Chapter 21
Source Tracking in Australia and New Zealand: Case Studies

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This chapter outlines the application of fecal source tracking (FST) methods in waterways in Australia and New Zealand. FST methods used in the case studies include biochemical fingerprinting (BF), antibiotic resistance analysis (ARA), anaerobic bacterial genetic markers, toxin genetic markers, viral markers, fluorescent whitening agents (FWAs), and fecal sterols. These methods were predominantly used to identify human fecal pollution in receiving waters sourced from defective septic systems or discharges from sewage treatment plants (STPs). In some cases, these methods were also used to identify the sources of elevated levels of fecal indicator bacteria in catchment waters. The earlier case studies employed library-dependent FST methods, whereas the recent studies focused on validation and application of library-independent methods. Several case studies reported the presence of human fecal pollution in environmental waters and suggested that genetic markers are appealing because of their high specificity and sensitivity to differentiate and detect human and animal fecal pollution. Few case studies also used a combination of methods and suggested that such an approach can compensate uncertainty when one marker fails to produce satisfactory results. However little is known regarding the persistence of these markers in relation to fecal indicators and pathogens. More research is required regarding the behaviors of these markers in the environments.

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21.1 Introduction

Pollution from animal and human waste is one of the major concerns about water bodies that are used for drinking water supply, recreational activities, and harvesting seafood worldwide due to possible exposure of water users to a wide array of pathogenic bacteria, protozoa, and viruses (Fong and Lipp 2005; Hörman et al. 2004). Unsafe drinking water, inadequate sanitation, or insufficient hygiene is to blame for 1.5 million deaths each year, most being deaths of children in the developing world (Prüss-Üstün et al. 2008). While the developing world is hit hardest by the waterborne illnesses, waterborne disease outbreaks also occur in developed countries (Hrudey and Hrudey 2004). Various sources such as agricultural runoff, wild animals, combined sewer overflows, STPs, defective septic systems, and industrial outlets are known to be potential sources of such pollution (Ahmed et al. 2005a, b; McLellan 2004; Parveen et al. 1997). Microbiological quality of water is generally assessed by enumerating indicator bacteria such as *Escherichia coli* and enterococci (USEPA 2000). Indicator bacteria such as these are commonly found in the feces of warm-blooded animals including humans. The presence of these bacteria in water bodies generally indicates pollution and the potential public health risks. However, the presence of fecal indicators does not provide information regarding their sources. The identification of the major polluting source(s) is vitally important to implement appropriate mitigation strategies to minimize pollution and subsequent public health risks (Scott et al. 2002). However, the identification and assigning of indicator bacteria to human and animal sources in environmental waters is difficult due to their cosmopolitan nature, i.e., they are shed in the waste of a wide variety of hosts (Field and Samadpour 2007). In addition, environmental waters are subjected to multiple sources of pollution, making it extremely difficult to implement management plans without knowing the sources. Economically feasible and simple methodologies to estimate water-related health risk and identify pollution sources are needed globally.

Over the last decade, researchers have developed a range of FST tools that can be used to distinguish human fecal pollution from animal. These methods are broadly categorized into library-dependent (i.e., phenotypic and genotypic), library-independent (i.e., molecular markers), and chemical (i.e., sterols, FWAs). The application of some of these methods has been evaluated rigorously. However, other methods require more validation before being used to identify the sources of fecal pollution in environmental waters. This chapter reviews the application of source tracking tools in practical situations in Australia and New Zealand.

21.2 Source Issues in New Zealand

New Zealand is well known for its vast and, seemingly, untouched landscapes. Indeed, the population of New Zealand is relatively low (four million) compared to its size (250,000 km^2). Placed deep in the Pacific, over 2,000 km from the nearest continent, the native fauna of New Zealand is characterized by low terrestrial

mammal diversity with only three species of bats considered as true native mammals. While the isolation has limited the mammalian diversity, a number of species have been introduced by both Polynesian and European settlers and are now major components of New Zealand wildlife. These include rabbits, rodents, and possums, which currently outnumber humans fifteen to one (King 2005). Furthermore, farming is a significant contributor to the New Zealand economy (agriculture contributing roughly 5% of the national gross domestic product GDP and most of export), and the livestock numbers are high with sheep (34.1 million), poultry (20 million), and beef and dairy cattle (9.7 million) being the dominant species (Statistics New Zealand 2009). It is estimated (Kirs' unpublished data) that approximately 2.2×10^5 and 4.3×10^4 T of wet weight matter per day is released by cattle and sheep, respectively, in New Zealand, which exceeds the contribution from the human population by 350 fold. Although this untreated waste is dispersed over a large area, livestock usually roam free and often have open access to waterways. Therefore, in the New Zealand context, livestock can be a major contributor to microbial pollution of streams, lakes, and coastal regions and may result in significant human health risks. Municipal sewage treatment facilities have progressively been upgraded in most cities, with high levels of treatment achieved in most areas. There has, however, been an increase in septic systems on the edges of urban centers as an increasing number of people move to "lifestyle" blocks or accept longer commuting distances in exchange for living in less urbanized environments.

21.3 Fecal Source Issues in Australia

Microbial pollution in Australian waters is caused by a combination of point sources (PS) and nonpoint sources (NPS) of pollution. Pollution from PS includes discharge from industries and STPs and defective septic systems. Septic systems are the only waste disposal option in nonsewered catchments throughout Australia. High numbers of septic systems are reported to be failing, are not monitored (Ahmed et al. 2005a), and have the potential to discharge microbial pollutants in surface and ground waters. NPS pollution also poses a significant threat to the catchment and recreational water quality. This is because NPS sources of pollution are comprised of diverse sources of microbial pollutants from undefined sites within a catchment. These include stormwater runoff, urban runoff from agricultural fields, livestock runoff, and defecation of wild animals and pets. With increasing urbanization of the nation's coastal areas, pollution of waterways by NPS is a growing concern.

21.3.1 Fecal Source Tracking Tools

The number of tools available to identify the possible sources of fecal pollution in environmental waters is rapidly increasing. A brief summary of these tools is given below.

21.3.2 Library-Dependent Source Tracking Tools

The majority of the early-developed MST methods are library-dependent, which requires the development of a database or "library" of *E. coli* or enterococci from suspected sources using various genotypic and phenotypic methods. The underlying assumption of these methods is that host specificity of microorganisms is influenced by selective pressure (Wiggins 1996). Phenotypic and genotypic patterns of target strains are then compared to the library to identify their likely sources (Harwood et al. 2000; Wiggins et al. 1996). The commonly used phenotypic library-dependent methods are ARA (Wiggins et al. 1999), carbon source utilization (CSU) (Hagedorn et al. 2003), and BF (Ahmed et al. 2005a, b). The most commonly used genotypic library-dependent methods include pulsed field gel electrophoresis (Moyda et al. 2003), repetitive extragenic palindromic PCR (rep-PCR) (Dombek et al. 2000), and ribotyping (Parveen et al. 1999; Scott et al. 2003). The case studies presented in this chapter include examples of the use of BF and ARA.

21.3.2.1 Biochemical Fingerprinting

This method uses quantitative measurements of the kinetics of several biochemical reactions of bacteria in microtiter plates with dehydrated substrates (Katouli et al. 1997; Möllby et al. 1993). The typing reagents used in this method are specifically chosen for different groups of bacteria to give an optimal discriminatory power and reproducibility (Möllby et al. 1993). For each bacterial isolate, it yielded a biochemical fingerprint made of several quantitative data which were used with the PhPlate software version 4001 (PhPlate system, PhPlate AB, Stockholm) to calculate the level of similarity between the tested isolates. Similarities between the isolates were calculated as correlation coefficients and clustered according to the unweighted-pair group method with arithmetic averages (UPGMA) (Sneath and Sokal 1973). All data handling, including optical readings, calculations of correlation coefficients, diversity indexes, and Sp values, as well as clustering and printing dendrograms, was performed using the PhPlate software.

21.3.2.2 Antibiotic Resistance Analysis

Antibiotics are used to prevent and treat infections in humans and domestic animals, as well as to promote growth in animals. Microorganisms develop resistance to antibiotics to which they are regularly exposed, and intrinsic resistance to certain antibiotics is found in many bacteria. The ARA method is based on the hypothesis that bacteria present in the intestine of different animals, subjected to different types and concentrations of antibiotics, would result in host-specific resistance profiles (Wiggins 1996). ARA fingerprints of unknown environmental isolates can be compared to a reference library of several known host groups. There is currently

no standard panel of antibiotics and concentrations used for this method. Antibiotics are basically selected on the basis of their uses in different host groups. This method has shown to be successful in discriminating *E. coli* and enterococci isolated from animal species (Parveen et al. 1997; Wiggins et al. 1996, 1999).

21.3.3 Library-Independent Source Tracking Tools

There are many microorganisms other than fecal coliforms, *E. coli* and enterococci present in feces, which have greater specificity to human and animal hosts. Difficulties in culturing and identifying some of these organisms have limited their useful application to source identification. An alternative approach is to extract total DNA from a water sample and examine the sample using PCR for DNA from source-specific organisms. These methods do not require the development of a library and, therefore, are known as library-independent methods. Library-independent methods could be categorized into three groups:

- Anaerobic bacterial genetic markers: Some members of the *Bacteroides* and *Bifidobacterium* genus appear to be host-associated (Allsop and Stickler 1985; Kreader 1995). These anaerobes constitute a larger portion of gut microflora than do coliforms and enterococci (Sghir et al. 2000) and have limited reproduction capacity in the environment. A research study reported the identification of human- and bovine-specific *Bacteroides-Prevotella* 16S rRNA gene markers using length heterogeneity PCR and terminal restriction fragment length polymorphism (T-RFLP) and concluded that these markers could be used to detect human or bovine origin pollution (Bernhard and Field 2000). Other researchers have also exploited this group of bacteria, developing PCR assays targeted at *Bacteroides* markers specific to human (Layton et al. 2006; Okabe et al. 2007; Reischer et al. 2007), dog (Kildare et al. 2007), ruminant (Reischer et al. 2006), pig, and horse (Dick et al. 2005) for the identification of pollution. Recently, quantitative PCR (qPCR) methods have been developed to quantify human and ruminant specific *Bacteroides* markers in sewage and environmental waters (Layton et al. 2006; Reischer et al. 2007; Seurinck et al. 2005). Other species can also be targeted. For example Devane et al. (2007) isolated from duck feces a novel bacteria (most closely related to members of the *Desulfovibrio* genus) and then designed PCR primers targeting the 16S rRNA of this organism, which could be used as wildfowl-specific marker.
- Viral markers: Viral genetic markers have been used to identify the sources of pollution in environmental waters. The most commonly used viral markers are human adenoviruses (Choi and Jiang 2005; Fong et al. 2005; Noble et al. 2003), human polyomaviruses (McQuaig et al. 2006, 2009), F+ specific RNA phages (Love and Sobsey 2007). Human-, bovine- and porcine-specific adenoviruses and polyomaviruses have been used to identify the sources of pollution in USA and Spain (Choi and Jiang 2005; Fong and Lipp 2005; Hundesa et al. 2006;

Table 21.1 General and host-specific PCR markers used in the case studies in Australia and New Zealand

Assays	Host-marker designation	References
Bacteroides general	General-Bac32	Bernhard and Field (2000)
Bacteroides human	HF183	Bernhard and Field (2000)
Bacteroides ruminant	CF128	Bernhard and Field (2000)
Bacteroides dog	Dog-BacCan	Kildare et al. (2007)
E. faecium human	Human-*esp*	Scott et al. (2005)
JCV–BKV human PCR	Human polyomavirus-JCV and BKV	McQuaig et al. (2006)
M. smithii human PCR	Human-*nifH*	Ufnar et al. (2006)
B. adolesscentis human	*B. adolesscentis*	Matsuki et al. (2004)

Maluquer de Motes et al. 2004; McQuaig et al. 2006; Pina et al. 1998). qPCR method has also been developed for the quantitative detection of these viruses in environmental waters.

- Bacterial toxin genetic markers: PCR assays have been developed to detect host-specific toxin genes in indicator bacteria such as *E. coli* and enterococci. These include the pig-specific ST1b (Khatib et al. 2003) and cattle-specific LTIIa*E. coli* toxin gene (Chern et al. 2004; Khatib et al. 2002), and the enterococcal surface protein (*esp*) gene found in *Enterococcus faecium* (Scott et al. 2005). A recent study reported the development of a qPCR assay for the quantitative detection of *esp* markers in sewage and environmental waters (Ahmed et al. 2008c).

A selection of these methods has been used in some of the case studies described in this chapter. These are detailed in Table 21.1

21.3.4 Chemical Source Tracking Methods

FWAs (also called optical brighteners) are man-made fluorescent organic compounds that absorb ultraviolet light and reemit most of the absorbed energy as blue light. To improve whiteness, FWAs are used in the manufacture of paper and clothing. As FWAs are lost from clothing during wear and washing, most laundry powders contain 0.10–0.15% (w/w) FWA to maintain whiteness. It is estimated that between 20 and 95% of the FWAs bind to the fabric during washing with the remainder being discharged with the washing liquor (Poiger et al. 1998, Stoll and Giger 1998). Most household plumbing mixes effluent from toilets with "grey water" from washing machines. Consequently, FWAs are usually associated with human fecal pollution in both septic tanks and community wastewater systems. FWAs absorb light at 350 nm (the excitation wavelength) and reemit the light as fluorescence at a higher wavelength (emission wavelength) in the range 430 nm (Poiger et al. 1993). The detection of FWAs, therefore, indicates the potential presence of human fecal pollution from a sewage system. In general, levels of FWA greater than 0.2 ppb typically

indicate recent or local source of human pollution, while lower levels indicate increasingly dilute or distant sources of human pollution. Levels below 0.1 ppb typically are not associated with local source of human pollution (Devane et al. 2006).

Fecal sterols are a group of C27-, C28-, and C29-cholestane-based sterols found mainly in animal feces. The sterol profile of feces depends on the interaction of three factors. First, the animal's diet determines the relative quantities of sterol precursors (cholesterol, 24-ethylcholesterol, 24-methylcholesterol, and/or stigmasterol) entering the digestive system. Second, animals differ in their endogenous biosynthesis of sterols (for example, human beings on a low cholesterol diet synthesize cholesterol). Third and perhaps most importantly, anaerobic bacteria in the animal gut biohydrogenate sterols to stanols of various isomeric configurations (Devane et al. 2006).

The sterol, cholesterol, can be hydrogenated to one or more of four possible stanols. In humans, cholesterol is preferentially reduced to coprostanol where it constitutes 60% of the total sterols found in human feces (Leeming et al. 1996). By contrast, cholesterol is predominantly reduced to cholestanol in the environment. Similarly, plant-derived 24-ethylcholesterol is reduced to 24-ethylcoprostanol and 24-ethylepicoprostanol in the gut of herbivores, whereas in the environment it is primarily reduced to 24-ethylcholestanol. As a consequence, analysis of the sterol composition of feces can generate a sterol fingerprint, which can be distinctive from one species to another – particularly in the case of differentiating human from animal pollution. In the case studies described in this chapter, ten different ratios are presented (Table 21.2). Ratio 1 (coprostanol/cholestanol) and ratio 2 (24-ethylcoprostanol/24-ethylcholestanol) are typically >0.5 in human and ruminant fecal material (Leeming et al. 1996). These ratios in wildfowl and canine feces may not exceed these thresholds. Ratios 3–6 are all indicative of human feces when thresholds are exceeded and are based around elevated relative levels of coprostanol (Grimault et al. 1990, Reeves and Patton 2001). Ratios 5–8 are indicative of herbivore feces (Leeming et al. 1998, Gilpin et al. 2002). High levels of ratio 8 (24-ethylcholesterol/24-ethylcoprostanol) suggest either plant decay or a diet of

Table 21.2 Fecal sterol ratios and data interpretation

Sterol ratio		Interpretation
Ratio 1	Coprostanol/cholestanol	>0.5 Fecal
Ratio 2	24-Ethylcoprostanol/24-ethylcholestanol	>0.5 Fecal
Ratio 3	Percent coprostanol	>5–6% Human fecal pollution
Ratio 4	Coprostanol/(coprostanol+cholestanol)	>0.7 Human fecal pollution
Ratio 5	Coprostanol/24-ethylcoprostanol	<1.0 Herbivore; ≥1.0 human
Ratio 6	Coprostanol/coprostanol+24-ethylcoprostanol	<30% Herbivore; >75% human
Ratio 7	Percent 24-ethylcoprostanol	>5–6% Herbivore
Ratio 8	24-Ethylcholesterol/24-ethylcoprostanol	<1.0 Herbivore; >4.0 plant decay
Ratio 9	24-Ethylcholestanol/(24-ethylcholestanol+24-ethylcoprostanol+24-ethylepicoprostanol)	>30% Avian
Ratio 10	Cholestanol/(cholestanol+coprostanol+epicoprostanol)	>67% Avian

plant material such as that seen in wildfowl. Ratios 9 and 10 when both exceeding thresholds suggest wildfowl source of sterols (B. Gilpin, unpublished).

Fecal sterols analysis in each of the case studies in this chapter was performed by filtering up to 4 L of river water onto glass fiber filters. Filters were stored frozen until they were analyzed. Solvent extraction was performed prior to hydrolysis, which was followed by back-extraction into hexane. The sterol fraction is eluted into methanol and silylated prior to analysis by GC-MS (Gregor et al. 2002). Each sterol and stanol detected is expressed in parts per trillion (ppt).

21.4 Case Studies

21.4.1 Library-Dependent Methods

21.4.1.1 Case Study 1: Biochemical Fingerprinting Method Identifies Human and Animal Pollution in Eudlo Creek, Southeast Queensland, Australia

Situation: Urban creek water samples were collected from five sites on Eudlo Creek, Southeast Queensland, Australia. The primary aim was to identify human sewage pollution in the creek, which may have entered via defective septic systems. A secondary aim was to identify domestic and wild animal pollution (Ahmed et al. 2005b).

Tools used: BF libraries comprising of 4,057 enterococci and 3,728 *E. coli* isolates from horses, cattle, sheep, pigs, ducks, chickens, deer, kangaroos, dogs and septic tanks were used to identify the sources of unknown environmental *E. coli* and enterococci using cluster analysis.

Results: A total of 248 enterococci biochemical phenotypes (BPTs) were obtained from creek water samples, of which 26 BPTs (10%) were identical to BPTs from septic tanks and 152 BPTs (61%) were identical to various animals (Table 21.3). Of the 282 *E. coli* BPTs from the same water samples, 36 BPTs (13%) were identical to

Table 21.3 Comparison of biochemical phenotypes (BPTs) from environmental water samples from sampling sites (EC1–EC5) on Eudlo Creek, Queensland, Australia with enterococci (Ent) and *E. coli* libraries

	No. of BPTs found		Septic UQ-BPTs		Animal BPTs		Unknown BPTs	
Sampling sites	Ent	*E. coli*	Ent	*E. coli*	Ent	*E. coli*	Ent	*E. coli*
EC1	60	71	9	11	38	37	13	23
EC2	72	84	8	11	42	47	22	26
EC3	71	85	8	8	45	51	18	26
EC4	22	13	1	3	14	5	7	5
EC5	23	29	0	3	13	11	10	15
Total	248	282	26	36	152	151	70	95

BPTs from septic tanks, and 151 BPTs belonged to animals. The sources of the remaining 70 enterococci BPTs and 95 *E. coli* BPTs could not be identified.

Conclusions: The study reports the use of BF method as a potential tool for MST studies. *E. coli* and enterococci libraries were capable of identifying the sources of more than 65% of indicator bacteria in the studied creek. The authors reported the presence of human fecal isolates in the studied creek originating from defective septic tanks as well as animal sourced isolates. However, the percentage of isolates that were identified as animals was higher than humans.

21.4.1.2 Case Study 2: Antibiotic Resistance Analysis for Detecting Pollution from Septic Systems in Surface Waters in Queensland, Australia

Situation: ARA was used to determine the significance of septic systems as a major contributor to fecal pollution in two mixed land-use catchments, Bonogin Valley and Tallebudgera Creek, in the Gold Coast region, Queensland, Australia (Carroll et al. 2005).

Tools used: Antibiotic resistance patterns (ARPs) were established for a library of 717 known source *E. coli* isolates obtained from human, domesticated animals, livestock ,and wild sources. Discriminant analysis (DA) was used to differentiate between the ARPs of isolates from various sources and to classify each isolate from water (unknown source) into a source category.

Results: A total of 256 (from five sites from Bonogin Creek catchment) and 169 (from three sites from Tallebudgera Creek catchment) isolates from water were subjected to ARA analysis. By applying DA to the water isolates, and utilizing the human vs. nonhuman source category, the percentage of human source isolates contained in each water sample was obtained. From the discriminant analysis of samples obtained from Bonogin Creek, 40, 55, 10, 52, and 56% of the isolates from sites BOS1 to BOS5, respectively, were classified as human source (Table 21.4). For Tallebudgera Creek, 24, 37 and 47% of isolates obtained from TA1 to TA3, respectively, were also classified as human source.

Table 21.4 Source identification of unknown environmental *E. coli* isolates from the Bonogin Valley and Tallebudgera Creeks by antibiotic resistance analysis in Queensland, Australia

Sampling sites	No. of unknown isolates	Source identification (%) of unknown source isolates	
		Human	Animals
BOS1	45	40	60
BOS2	48	55	46
BOS3	23	10	90
BOS4	93	52	48
BOS5	46	56	44
TA1	51	24	76
TA2	74	37	63
TA3	43	47	53

Conclusions: The results suggested the presence of human fecal pollution within the investigated catchments originating from septic systems. From the other classified sources, it was evident that in the upper regions of both catchments, the major source of pollution was from animals. The information obtained through this study has been utilized by the local regulatory authority to implement more appropriate management practices to reduce the human fecal pollution of water resources caused by high numbers of failing septic systems.

21.4.1.3 Case Study 3: Biochemical Fingerprinting and Antibiotic Resistance Analysis to Identify Dominant Sources of Pollution in a Coastal Lake, Southeast Queensland, Australia

Situation: Water samples were collected from five sampling sites on the Tooway, a recreational coastal lake, Queensland, Australia to identify the sources of elevated levels of indicator bacteria (Ahmed et al. 2008b).

Tools used: BF and ARA were used to identify the sources of enterococci and *E. coli* in the studied lake. A population similarity (Sp) analysis was used to compare the overall similarity between bacterial populations from suspected sources with those found in the environmental water samples.

Results: Five sampling sites (T1–T5) were chosen at various points along the length of the lake. Water samples ($n=20$) were collected fortnightly on four occasions. The BPTs of enterococci and *E. coli* isolates from each site were compared to the BPTs of the suspected sources and host groups. However, only *E. coli* isolates from water samples were typed by ARPs and were classified according to host source by ARA. BF of enterococci populations showed that isolates from waterfowl were most similar (showed the highest Sp-coefficient) (0.46 ± 0.09) to water samples and showed the next highest similarity to isolates from STPs (0.31 ± 0.06) (Table 21.5). Similar patterns were also observed when *E. coli* were subjected to BF (0.32 ± 0.03; waterfowl, and 0.27 ± 0.09; STP). Both bacterial populations from all sampling sites showed the highest similarities with those of waterfowl. In contrast, bacterial populations from dogs and chickens generally showed low similarities to water samples. High similarity values were also observed for both bacterial populations from STP and water samples with higher values found in sites T2 and T3 located below the submerged sewerage pipes collecting domestic wastewater. When *E. coli* populations from each site were compared to those of the ARPs, the highest similarity (0.27 ± 0.07) was found between STP and water samples followed by waterfowls and water samples (0.16 ± 0.07) (Table 21.6). *E. coli* populations from dogs and chickens generally showed low similarities with those from water samples.

Conclusions: BF identified waterfowl as a major source of contamination. Each method individually also identified the STP as a source of pollution. The author concluded that Sp-analysis is a simple, rapid, and reliable approach and could be used for comparing bacterial populations from known fecal sources with those from water samples to predict the sources of pollution. However, this approach should be limited to small catchments with limited possible sources of pollution.

Table 21.5 Comparison of population similarity (Sp) coefficient based on biochemical fingerprinting of enterococci (Ent) and *E. coli* isolates from sources and water samples collected from sites T1 to T5 on Tooway Lake, Queensland, Australia

	Population similarity (Sp) coefficient to water samples									
	T1		T2		T3		T4		T5	
Sources	Ent ($n=116$)	*E. coli* ($n=85$)	Ent ($n=97$)	*E. coli* ($n=92$)	Ent ($n=97$)	*E. coli* ($n=87$)	Ent ($n=98$)	*E. coli* ($n=83$)	Ent ($n=100$)	*E. coli* ($n=88$)
STP	0.32	0.22	0.27	0.38	0.35	0.29	0.40	0.31	0.24	0.14
Waterfowl	0.26	0.32	0.48	0.37	0.46	0.31	0.47	0.27	0.46	0.34
Dog	0.09	0.03	0.15	0.39	0.11	0.17	0.13	0.07	0.10	0.13
Chicken	0.07	0.11	0.13	0.09	0.16	0.03	0.06	0.04	0.04	0.02

Table 21.6 Comparison of population similarity (Sp) coefficient based on antibiotic resistance patterns (ARPs) of *E. coli* isolates from sources and water samples collected from sites T1 to T5 on Tooway Lake, Queensland, Australia

	Population similarity (Sp) coefficient to water samples				
Sources	T1 ($n=31$)	T2 ($n=69$)	T3 ($n=46$)	T4 ($n=52$)	T5 ($n=26$)
STP	0.19	0.35	0.29	0.34	0.20
Waterfowl	0.11	0.14	0.27	0.09	0.29
Dog	0.02	0.11	0.07	0.04	0.22
Chicken	0.06	0.05	0.06	0.01	0.03

Table 21.7 Quantitative PCR results of the enterococci surface protein (*esp*) marker in environmental waters collected from Ningi Creek, Queensland, Australia

Sampling sites (event 1)	Enterococci	Gene copies/100 mL	Sampling sites (event 2)	Enterococci	Gene copies/100 mL
NC1	4.1×10^3	1.1×10^2	NC1	3.7×10^3	–
NC2	3.2×10^3	–	NC2	1.0×10^2	–
NC3	1.3×10^4	1.6×10^2	NC3	3.9×10^3	–
NC4	1.9×10^4	5.3×10^2	NC4	5.6×10^4	4.3×10^2
NC5	4.3×10^4	–	NC5	3.9×10^4	3.1×10^2
NC6	2.8×10^4	5.2×10^2	NC6	2.1×10^3	–
NC7	3.9×10^3	–	NC7	9.1×10^2	–
NC8	1.4×10^3	–	NC8	9.2×10^2	–

21.4.2 Library-Independent Methods

21.4.2.1 Case Study 4: Quantitative PCR Assay for the Quantitative Detection of Human-Specific Enterococci Surface Protein (*esp*) Marker in Queensland's Environmental Waters

Situation: Quantitative PCR (qPCR) was used to estimate the levels of human-specific *esp* markers in environmental waters in Ningi Creek, Southeast Queensland, Australia. Environmental samples ($n=16$) were collected after storm events and tested with the qPCR along with the enumeration of enterococci for the quantitative detection of human pollution (Ahmed et al. 2008c).

Tools used: qPCR of sewage associated enterococcal surface protein (*esp*) markers from *E. faucium*.

Results: The specificity of the *esp* marker to distinguish between human and animal pollution was determined by screening a large number of human and animal samples. The *esp* marker was detected in 90.5% of combined sewage and septic tank samples ($n=42$) and was not detected in any of the fecal samples ($n=155$) from the nontarget animals tested. The overall specificity of this marker to distinguish between sewage and animal pollution was 1.0 (100%). The concentration of culturable enterococci in water samples collected from the studied creek ranged between 9.1×10^2 and 4.3×10^4 cfu/100 mL (Table 21.7). Of the 16 samples tested, six (38%)

were positive for the *esp* marker, and the concentration ranged between 1.1×10^2 and 5.3×10^2 gene copies/100 mL of water.

Conclusions: The evidence presented in this study demonstrated that the *E. faecium esp* marker appears to be host-specific and promising for human pollution tracking in environmental waters in Southeast Queensland, Australia. The study successfully demonstrated the application of a newly developed qPCR assay to quantify the *esp* marker in environmental waters.

21.4.2.2 Case Study 5: Application of Human-Specific HF183 and HF134 *Bacteroides* Markers for the Detection of Human Pollution in Nonsewered Catchments in Southeast Queensland

Situation: Stormwater samples were collected from the Bergin Creek, Four Mile Creek and River Oaks Drive nonsewered catchments within the Pine Rivers Shire in Southeast Queensland. The primary aim of this study was to assess whether human-specific *Bacteroides* markers (indicative of human pollution) could be detected in stormwater samples potentially contaminated by defective septic systems (Ahmed et al. 2008d).

Tools used: PCR detection of human-specific *Bacteroides* HF183 and HF134 markers.

Results: Prior to field application, the specificity of each marker was tested by screening a large number of samples from nontarget fecal species. The overall specificity of the tested markers to differentiate between human and animals was 1.0 (HF183) and 0.95 (HF134), respectively, as the HF134 marker was detected in a number of dog samples. The number of *E. coli* and enterococci in storm water samples collected from the three catchments is shown in Table 21.8. Of the four samples tested from the Bergin Creek on four occasions, three were positive for both the markers. Of the three samples tested from the Four Mile Creek on three occasions, two were positive for the HF134.

Table 21.8 The number of *E. coli* and enterococci and PCR positive/negative results of human-specific *Bacteroides* markers in environmental water samples collected from three nonsewered catchments

Catchments	Events	*E. coli*	Enterococci	HF183	HF134
Bergin Creek	Event 1	2.6×10^3	2.7×10^3	+	+
	Event 1	3.9×10^3	4.3×10^3	+	+
	Event 2	4.0×10^3	3.1×10^3	+	+
	Event 3	4.1×10^3	3.4×10^3	–	+
Four mile Creek	Event 1	1.4×10^3	1.8×10^3	–	+
	Event 2	9.6×10^3	8.5×10^3	+	+
	Event 3	2.6×10^3	2.5×10^3	–	–
River Oaks	Event 1	2.7×10^3	2.4×10^3	–	+
	Event 2	2.1×10^3	1.8×10^3	–	–
	Event 3	1.6×10^3	1.4×10^3	–	–

Conclusions: The HF183 marker is specific to human sewage and is a reliable marker for detecting human fecal pollution in Southeast Queensland, while the use of HF134 marker alone may not be sufficient enough to provide evidence of human pollution because of its presence in dog feces.

21.4.2.3 Case Study 6: Application of Human Associated JCV and BKV Polyomaviruses for the Detection of Sewage Pollution in a Coastal River in Southeast Queensland, Australia

Situation: Environmental water samples were collected from five locations (MR1 – MR5) in Maroochy River, Sunshine Coast Region, Queensland, Australia. The primary aim of this study was to evaluate the host specificity of a PCR method to detect JCV and BKV polyomaviruses, and a secondary aim was to identify sewage pollution in the studied river (Ahmed et al. 2010).

Tools used: PCR detection of human-specific JCV and BKV polyomaviruses.

Results: The host specificity of the markers was tested by screening wastewater/samples from nontarget sources such as chickens, dogs, ducks, kangaroos, wild birds, cattle, pigs, and sheep. The overall host specificity of the JCV and BKV PCR assay to differentiate between human and animal wastewater/samples was 0.99. The concentration of *E. coli* in water samples ranged between <1 and 2,906 ± 300 cfu/100 mL of water (Table 21.9). The concentration of enterococci in water samples ranged between <1 and 1,586 ± 180 cfu/100 mL of water. Of the 20 samples tested, five (25%) were positive for JCV and BKV indicating the presence of human wastewater in various sites in the Maroochy River.

Table 21.9 Concentrations of *E. coli* and enterococci along with PCR positive results for sewage-specific JCV and BKV polyomaviruses at sampling sites on Maroochy River, Southeast Queensland, Australia

Sampling sites (occasion)	*E. coli* (cfu/100 mL)	Enterococci (cfu/100 mL)	PCR positive results for HS-PVs
MR1 (1)[a]	$4.8\times10^{1}\pm1.1\times10^{1}$	$7.3\times10^{1}\pm1.2\times10^{1}$	–
MR2 (1)[a]	$5.2\times10^{1}\pm3.0\times10^{1}$	$1.2\times10^{2}\pm2.0\times10^{1}$	+
MR3 (1)[a]	$1.5\times10^{2}\pm5.5\times10^{1}$	$2.3\times10^{2}\pm3.0\times10^{1}$	–
MR4 (1)[a]	$3.6\times10^{2}\pm8.0\times10^{1}$	$4.0\times10^{2}\pm1.0\times10^{2}$	–
MR5 (1)[a]	$2.9\times10^{3}\pm3.0\times10^{2}$	$1.6\times10^{3}\pm1.8\times10^{2}$	–
MR1 (2)	$1.7\times10^{1}\pm1.0\times10^{1}$	$1.6\times10^{2}\pm3.8\times10^{1}$	–
MR2 (2)	$1.3\times10^{1}\pm5.0\times10^{0}$	$2.2\times10^{2}\pm6.0\times10^{1}$	–
MR3 (2)	$2.1\times10^{1}\pm7.0\times10^{0}$	$3.3\times10^{1}\pm8.0\times10^{0}$	–
MR4 (2)	$3.3\times10^{1}\pm7.0\times10^{0}$	$6.2\times10^{1}\pm1.3\times10^{1}$	–
MR5 (2)	$2.1\times10^{1}\pm6.0\times10^{0}$	$1.3\times10^{2}\pm5.7\times10^{1}$	–
MR1 (3)	$<1.0\times10^{0}$	$6.0\times10^{1}\pm2.0\times10^{1}$	+
MR2 (3)	$4.4\times10^{1}\pm5.0\times10^{0}$	$2.0\times10^{1}\pm5.0\times10^{0}$	–
MR3 (3)	$1.5\times10^{1}\pm5.0\times10^{0}$	$3.0\times10^{1}\pm1.0\times10^{1}$	+
MR4 (3)	$7.0\times10^{1}\pm1.5\times10^{1}$	$6.5\times10^{1}\pm4.1\times10^{1}$	+
MR5 (3)	$1.0\times10^{2}\pm1.1\times10^{1}$	$< 1.0\times10^{0}$	–

(continued)

Table 21.9 (continued)

Sampling sites (occasion)	*E. coli* (cfu/100 mL)	Enterococci (cfu/100 mL)	PCR positive results for HS-PVs
MR1 (4)	$1.3\times10^{1}\pm5.0\times10^{0}$	$4.0\times10^{1}\pm1.0\times10^{1}$	–
MR2 (4)	$6.0\times10^{0}\pm4.0\times10^{0}$	$1.5\times10^{1}\pm6.0\times10^{0}$	–
MR3 (4)	$<1.0\times10^{0}$	$1.8\times10^{1}\pm1.1\times10^{1}$	–
MR4 (4)	$6.7\times10^{1}\pm1.5\times10^{1}$	$4.0\times10^{1}\pm1.4\times10^{1}$	–
MR5 (4)	$6.0\times10^{0}\pm2.0\times10^{0}$	$4.2\times10^{1}\pm7.0\times10^{0}$	–

ND not detected
[a]Study area received >100 mm rainfall 2 days prior sampling

Conclusions: JCV and BKV viruses are highly specific to human sewage, and they proved a reliable marker for detecting human fecal pollution in this coastal river in Southeast Queensland, Australia. The presence of JCV and BKV viruses in water samples indicate potential public health risks as the studied river is used for recreational activities including swimming, fishing, and water sports.

21.4.2.4 Case Study 7: Application of a Suite of MST Markers in an Urbanized Waterway, New Zealand

Situation: A MST study was initiated due to the chronic elevated fecal indicator bacteria concentrations in the urban sections of the lower Maitai River, New Zealand.

Tools used: Water samples were collected along the river on three separate dates and tested for a suite of MST markers (human-associated *E. faecium esp* gene, human- and ruminant-associated *Bacteroides* spp. (HF183 and CF128), human-associated *Methanobrevibacter smithii nifH* gene (*nifH*), and human JCV and BKV polyomaviruses by end-point PCR analyses.

Results: All samples collected expressed a signal of ruminant-associated fecal pollution; however, a strong human component was detected in all samples collected at or downstream of stormwater drains entering from the urbanized side of the river. The human-associated fecal pollution detection was supported by a minimum of two different human markers at those sites and was coupled with elevated fecal indicator bacterial concentrations (Table 21.10). Therefore, while the upstream land runoff was probably the major source of ruminant-associated fecal pollution, leaking sewage collection systems or cross-connections that impact the groundwater or stormwater systems was indicated as the major source of human-associated fecal pollution in the river.

Conclusions: While the actual source at the time of this study was not clear, follow-on engineering work commissioned by the regional council located an aged and leaking sewage drainage system in a historic residential area upstream of the stormwater drainage entering the city side of the river. This outcome highlights the utility of using MST markers in association with traditional fecal indicator bacteria tests to assist in confirming and pinpointing a fecal pollution problem area and also reveals the risk of fecal pollution from aging sewage reticulation systems that many

Table 21.10 Fecal indicator bacteria concentrations and presence/absence of MST markers (positive signal/number of samples tested) in water samples collected in the Maitai River (New Zealand). More than one number is given where multiple samples for fecal indicator bacterial concentrations were collected.

	Fecal indicator concentrations		General	Ruminant	Human			
Sites Maitai River	Fecal coliforms (cfu/100 mL)	Enterococci (cfu/100 mL)	Bac32	CF128	HF183	*esp*	*nifH*	JCV-BKV
1	1.4×10^3	2.9×10^2	1/1	1/1	1/1	0/1	0/1	0/1
2	5.0×10^2	2.2×10^2	3/3	3/3	2/3	0/3	0/3	0/3
3	5.7×10^2	2.3×10^2	2/2	2/2	2/2	1/2	1/2	2/2
4	4.0×10^2	4.0×10^2	1/1	0/1	1/1	1/1	1/1	1/1
5A[a]	1.4×10^3	1.6×10^3	2/2	2/2	1/2	1/2	1/2	2/2
5B[a]	$>2.5\times10^4$	$>1.0\times10^4$	1/1	1/1	1/1	1/1	1/1	1/1
6	2.5×10^3	1.7×10^3	1/1	1/1	1/1	1/1	1/1	1/1
7[a]	NA	1.3×10^2	1/1	0/1	1/1	0/1	0/1	0/1
8	4.4×10^2	1.2×10^2	1/1	1/1	1/1	0/1	0/1	1/1
9	1.1×10^2	8.7×10^1	1/1	1/1	1/1	1/1	1/1	1/1
10[a]	9.2×10^1	6.3×10^1	1/1	1/1	1/1	1/1	0/1	1/1

[a]Indicates samples collected in stormwater outfalls

older developed areas may face in the future. Frequent seismic activity can be a contributing factor in New Zealand.

21.4.3 Combinations of Methods

21.4.3.1 Case Study 8: Application of FWAs and Molecular Methods for Determination of Fecal Sources in a Large River in Two New Zealand Rivers

Situation: An investigation of fecal sources was undertaken on two New Zealand rivers. The primary interest for both rivers was whether human sources of fecal pollution were present, and secondarily, whether dairy cows further up the catchment of each river were contributing to fecal pollution of each river.

Tools used: Five samples were analyzed from various sites on river A, and three samples from river B. Samples were analyzed for the presence of FWAs, and with PCR-based assays for *Bacteroides* general, human, and ruminant markers. River B water samples were also tested for the presence of the *B. adolescentis* PCR marker.

Results: Levels of the microbial indicator *E. coli* differed markedly between the two rivers. All sites on river B contained high levels of *E. coli* ($>5.0\times10^3$ *E. coli*/100 mL), while in River A only site 5 contained similar levels.

FWAs were detected in all samples from river B, only from the site 2 samples in river B (Table 21.11). These levels all support a human source of fecal contamination. The general *Bacteroides* marker was detected in all samples. The human *Bacteroides* marker was present in all river B samples, but from river A, only in the site 2 sample.

Table 21.11 *E. coli*, FWAs and molecular markers detected in rivers A and B

Sample	*E. coli*[a]	General	Human	*B. adolescentis*	Ruminant	FWA[b]
River A, site 1	5.2×10^{1}	+	–	Not tested	–	<0.01
River A, site 2	1.5×10^{2}	+	+	Not tested	–	0.2
River A, site 3	9.8×10^{1}	+	–	Not tested	+	<0.01
River A, site 4	6.3×10^{1}	+	–	Not tested	–	<0.01
River A, site 5	8.7×10^{3}	+	–	Not tested	+	<0.01
River B, site 1	1.5×10^{4}	+	+	+	–	0.38
River B, site 2	5.6×10^{3}	+	+	+	–	0.45
River B, site 3	7.5×10^{3}	+	+	+	–	0.55

[a]MPN/100 mL
[b]Parts per billion (ppb) equivalent to μg/L

This human source was supported in river B samples by the presence of the *B. adolescentis* marker. The ruminant marker in contrast was not detected in any of the river B samples. It was, however, detected in river A site 3 and 5 samples.

Conclusion: River A: At the time of sampling, *E. coli* levels were very low in samples from four of the sites, with the fifth (site 5) containing very high levels of *E. coli*. This site was negative for human-specific PCR marker, negative for FWAs and positive for the ruminant marker. Subsequent investigation identified that this site was a drain which was being used for illegal dumping of stock effluent. Of the other four sites with low levels of pollution, the general *Bacteroides* marker could be detected in all of these samples, with evidence of human source of pollution at site 2 (human-associated *Bacteroides* and elevated FWAs). Site 2 is impacted directly by a stormwater drain from a residential area, and these results indicate that human sewage is likely to be entering this system. The low levels of *E. coli* in samples from other sites limit the conclusions that can be made, although site 3 was positive for the ruminant marker.

River B: All sites sampled on this river contained significant evidence of the entry of human sewage into this stream and the very high levels of *E. coli* reinforce the likely health risk. Further investigation of site 1 identified evidence of recent construction of a retaining wall, which may have disrupted sewage lines.

21.4.3.2 Case Study 9: Application of FWAs, Fecal Sterols, and Molecular Methods for Determination of Fecal Sources in Two Streams in Auckland, New Zealand

Situation: A series of small streams run between residential properties in the city of Auckland. These discharge into the marine environment. In this study, three sites on stream C and 4 sites on stream D were sampled. For comparison, a duck pond adjacent, but not visibly connected, to stream D was also tested.

Tools used: Fecal sterols, FWAs, and PCR markers for human-associated *Bacteroides* and *B. adolescentis*.

Results: *E. coli* levels were relatively low in stream C, while in stream D levels were tenfold higher. FWAs were detected at low levels in stream C but were at or below the

Table 21.12 Measured levels of microbial indicators, FWAs, and human DNA markers in streams C and D

	Microbial Indicators	Human effluent indicators		
Sample	*E. coli*[a]	FWAs[b]	*Bacteroidetes*	*B. adolescentis*
Stream C, site 1(3)	5.2×10^2	0.03	+	+
Stream C, site 2	5.2×10^2	0.04	+	+
Stream C, site3(1)	3.1×10^2	0.02	+	+
Stream D, site 1	7.8×10^3	<0.01	–	–
Stream D, site 2	4.8×10^3	0.01	–	–
Stream D, site 3	3.6×10^3	<0.01	–	–
Stream D, site 4	3.6×10^3	<0.01	–	–
Duck pond	2.2×10^3	<0.01	–	–

[a]MPN/100 mL
[b]Parts per billion (ppb) equivalent to μg/L

detection limit in stream D. The human-associated *Bacteroides* and *B. adolescentis* markers were both detected in all stream C sites, but not in stream D sites. The duck pond while containing high levels of *E. coli*, contained no detectable FWAs or human-associated *Bacteroidetes* and *B. adolescentis* markers (Table 21.12). Total levels of fecal sterols contrasted between streams C and D. Stream C contained high levels of sterols, with ratios strongly supporting the presence of human fecal material (Table 21.13). The duck pond also contained high levels of sterols but with quite different ratios, clearly not indicative of a human source. The stream D samples contained much lower levels of sterols, particularly of coprostanol and 24-ethylcoprostanol. The low levels of these two sterols makes interpretation of some of the ratios difficult, but on the basis of the ratios of sterols present, a human source is not indicated.

Conclusions: These two streams contrasted markedly in levels of *E. coli* and the presence of human-associated markers. For stream C, the three source specific tools used all indicated the presence of human effluent, although the levels of FWAs are at very low levels. The low level of FWAs may be indicative of the lack of use of washing powders at the time of sampling, and may suggest relatively few contributing households. In light of the source specific indicators, it would seem likely most of the *E. coli* present were of human origin. Stream D samples contained no evidence of human effluent in the samples analyzed. The absence of FWAs, human specific molecular markers, and fecal sterols, all suggested a nonhuman source of the *E. coli* present.

21.4.3.3 Case Study 10 Application of FWAs, Fecal Sterols, and Molecular Methods for Determination of Fecal Sources in a Rural River in the South Island of New Zealand

Situation: This investigation focussed on two sites on a river in the lower South Island of New Zealand. Possible fecal sources included human septic tanks, dairy cows, and wildfowl. An adjacent duck pond was also sampled for comparison.

Table 21.13 Fecal sterol analysis of streams C, D, and a duck pond

	Stream C			Stream D					
Sterol[a]	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 4	Duck pond	Interpretation
Coprostanol	3,620	2,150	2,410	35	50	46	34	1,110	
24-Ethylcoprostanol	1,460	914	941	52	38	46	46	4,380	
Epicoprostanol	29	52	56	21	33	23	31	630	
Cholesterol	5,950	4,920	5,110	1,310	1,060	1,150	2,500	14,890	
Cholestanol	414	432	467	270	223	213	289	5,640	
24-Methylcholesterol	932	715	669	466	334	391	632	6,170	
24-Ethylepicoprostanol	24	5	5	28	5	5	5	1,000	
Stigmasterol	457	329	322	339	275	289	387	7,640	
24-Ethylcholesterol	2,860	2,120	2,180	1,640	1,000	1,330	2,190	31,920	
24-Ethylcholestanol	391	340	362	207	269	210	554	8,490	
Total sterols	16,137	11,977	12,522	4,368	3,287	3,703	6,668	81,870	
Ratio 1	8.74	4.98	5.16	0.13	0.22	0.22	0.12	0.20	>0.5 Fecal
Ratio 2	3.73	2.69	2.60	0.25	0.14	0.22	0.08	0.52	>0.5 Fecal
Ratio 3	22.4	18.0	19.2	0.8	1.5	1.2	0.5	1.4	>5–6% Human contamination
Ratio 4	0.90	0.83	0.84	0.11	0.18	0.18	0.11	0.16	>0.7 Human contamination
Ratio 5	2.48	2.35	2.56	0.67	1.32	1.00	0.74	0.25	<1.0 Herbivore; ≥1.0 human
Ratio 6	71.26	70.17	71.92	40.23	56.82	50.00	42.50	20.22	<30% Herbivore; >75% human
Ratio 7	9.0	7.6	7.5	1.2	1.2	1.2	0.7	5.3	>5–6% Herbivore
Ratio 8	1.96	2.32	2.32	31.54	26.32	28.91	47.61	7.29	<1.0 Herbivore; >4.0 plant decay
Ratio 9	20.85	27.01	27.68	72.13	86.22	80.46	91.57	61.21	>30% Avian
Ratio 10	10.19	16.40	15.92	82.82	72.88	75.53	81.64	76.42	>67% Avian

[a]Results all parts per trillion

Table 21.14 *E. coli*, molecular markers and fecal sterols detected in water samples from two sites on a South Island River and an adjacent duck pond

Analyte	Site 1	Site 2	Duck pond	Interpretation
E. coli[a]	2.8×10^3	1.0×10^3	5.4×10^3	
PCR markers				
General *Bacteroides*	+[b]	+	+	
Herbivore *Bacteroides*	+	–	–	
Human *Bacteroides*	–	+	–	
Fecal sterols				
Coprostanol	362[c]	4,243	561	
24-Ethylcoprostanol	1,425	2,410	961	
Epicoprostanol	63	295	349	
Cholesterol	1,914	6,672	11,795	
Cholestanol	296	952	5,143	
24-Methylcholesterol	562	2,820	4,422	
24-Ethylepicoprostanol	424	486	246	
Stigmasterol	627	2,680	4,631	
24-Ethylcholesterol	1,712	6,036	14,397	
24-Ethylcholestanol	1,095	1,268	1,106	
Total sterols	8,480	27,862	43,611	
Ratio 1	1.22	4.46	0.11	>0.5 Fecal
Ratio 2	1.30	1.90	0.87	>0.5 Fecal
Ratio 3	4.3	15.2	1.3	>5–6% Human fecal pollution
Ratio 4	0.55	0.82	0.10	>0.7 Human fecal pollution
Ratio 5	0.25	1.76	0.58	<1.0 herbivore; ≥1.0 human
Ratio 6	20.26	63.78	36.86	<30% All herbivore; >75% all human
Ratio 7	16.8	8.6	2.2	>5–6% Herbivore
Ratio 8	1.20	2.50	14.98	<1.0 Herbivore; >4.0 plant decay
Ratio 9	37.19	30.45	47.82	>30% Wildfowl
Ratio 10	41.05	17.34	84.97	>67% Wildfowl

[a]MPN/100 mL
[b]+ equals detection of marker, – equals not detected
[c]Sterol results all parts per trillion

Tools applied: Samples were analyzed for presence of fecal sterols, and for PCR markers specific for *E. coli*, human, ruminant, and general *Bacteroides* markers (Table 21.1).

Results: All three sites contained fairly similar levels of *E. coli* and elevated levels of sterols (Table 21.14).

Fecal sterol ratios 1 and 2 were both elevated above the typical human and herbivore fecal thresholds at both these sites. The sterols at site 1 didn't meet any of the human-associated ratio thresholds (ratios 3–6), while the herbivore indicative

ratios 3–5, were satisfied. While ratio 6 was not below 1 in site 1, it is close to this point. In contrast at site 2 the ratios exceeded thresholds for human ratios 3–5. Ratio 10 was significantly less than 67% in both sites 1 and 2, indicating that wildfowl contribution to pollution is not significant. Analysis of the duck pond samples confirmed that the fecal sterol profile of this water was quite different from either of the water samples. These conclusions were also supported by the PCR analysis with the human-associated marker only detected at site 2, while the herbivore specific marker was only detected in site 1.

Conclusions: These two sites provided a strong contrast in terms of the identified source of fecal pollution. The molecular and fecal sterol signatures in site 1 were consistent with an herbivore source of pollution, while site 2 samples produced a profile consistent with a human source. Sterols and molecular markers analyzed from the duck pond confirmed that water containing feces from ducks would not falsely be identified as being either of human or herbivore origin.

21.4.3.4 Case Study 11: A Combination of Source Tracking Methods to Identify Human Sourced Pollution in Stormwater via Defective Septic Systems in Pine Rivers Shire, Queensland, Australia

Situation: Storm water samples were collected from Bergin Creek, Four Mile Creek and River Oaks Drive to determine whether the water was contaminated by human pollution from possible defective septic systems (Ahmed et al. 2007).

Tools used: A battery of methods, (1) library-dependent BF of *E. coli* and enterococci (2) human-specific *Bacteroides* HF183, HF134 and (3) human-specific enterococci surface protein (*esp*) markers, were used to detect human sourced pollution in the nonsewered, residential catchments studied.

Results: In all, 550 *E. coli* and 700 enterococci were isolated and biochemically fingerprinted to compare these fingerprints with existing libraries (Ahmed et al. 2005b). Of the 18 samples tested, 7 samples were also analyzed for the presence of human-specific markers using PCR methods. A total of 305 *E. coli* BPTs and 299 enterococci BPTs were obtained from water samples. The source of 105 *E. coli* BPTs and 93 enterococci BPTs were identified in water samples from River Oaks Drive catchment. Of these, 10 and 9% were identified as human-source *E. coli* and enterococci BPTs, respectively. Similarly, of the 83 *E. coli* BPTs and 93 enterococci BPTs from the Bergin Creek catchment site, 8% *E. coli* BPTs and 9% enterococci BPTs were identified as human-source isolates. The number of *E. coli* and enterococci assigned to human origin in the Four Mile Creek site were 4 and 3% respectively. Of the seven samples tested, both HF134 and *esp* markers were detected in five samples, and the HF183 marker was detected in four samples (Table 21.15). Human fecal pollution was detected in six out of seven water samples by at least one of these markers. The methods were not always in agreement in detecting human fecal pollution in water samples.

Table 21.15 Detection of human fecal pollution using library-dependent and library-independent methods

Catchments	Storm events	*E. coli*	Ent	*Bacteroides* HF183	*Bacteroides* HF134	Enterococci surface protein (*esp*)
BC	1	–	+	Not tested	Not tested	Not tested
	3	+	+	Not tested	Not tested	Not tested
	5	–	–	+	+	+
	5	–	+	+	+	+
	6	–	–	+	+	–
FMC	1	–	–	Not tested	Not tested	Not tested
	2	–	+	Not tested	Not tested	Not tested
	3	–	–	Not tested	Not tested	Not tested
	4	–	–	Not tested	Not tested	Not tested
	4	+	+	Not tested	Not tested	Not tested
	5	–	+	–	+	+
	6	+	+	+	+	+
ROD	1	+	+	Not tested	Not tested	Not tested
	2	+	+	Not tested	Not tested	Not tested
	3	+	–	Not tested	Not tested	Not tested
	4	+	+	Not tested	Not tested	Not tested
	5	+	–	–	–	+
	6	+	–	–	–	–

Ent enterococci

Conclusions: The results suggested that human fecal pollution is present in stormwater from these catchments. The *E. coli* and enterococci libraries used in this study were capable of detecting human fecal pollution. The presence of host-specific markers further confirmed the presence of human fecal pollution. This study demonstrated the value of a combination of methods for source tracking to obtain a better understanding regarding the pollution sources.

21.4.3.5 Case Study 12: Detection of Human and Animal Fecal Pollution in a Coastal Creek Located in Southeast Queensland, Australia Using Multiple Host-Specific PCR Markers

Situations: Environmental samples ($n = 16$) were collected from Ningi Creek urban catchment to identify the sources of fecal pollution using PCR along with the enumeration of *E. coli* and enterococci (Ahmed et al. 2008e).

Tools used: PCR detection of the human-specific HF183, HF134, *esp* markers, cattle-specific markers, and dog-specific markers.

Results: The specificity of these markers were determined by testing 197 samples from sewage/septage, ducks, kangaroos, cattle, horses, dogs, chickens, pigs, pelicans, goats, deer, wild birds, and sheep. The overall specificity of the *Bacteroides* HF183 and HF134 markers to differentiate between sewage/seepage and animal host groups was 1.0 and 0.95, respectively. The *Bacteroides* CF128 markers also showed high specificity (0.93) for ruminant feces, which included cattle.The *Bacteroides* BacCan

Table 21.16 Concentrations of *E. coli* and enterococci and detection of MST markers signifying human or animal pollution in water samples from Ningi Creek, Queensland, Australia

Sampling sites	*E. coli*	Enterococci	HF183	HF134	CF128	BacCan	*esp*
Occasion 1							
NC1	2.1×10^3	4.1×10^3	+	–	–	–	+
NC2	3.6×10^3	3.2×10^3	+	–	+	+	–
NC3	4.9×10^3	1.3×10^3	+	+	+	+	+
NC4	4.1×10^3	1.9×10^3	+	+	–	+	+
NC5	1.2×10^4	4.3×10^4	+	+	+	–	–
NC6	3.9×10^3	2.8×10^4	+	–	+	–	+
NC7	3.1×10^3	3.9×10^3	–	–	+	–	–
NC8	3.4×10^3	1.4×10^3	–	–	+	–	–
Occasion 2							
NC1	3.1×10^3	3.7×10^3	–	–	+	–	–
NC2	9.1×10^2	1.0×10^2	+	–	–	–	–
NC3	4.9×10^4	3.9×10^3	+	+	–	+	–
NC4	4.4×10^4	5.6×10^4	+	+	+	+	+
NC5	4.2×10^4	3.9×10^4	+	+	+	–	+
NC6	1.1×10^3	2.1×10^3	–	–	+	–	–
NC7	1.6×10^3	3.1×10^2	–	–	+	–	–
NC8	2.1×10^3	1.2×10^2	–	–	–	–	–

marker (i.e., dog markers) was detected in samples from sewage/septic, chickens and pigs and the specificity was low (i.e., 65%). The *esp* marker also exhibited high specificity for differentiation between human and animal feces. The concentrations of FIB in the water samples ranged between 9.1×10^2 and 1.2×10^4 cfu/100 mL (for *E. coli*), and 1.2×10^2 and 5.6×10^4 cfu/100 mL (for enterococci) (Table 21.16). At least one host-specific marker was detected in 14 (87%) out of 16 samples. Human-specific *Bacteroides* HF183 and HF134 markers were detected in 9 (56%) and 6 (37%) samples, respectively. This figure for human-specific *esp* marker was also 6 (37%). Cattle-specific marker CF128 was detected in 11 (69%) samples, whereas dog-specific marker BacCan was detected in 5 (31%) samples.

Conclusion: The host-specific PCR markers are reliable tools for detection of fecal pollution from humans and animals. Among all markers, *Bacteroides* HF183 and *esp* performed well in terms of specificity and identifying the sources of human fecal pollution. However, a combination of multiple human-specific markers provides greater reliability regarding the presence/absence of human fecal pollution when one marker is not sufficient to identify human fecal pollution. The CF128 marker also performed well in identifying ruminant fecal pollution.

21.5 Conclusions

This series of case studies conducted in Australia and New Zealand demonstrate the application of FST tools in a range of water systems. The primary question that arises in many situations is whether a water body contains human derived fecal

pollution. Human fecal pollution usually is considered to represent the greatest health risk (Field and Samadpour 2007; Leclerc et al. 2002), has the lowest threshold of public acceptability, and once identified often provides an opportunity to rectify the situation. This corrective action may involve considerable expenditure, and therefore, avoidance of false-positive results is very important. Indeed, demonstrating an absence of human fecal pollution allows water managers to prioritize resources to other areas and thereby achieve improvements in water quality.

Library-dependent methods such as BF and ARA, as illustrated in case studies 1 and 2, can be effective in source identification. However, the need to generate a large source library and potential concerns over validity of a library beyond the spatial and temporal constraints in which it was derived from can make library-dependent methods both time-consuming and expensive.

Library-independent source tracking methods, such as source specific PCR marker approaches, are appealing as in theory these markers may be temporally and spatially more stable than libraries. The case studies in this chapter indicate that the tested markers indeed exhibit similar sensitivity and specificity in Australia and New Zealand compared to results obtained overseas. Most of the markers showed higher specificity, although the sensitivity was not always high. For example, the *esp* markers in the case study 4 could not be detected in all wastewater samples collected from septic tanks. Some cross-reactivity has been observed for some markers as in case study 5, where HF134 markers were detected in dog fecal samples. Nonetheless, the application of an array of markers and/or combination of MST techniques can compensate for any uncertainty associated with a single marker. FWAs are useful indicators of human pollution, as are fecal sterols. Increasing use of sterols in water-quality analysis is also improving our understanding of these chemicals in nonhuman sources (Devane et al. 2006).

The cost of assays often limits the willingness of water managers to invest in sufficient replication of analysis to be able to understand variability of results. "Murphy's Law" and the inherent variability of aquatic systems can also create the situation where a river with historically high levels of pollution may have low levels when samples are taken for analysis by FST tools. This is demonstrated in case studies 8 and 9, and as illustrated in case study 9, the generation of meaningful results may still be possible in this situation. However, these must be interpreted with care. Are the sources of pollution at lower *E. coli* levels the same as at higher levels, or do the higher levels of pollution come from a different, intermittent source? When a human source is detected as in case study 9, this may be sufficient evidence for water managers to take action even if water standards are not exceeded. Unless all pathogens have been removed, and only indicators are present, any human pollution is usually unacceptable. Certainly, the avoidance of the use of this water for recreational or aquaculture is preferable. If used for drinking water, a very high level of treatment is required to ensure that any possible viral or protozoan pathogens in particular are inactivated.

While we are beginning to build up knowledge on the degradation, absorption, sedimentation, and transport of these new fecal source indicators (Bae and Wuertz 2009; Okabe and Shimazu 2007; Walters and Field 2006; Walters and Field 2009),

our understanding is incomplete and in most cases untested in real-life situations. Preferential absorption or transport may result in some markers behaving differently compared to traditional *E. coli* indicators and also behaving differently compared to pathogens that are of ultimate concern. The adoption of multiple source indicators does to a degree counter this issue. Additional tools do, however, increase both the time and expense in analyzing an area and may not be available in some areas. Cost-effectiveness of the use of multiple tools must also be considered. Devane et al. (2008) explored the development of decision trees to begin addressing these issues. While a work in progress, they do provide guidance, enabling users of these tools to compare various scenarios and identify cost-effective implementation strategies. This is critical as the cost and complexity have been the key factors hampering the implementation of MST technologies in water-management programs (Sagarin et al. 2009).

Collectively, these case studies indicate that current MST technology can successfully be applied for source identification and lead to meaningful and productive management decisions. There is room for significant refinement of these tools, and a continued investment in research to achieve these improvements is required. However, MST technology, even in its current, developing form can and is being used to improve water-quality outcomes.

References

Ahmed W, Neller R, Katouli M (2005a) Evidence of septic system failure determined by a bacterial biochemical fingerprinting method. J Appl Microbiol 98:910–920

Ahmed W, Neller R, Katouli M (2005b) Host-species specific metabolic fingerprint database for enterococci and *Escherichia coli* and its application to identify sources of fecal contamination in surface waters. Appl Environ Microbiol 71:4461–4468

Ahmed W, Stewart J, Gardner T, Powell D, Brooks P, Sullivan, D, Tindale, N (2007) Sourcing fecal pollution: A combination of library-dependent and library independent methods to identify human fecal pollution in non-sewered catchments. Water Res 41:3771–3779

Ahmed W, Stewart J, Powell D, Gardner T (2008a) Evaluation of host-specificity and prevalence of enterococci surface protein (*esp*) marker in sewage and its application for sourcing human faecal pollution. J Environ Qual 37:1583–1588

Ahmed W, Hargreaves M, Goonetilleke A, Katouli, M (2008b) Population similarity analysis of indicator bacteria for source prediction of fecal pollution in a recreational coastal lake. Mar Pollut Bull 56:1469–1475

Ahmed W, Stewart J, Gardner T, D Powell (2008c) A real-time polymerase chain reaction assay for the quantitative detection of the human-specific enterococci surface protein marker in sewage and environmental waters. Environ Microbiol 10:3255–3264

Ahmed W, Stewart J, Powell D, Gardner T (2008d) Evaluation of Bacteroides markers for the detection of human faecal pollution. Lett Appl Microbiol 46:237–242

Ahmed W, Powell D, Goonetilleke A, Gardner T (2008e) Detection and source identification of faecal pollution in non-sewered catchment by means of host-specific molecular markers. Water Sci Technol 58:579–586

Ahmed W, Wan C, Goonetilleke A, Gardner T (2010) Evaluation of human associated JCV and BKV polyomaviruses for the detection of sewage pollution in a coastal river. J Environ Qual 39:1743–1750

Allsop K, Stickler JD (1985) An assessment of *Bacteroides fragilis* group organisms as indicators of human faecal pollution. J Appl Bacteriol 58:95–99

Bae S, Wuertz S (2009) Rapid decay of host-specific fecal *Bacteroidales* cells in seawater as measured by quantitative PCR with propidium monoazide. Water Res 43:4850–4859

Bernhard AE, Field KG (2000) A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. Appl Environ Microbiol 66:4571–4574

Carroll S, Hargreaves M, Goonetilleke A (2005) Sourcing faecal pollution from onsite wastewater treatment systems in surface waters using antibiotic resistance analysis. J Appl Microbiol 99:471–482

Chern EC, Tsai YL, Olson BH (2004) Occurrence of genes associated with enterotoxigenic and enterohemorrhagic *Escherichia coli* in agricultural waste lagoons. Appl Environ Microbiol 70:356–362

Choi S, Jiang SC (2005) Real-time PCR quantification of human adenoviruses in urban rivers indicates genome prevalence but low infectivity. Appl Environ Microbiol 71:7426–7433

Devane M, Saunders D, Gilpin B (2006) Faecal sterols and fluorescent whiteners as indicators of the source of faecal contamination. Chemistry in New Zealand 70:74–77

Devane ML, Robson B, Nourozi F, Scholes P, Gilpin BJ (2007) A PCR marker for detection in surface waters of faecal pollution derived from ducks. Water Res 41:3553–3560

Devane M, Gilpin B, Robson B, Nourozi F, Wood, D (2008) Decision analysis trees for identification of faecal sources in water. The NZWWA Journal 157:23–31

Dick LK, Bernhard AE, Brodeur TJ, Santo Domingo JW, Simpson JM, Walters SP, Field KG (2005) Host distributions of uncultivated fecal *Bacteroidales* reveal genetic markers for fecal source identification. Appl Environ Microbiol 71:3184–3191

Dombek PE, Johnson LK, Zimmereley ST, Sadowsky MJ (2000) Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Appl Environ Microbiol 66:2572–2577

Field KG, Samadpour M (2007) Fecal source tracking the indicator paradigm and managing water quality. Water Res 41:3517–3538

Fong TT, Griffin DW, Lipp EK (2005a) Molecular assays for targeting human and bovine enteric viruses in coastal waters and their application for library-independent source tracking. Appl Environ Microbiol 71:2070–2078

Fong TT, Lipp EK (2005b) Enteric viruses of humans and animals in aquatic environments: health risks detection and potential water quality assessment tools. Microbiol Mol Biol Rev 69:357–371

Gilpin BJ, Gregor JE, Savill MG (2002) Identification of the source of faecal pollution in contaminated rivers. Water Sci Technol 46:9–15

Gregor J, Garrett N, Gilpin B, Randall C, Saunders D (2002) Use of classification and regression tree (CART) analysis with chemical faecal indicators to determine sources of contamination. NZ J Mar Freshwater Res 36:387–398

Grimault JO, Fernandez P, Bayona JM, Albaiges J (1990) Assessment of faecal sterols and ketones as indicators of urban sewage inputs to coastal waters. Environ Sci Technol 24:357–363

Hagedorn C, Crozier JB, Mentz KA, Booth AM, Graves AK, Nelson NJ, Reneau Jr RB (2003) Carbon source utilization profiles as a method to identify sources of fecal pollution in water. J Appl Microbiol 94:792–799

Harwood, VJ, Whitlock J, Withington, V (2000) Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical waters. Appl Environ Microbiol 66:3698–3704

Hrudey SE, Hrudey EJ (2004) Safe drinking water: Lessons from recent outbreaks in affluent nations. IWA Publishing, London

Hundesa A, Maluquer de Motes C, Bofill-Mas S, Albinana-Gimenez N, Girones R (2006) Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. Appl Environ Microbiol 72:7886–7893

Hörman A, Rimhanen-Finne R, Maunula L, von Bonsdorff C-H, Torvela N, Heikinheimo A, Hanninen M-L (2004) *Campylobacter* spp. *Giardia* spp. *Cryptosporidium* spp. Noroviruses

and indicator organisms in surface water in south-western Finland 2000–2001. Appl Environ Microbiol 70:87–95

Katouli M, Lund A, Wallgren P, Kuhn I, Sodermind O, Mollby R (1997) Metabolic fingerprinting and fermentative capacity of the intestinal flora of pigs during pre- and post-weaning periods. J Appl Microbiol 83:147–154

Khatib LA, Tsai YL, Olson BH (2002) A biomarker for the identification of cattle fecal pollution in water using the LTIIa toxin gene from enterotoxigenic *E. coli.* Appl Microbiol Biotechnol 59:97–104

Khatib LA, Tsai YL, Olson BH (2003) A biomarker for the identification of swine fecal pollution in water using the STII toxin gene from enterotoxigenic *E. coli*. Appl Microbiol Biotechnol 63:231–238

Kildare BJ, Leutenegger CM, McSwain BS, Bambic DG, Rajal VB, Wuertz S (2007) 16S r RNA-based assays for quantitative detection of universal human- cow- and dog-specific fecal *Bacteroidales*: a Bayesian approach. Water Res 41:3701–3715

King CM (Ed) (2005) The Handbook of New Zealand Mammals, Second Edition. Oxford University Press, USA

Kreader CA (1995) Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. Appl Environ Microbiol 61:1171–1179

Layton A, McKay L, Williams D, Garrett V, Gentry R, Sayler G (2006) Development of *Bacteroides* 16S rRNA gene TaqMan based real-time PCR assays for estimation of total human and bovine fecal pollution in water. Appl Environ Microbiol 72:4214–4224

Leclerc H, Schwartzbrod L, Dei-Cas E (2002) Microbial agents associated with waterborne diseases. Crit Rev in Microbiol 28:371–409

Leeming R, Ball A, Ashbolt N, Nichols P (1996) Using faecal sterols from humans and animals to distinguish faecal pollution in receiving waters. Water Res 30:2893–2900

Leeming R, Bate N, Hewlett R, Nichols PD (1998) Discriminating faecal pollution: A case study of stormwater entering Port Phillip Bay Australia. Water Sci Technol 38:15–22

Love DC, Sobsey MD (2007) Simple and rapid F+ coliphage culture, latex agglutination and typing assay to detect and source track fecal contamination. Appl Environ Microbiol 73: 4110–4118

Maluquer de Motes C, Clemente-Casares P, Hundesa A, Martin M, Girones R (2004) Detection of bovine and porcine adenoviruses for tracing the source of fecal contamination. Appl Environ Microbiol 70:1448–1454

Matsuki T, Watanabe K, Fujimoto J, Kado Y, Takada T, Matsumoto K, Tanaka R (2004) Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. Appl Environ Microbiol 70:167–173

McLellan SL (2004) Genetic diversity of *Escherichia coli* isolated from urban rivers and beach water. Appl Environ Microbiol 70:4658–4665

McQuaig SM, Scott, TM, Harwood VJ, Farrah, SR, Lukasik JO. 2006. Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. Appl Environ Microbiol 72:7567–7574

Mcquaig SM, Scott TM, Harwood VJ, Farrah SR, Lukasik JO. 2009. Quantification of human polyomaviruses JC virus and BK virus bt Taqman Quantittaive PCR and comparison to other water quality indicators in water and fecal samples. Appl Environ Microbiol 75:3379–3388

Möllby R, Kühn I, Katouli, M (1993) Computerized biochemical fingerprinting – a new tool for typing of bacteria. Rev Med Microbiol 4:231–241

Moyda SP, Carson CA, Fuhrmann JJ, Hahm BK, Hartel PG, Kuntz RL, Nakatsu CH, Sadowsky MJ, Samadpour M, Yampara-Iquise, H (2003) Comparing genotypic bacterial source tracking methods that require a host origin database. J Wat and Health 1:167–180

Noble RT, Allen SM, Blackwood AD, Chu W, Jiang SC, Lovelace GL, Sobsey MD, Stewart JR, Wait DA. 2003. Use of viral pathogens and indicators to differentiate between human and non-human fecal contamination in a microbial source tracking comparison studu. J Water Health 1:195–207

Okabe S, Shimazu Y (2007) Persistence of host-specific *Bacteroides*-Prevotella 16S rRNA genetic markers in environmental waters: effects of temperature and salinity. Appl Microbiol Biotechnol 76:935–944

Okabe S, Okayama N, Savichtcheva O, Ito T (2007) Identification and quantification of host-specific *Bacteroides-Prevotella*16S rRNA genetic markers for assessment of fecal pollution in freshwaters. Appl Microbiol Biotechnol 74:890–901

Parveen S, Murphree RL, Edminston L, Kaspar CW, Portier KM, Tamplin ML (1997) Association of multiple antibiotic resistance profiles with point and nonpoint sources of *Escherichia coli* in Apalachicola Bay. Appl Environ Microbiol 63:2607–2612

Parveen S, Portier KM, Robinson, K, Edmiston L, Tamplin ML (1999) Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. Appl Environ Microbiol 65:3142–3147

Pina S, Puig M, Lucena F, Jofre J, Girones R (1998) Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. Appl Environ Microbiol 64:3376–3382

Poiger T, Field JA, Field TM, Giger W (1993) Determination of detergent-derived fluorescent whitening agents in sewage sludges by liquid chromatography. Analytical Methods and Instrumentation 1:104–113

Poiger T, Field JA, Field TM, Siegrist H, Giger W (1998) Behaviour of fluorescent whitening agents during sewage treatment. Water Res 32:1939–1947

Prüss-Üstün AR, Bos F, Gore, J Bartram (2008) Safer Water Better Health: Costs Benefits and Sustainability of Interventions to Protect and Promote Health. Geneva: World Health Organization

Reeves AD, Patton D (2001) Measuring change in sterol input to estuarine sediments. Physics and Chemistry of the Earth Part B: Hydrology Oceans and Atmosphere 26:753

Reischer GH, Kasper DC, Steinborn R, Farnleitner AH, Mach RL (2007) A quantitative real-time PCR assay for the highly sensitive and specific detection of human faecal influence in spring water from a large alpine catchment area. Lett Appl Microbiol 44:351–356

Reischer GH, Kasper DC, Steinborn R, Mach RL, Farnleitner AH (2006) Quantitative PCR method for sensitive detection of ruminant fecal pollution in freshwater and evaluation of this method in Alpine Karstic regions. Appl Environ Microbiol 72:5610–5614

Sagarin R, Carlsson J, Duval M, Freshwater W, Godfrey MH, Litaker W, Munoz R, Noble R, Schultz T, Wynne B. (2009) Bringing molecular tools into environmental resource management: untangling the molecules to policy pathway. PloS Biol 7:e69

Scott TM, Jenkins TM, Lukasik J, Rose JB (2005) Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human pollution, Environ Sci Technol 39:283–287

Scott TM, Rose JB, Jenkins TM, Farrah SR, Lukasik J (2002) Microbial source tracking: current methodology and future directions. Appl Environ Microbiol 68:5796–5803

Scott TM, Parveen S, Portier KM, Rose JB, Tamplin ML, Farrah SA, Koo A, Lukasik J (2003) Geographical variation in ribotype profiles of *Escherichia coli* isolates from humans swine poultry beef and dairy cattle in Florida. Appl Environ Microbiol 69:1089–1092

Seurinck S, Defoirdt T, Verstraete W, Siciliano SD (2005) Detection and quantification of the human-specific HF183 *Bacteroides* 16S rRNA genetic markers with real-time PCR for assessment of human fecal pollution in freshwater. Environ Microbiol 7:249–259

Sghir A, Gramet G, Suau A, Rochet V, Pochart P, Dore J (2000) Quantification of bacterial groups within human faecal flora by oligonucleotide probe hybridization. Appl Environ Microbiol 66:2263–2266

Sneath PHA, RR Sokal (1973) Numerical taxonomy: the principles and practice of numerical classification. W H Freeman, San Francisco, CA

Stoll JMA, Giger W (1998) Mass balance for detergent-derived fluorescent whitening agents in surface waters of Switzerland. Water Res 32:2041–2050

Ufnar JA, Wang SY, Christiansen JM, Yampara-Iquise H, Carson CA, Ellender RD (2006) Detection of the *nifH* gene of *Methanobrevibacter smithii:* a potential tool to identify sewage pollution in recreational waters. J Appl Microbiol 101:44–52

US Environmental Protection Agency (2000) Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and *Escherichia coli* EPA/821/R-97/004. Office of Science and Technology, Washington DC, 55 pp

Walters SP, Field KG (2006) Persistence and growth of fecal *Bacteroidales* assessed by bromodeoxyuridine immunocapture. Applied and Environmental Microbiology 72:4532–4539

Walters SP, Field KG (2009) Survival and persistence of human and ruminant-specific faecal *Bacteroidales* in freshwater microcosms. Environmental Microbiology 11:1410–1421

Wiggins BA (1996) Discriminant analysis of antibiotic resistance patterns in fecal streptococci a method to differentiate human and animals sources of fecal pollution in natural waters. Appl Environ Microbiol 62:3997–4002

Wiggins BA, Andrews RW, Conway RA, Corr CL, Dobratz EJ, Dougherty DP, Eppard JR, Knupp SR, Limjoco MC, Mettenburg JM, Rinehardt JM, Sonsino J, Torrijos RL, Zimmerman ME (1999) Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution. Appl Environ Microbiol 65:3483–3486