, 65% of them were unique (Table 3). Therefore to comprehensively represent all types in a catchment, a very large library of several thousand isolates is required. There is also the concern that such libraries over represent domestic sources of *E. coli*, such as humans, dogs and farm animals compared with indigenous sources (feral animals and birds). The cost and time delays in establishing such large databases, is a clear drawback to any method based on comparison of isolates from unknown sources with known sources. Several studies of libraries of ribotypes of *E. coli* have also noted the variability of ribotypes over time and geographical distance (Scott *et al.*, 2002b).

Hartel (2002) suggested the size of the reference library could be minimised by building a site specific library based on suspected sources. In this approach, local knowledge and targeted sampling over one day from within a restricted geographical area would allow a focus on a minimal number of sites of contamination. Investigators would then build a source-specific library from potential sources sampled on same day as the unknowns.

If however a sufficiently representative library is built, the second main limitation is the number of isolates that need to be analysed. All the subtyping methods require the isolation of individual colonies which usually require purification and identification before subtyping. In a sample with a 1,000 or more *E. coli* or enterococci, phenotypic or genotypic subtyping based on characterisation of 1-5 isolates per sample may under represent the number of different subtypes in a sample. Hagedorn (2003) recently evaluated - by both statistical analysis (Analysis of Variance, Chi square, kappa, likelihood ratio and Pearson's confidence analysis) and examination of data sets - the number of isolates that need to be analysed within a single sample to accurately classify faecal sources. Statistically at least eight isolates must be analysed within any one sample to achieve significance at the 0.90 level of confidence. Together with

examination of datasets Hagedorn concluded that 24 isolates per sample is necessary for accurate analysis.

5.5 Conclusions

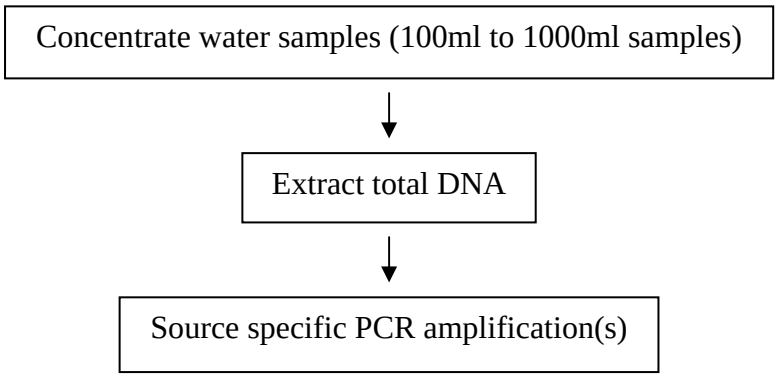
The necessity for isolating and analysing 24 colonies from each sample, and establishing sufficiently large database to compare isolates with, together make genotyping and phenotyping methods cumbersome, expensive. Analysis of fewer samples against smaller libraries is potentially misleading.

6 Host specific molecular markers

A growing field, which researchers in ESR and elsewhere are actively exploring, is the development of PCR assays to detect host specific molecular markers. Some of these PCR reactions target organisms previously proposed as source specific, while others have been identified through a screening process of DNA from different faecal sources.

Molecular markers do not require culturing, and are potentially rapid and precise means of faecal source discrimination. The work processing flow for analysis is shown in Figure 1.

Figure 1. Analysis of molecular markers



We are evaluating molecular markers published in the literature (Bernhard & Field, 2000b; Bernhard et al., 2003) and developing our own markers. The most useful markers we have evaluated to date are described in Table 4, and described in greater detail below.

Table 4. Source specific PCRs in development

PCR amplification	Source specificity[1]	Survival[2]
Bifidobacterium 16S	None – positive control	Short
Ruminococcus	None – positive control	?
Bifidobacterium adolescentis	Human	Short
Bifidobacterium dentium	Human	Short
Fibrobacter	Dairy effluent, pig, sheep	?
Bacteroides	Human	
Rhodococcus coprophilus	Dairy effluent	Long
E. coli pig biomarker	Pig	Medium
?[3]	Dog	
?[3]	Bird	

[1]Specificity only partially tested.
[2]Estimated survival compared to E. coli. Requires experimental confirmation.
[3]Actively looking for markers.

6.1 Rhodococcus coprophilus

R. coprophilus (as described in section 4.4) is a natural inhabitant of the faeces of herbivores. A major impediment to the use of *R. coprophilus* as an indicator of animal faecal pollution is the cumbersome and time-consuming culturing method associated with this organism (Rowbotham and Cross, 1977; Mara and Oragui, 1981; Sneath *et al.*, 1986).

Savill *et al.* (2001) developed a PCR assay for the detection of *R. coprophilus* in animal faecal samples and converted this assay into a quantitative TaqMan™ PCR assay. Quantitative, "real time", 5'-nuclease or TaqMan™ PCR exploits the 5' nuclease activity of *Taq* polymerase to cleave a fluorogenic probe that is annealed to the target sequence during amplification. During PCR, the fluorogenic probe is cleaved generating a fluorescent emission. Measurements of this fluorescence increase throughout the PCR cycles enabling quantitative estimates of the initial quantity of DNA to be made.

The detection limit of the *R. coprophilus* PCR assays (60 CFU per conventional and 1 CFU for TaqMan™ PCR) is sufficiently sensitive to detect *R. coprophilus* in faecal samples, since 1 g of faecal material contains between 5.5×10^3 and 3.6×10^6 CFU depending on the animal. We detected *R. coprophilus* by PCR in all faecal samples where it was identified by culturing (Section 4.4).

We have detected *R. coprophilus* by PCR in dairy faecal samples, dairy effluent and river water samples. We did not observe it in human effluent or faecal material.

The longevity of *R. coprophilus* however remains a potential limitation. The organism appears to be more persistent in the environment than traditional faecal indicators such as *E. coli* and faecal streptococci (Mara and Oragui, 1983), and since PCR may also detect non-viable *R. coprophilus*, the PCR assay may be an even more persistent marker.

Conclusions: PCR assay for *R. coprophilus* appears specific for farmed herbivore faecal pollution. A key limitation may be the extended survival of the organism.

6.2 Bifidobacteria

Bifidobacteria (as described in section 4.5) are present in high numbers in the human gut (Kaneko & Kurihara, 1997), typically 10-100 times greater than those of the coliform bacteria (Carrillo *et al.*, 1985).

Various PCR primer systems (Dong *et al.*, 2000; Matsuki *et al.*, 1998; Wang *et al.*, 1996) have been developed which differentiate between various bifidobacterium species based on the 16S rRNA sequence. However all of these PCR systems also amplify *B. ruminantium* a species isolated from the rumen of cows (Biavati & Mattarelli, 1991). Limited DNA sequencing has been performed on this novel species but the 16S rRNA sequence does provide one region of dissimilarity between *B. adolescentis* and *B. ruminantium*, which can be targeted for a new PCR primers system to differentiate the two species. Another molecular method for differentiating between the various bifidobacteria species is based on the amplified ribosomal DNA restriction analysis (ARDRA) method of (Ventura *et al.*, 2001). This method can also be adapted to distinguish between the different restriction enzyme sites of *B. adolescentis* and *B. ruminantium*. This method is based on amplification of a PCR product, which is subsequently digested with restriction enzymes, which cut the DNA at specific sites. The resulting DNA fragments can be visualised and separated on an agarose gel as for a PCR product.

Nebra *et al.* (2003) have reported a DNA-DNA probe hybridisation method for *B. dentium*, another potential candidate marker for human faecal pollution. The probe targets the 16S rRNA gene of *B. dentium*, which is a *Bifidobacterium* of exclusively human origin (Biavati *et al.*, 1991). The method requires PCR amplification prior to hybridisation to increase the sensitivity of the technique from 10^8 cfu/ml to 10^3 cfu/ml. In mixed bacterial suspensions, the probe was able to detect one *B. dentium* cell in 10,000 non target cells.

Testing of the *B. dentium* probe was performed on environmental samples derived from various sources of sewage and slaughterhouse effluent, and from human and

animal faeces. One of the 29 urban sewage samples and one of the two hospital sewage samples did not hybridise with the probe. Sanitizers used in hospital disinfection routines may have altered the bacterial population of the sewage resulting in low numbers of bifidobacteria, which fell below the detection threshold of this sample. The negative result from the one urban sewage sample may be explained by similarly low bifidobacterial counts as nine of the 28 human faecal samples tested were positive for hybridisation with the probe. This suggests that *B. dentium* may not be present in all human faecal specimens at levels above the detection limit; however this species can still be detected in human sewage, which can be interpreted as a pool of human derived faecal sources.

All samples from animal faeces and the slaughterhouses were negative with the *B. dentium* probe, except for one cattle slaughterhouse effluent that subsequent investigation revealed also had human sewage from toilets (Nebra *et al.*, 2003). The results of the environmental sampling suggest that the *B. dentium* probe is applicable for use as a molecular marker to detect human sources of faecal pollution.

The 16S rRNA sequences of both *B. adolescentis* and *B. dentium* could be utilised to extend the current primer (*B. adolescentis*) and probe hybridisation system (*B. dentium*) to allow for detection by the TaqMan PCR system for either organism. Advantages of this system include the increased specificity over normal PCR, quantitative ability and more rapid format.

Conclusions: *B. adolescentis* PCR is specific for human faeces and raw or primary human effluent. *B. dentium* may be a useful additional marker. There is however a lack of data relating to survival characteristics of Bifidobacteria and particularly their correlation to coliform survival. Initial studies have suggested that there is an initially high die-off rate of Bifidobacteria, but that this rate slows down and is reduced at lower water temperatures.

6.3 LTIIa toxin biomarker for enterotoxigenic *E. coli*

The LTIIa toxin is a heat labile enterotoxin of *E. coli*, which is composed of the products of two genes (Pickett *et al.*, 1987). Strains of ETEC are considered to be host specific due to their requirement for host cell receptor recognition sites within the intestinal wall of the host animal (Krogfelt, 1991). If the receptor sites are not recognised by the ETEC strain then the bacterium is unable to colonise the intestinal wall and cause infection. This degree of strain specification for the host animal has been exploited to develop a cattle specific biomarker for the detection of cattle faecal pollution (Khatib *et al.*, 2002). The biomarker is based on the unique DNA sequence of a portion of the LTIIa genes of ETEC strains that colonise cattle. DNA primers that target this unique sequence have been developed and allow the PCR amplification of a portion of the LTIIa genes.

Specificity of the PCR product to ETEC strains colonising cattle was confirmed by the absence of the PCR product from faecal sources other than cattle pollution. These faecal sources included human sewage, farm and domestic animals and birds. The LTIIa gene also has low identity to the nucleotide sequence of other closely related toxin genes, such as cholera and LTI. This was confirmed by the absence of a PCR product when *E. coli* strains containing plasmids with related toxin genes were screened against the target primers.

Conclusions: Initial studies of LTIIa in New Zealand cattle herds suggests a low prevalence of the LTIIa genes in the cattle population. Additional LTII PCR primers specific to animal groups have been developed, and we have successfully amplified this from pig effluent, and are continuing evaluation.

6.4 Bacteroides

Bernhard and Field (2000a; 2000b) have designed a PCR assay that distinguishes between human and ruminant sources of faecal contamination. The PCR markers are based on the rDNA of the bacterial group Bacteroides-Prevotella, which are strict anaerobes that reside exclusively in the gut of warm-blooded animals. Their anaerobic nature means they are difficult to culture in the laboratory and yet ideal as faecal indicators because they do not survive for long periods once released into an aquatic environment. The difficulty in culturing makes them ideal candidates for detection by molecular methods, which are rapid and circumvent the need for detection methods based on isolation by culturing.

Bernhard *et al.* (2003) evaluated their PCR marker assay on water samples collected from Tillamook Bay in Oregon. Tillamook Bay is a shallow estuary whose watershed covers nearly 150,000 hectares and is drained by five major rivers. This estuary has a long history of high levels of faecal contamination, which have resulted in closure of the bay for recreational and commercial oyster farming operations. Potential sources of faecal contamination include farm animal waste from the estimated 22,000 dairy cattle in the area; sewage treatment plants and septic tank systems. The researchers tested the efficiency of PCR amplification in the presence of varying saline concentrations similar to those encountered in an estuarine environment. They concluded that salinity levels did not affect the assay.

The PCR assay used two specific PCR markers to detect ruminant faecal contamination (including elk and deer) and one PCR marker to detect human faecal sources. Five of the six urban sampling sites and three of the four sewage treatment plants/outfalls were positive for the human marker. Three of the 14 rural sampling sites were positive for the human marker. One of these sites was close to a trailer park and the other two were nearby to or downstream of a sewage treatment plant. The sites that tested

positive for either the ruminant or human specific markers were located in the upper areas of the bay, where previous testing had indicated consistently high faecal bacterial concentrations. Samples from upriver sites and the mouth of the estuary, where the impact of farming and sewage were unlikely, tested negative for all of the faecal contamination markers. There was a discrepancy between detection by the two ruminant specific markers. One of the markers tested positive in 14 water samples, but only eight of these same samples were positive by the second ruminant specific marker. In a previous study (Bernhard & Field, 2000b) both markers had been detected in all ruminant faeces tested, however there is still the possibility that not all ruminants carry both markers. The researchers suggested that further investigation is required to determine if differential survival of the Bacteroides-Prevotella group of bacteria and/or sensitivity of the two ruminant specific PCR assays affects the detection system. Overall they concluded that the PCR assay has demonstrated an ability to distinguish between potential non-point sources of faecal contamination derived from human and ruminant sources.

Conclusions: Initial evaluation of these primers with New Zealand effluent (data not shown) suggests source specificity. Further evaluation is required, and is currently being undertaken.

6.5 Conclusions

We are still building a comprehensive set of PCR assays to test DNA extracted from water samples. Preliminary assays of faecal material and effluent suggest that discrimination is possible. Evaluation in a wide range of environmental water samples is now required, with appropriate concentration of water samples perhaps the key potential limitation.

7 Chemical indicators

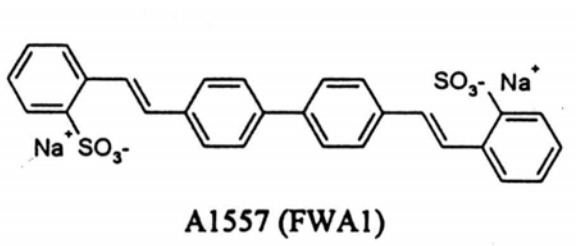
There are a number of chemicals that can be used to indicate faecal sources.

7.1 Fluorescent Whitening Agents

Fluorescent whitening agents (FWAs), which are also called "optical brighteners", are organic compounds that absorb ultraviolet light and re-emit most of the absorbed energy as blue fluorescent light. FWAs are used in manufacturing textiles and paper. They are also added to most washing powders to replace FWAs lost from clothing during wear and washing where they adsorb to the fabric and brighten clothing. Washing powders contain 0.03% to 0.3% (dry weight) of FWAs, with 20-95% bound to fabrics during washing, while the rest is discharged with the washing water. Most household plumbing mixes effluent from toilets with this "grey water" from washing machine. As a consequence, in both septic tanks and community wastewater systems, FWAs are usually associated with human faecal contamination.

There are a range of FWAs, but only one (4,4'-bis[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)-amino]stilbene-2,2'-disulfonate) is used in New Zealand (Figure 2).

Figure 2. FWA1 - 4,4'-bis(2-sulfostyryl)biphenyl



The relevant features of FWAs include:

- FWAs are not known to occur as a natural product.
- Their highly polar nature means FWAs adsorb strongly to polysaccharides in paper and clothing.
- Irradiation by sunlight causes FWAs to bind irreversibly to cellulose of protein, enabling them to bind to cotton and nylon fabrics.

- FWAs are highly water soluble.
- They undergo photochemical degradation, with half-life of several hours under summer noon sunlight.
- FWAs adsorb to the soil, only photodegradation in the topsoil. Below photic zone, FWAs are assumed to be persistent.
- Not readily biodegradable.
- Accumulate on sewage sludge with removal rates of 55-98% in sewage treatment plants.
- No known health effects of FWAs at levels seen in effluent or water.

In New Zealand, Gregor *et al.* (2002) detected FWAs in septic tank and community wastewater at levels between 10 and 70 μg/litre. With a detection limit of 0.01 μg/litre, this allows dilution factor of perhaps 1,000 fold. Close *et al.* (1989) were able to identify septic tank contamination in wells based on the presence of FWAs.

In many of the studies carried out in ESR, we have detected low levels (<0.01 to 0.06 μg/litre) of FWAs in many samples without clear supporting evidence of human pollution. This probably reflects low levels of human effluent which may or may not have health implications. Low levels of FWAs may also reflect upstream events.

Levels of FWAs greater than 0.1 μg/litre suggest a more significant level of human sewage input. As a general recommendation, levels exceeding 0.2 μg/litre are strong indication of human sewage. While samples with higher levels of FWAs generally also contained high levels of *E. coli* a direct linear relationship between the two was not always evident.

In our hands FWAs are the best indicator of potential human effluent, and are useful to identify points to focus attention on. They do not however indicate whether human pathogenic, or even indicator bacteria such as *E. coli*, were also transported with the FWAs and contributed to the microbial population in the river.

Conclusion: FWAs are currently the most practical indicator of human faecal pollution. Studies of the movement and degradation of FWAs relative to microbial pathogens and indicators are required.

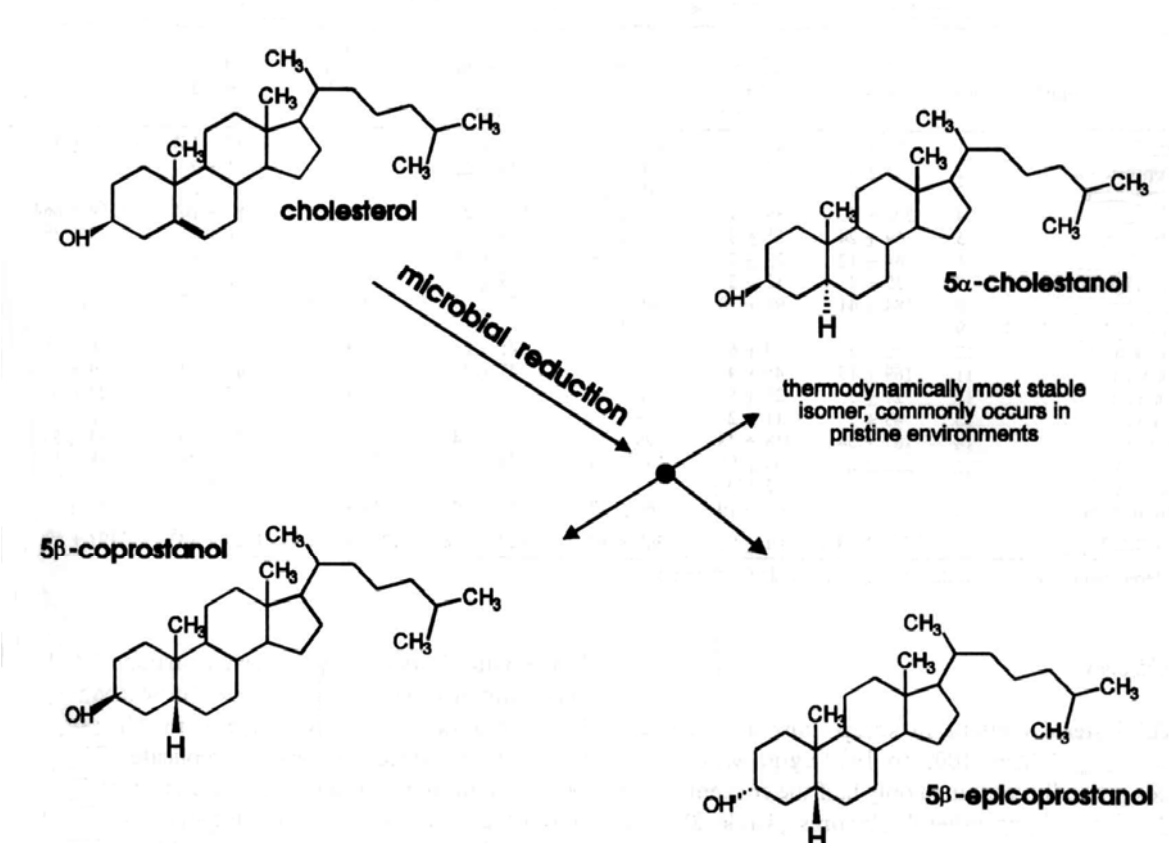
7.2 Faecal Sterols

Faecal sterols are a group of C27, C28 and C29 cholestane-based sterols found in mainly in animal faeces. The sterol profile present in faeces is dependent on interaction of three factors. Firstly, the animals diet determines the relative quantities of sterol

precursors (cholesterol, 24-ethylcholesterol, 24-methylcholesterol, and/or stigmasterol) entering the digestive system. Secondly animals differ in endogenous biosynthesis of sterols (humans on a low cholesterol diet synthesise cholesterol). Perhaps the most important factor is that the anaerobic bacteria in the animal gut biohydrogenate sterols to stanols of various isomeric configuration (Elhmmali *et al.*, 2000; Glipin *et al.*, 2002; Leeming *et al.*, 1996).

The sterol cholesterol can be hydrogenated to one or more of four possible stanols. In humans, cholesterol is preferentially reduced to coprostanol (Figure 3), whereas in the environment cholesterol is predominately reduced to cholestanol. Similarly, plant-derived 24-ethylcholesterol is reduced in the gut of herbivores to 24-ethylcoprostanol and 24-ethylepicoprostanol, whereas in the environment it is primarily reduced to 24-ethylcholestanol (Nichols *et al.*, 1993).

Figure 3. Example of faecal sterol biotransformation.



As a consequence analysis of sterol composition of animal faeces can generate a sterol fingerprint which can be quite distinctive. Faecal sterol analysis generates a lot of data, the interpretation of which can be quite complex. Some generalisations or guidelines to the individual analytes are described in Table 5.

Table 5. Descriptions of faecal sterols and stanols

Coprostanol	Principal human biomarker, high relative amounts indicate fresh human faecal material. Constitutes 60% of the total sterols found in human faeces. Dogs and birds have either no coprostanol or only trace amounts, present in their faeces. Not found in unpolluted fresh or marine waters or in fully oxic sediments (only anaerobic bacteria can hydrogenate cholesterol to coprostanol). However under conditions of anoxia, small amounts can be found in sediments not contaminated by faecal pollution.
Epicoprostanol	Found in trace amounts (relative to coprostanol) in human faeces. Increases in relative proportions in digested sewage sludges perhaps through epimerisation of coprostanol to epicoprostanol.
24-ethylcoprostanol	Principal herbivore indicator.
24-ethylepicoprostanol	Usually also present in herbivore faeces, often at similar level to 24-ethylcoprostanol.
Cholesterol	Precursor to coprostanol and epicoprostanol. Also comes from domestic waste, food scraps, algae etc.
Cholestanol	Most stable isomer, ubiquitous and occurs in pristine environments.
24-methylcholesterol	Plant sterol.
24-ethylcholesterol	Precursor to 24-ethylcoprostanol and 24-ethylepicoprostanol.
Stigmasterol	Plant sterol.

The absolute levels of each sterol or stanol can be dependent on many factors. The ratios of each stanol are however less concentration dependent, and fairly stable due to equivalent stability of stanols.

7.2.1 Key Ratios of faecal sterols

Coprostanol:24-ethylcoprostanol: These stanols are present in both human and herbivore faeces, but in significantly different amounts. Therefore relative contributions of each can be determined by examining ratio. Human faecal pollution typically has a ratio greater than 1.

Coprostanol:epicoprostanol: High relative amounts of epicoprostanol compared to coprostanol suggests older human faecal contamination. However if the ratio of coprostanol:cholestanol is less than 0.3, the epicoprostanol may be derived from non-preferential production of anaerobic bacteria in anoxic sediments.

Coprostanol:cholestanol: The ratio of coprostanol:cholestanol can indicate whether the coprostanol present is of faecal origin. A ratio greater than 0.5 suggests faecal contamination (preferential reduction from sterol by gut microbiota), whereas a ratio of less than 0.3 may suggest environmental reduction by, for example, anaerobic bacteria in sediments.

24 ethylcoprostanol:24ethylcholestanol: If the ratio is less than 0.5 then the stanols may not be of herbivore faecal origin.

7.2.2 Differences between animal sources

Human faecal material has considerably larger quantities of faecal sterols than other animals analysed (Table 6). The relative levels of the sterols and stanols also differ considerably. So human faecal material has a predomination of coprostanol, while dogs and birds have almost no coprostanol. In contrast cholesterol can constitute up to 70% of the total sterols of these animals. Dog and birds exhibit little evidence of sterol reduction, probably due to absence of bifidobacteria which can reduce cholesterol.

Table 6. Relative levels of faecal sterols in faeces of selected animals.

	human	pig	cow	sheep	horse
Total sterols ug/g	5614	1289	1427	1308	681
Coprostanol	61%	27%	15%	13%	6%
24-ethylcoprostanol	22%	26%	13%	19%	13%
Cholesterol	5%	10%	14%	8%	18%
Other plant sterol/stanols	4%	12%	41%	31%	48%
Coprostanol:24-ethylcop	2.8	1.1	1.2	0.7	0.5

	dog	cat	possum	duck	seagull
Total sterols ug/g	2193	1769	1427	1285	1015
Coprostanol	0%	22%	15%	2%	1%
24-ethylcoprostanol	0%	5%	40%	3%	13%
Cholesterol	72%	42%	8%	27%	73%
Other plant sterol/stanols	17%	21%	28%	53%	8%
Coprostanol:24-ethylcop	0.0	4.1	0.4	0.7	0.1

Faecal sterols are non-polar, non-ionic, and water insoluble, and therefore become associated with fine grain particles and sediments. They undergo aerobic degradation by bacteria. They exhibit a ubiquitous occurrence, albeit at trace concentrations, in soils

and sediments. Previous studies in ESR indicated that similar results were obtainable from faecal sterol analysis of particulate and aqueous phases. Faecal sterols from sediments would be clearly particle associated, and older faecal sterols, would be more likely to associate with particulate over time. It was therefore reasoned that analysis of the aqueous phase would be more representative of recent or fresh faecal contamination that was not yet particle associated.

There are different potential advantages to the analysis of the particulate and aqueous phases:

Analysis of the aqueous phase may:

- Be more representative of recent or fresh faecal contamination that was not yet particle associated
- Be more prone to variations
- Not be practical for volumes greater than one litre.

Analysis of particulates may:

- Be more representative of the ongoing nature of faecal contamination
- Be more easily "scaled up" to more than 1 litre analysis
- Allow easier transport of samples once filtered
- Represent situation at upstream site some distance away,

On balance, the analysis of particulates seems to be the best option, where analysis of more than 1 litre is desired. Onsite filtering and then freezing of filters maintains the optimum integrity of sterols, and minimises transport costs.

Section 8 contains numerous examples of the application of faecal sterol analysis.

Conclusion: Faecal sterol analysis is the most time consuming and expensive assay we have used for faecal source identification. It does provide useful evidence of human faecal pollution. However, in our hands, it is less definitive in the identification of other sources. Faecal sterol analysis is best utilised selectively based on indications of microbial indicators, FWAs, and potentially molecular markers – all of which would be faster and easier to assay, and cheaper.

7.3 Long-Chain Alkylbenzenes

Long-chain alkylbenzens (LABs) having C10 –C14 normal alkyl chains are sulfonated in the industrial production of linear akylbenzene sulfonates. They are widely used as anionic surfactants in commercial detergents (Eganhouse, 1986). A number of studies have found LABs in the waters and sediments exposed to sewage. LABs are purely synthetic and are derived solely from direct industrial discharges and domestic wastes (Eganhouse, 1986). They are therefore strongly indicative of human sources. They are also generally present in concentrations up to one order of magnitude lower than the corresponding faecal sterol concentrations in human derived wastes (Sinton *et al.*, 1998). Gregor *et al.* (2002) found FWAs to be present at higher detectable levels in effluent than LABs

Conclusions: FWAs are a better human source indicator than LABs.

7.4 Bile acids

Bile acids are C_{24}, C_{27}, and C_{28} steroidal acids produced in the digestive system of animals. They assist in enzyme mediated digestion of dietary fats and they maintain body cholesterol levels via faecal elimination of excess sterols from the body (Elhmmali *et al.*, 2000).

Ruminants produce predominantly deoxycholic acid, while ominivores (canines and humans) also produce significant quantities of lithocholic acid. Porcine faecal material has an absence of deoxycholic acid and has instead hyocholic acid (Bull *et al.*, 2002).

Gas chromatography allows detection at nanogram detection limits, but the bile acid extraction procedure is more complex than for faecal sterols and stanols (Bull *et al.*, 2002).

Conclusions: The analytical procedure appears too complex and costly for this approach to be practical. But bile acid analysis may be of value if pig faecal pollution needs to be distinguished from human.

7.5 Caffeine

Caffeine is present in coffee, tea, some carbonated drinks, and in some pharmaceutical products. Caffeine and its metabolites are excreted in the urine of individuals who have consumed caffeine containing products. Although caffeine is extensively metabolized, with only 3 percent of ingested caffeine excreted unmetabolized in the urine (Tang-Liu *et al.*, 1983), caffeine has been detected in domestic wastewater effluent,

environmental surface water samples, ground water and drinking water at between 0.01 and 300 μg/L (Rogers, 1986; Standley *et al.*, 2000).

Although caffeine has been extensively detected in environments exposed to human wastes, there are only a small number of studies that can be used to estimate the probable concentrations of caffeine that might result from sewage spills, and its sensitivity as a marker of human faecal pollution is unknown. Therefore, further investigations are required.

Conclusion: Low levels limit its potential usefulness.

7.6 Conclusions

Chemical indicators have been extensively investigated and successfully used to identify human effluent in particular. However there remains a need to clarify the relative movement and persistence of these chemical indicators relative to microbial pathogens and indicators.

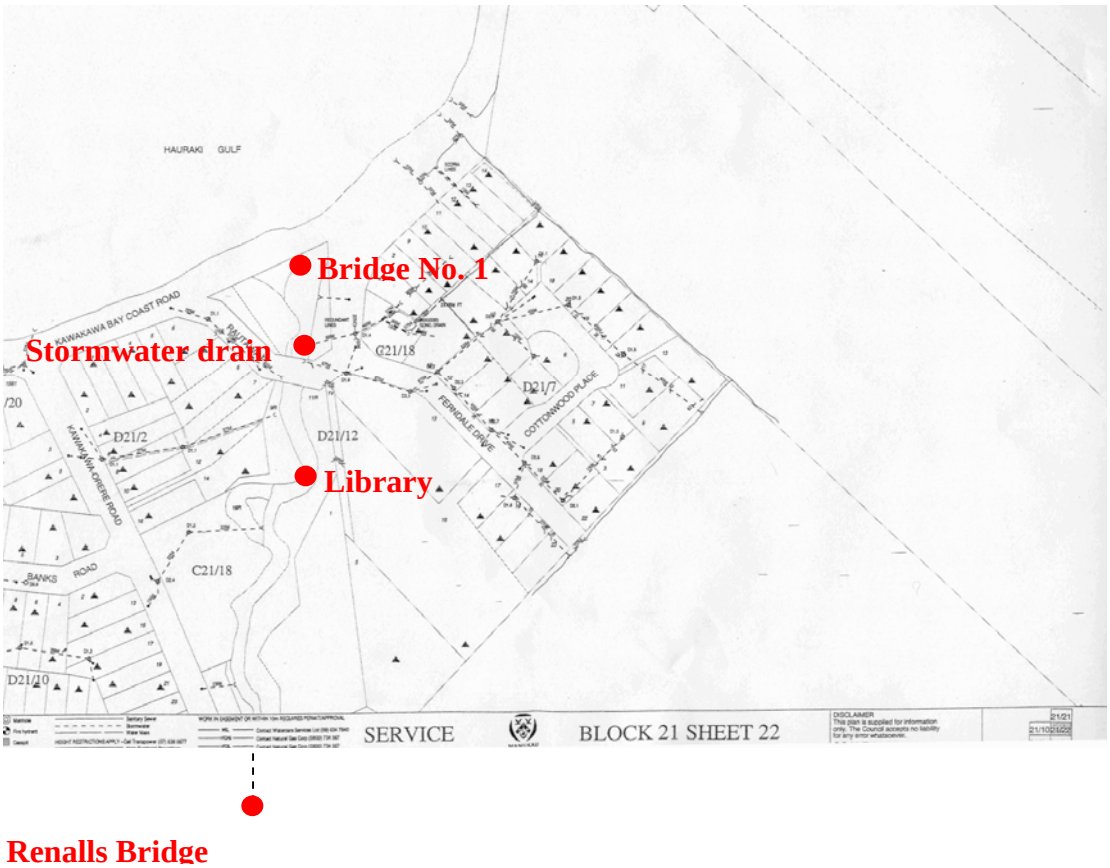
8 New Zealand case studies

A number of New Zealand studies to evaluate advanced faecal source discrimination tools have been carried out by ESR in the last few years. Selected examples are included here:

8.1 Kawakawa Bay

Previous sampling of stormwater drain at Kawakawa Bay indicated the presence of high levels of faecal coliforms. While assumed to be from local septic tanks, advanced indicators of faecal pollution were used to confirm human origin of faecal contamination. A "5 in 30" sampling protocol was undertaken at four sites in Kawakawa Bay with sampling occurring on 17/09/01, 1/10/01, 4/10/01, 15/10/01 and 18/10/01.

Figure 4. Map of sampling sites at Kawakawa Bay



Samples were taken from the river, on the upstream side of the road by Renalls Bridge (Figure 5 a, b). This is a rural area with cows visible from the sampling site. There may also be farm inputs to the river further upstream. Samples taken near Kawakawa Library

were taken from a narrow part of the river slightly upstream of the Kawakawa Library (Figure 5 c). There is a stormwater drain input upstream of this site. The actual stormwater outlet (Figure 5 d) drains the stormwater from Ferndale Drive and Cottonwood Place discharging into the river (Figure 5 e). The last set of samples was taken adjacent to Bridge No. 1, on the opposite side of the river to the stormwater discharge, approximately 100 metres downstream.

Figure 5. Photographs of sampling sites at Kawakawa Bay

Sample Analysis

E. coli levels were clearly elevated in the stormwater samples (Table 7).

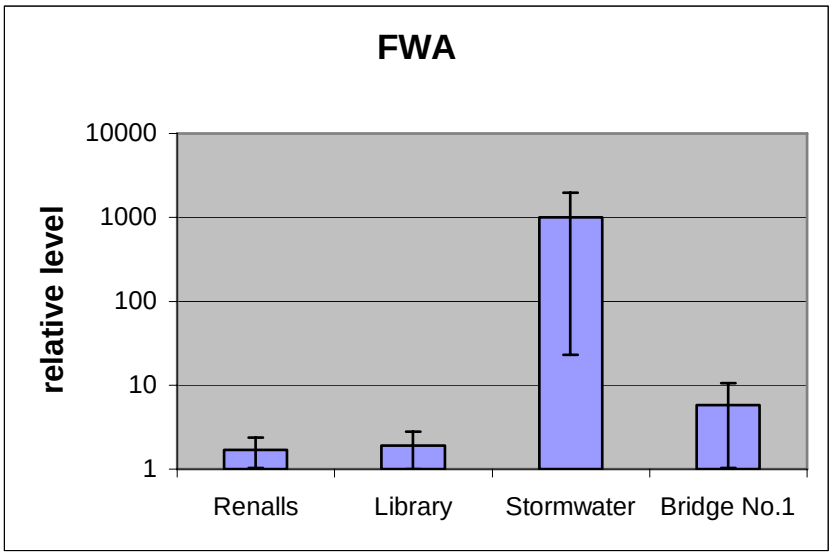
Table 7. Mean microbial and chemical indicators samples from Kawakawa Bay

	Mean E. coli/100mL	Total ammonia mg/L	Ammonia:nitrate	Dissolved reactive phosphorous mg/L
Renalls Bridge	264	0.014	0.4	0.02
Library	649	0.036	0.3	0.02
Stormwater	156,000	4.98	3.7	1.7
Bridge No. 1	1,300	0.058	0.3	0.02

Fluorescent whitening agents (FWAs)

FWAs were detected in all samples except for one sample at Renalls Bridge, and one sample at the library (Figure 6). The levels at Renalls Bridge and at the library were all close to the detection limit (0.01 μg/100ml) and are probably not significant. In contrast the detected levels of FWAs in the stormwater outlet ranged from 1.66 to 26.29 μg/100ml. These levels clearly indicate the presence of domestic sewage. Three of the five Bridge No. 1 samples contained significantly elevated FWA levels, with sample 16 (15/10/2001) containing 0.14 μg/100ml of FWAS. This was also the sample with the highest faecal coliform counts at this site.

Figure 6. Mean relative levels of FWAs at Kawakawa Bay



Faecal Sterols

The levels of faecal sterols were elevated in the stormwater samples in line with the traditional indicators. Comparison of the ratios of the sterols and stanols all support human faecal contamination at the stormwater drain.

The ratio of the principal human marker coprostanol to the principal herbivore maker 24-ethylcoprostanol was in the stormwater samples consistently elevated indicating human faecal pollution (Figure 7). The ratio of coprostanol to the minor human sterol epicoprostanol was also high in the stormwater sample suggesting *fresh* human faecal contamination (Figure 8). In older sewage the relative levels of epicoprostanol tends to increase. The ratio of coprostanol:cholestanol was, in most of the samples above 0.5 (Figure 9) indicating that the coprostanol present was of faecal origin, and present as a result of preferential reduction from sterols by gut microbiota. The low levels at Renalls Bridge, where the ratio was less than 0.3, may suggest environmental reduction by for example anaerobic bacteria in sediments. Cholesterol levels increased dramatically in stormwater drain, but the ratio of coprostanol to cholesterol showed an even more significant increase (Figure 10).

Figure 7. Mean ratio of coprostanol:24-ethylcoprostanol

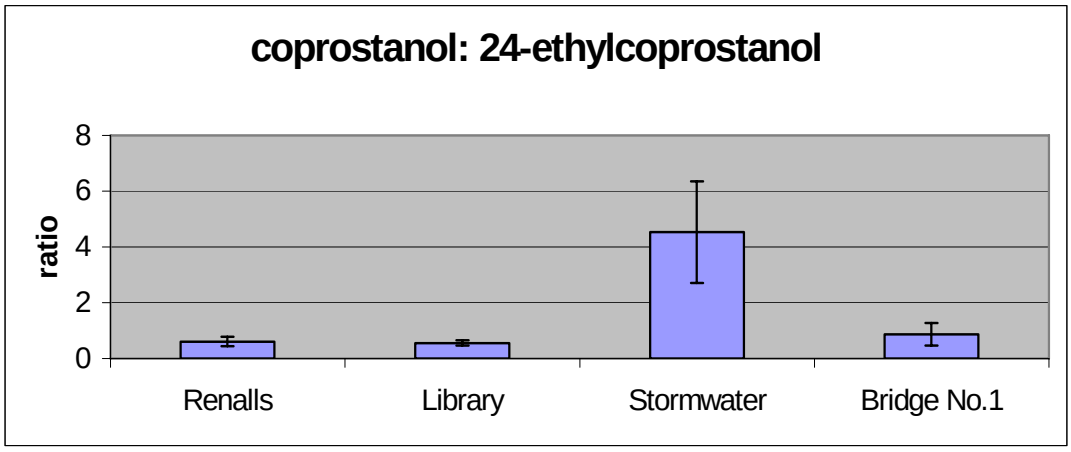


Figure 8. Mean ratio of coprostanol:epicoprostanol at Kawakawa Bay

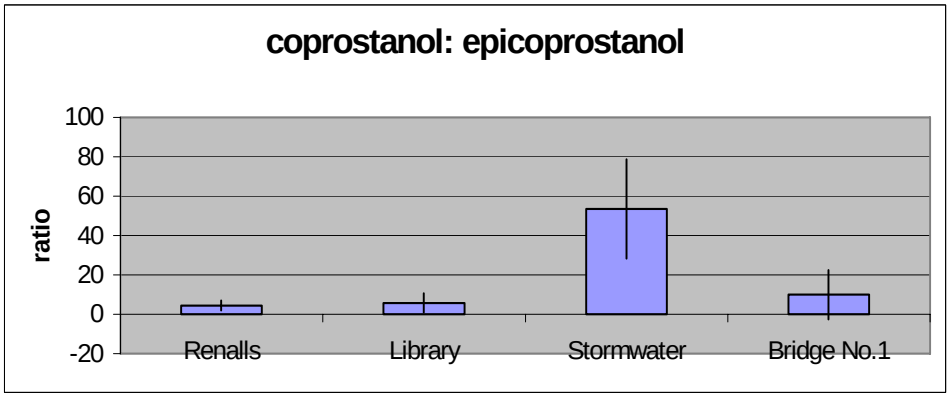


Figure 9. Mean ratio of coprostanol:cholestanol at Kawakawa Bay

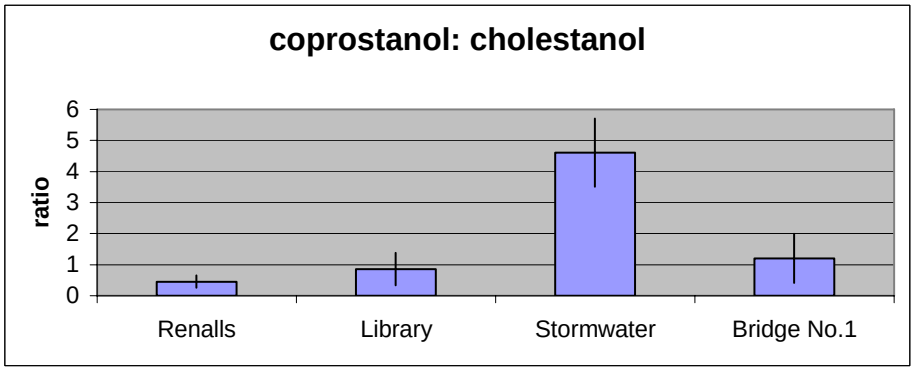
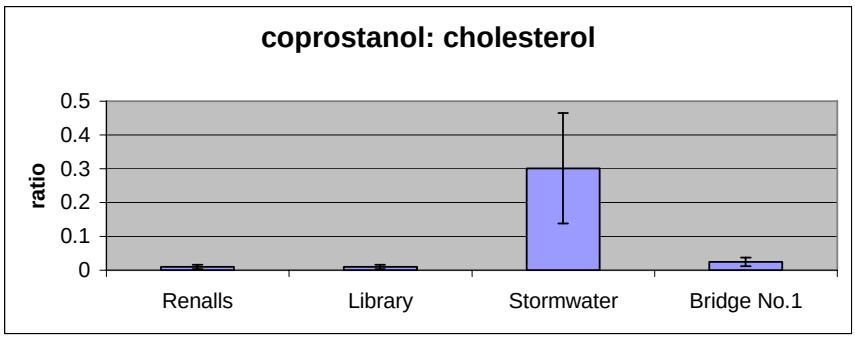


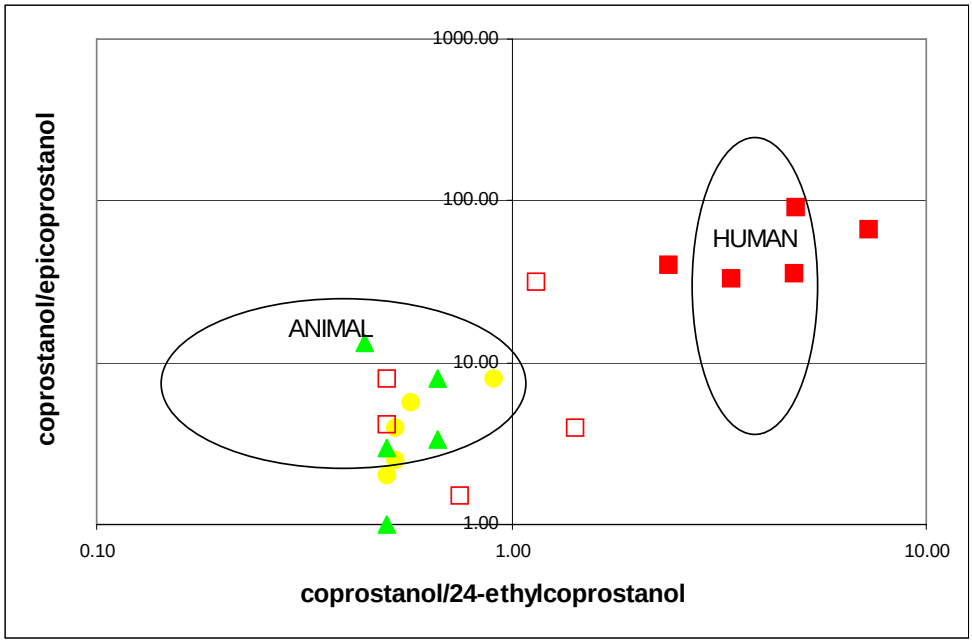
Figure 10. Mean ratio of coprostanol:cholesterol at Kawakawa Bay



When the ratios of coprostanol:epicoprostanol to coprostanol:24-ethylcoprostanol are plotted for each sample, a clear separation of the sampling sites is evident (Figure 11).

When compared with previously analysed human and animal effluent, the human nature of the stormwater samples is clear. The open circles indicate the clustering points of previously analysed animal effluent (beef and sheep meatworks), and human effluent (septic tanks, wastewater processing plants). The stormwater samples (closed red squares) all cluster with human effluent. The Renalls Bridge (yellow circles) and Library samples (green triangles) all cluster closer to the previous animal effluent. Three of the Bridge No. 1 samples cluster with or close to the animal samples (open red boxes), while sample 16 falls in between (higher open red box), as does sample 20 (lower open red box).

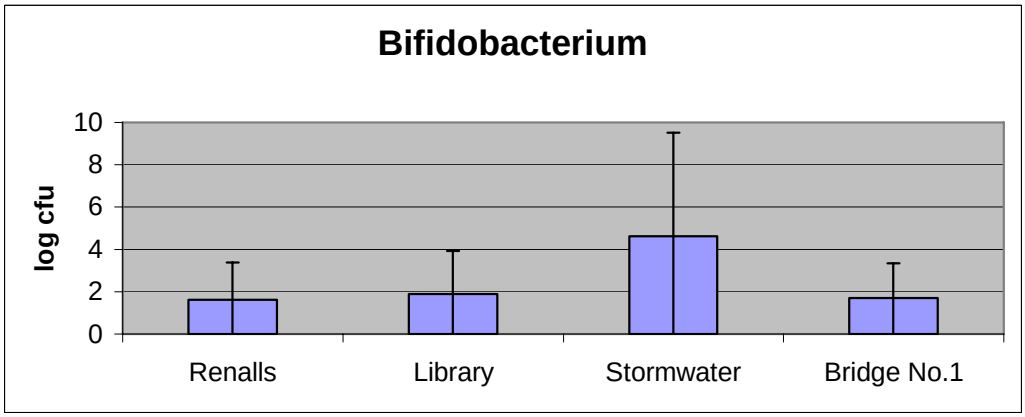
Figure 11. Ratios of coprostanol:epicoprostanol to coprostanol:24-ethylcoprostanol plotted alongside previously analysed human and animal effluent



Bifidobacteria

Bifidobacteria were enumerated on BFM agar. Colony counts increased dramatically in the stormwater drain samples, (Figure 12), indicating likely human faecal pollution.

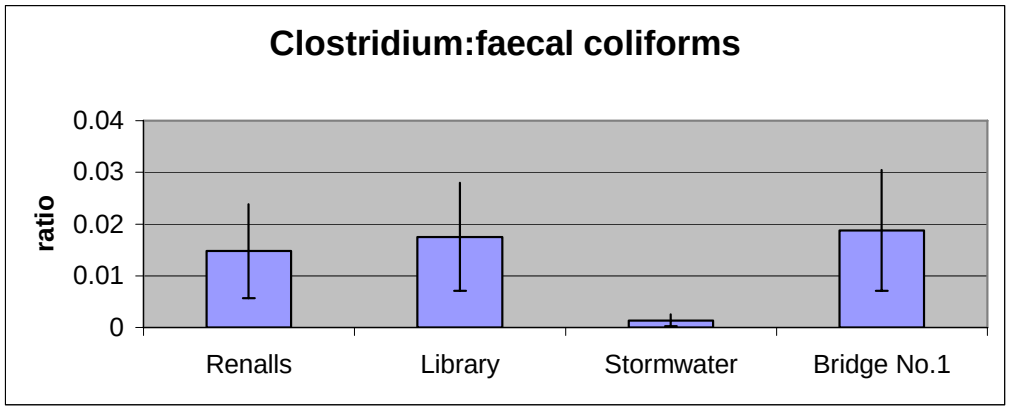
Figure 12. Presumptive Bifidobacteria at Kawakawa Bay



Clostridium perfringens

The ratio of *Clostridium perfringens* to faecal coliforms was low in all samples (Figure 13) suggesting that canine faecal contamination was not a significant issue. The low level of this ratio in the stormwater sample reinforces the likelihood of human contamination in this sample.

Figure 13. Mean levels of *C. perfringens* at Kawakawa Bay



Conclusions

The evidence examined all suggests that the increase in traditional microbiological faecal indicators in the storm water drain is due to human faecal input.

Key support for this statement is:

- Significant levels of FWAs in stormwater at levels of at least 100-fold greater than upstream samples. FWAs are man-made chemicals with no known environmental sources, used in washing powders.
- Faecal sterol analysis in both gross amount, and ratios, all support human faecal contamination. Stormwater samples cluster with previously analysed human effluent in terms of faecal sterol characteristics.
- Bifidobacterium, of which the isolates detected were most likely human specific, were significantly higher in stormwater.
- Ratio of *C. perfringens* to faecal coliforms was low in the stormwater sample, suggesting input from dogs was not a significant contributor.

8.2 Mathesons Bay

The available chemical and microbial faecal source discrimination tools were evaluated at Mathesons Bay in the Rodney District. One day sampling was undertaken, with twelve samples collected from Mathesons Bay on 12 February 2002. The weather on the day of sampling was overcast with light rain. The previous day also had light rain.

Table 8. Sampling site at Mathesons Bay

Number	Site	Description
M1	Stream at main road culvet	Predominantly rural and farmhouses
M2	Upper lagoon/ stream	Houses and stormwater inputs
M3	Stormwater drains feeding lagoon	Urban stormwater
M4	Lagoon main body @ new bridge EAST	sum of 1+2+3 inputs
M5	Lagoon main body @ new bridge MIDDLE	sum of 1+2+3 inputs
M6	Lagoon main body @ new bridge WEST	sum of 1+2+3 inputs
M7	Lagoon exit T=0	
M8	Lagoon exit T=15 min	
M9	Lagoon exit T=30 min	
M10	Lagoon exit T=45 min	
M11	Lagoon exit T=60 min	
M12	Beach sample	Knee depth in mixing zone

Sample Analysis

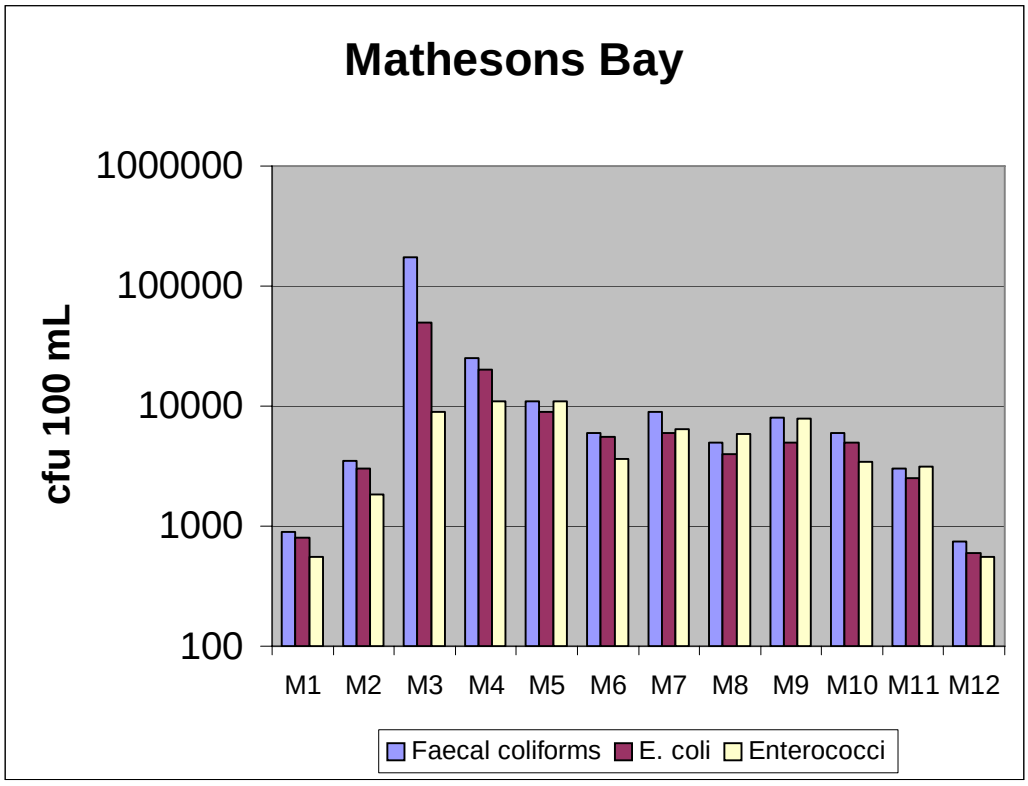
Traditional Microbial Indicators

All the samples taken contained levels of *E. coli* and enterococci which exceeded both fresh and marine water bathing guidelines (Figure 14) (regardless of how the water was classified) at the ACTION/RED MODE. The guidelines suggest:

- Increase sampling to daily
- Undertake a sanitary survey, identify sources of contamination
- Erect warning signs
- Inform the public through the media that a public health problem exists.

Sample M1, from the stream at the main road with predominant rural and farmhouse inputs, contained 800 MPN *E. coli*/100 mL. The peak level of *E. coli* was detected in the stormwater drain, with 50,000 MPN *E. coli*/100 mL.

Figure 14. Microbial indicators at Mathesons Bay



Chemical Indicators

Levels of nitrate were below detection limit (less than 0.002 mg/L) in all samples except for M3 (Stormwater drains) where 0.523 mg/L was detected. This also indicates a minimum 300-fold dilution of this stormwater input at any of the downstream points sampled. Ammonia (Figure 15) and phosphate (Figure 16) levels were low in all samples. The maximum phosphorus level was detected in sample M1, and particularly in view of the absence of ammonia, may represent runoff from agricultural use of fertilisers.

Figure 15. Ammonia levels in Mathesons Bay samples

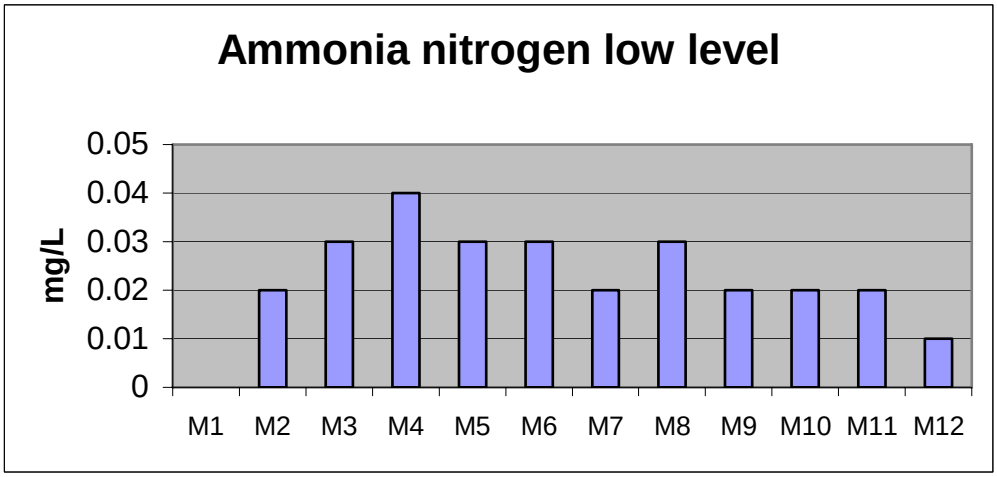
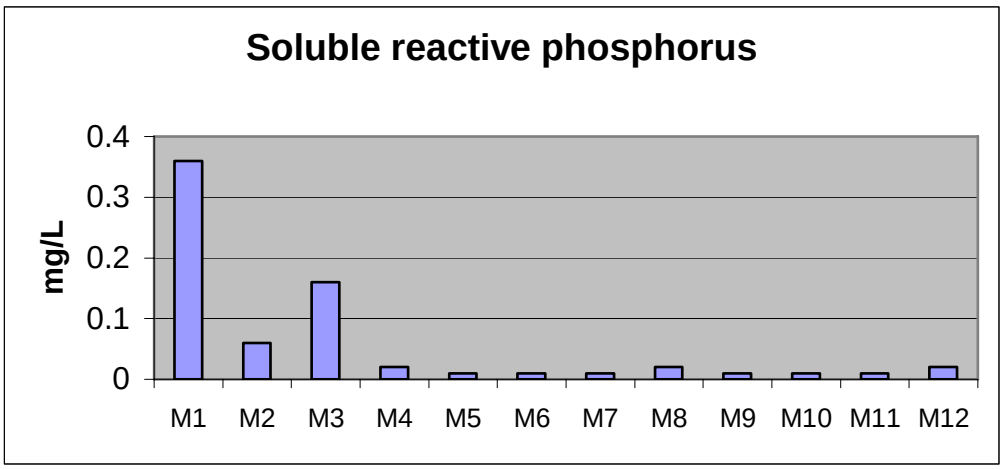


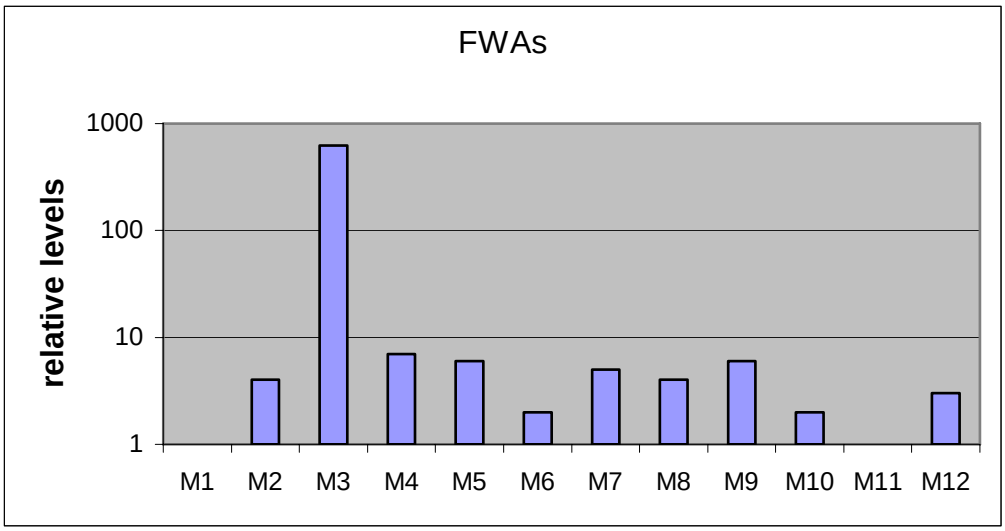
Figure 16. Phosphate detected in Mathesons Bay samples



Fluorescent whitening agent (FWA)

FWAs were detected in all samples except for M1 and M11. The absence of FWA from M1, probably confirms that the slightly elevated levels of phosphate detected in M1, are of agricultural origin, rather than from washing powders. The highest level of FWAs was detected from the stormwater outlet and indicated the entrance of "greywater" into the stormwater system.

Figure 17. Relative levels of FWAs at each site (Log$_{e}$).



Faecal Sterols

The levels of faecal sterols detected were generally low. Samples M8 and M12 in particular had levels of coprostanol, and other biomarkers, below the level of detection. As such little can be inferred in these samples about the sterol profile. Only the stormwater sample contained a ratio of coprostanol:cholestanol significantly above 0.5, indicating that at least some of the coprostanol present was of human faecal origin, and present as a result of preferential reduction from sterols by human gut microbiota. The low levels in most of the samples may suggest environmental reduction by for example anaerobic bacteria in sediments.

The ratios of coprostanol:24-ethylcoprostanol were less than one in all samples, with ratio of 0.9 in sample M3, three times that of any other sample. Calculations based on coprostanol and 24-ethylcoprostanol ratios would suggest that all samples do not contain appreciable human effluent, except for M3, of which approximately 30% of the faecal sterols could be attributed to human effluent. The stormwater sample (M3) also

contained a high ratio of **coprostanol:epicoprostanol** suggesting fresh human faecal contamination.

Figure 18. Ratio of coprostanol to cholestanol in Mathesons Bay samples

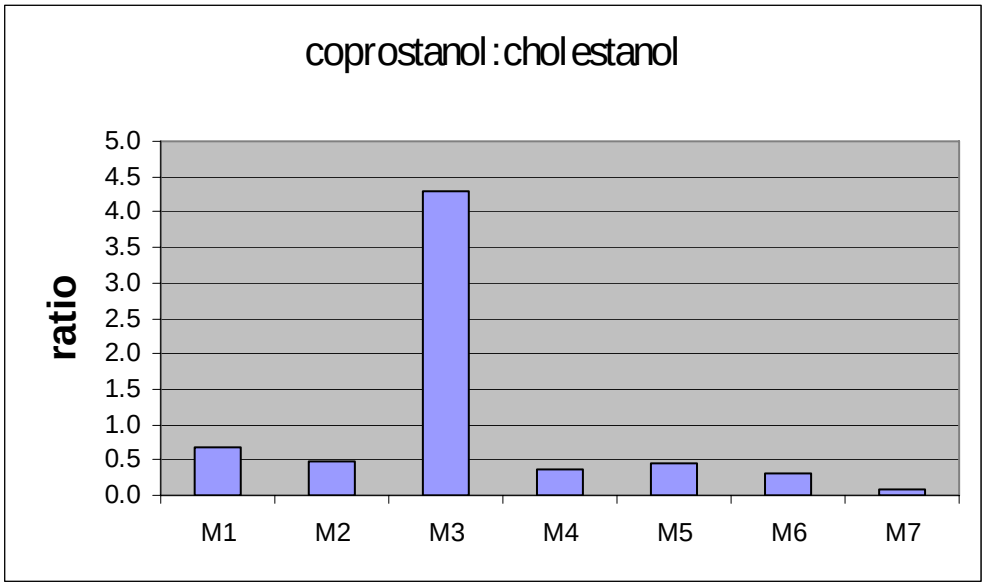
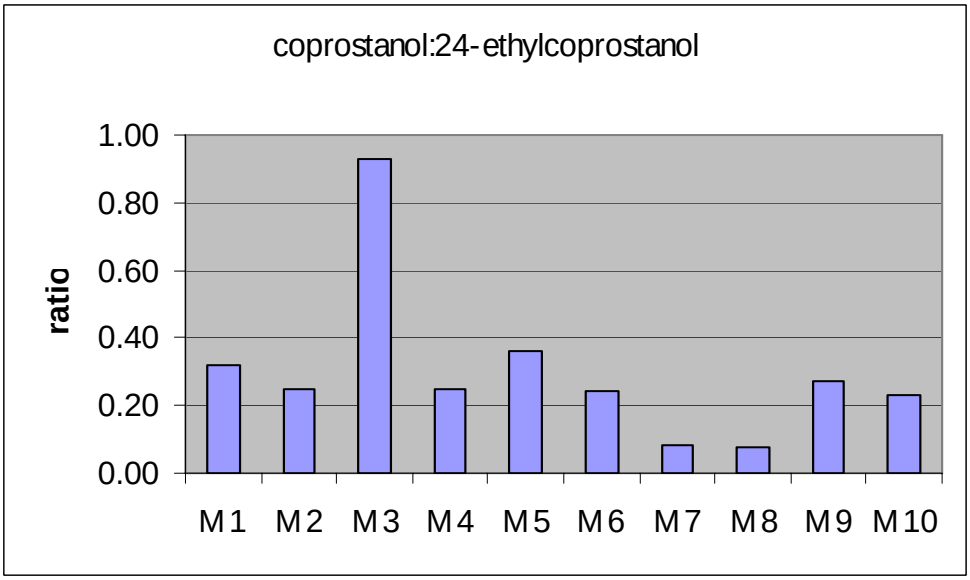


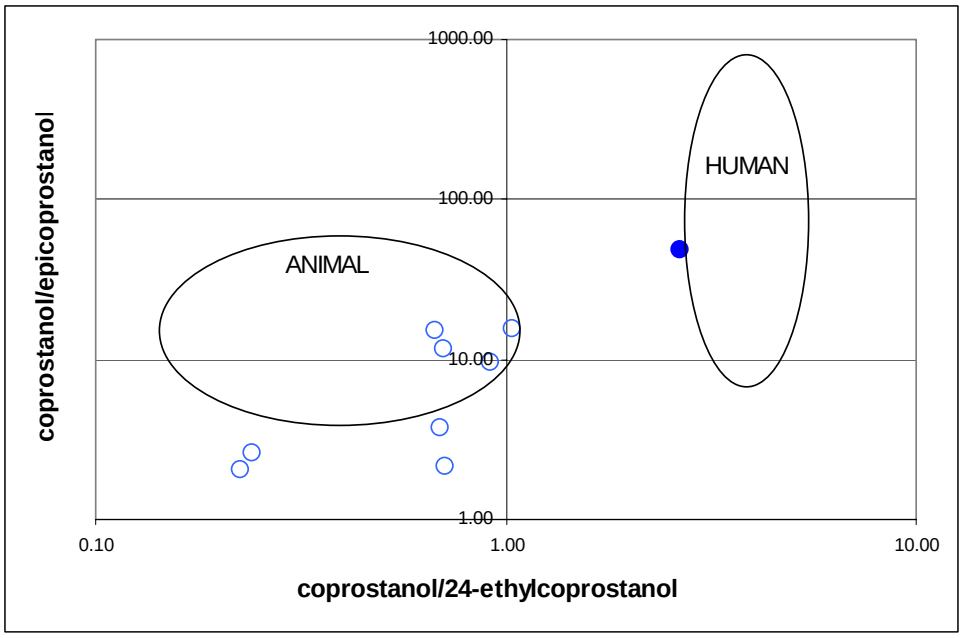
Figure 19. Ratio of coprostanol to 24-ethylcoprostanol in Mathesons Bay samples



When the ratios of coprostanol/epicoprostanol to coprostanol/24-ethylcoprostanol are plotted alongside previously analysed human (septic tanks, wastewater processing plants), and animal effluent (beef and sheep meatworks), all of the samples cluster with

animal effluent (open circles) except for the M3 sample (closed circle) which groups with the human effluent.

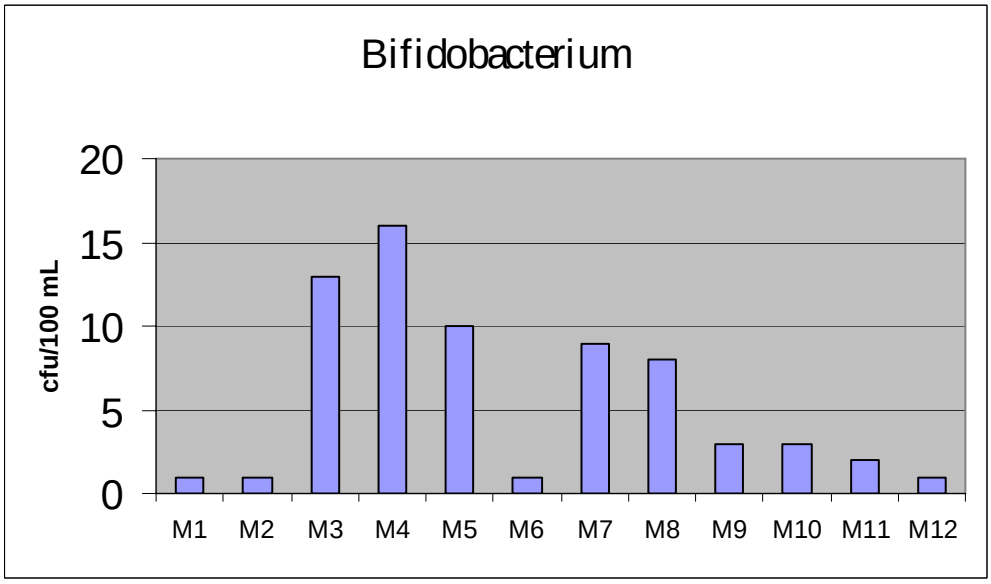
Figure 20. Ratios of coprostanol:epicoprostanol to coprostanol:24-ethylcoprostanol plotted alongside previously analysed human and animal effluent



Bifidobacteria

Bifidobacterium numbers were low in all samples (Figure 21). A ten-fold increase was observed in the stormwater drain and in some of the downstream samples. Bifidobacterium are less hardy, and have lower survival period than faecal coliforms. There presence at low levels relative to the faecal coliform indicators, may suggest that the faecal pollution is not fresh effluent, and may have undergone some treatment for preferential removal of Bifidobacteria.

Figure 21. Bifidobacteria in Mathesons Bay samples

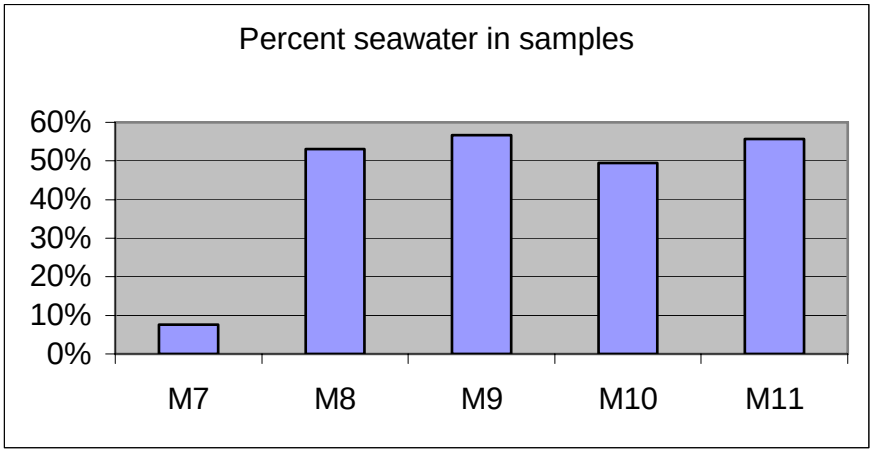


Temporal Sampling

Any grab sample is purely an indication of water quality in that particular bit of water, at the time sampled. To evaluate to a small extent expected differences, five samples were collected from the same site at 15 minute intervals.

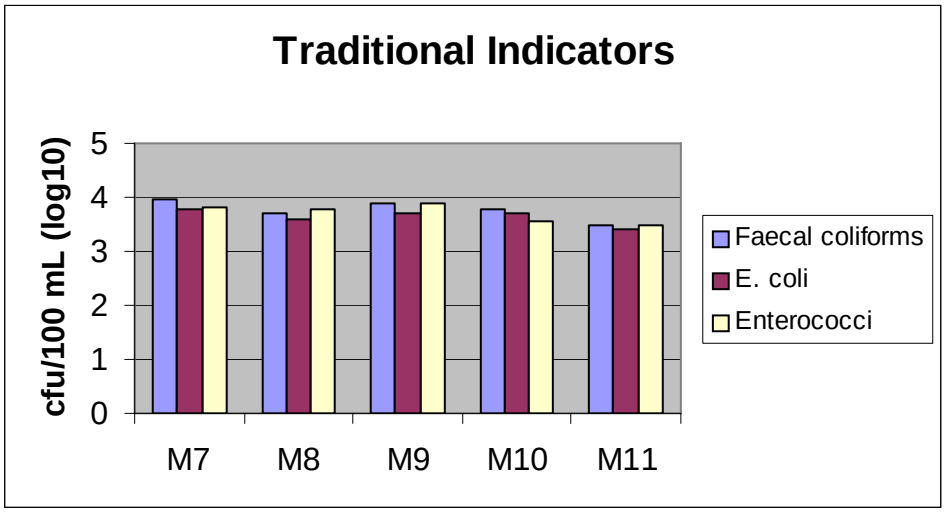
The seawater composition of each of these samples, calculated from conductivity indicated a significant difference between the first sample and the following four. Excluding the first sample however, a range of less than 6% was observed for the remaining samples (Figure 22).

Figure 22. Percent seawater in temporal Mathesons Bay Samples



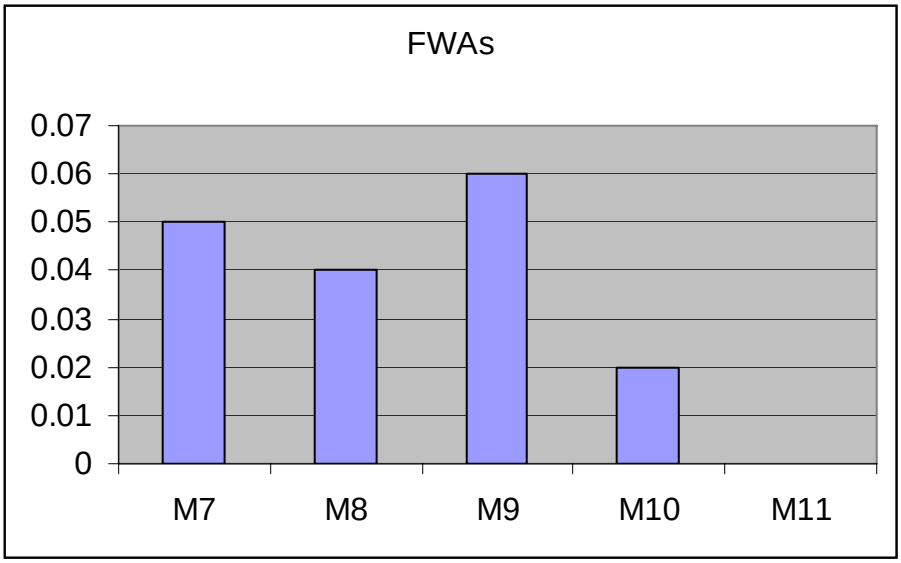
The traditional microbial indicators were fairly consistent in the samples, although the log scale minimises the apparent variation (Figure 23).

Figure 23. Traditional indicators in temporal sampling of Mathesons Bay



FWAs were not detected in the final sample of the set, which also had the lowest microbial counts (Figure 24).

Figure 24. FWAs in temporal Mathesons Bay Samples



Faecal sterols were detected at low levels, which amplify the apparent variability. The levels however were generally in agreement, and conclusions would not appreciably differ at the sampling points (Table 9).

Table 9. Faecal sterols in Matheson Bay temporal samples

	M7	M8	M9	M10	M11
	Lagoon T=0	exit Lagoon T=15 min	exit Lagoon T=30 min	exit Lagoon T=45 min	exit Lagoon T=60 min	exit
coprostanol	0.0054	Nd	0.0043	0.0077	0.0046	
24-ethylcoprostanol	0.0638	0.0130	0.0157	0.0329	nd	
epicoprostanol	0.0021	nd	0.0061	nd	nd	
cholesterol	1.4300	0.1500	0.3200	0.2800	0.1900	
cholestanol	0.0649	0.0078	0.0063	0.0049	0.0076	
24-methylcholesterol	0.0200	0.0200	0.0050	0.0400	0.0100	
stigmasterol	0.0300	0.0200	0.0400	0.0700	0.0300	
24-ethylcholesterol	0.3700	0.0500	0.1000	0.1800	0.1100	

Conclusions

This sampling represents only a single sampling, and conclusions are therefore only suggestive, and would require further sampling for confirmation.

All samples examined contained high levels of traditional microbial indicators. The stormwater drain (M3) contained 50,000 *E. coli*/100mL which is a level of considerable concern. Indeed all samples exceeded guidelines for use as bathing waters.

Despite the high levels of faecal pollution, relatively low levels of faecal sterols were detected in most of the samples except for the stormwater drain. This may suggest that many of the sources of microbial inputs are not human, but may be of from diffuse sources. Birds can have low levels of faecal sterols. Regrowth of faecal indicators in the environment may also be significant.

Sampling across the lagoon, suggests that the maximum levels of faecal pollution may be on the Eastern bank.

For faecal sterol analysis, a higher level of sensitivity is required in most of these samples. This could be achieved in future sampling by increasing the volumes

analysed. Samples should also be analysed for *C. perfringens*, which in conjunction with *E. coli*, may indicate canine input.

8.3 Little Oneroa Bay, Waiheke Island

Samples were collected on four occasions from four sites on the river which feeds into Little Oneroa Bay on Waiheke Island (Figure 25). Fortuitously the sampling surrounded a "weather bomb" of heavy rainfall which occurred in June. This divides the samples into three groups – Before weather bomb (first two sampling days in May), immediately after/during the weather bomb (20 June), and post weather bomb (23 June). In general the weather bomb samples showed massive increase in microbial indicators, followed by decrease on the next sampling occasion perhaps due to washout effect.

FWA analysis suggested that the Frank Street site contained potentially the most direct input of septic tank greywater, and is perhaps best site for some more in-depth investigation.

Samples were collected from 4 sites (Figure 25), on four occasions (22 May, 26 May, 20 June and 23 June 2002).

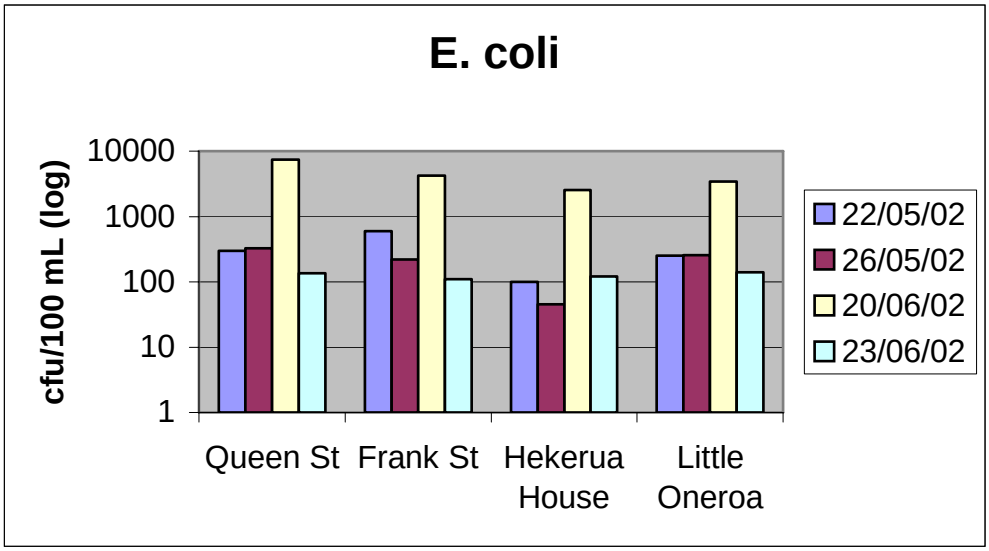
Figure 25. Sampling sites on Waiheke Island:

56 – Queen Street; 55 – Frank Street; 54 – Hekerua House; 53 – Little Oneroa

Traditional Microbial Indicators

The May samples contained levels of *E. coli* which ranged from 45 – 600 *E. coli*/100 ml (Figure 26).

Figure 26. *E. coli* levels in samples from Waiheke Island



Immediately after the weather bomb, a dramatic increase in *E. coli* was evident to levels between 2550 and 7450 MPN / 100 mL. Within 3 days these levels had retreated to less than half of the pre-weather bomb levels.

Fluorescent whitening agent (FWA)

In the May samples FWAs were below the level of detection in samples taken from Queen Street site suggesting local septic tanks were not a significant source of contamination. On the 20 June sample from Queen Street higher level of FWA was observed as a consequence of the increased water load from the weather bomb. Three days later this had reduced.

The highest levels of FWAs were observed at the Franks Street site before the weather bomb. Dilution effect reduced these levels immediately after the weather bomb, although these were increased again three days later. The FWAs at this site clearly suggest flow from septic tanks, and further investigation of this site. The other two sites contained 0.2μg/L or less of FWAs. These may be the Frank St site inputs, or may also include new inputs from this site.

Figure 27. Ratio of atypical to total coliforms

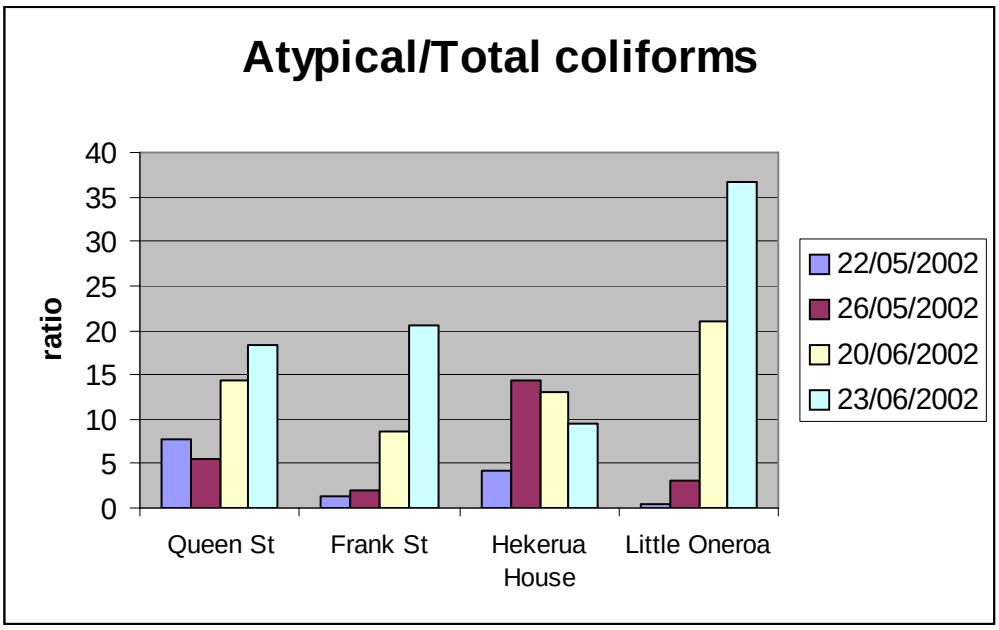
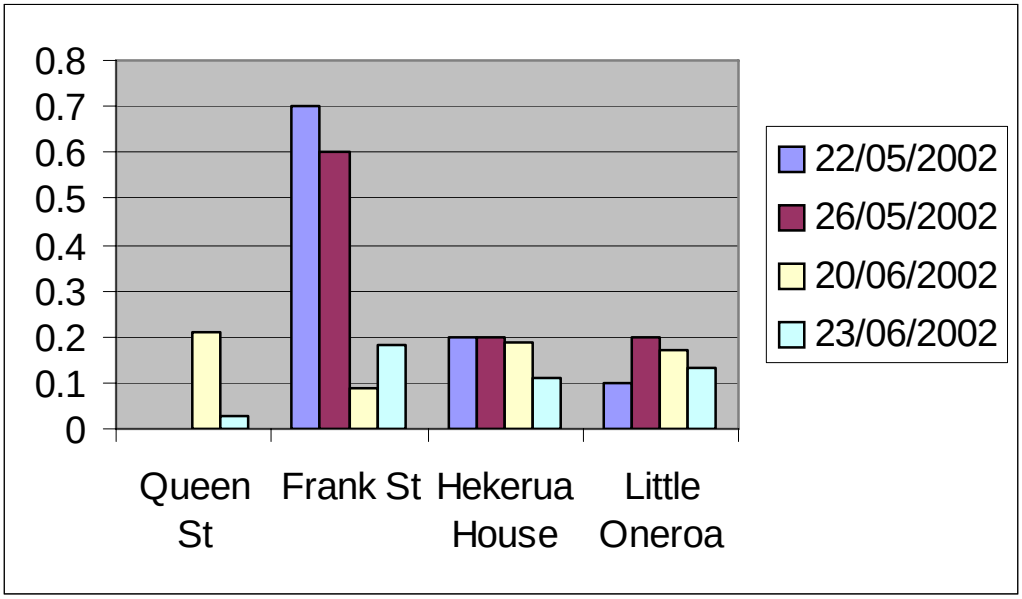


Figure 28. Levels of FWAs at each site (*u*g/L).



Faecal Sterols

Initial faecal sterol analysis was performed with total solvent extraction of five litres of the water sample. Five litres of each sample was filtered, and then the aqueous phase solvent extracted, and analysed. Previous analysis had just used one litre, but in these experiments, and increase in sensitivity was desired for which larger volume was one approach. This was however a failure, with no useable data. Re-extraction of frozen one litre sample was also not successful.

Fortunately however the filters used were stored, and we reanalysed selected samples taken on 26 May and 20 June by solvent extraction of the filters.

Ratios of coprostanol/cholestanol above 0.5 suggest sources of faecal sterols are due to faecal contamination. The samples from Frank Street and Little Oneroa exceed this level, while only the post-weather bomb at Queen Street, approaches this level (Figure 29). Ratios of coprostanol/24-ethylcoprostanol above 1, suggest human faecal contamination which again Frank Street and Little Oneroa reached (Figure 29).

Calculation of the proportion of human faecal contamination suggests between 50 and 85% human contribution to faecal sterols (Figure 30).

Figure 29. Ratios of coprostanol/24-ethycoprostanol and coprostanol/cholestanol in water samples.

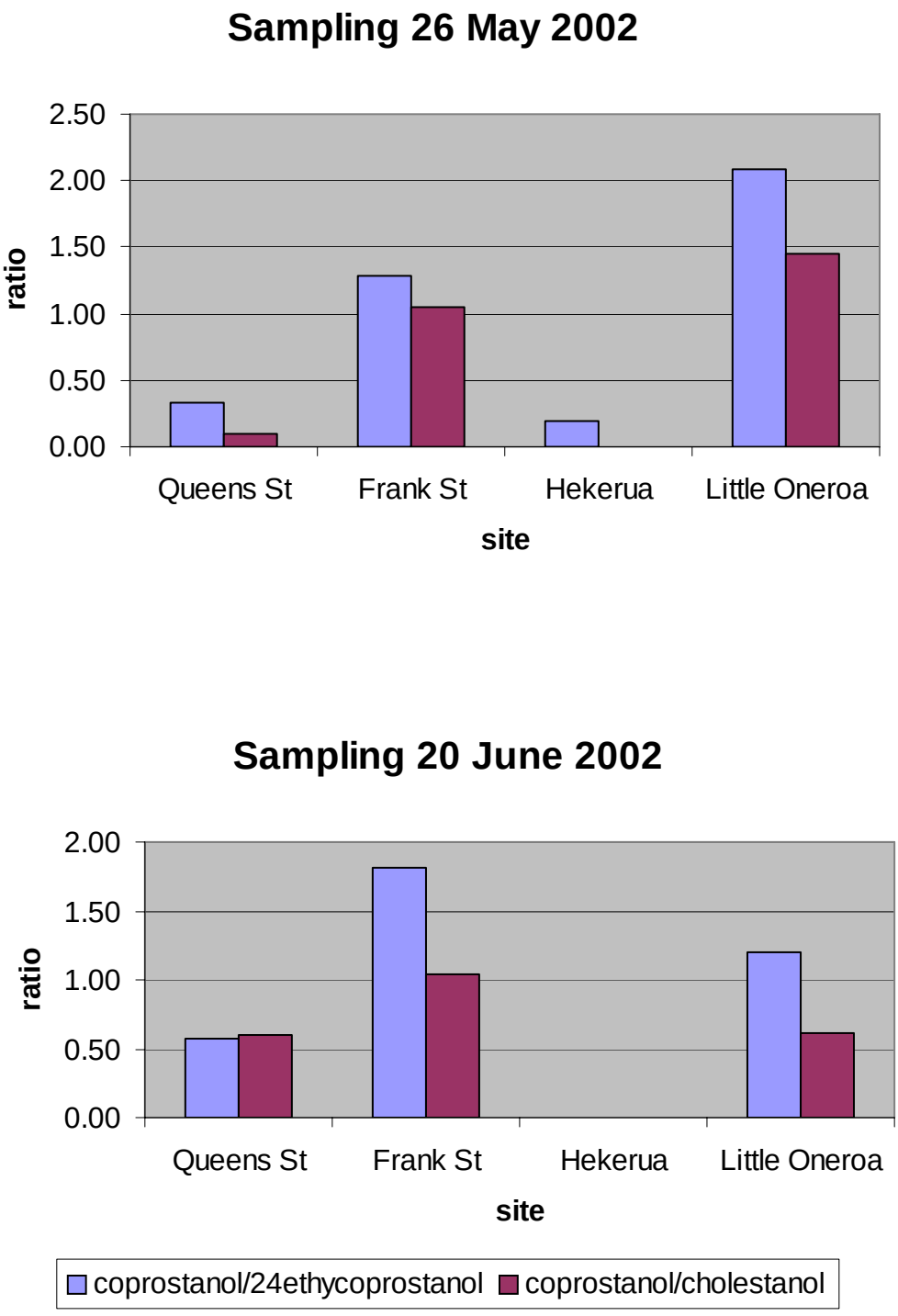
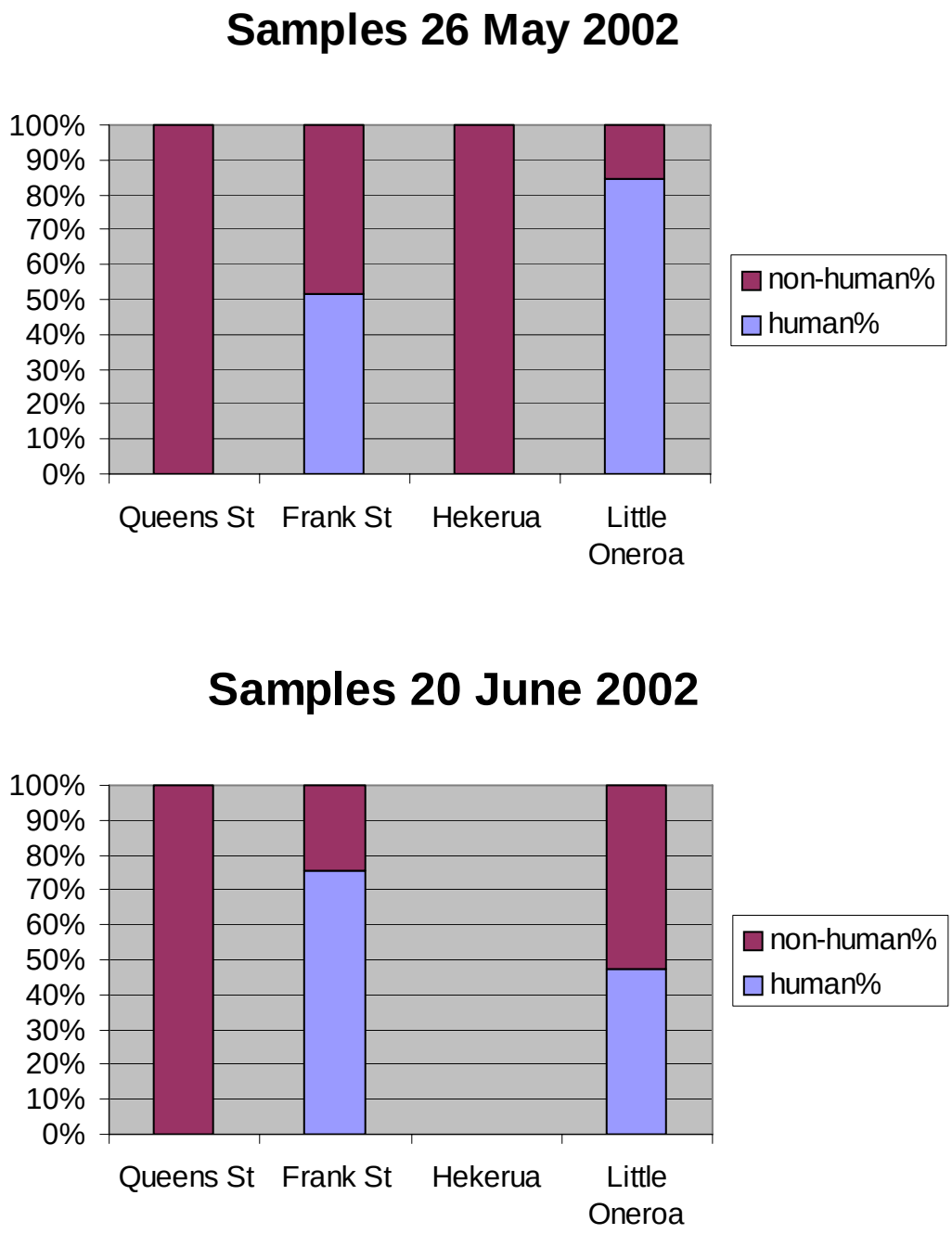


Figure 30. Calculated human and non-human proportions of faecal sterols analysed



The following conclusions could be made regarding the samples taken from Little Oneroa.

Queen Street

There is little evidence of significant human faecal contamination at this site. Prior to the weather bomb there was no evidence of human faecal contamination. The absence of FWAs (Figure 28) and the ratios of faecal sterols (Figure 29 and Figure 30) both suggest non-human sources of microbial indicators. The highest counts of *E. coli* of any site (7,450 MPN/100ml) were observed in the 20 June sample immediately after the weather bomb. The FWAs were detectable in this sample, but at a fairly low level, and could indicate human related overflow form for example septic tank drainfield flooding or sewer overflow. However although the absolute levels of faecal sterols were increased at least five fold, the ratios continued to suggest non-human sources of sterols. The rural nature of this site suggests that farm animals, wild animals or birds may be the main sources of contamination.

Frank Street

Human faecal contamination is identifiable at this site. Highest levels of FWAs were observed in the pre-weather bomb samples, with faecal sterol analysis indicating human faecal contamination.

Hekerua House

There is little evidence of significant human faecal contamination at the this site.

This site had the lowest levels of *E. coli* on each of the sampling occasions, with pre weather bomb samples containing 100 and 45 *E. coli*/100 ml. Low levels of FWAs were detected in all samples, but faecal sterol analysis, which was only done for 26 May sample, indicated no human faecal contamination.

Little Oneroa

Like the Frank Street samples, the Little Oneroa samples contained evidence of human faecal contamination. The FWA levels were similar to Hekerua House, but the faecal sterol ratios suggested human faecal pollution. After the weather bomb the proportion of human decreased, suggesting proportionally more non-human sources of the increased microbial load.

Weather bomb

High levels of rainfall will increase microbial load of rivers dues to both overflow of sewage systems, overland wash-down of faecal material, and re-suspension of sediments. Limited results presented here would indicate that although this undoubtedly poses significant health risk, within three or four days, microbial load may well return to levels below "normal". Except for the Queen Street site, levels of FWAs actually decreased, perhaps due to dilution, while faecal sterols generally increased. This increase may be due to input of animal faecal material, or the re-suspension of sediments containing animal faeces.

9 CONCLUSIONS

Faecal contamination of our rivers, lakes, oceans, groundwater, and drinking-water degrades their value for recreation and consumption. Even where these waters aren't directly used, there is a growing acceptance that human sewage and animal faeces derived from human activities (farming) should have minimal impact on the environment. The detection of coliform group of bacteria has, for the last 100 years, enabled the identification of faecally contaminated water. Where a point source was obvious, and could be corrected this has worked well. When however it has been necessary to identify the animal source of the faeces – human, farmed, domestic or wild animals or birds – less success has been had. The primary concern for most managers, and the public, is the presence of human faecal pollution in water. Therefore the most basic level of discrimination necessary is the ability to detect human faecal pollution usually in a background of non-human pollution. Fluorescent whitening agents (FWAs) are the primary tool recommended for this purpose.

FWAs are components of most washing powders and in both septic tanks and community effluent, become associated with human faeces. As such, FWAs are a useful indicator of human effluent. FWAs are relatively easy to assay and in a number of studies have proven their usefulness. However as chemicals they may have different movement and survival characteristics to microbial pathogens that are usually the source of concern. As such, FWAs should be supported by additional source specific indicators.

There are a number of micro-organisms such as *Bifidobacterium adolescentis*, and some strains of Bacteroides which appear specific to humans. Other organisms such as *Rhodococcus coprophilus* appear restricted to herbivores. Traditional culturing and identification is not practical for many of these organisms. The polymerase chain reaction (PCR) makes the specific detection of these and other source specific organisms possible. Total DNA can be extracted from a water sample, and screened with a suite of PCR assays specific to different organisms. ESR is currently evaluating and developing this methodology which should not only allow the confirmation of human effluent, but the identification of non-human components.

Faecal sterol analysis is also useful, particularly for the identification of human effluent. While less distinctive, animal faecal pollution can also be identified to a limited extent. Faecal sterol analysis is however time consuming and expensive, and therefore best suited to very targeted sample evaluation.

There remain a number of key research questions that need answering. Studies of degradation and movement of faecal source indicators through soils, and different water types is required. Another key question is how far downstream from the point of entry will each indicator be detectable?

A number of other faecal source indicators are in various stages of development, but currently are not recommended as offering significant benefits to faecal source discrimination in New Zealand. Additional and improved tools will no doubt emerge, and continued evaluation of literature is prudent.

9.1 Recommendations for faecal source identification studies

The traditional microbial indicators remain the best tools for routine evaluation of microbial water quality. When elevated levels of faecal indicators are detected, and identification or confirmation of the source is required, the following general strategy (illustrated in Figure 31) is recommended:

1 Undertake a thorough site examination around the areas of concern. Utilise local knowledge from residents, farmers, local regulators and others to design a sampling strategy to cover temporal and spatial variations. Further sampling for traditional indicators to confirm the severity and consistency of the contamination is recommended. Identification of sampling locations by GPS, and with digital photographs is useful.

2 Undertake a preliminary collection of samples, and analyse for E. coli or enterococci, molecular markers, and FWAs.

	Results available	Cost	Source discrimination
Total coliforms and E. coli	1–2 days	$30	none
Molecular marker analysis	5 days	$150?	multiple
FWA analysis	10 days	$150?	human

Where the most significant contamination is identified, resample with a more comprehensive and targeted strategy. Repeat analysis for indicators in step 2, and collect, and filter water samples (4 litres or more) for later faecal sterol analysis if required.

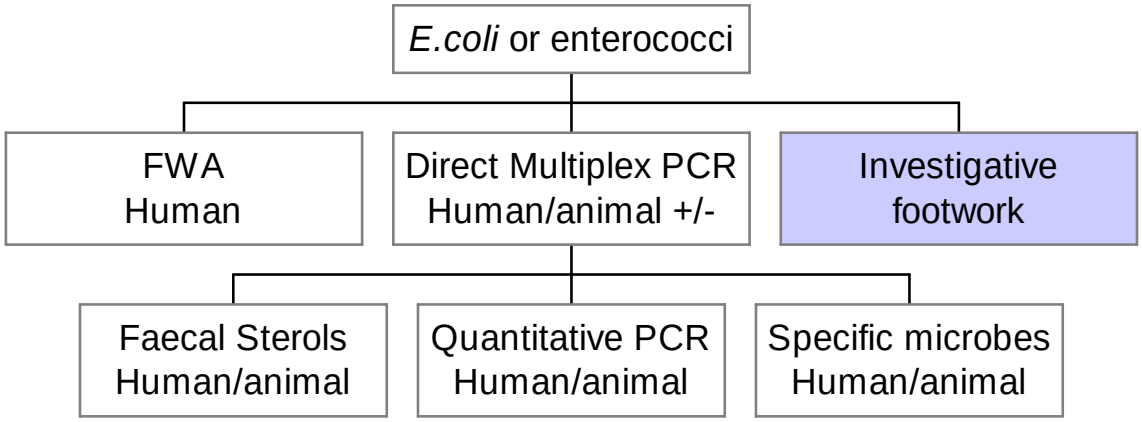
	Results available	Cost	Source discrimination
Faecal sterol analysis	14-28 days	$500	human & other

Where sewage pipe leakage is suspected, additional evidence could be gathered through the use of television camera inspections of sewage pipes, and rhodamine WT tracers to follow exfiltration. In addition, determination of flow rates in pipes could also assist with evaluation of exfiltration.

To confirm suspected sources, additional source discrimination tools may be useful to clarify or exclude potential sources. For example *C. perfringens* in comparison with faecal coliforms may indicate canine pollution, while individual bile salts are specific to pigs.

Additional evidence could be gathered through the use of television camera inspections, and rhodamine WT tracers to follow exfiltration. Determination of flow rates in pipes could also assist.

Figure 31. Strategy for use of faecal source tools.



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GLOSSARY

Genome

The genetic constitution of a microorganism.

Genotype

The recognised "subtype" of a micro-organism's genome.

Penner Serotyping

Penner Serotyping is a phenotypic subtyping method, which relies on the detection of antigens present on the surface of microorganisms. It was developed by Penner and Hennessy (1980). It uses the technique of passive haemagglutination to differentiate *Campylobacter* species isolates on the basis of their soluble heat-stable (HS) antigens.

Phenotype

The measurable, expressed, physical and biochemical characteristics of an organism, which are a result of the interaction between its genotype and environment.

Polymerase Chain Reaction (PCR)

Two oligonucleotide primers, complementary to two regions of the target DNA to be amplified, are added to the target DNA, in the presence of excess deoxynucleotides and a heat-stable DNA polymerase. In a series of temperature cycles (typically 30), the target DNA is repeatedly denatured at 95°C, annealed to the primers at 50-60°C and a daughter strand extended from the primers, at 72°C. As the daughter strands, themselves, act as templates for subsequent cycles, DNA fragments matching both primers are amplified exponentially, rather than linearly.

Restriction Enzyme

Enzymes produced by micro-organisms which each recognise specific short palindromic base sequences in DNA. They cut the DNA helix at a particular point within the recognised sequence.

Taqman™ PCR System

Taqman™ PCR exploits the 5' nuclease activity of Taq polymerase to cleave a dual-labelled fluorogenic probe that is annealed to the target sequence during amplification.The 5'end of the probe is labelled with the fluorescent reporter dye FAM and the 3' end is labelled with TAMARA which acts as a quencher dye. During the PCR reaction, the fluorogenic probe is cleaved, separating the reporter dye from the quencher and thereby generating a fluorescent emission. This fluorescent signal increases with each PCR cycle and therefore, quantitative estimates of the initial quantity of DNA present may be made (Savill *et al.*, 2001).