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Sourcing faecal pollution: A combination of library-dependent and library-independent methods to identify human faecal pollution in non-sewered catchments

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ABSTRACT

Library-dependent (LD) (biochemical fingerprinting of *Escherichia coli* and enterococci) and library-independent (LI) (PCR detection of human-specific biomarkers) methods were used to detect human faecal pollution in three non-sewered catchments. In all, 550 *E. coli* isolates and 700 enterococci isolates were biochemically fingerprinted from 18 water samples and compared with metabolic fingerprint libraries of 4508 *E. coli* and 4833 enterococci isolates. *E. coli* fingerprints identified human unique biochemical phenotypes (BPTs) in nine out of 18 water samples; similarly, enterococci fingerprints identified human faecal pollution in 10 water samples. Seven samples were tested by PCR for the detection of biomarkers. Human-specific HF134 *Bacteroides* and enterococci surface protein (*esp*) biomarkers were detected in five samples. Four samples were also positive for HF183 *Bacteroides* biomarker. The combination of biomarkers detected human faecal pollution in six out of seven water samples. Of the seven samples analysed for both the indicators/markers, at least one indicator/marker was detected in every sample. Four of the seven PCR-positive samples were also positive for one of the human-specific *E. coli* or enterococci BPTs. The results indicated human faecal pollution in the studied sub-catchments after storm events. LD and LI methods used in this study complimented each other and provided additional information regarding the polluting sources when one method failed to detect human faecal pollution. Therefore, it is recommended that a combination of methods should be used to identify the source(s) of faecal pollution where possible.

1. Introduction

Faecal pollution from both human and animal sources poses serious public health risks to recreational water users and shellfish consumers, due to exposure to a variety of pathogenic microorganisms (Baker and Herson, 1999; Griffin et al., 1999). The identification of major sources of faecal pollution is, therefore, necessary for the improved management of water quality and to minimise public health risks associated with such pollution. The microbiological quality of water is generally assessed by monitoring faecal indicator bacteria such as faecal coliforms and enterococci (US EPA, 2003). These indicator bacteria are found in warm-blooded animals in high numbers, and their presence in natural waters generally indicates faecal pollution and potential presence of pathogenic microorganisms (Dufour, 1984). However, the presence of these bacteria in aquatic environments does not provide definitive information regarding their possible

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sources (Harwood et al., 2000; Kühn et al., 1991). To overcome this limitation, numerous microbial source tracking (MST) methods are now being used in order to identify the sources of faecal pollution in various aquatic systems (Bernhard and Field, 2000a; Harwood et al., 2000; Scott et al., 2005). These methods can be classified as library-dependent (LD) and library-independent (LI) methods. LD methods require the development of a library, or host-origin database of phenotypic or genotypic attributes of *Escherichia coli* or enterococci from suspected host groups. Unknown environmental bacterial isolates are compared to this library to identify the likely sources. In contrast, LI methods, which are mainly polymerase chain reaction (PCR) based, do not require development of a library. Instead, they detect the presence of specific genes associated with certain groups of bacteria from human and animal sources. The performance of the LD methods can be limited by library size and representativeness, discriminatory power, lack of stability of bacterial characters, and statistical analyses and accuracy (Gordon, 2001; Harwood et al., 2003; Ritter et al., 2003; Stoeckel et al., 2004; Wiggins et al., 2003). On the other hand, LI methods have several advantages: these methods are rapid, sensitive and have shown higher accuracy in method comparison studies in California (Griffith et al., 2003). However, some of the LI methods are not quantitative and cannot be used to differentiate among multiple sources of pollution(s). In view of this, it has been recommended (Seurinck et al., 2005) that a combination of methods should be used (where possible) in order to obtain confirmatory results, as any error in identifying the sources may not be acceptable for regulatory purposes.

The primary objective of this study was to use a combination of LD (i.e. *E. coli* and enterococci metabolic fingerprint libraries) (Ahmed et al., 2005b) and LI methods to detect human faecal pollution in non-sewered residential catchments in southeast Queensland, Australia. LI methods were host-specific PCR of *Bacteroides* 16S rRNA gene biomarkers (Bernhard and Field, 2000b), and PCR of a human-specific *Enterococcus* surface protein [*esp*] marker found in *Enterococcus faecium*) (Scott et al., 2005).

2. Materials and methods

2.1. Study area and water sampling

Three non-sewered catchments—Bergin Creek (BC), Four Mile Creek (FMC) and River Oaks Drive (ROD) in Pine Rivers City in southeast area of Queensland, Australia (Fig. 1) were selected for stormwater monitoring and identification of the presence of human faecal pollution. These sub-catchments were chosen from a selection of 14 short listed sub-catchments which were identified through Geographical Information System (GIS) analysis on the basis of limited non-point sources of faecal pollution (i.e. no animal farms), overall density of septic systems in each sub-catchment, and the number of septic systems within 100 m of drainage lines. The selected residential sub-catchments are generally small in size, ranging between 96 and 429 ha and contain between 60 and 243 septic systems. The landscape is undulating with allotments featuring typical suburban houses. The creeks in these areas discharge during storm events and the lack of base flow between events indicate that the groundwater table may not be shallow.

A sanitary survey was carried out to identify the possible sources of faecal pollution. Few domestic animals such as horses (<3) were identified within these sub-catchments. Other likely sources of pollution included domestic dogs, wild birds and wild animals, particularly kangaroos. A total of 18 water samples were collected from the BC, FMC and ROD sub-catchments between January and March 2006 and during November 2006, representing six separate storm events. Among the 18 water samples, 16 were hydrograph recession grab samples and two were rising stage samples. Samples were collected in sterilised 2.5 L containers and transported to the laboratory on ice and processed within 8 h.

2.2. Isolation, identification and enumeration of indicator bacteria

The membrane filtration method was used to process all the water samples for bacterial enumeration. Serial dilutions were made and filtered through 0.45 μm pore size (47 mm diameter) nitrocellulose membranes (Advantec, Tokyo, Japan) and placed on m-enterococcus (Difco, Detroit, USA) and RAPID' *E. coli* 2 (REC 2) agar with supplement (Bio-rad, California, USA). Plates were then incubated at 37 °C for 48 h (for faecal streptococci), and at 44 °C for 24 h (for faecal coliforms and *E. coli*). The REC 2 medium used for isolation of *E. coli* is based on the detection of two enzyme activities; β-D-glucuronidase and β-D-galactosidase. The hydrolysis of chromogenic substrates results in purple *E. coli* and blue coliform colonies. Single purple colonies from this medium were streaked on McConkey agar (Oxoid, London, UK) for purity and also tested for indole production and citrate cleavage. Indole-positive and citrate-negative isolates were identified as *E. coli*. All isolates from m-enterococcus agar plates were also tested for esculin hydrolysis using Bile Esculin agar (BEA) (Oxoid, London, UK) and incubated at 45 °C for 1 h to confirm their identification as enterococci (Manero and Blanch, 1999).

2.3. MST with biochemical fingerprinting

To identify the sources of faecal indicator bacteria in storm water samples, we used existing metabolic fingerprinting libraries of *E. coli* and enterococci developed in this region for faecal pollution tracking (Ahmed et al., 2006). These libraries consist of 4508 *E. coli* and 4833 enterococci isolates from 10 host groups (i.e. cattle, chickens, deer, dogs, ducks, horses, humans (i.e. via septic tanks), kangaroos, pigs and sheep. The libraries were developed in another catchment located approximately 80 km distance from the present sub-catchments, and contain *E. coli* and enterococci fingerprints which are unique to host groups (Ahmed et al., 2006). For this study, we used two types of micro plates specifically developed for typing of *E. coli* (PhP-RE plates) and enterococci strains (PhP-RF plates) (PhPlate system, PhPlate AB, Stockholm, Sweden). The 11 substrates used for *E. coli* and enterococci have been described before (Iversen et al., 2002; Kühn et al., 1991). The fingerprinting procedures were performed according to the manufacturer's instruction. In brief, *E. coli* and

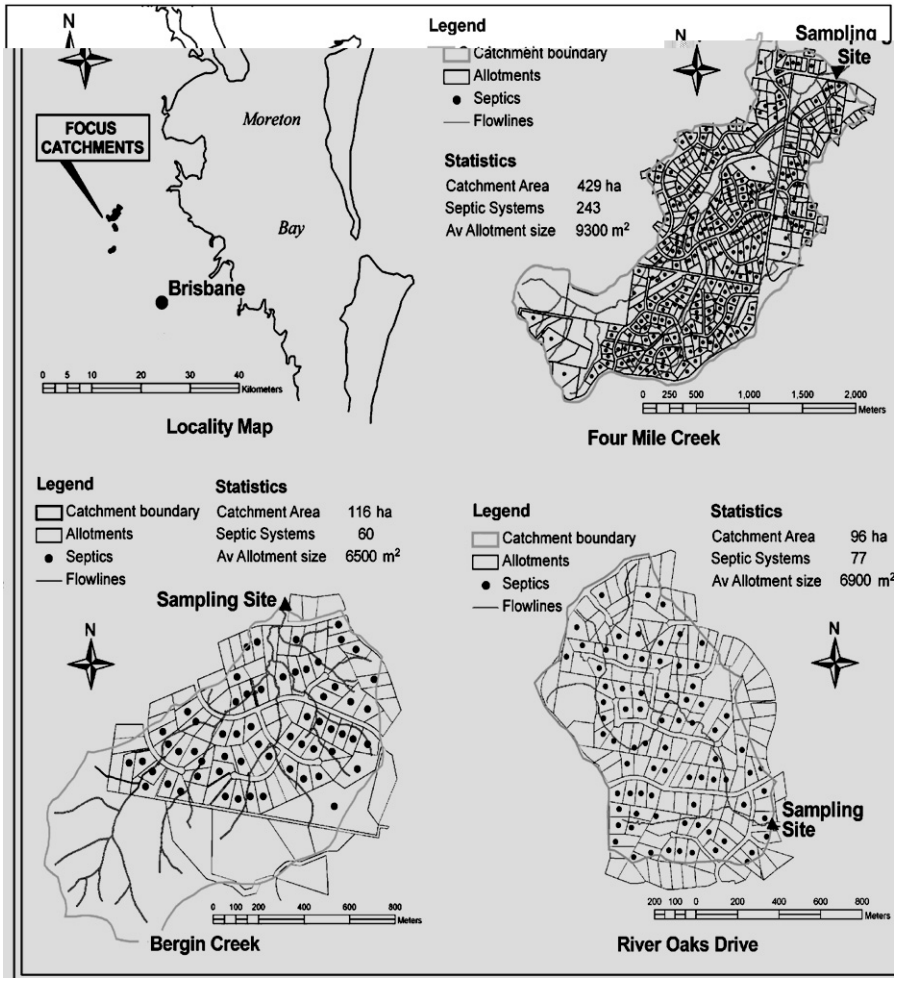

Fig. 1 – Map of the Bergin Creek, Four Mile Creek and River Oaks Drive sub-catchments showing sampling sites and the septic tanks.

enterococci colonies were suspended in the first well of each row containing only 350 μl of growth medium. Aliquots of 25 μl of bacterial suspensions were transferred into each of the other 11 wells containing 150 μl growth medium. Plates were then incubated at 37 °C and A_{620} was measured at 7, 24 and 48 h for *E. coli* and at 16, 40 and 64 h for enterococci using a micro plate reader (Lab-systems Multiskan, Helsinki, Finland). After the final reading, the mean value for all three readings was calculated for each isolate (biochemical fingerprint) (Kühn et al., 1991). The isolates were compared pair-wise and the obtained similarity matrix was clustered according to the unweighted pair group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973). An identity level of 0.965 was established based on the reproducibility of the system. Isolates showing similarity to each other above the ID level were regarded as identical and were assigned to the same biochemical phenotype (BPT). All data handling including optical readings, calculations of similarities among fingerprints of strains as well as clustering and printing of dendrograms, were performed using the PhPlate software version 4001 (PhPlate system). From each water sample, a maximum of 39 *E. coli* (where possible) isolates and 40 enterococci (where possible) isolates were typed with the PhPlate system for comparison with the libraries.

2.4. *MST using host-specific biomarkers*

The samples from the fifth and sixth storm events were chosen for further analysis by PCR. Detection of human-specific *Bacteroides* markers HF183 and HF134 was undertaken according to published methods (Bernhard and Field, 2000b; Shanks et al., 2006), with a few changes. Briefly, 300 ml of water samples were filtered through 0.45-μm-pore size membranes (Advantec). The filters were lifted and suspended in sterile 1.5 ml tubes containing 500 μl of GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8.0], 0.5% Sarkosyl) and stored overnight at −80 °C. DNA was extracted by using QIAamp stool DNA kit (Qiagen Inc. Valencia, CA, USA) according to the manufacturer's instructions.

For the detection of the *esp* marker found in *E. faecium*, 300 ml of each water sample was filtered through 0.45-μm-pore size membranes and enterococci were concentrated as described by Scott et al. (2005). In brief, filters papers were

placed on m-enterococcus agar with indoxyl substrate (Difco, Detroit, USA) and incubated at 41 °C for 48 h. After 48 h, filter papers were transferred to sterile tubes containing 10 ml tryptic soy broth (TSB) (Oxoid, London, UK) and incubated at 41 °C for 3 h for enrichment. QIAamp DNA kit (Qiagen) was used to extract DNA from 1 ml of culture.

Human-specific *Bacteroides* HF183 and HF134 markers were amplified with forward primers (5′-ATC ATG AGT TCA CAT GTC CCG-3′) for HF183 and (5′-ATC ATG AGT TCA CAT GTC CCG-3′) for HF134, coupled with the Bac708 reverse primer (5′-CAA TCG GAS GTT CTT CGT G-3′) (Bernhard and Field, 2000a). The human-specific *esp* marker was amplified by using forward primer (5′-TAT GAA AGC AAC AGC ACA AGT T-3′) specific for *E. faecium* (Scott et al., 2005) and an enterococcus reverse primer (5′-ACG TCG AAA GTT CGA TTT CC-3′) (Hammerum and Jensen, 2002). For all markers, PCR was carried out in a volume of 50 μl reaction containing 45 μl Platinum Blue Supermix (Invitrogen, Carlsbad, CA, USA), 0.3 μM of forward and reverse primers and 2 μl of template DNA.

PCR was optimized and performed using a Mastercycler gradient (Eppendorf, Hamburg, Germany). For all biomarkers, cycling parameters were 15 min at 95 °C for initial denaturation and 30 cycles of 94 °C for 30 s, 59 °C for 1 min for annealing and 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. To detect amplified products, 5 μl aliquot of the PCR product was visualized by electrophoresis through 2% E-Gel® (Invitrogen) and exposure to UV light. For human-specific *Bacteroides*, positive controls (i.e. DNA from sewage samples) specific to each marker and negative controls (no DNA) were included in each assay. Samples were recorded as positive when the visible band was the same size as the positive control (i.e. 520 bp for HF183 and 570 bp for HF134). For the *esp* marker, a positive control (*E. faecium* C68 strain, provided by Dr. Louis B. Rice of the Louis Stokes Cleveland Veterans Affairs Medical Center in Cleveland, OH, USA), and negative controls (no DNA) were included in each assay. Samples were recorded as positive when the visible band was the same size as positive control (i.e. 680 bp).

2.5. DNA cloning and sequencing

To verify the identity of the PCR product obtained using human-specific HF183 and HF134 *Bacteroides* primers, the PCR amplified sequences from either HF183 or HF134 were purified using the QIAquick PCR Purification Kit (Qiagen) and cloned, in duplicate, into the pGEM®-T Easy Vector System (Promega, Madison, WI, USA) as recommended by the manufacturer. Plasmids were extracted using the QIAprep Spin® Miniprep kit (Qiagen). Bidirectional sequences were obtained using T7 and SP6 long sequencing primers targeting sites on either side of the insert. DNA sequencing was carried out at the Australian Genome Research Facility (St Lucia, Queensland, Australia). Sequences were analysed using Bioware Jellyfish Software.

3. Results

3.1. Water quality indicators

In all, 18 water samples representing six storm events were collected from three sub-catchments. The number of faecal coliforms in water samples was high, ranging from 3100 to 43,060 colony forming units (cfu)/100 ml. For *E. coli* and enterococci, these figures ranged from 480 to 4360 and 750 to 8500 cfu/100 ml, respectively (Table 1). The abundance of indicator bacteria in all water samples exceeded the Australia and New Zealand Environment and Conservation Council (ANZECC) recreational water quality guidelines for fresh and marine waters of 150 faecal coliforms/100 ml and 35 enterococci/100 ml for primary contact (ANZECC, 2000).

3.2. Phenotypic diversity of E. coli and enterococci

Up to 39 *E. coli* isolates (where possible) and 40 enterococci isolates (where possible) were biochemically fingerprinted from each water sample (Table 2). The mean diversities of *E. coli* (0.93 ± 0.02) and enterococci (0.89 ± 0.08) were high (out of a maximum of 1) in water samples tested from the BC sub-catchment, indicating diverse indicator bacterial populations. The diversities were also high in water samples from the FMC (0.87 ± 0.05 for *E. coli*; 0.85 ± 0.08 for enterococci) and the ROD (0.89 ± 0.05 for *E. coli*; 0.81 ± 0.14 for enterococci) sub-catchments.

3.3. Human faecal pollution tracking

In total, 550 *E. coli* isolates and 700 enterococci isolates were typed with the biochemical fingerprinting method from 18 water samples. In all, 305 *E. coli* BPTs and 299 enterococci BPTs

Table 1 – The range and geometric mean of faecal coliforms, *E. coli* and enterococci (colony forming unit/100 ml) concentration at Bergin Creek (BC), Four Mile Creek (FMC) and River Oaks Drive (ROD) sub-catchments in Pine River Shire tested between January and March 2006 and in November 2006

Sub-catchments	Faecal coliforms		*E. coli*		Enterococci	
	Range	Geometric mean	Range	Geometric mean	Range	Geometric mean
BC	4900–26 700	11 201	2600–4000	3509	2700–5950	3762
FMC	3100–23 020	7913	480–2680	1590	750–8500	2170
ROD	4300–43 060	10 401	1300–4360	2077	866–3700	2111

Table 2 – Number of *E. coli* isolates and enterococci isolates tested with the biochemical fingerprinting from Bergin Creek (BC), Four Mile Creek (FMC) and River Oaks Drive (ROD) sub-catchments over six storm events and their phenotypic diversity

Sub-catchments	Storm events (month)	No of isolates tested (no. of biochemical phenotypes found)		Phenotypic diversity	
		E. coli	Enterococci	*E. coli*	Enterococci
BC	1 (January)	39 (23)	39 (20)	0.96	0.88
	3 (February)	27 (14)	39 (13)	0.93	0.75
	5 (November)	21 (13)	39 (19)	0.92	0.94
	5 (November)[a]	23 (14)	39 (24)	0.96	0.94
	6 (November)	39 (19)	39 (17)	0.90	0.94
FMC	1 (January)	37 (21)	37 (18)	0.90	0.94
	2 (February)	22 (11)	39 (12)	0.81	0.77
	3 (February)	33 (17)	39 (6)	0.90	0.74
	4 (March)	28 (19)	39 (18)	0.93	0.87
	4 (March)[a]	35 (21)	40 (21)	0.93	0.92
	5 (November)	17 (9)	39 (17)	0.80	0.82
	6 (November)	35 (16)	39 (23)	0.84	0.95
ROD	1 (January)	31 (19)	38 (15)	0.94	0.88
	2 (February)	28 (10)	39 (21)	0.84	0.96
	3 (February)	35 (17)	39 (13)	0.87	0.69
	4 (March)	36 (22)	39 (19)	0.94	0.93
	5 (November)	33 (17)	39 (12)	0.84	0.83
	6 (November)	31 (20)	39 (11)	0.94	0.59
Total	18	550 (305)	700 (299)	0.89 ± 0.05[b]	0.85 ± 0.10[b]

[a] Rising stage sample.
[b] Overall mean diversity.

were identified among these isolates (Table 2). All BPTs from water samples were compared to the existing metabolic fingerprinting libraries of *E. coli* and enterococci. The libraries contain 303 *E. coli* and 308 enterococci BPTs, which are unique to the host groups including humans (Ahmed et al., 2006). A total of 105 *E. coli* BPTs were identified among six water samples from the ROD site, of which 11 (10%) were identified as human unique BPTs and 50 (48%) were identified as general animals. The remaining 44 (42%) *E. coli* BPTs could not be identified to any host groups (Fig. 2). Of the 93 enterococci BPTs obtained from the same site eight (9%) were identified as humans and 59 (65%) identified as animals. The remaining 24 (26%) could not be identified. Similarly, of the 83 *E. coli* BPTs and 93 enterococci BPTs found in the BC site, seven (8%) *E. coli* BPTs and eight (9%) enterococci BPTs were of humans and 30 (35%) *E. coli* BPTs and 53 (57%) enterococci were of animals. Unidentified proportions of *E. coli* and enterococci BPTs were 57% and 34%, respectively. The number of *E. coli* and enterococci of human origin in the FMC site were five (4%) *E. coli* BPTs and five (4%) respectively. In this sub-catchment, 57 (50%) *E. coli* and 75 (65%) enterococci BPTs belonged to animals. Unidentified proportions of *E. coli* and enterococci BPTs were 44% and 31%, respectively (Fig. 2).

Using only the *E. coli* fingerprint library, nine (50%) out of 18 water samples were positive for human faecal pollution and this figure for the enterococci fingerprint library was 10 (56%) out of 18 samples. However, combining the data from both *E. coli* and enterococci fingerprint libraries showed that 13 (72%) out of 18 water samples were polluted with human faeces (Table 3).

The seven samples from the fifth and sixth storm events were chosen for PCR analysis using human-specific *Bacteroides* (HF183 and HF134) and *esp* biomarkers. HF134 and *esp* markers were detected in five samples and HF183 marker was detected in four samples (Table 3). Human faecal pollution was detected in six out of seven water samples by at least one of these markers. In four of the seven samples, PCR results were in unanimous agreement (i.e. either all detected or all non-detected). In all but one of the human-positive samples, more than one PCR marker was detected. The PCR-amplified sequences of the human-specific HF183 and HF134 *Bacteroides* 16S rRNA genetic markers found in the sewage samples were verified as $\geqslant$98.7% identical to the marker sequences described by Bernhard and Field (2000a).

Four of the seven PCR-positive samples were also positive for one of the human-specific *E. coli* or enterococci BPTs. One sample (i.e. event 6, November from the FMC) was positive by all indicators/markers tested in this study. The *E. coli* fingerprint library detected human faecal pollution in one sample from the ROD site (i.e. event 6, November) although the enterococci fingerprint library and all markers could not detect any human faecal pollution in this sample.

4. Discussion

We used emerging MST methods to identify whether human faecal pollution is being transported to catchment outlets during storm events. The studied residential sub-catchments

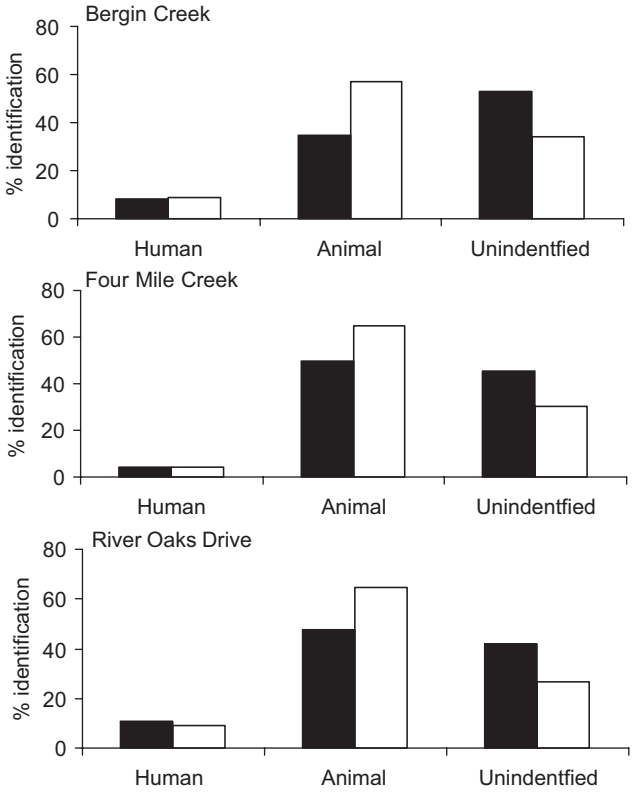

Fig. 2 – Percentage identification of human unique and animal *E. coli* (■) and enterococci (□) biochemical phenotypes (BPTs) found in water samples from the Bergin Creek, Four Mile Creek and River Oaks Drive sub-catchments.

are generally small in size and characterized by a high density of septic systems with close proximity to surface catchment drainage lines and a limited number of other non-point sources of faecal pollution. The concentrations of faecal coliforms, *E. coli* and enterococci in all storm water samples exceeded relevant water quality guidelines (ANZECC, 2000). This is not surprising, as water samples were collected during and after storm events when a large number of bacteria are washed into the creek via surface run-off. The number of faecal indicator bacteria is generally higher for catchments with higher densities of septic systems compared to catchments which are serviced by STPs (Beard et al., 1994). Phenotypic diversity of *E. coli* and enterococci in water samples from all sub-catchments was high, ranging from 0.85 to 0.89 (maximum of 1). In general, *E. coli* showed higher diversity than enterococci, probably because *E. coli* strains are more diverse in the environment (Ahmed et al., 2005b).

To identify faecal pollution, initially we used our existing metabolic fingerprint libraries of *E. coli* (4508 isolates) and enterococci (4833 isolates). These libraries were developed by using a stringent sampling protocol in order to capture the diverse phenotypic attributes of faecal indicator bacteria from 10 host groups (representing more than 250 farms and 300–350 individual animal) with the aim to identify the sources of faecal pollution in the southeast Queensland region (Ahmed et al., 2005b). These libraries were "decloned" (identical strains were removed from the analysis) and then the unique fingerprints (i.e. BPTs) were identified for each host group by comparing their fingerprints with each other (Ahmed et al., 2005b). These libraries have been previously

Table 3 – Detection of human faecal contamination at each sampling site using both library-dependent and independent methods

Sub-catchments	Storm events (month)	Biochemical fingerprint			Human-specific gene biomarkers	
		E. coli	Enterococci	*Bacteroides* HF183	*Bacteroides* HF134	Enterococci surface protein (*esp*)
BC	1 (January)	–	+	NT	NT	NT
	3 (February)	+	+	NT	NT	NT
	5 (November)	–	–	+	+	+
	5 (November)[a]	–	+	+	+	+
	6 (November)	–	–	+	+	–
FMC	1 (January)	–	–	NT	NT	NT
	2 (February)	–	+	NT	NT	NT
	3 (February)	–	–	NT	NT	NT
	4 (March)	–	–	NT	NT	NT
	4 (March)[a]	+	+	NT	NT	NT
	5 (November)	–	+	–	+	+
	6 (November)	+	+	+	+	+
ROD	1 (January)	+	+	NT	NT	NT
	2 (February)	+	+	NT	NT	NT
	3 (February)	+	–	NT	NT	NT
	4 (March)	+	+	NT	NT	NT
	5 (November)	+	–	–	–	+
	6 (November)	+	–	–	–	–

[a] Rising stage sample; NT: not tested.

used to demonstrate evidence of septic system failure and to identify non-point sources of faecal pollution in various catchments in southeast Queensland (Ahmed et al., 2005a, b). The efficacy of these libraries has also been evaluated in a cross-catchment study after testing their representativeness (Ahmed et al., 2006). We were also interested in investigating whether the human isolates in our libraries have reached their saturation level. To investigate this, we have updated our existing libraries (only human isolates) by comparing with more than 500 new human isolates that were external to the libraries. Clustering of this data indicated 96% of the enterococci BPTs and 91% of *E. coli* BPTs were already present in our libraries, which can be considered as highly representative (Wiggins et al., 2003).

E. coli and/or enterococci fingerprint libraries identified human faecal pollution in 13 water samples based on comparison with the human unique BPTs, indicating the presence of human faecal pollution during or after storm events. Of these, six contained both *E. coli* and enterococci human BPTs; the other seven contained human BPTs from either *E. coli* or enterococci. However, the percentage of isolates identified as human by both libraries was low (4–10%). There are several possible explanations for this result. First, it could be because our analysis was based on the comparison of unknown environmental BPTs with human unique BPTs. Septic tanks may contain certain unique (human-specific) BPTs of *E. coli* and/or enterococci as well as numerous shared BPTs; these shared ones would also occur in animal host groups (Ahmed et al., 2005b). Shared BPTs cannot discriminate between human and animal sources of faecal pollution and for this reason they were not included in our analysis. However, certain shared BPTs in water samples may have originated from defective septic systems. Because we compared the environmental isolates only to unique BPTs, we could have underestimated the human faecal pollution in these sub-catchments. It has previously been reported that the diversity of unique *E. coli* strains in host groups is higher than environmental samples (McLellan, 2004). The author also reported under representation of unique gull strains in beach water, known to be highly polluted with gull faeces. It is also possible that there are geographic differences in *E. coli* and enterococci BPTs between the sub-catchments in this study and the sub-catchments used for library development. The geographical variability of faecal indicators especially *E. coli*, has been reported by several studies (Gordon, 2001; Hartel et al., 2002; Scott et al., 2003). However, this could not be tested as we did not have access to any septic systems in the studied sub-catchments. Nonetheless, this seems unlikely given the number of isolates in our libraries and the geographical distance (<80 km) between two study areas. Finally, investigators have repeatedly observed that environments are dominated by environmental strains of indicators. These differ from genotypes or phenotypes found in faeces, even when the environments in question are heavily contaminated with faeces (Anderson et al., 2005; McLellan, 2004; Whittam, 1989). Our LD analysis may have identified the human-specific isolates; the others may have been environmentally adapted strains. On the other hand, the isolates identified as general animal faecal pollution by both the libraries was high (35–65%) as expected, because when we calculated the contribution from general animals, we compared environmental BPTs not only with unique BPTs but also with BPTs shared by multiple animal host groups, which increases the percentage identification. Both libraries could not identify around 26–57% BPTs in water samples, indicating the presence of other potential sources (animals such as wild bird and possums) of faecal pollution in these sub-catchments.

In this study, we also tested seven samples for the presence/absence of three human-specific biomarkers, in addition to the fingerprint libraries of *E. coli* and enterococci. These markers included human-specific *Bacteroides* HF183 and HF134 markers developed by Bernhard and Field (2000b), and *esp* marker developed by Scott et al. (2005). The application of these biomarkers for human faecal pollution tracking has been assessed in the US (Bernhard and Field, 2000a, b; Bernhard et al., 2003; Scott et al., 2005; Shanks et al., 2006). Although the *Bacteroides* method is in use worldwide (Gilpin et al., 2003; Seurinck et al., 2006), nothing has been reported regarding the geographical distribution and host specificity of these markers in Australia. In view of this, we rigorously evaluated the host specificity and geographical distribution of these markers by testing more than 150 samples from 13 host groups including humans. The results indicated that these markers are indeed specific to humans and suitable for identifying human faecal pollution in southeast Queensland region (data not shown).

Of the seven samples analysed with both *E. coli* and enterococci fingerprint libraries and PCR detection of biomarkers, at least one human-specific indicator or marker was detected in every sample. In a single sample, only an indicator (human-specific *E. coli* BPT) was detected; no PCR markers were detected. In two samples, only PCR markers were detected. Four samples were positive for at least one indicator/biomarker. These results confirm the presence of human faecal pollution by both LD and LI methods and also indicate that our library containing unique *E. coli* and enterococci BPTs are capable of detecting human faecal pollution. The lack of complete agreement among the different methods could be because each is detecting a different, possibly uncommon target; in samples where all or most of the tests were positive for human faecal pollution, it is likely that a large amount of human contamination was present.

Results obtained by using a LD method should be interpreted with care, as this method may not be highly sensitive to detect faecal pollution. For example, in this study, both *E. coli* and enterococci libraries did not identify human-specific isolates in two samples from Bergin Creek (events 5 and 6, November). However, these samples were positive for two or more human faecal PCR markers. This suggests that PCR detection of host-specific biomarkers is more sensitive in detecting faecal pollution. Most of the LD methods generally test a small number of isolates from a water sample that may actually contain thousands of different isolates from different sources (if highly polluted). As a result, some human-sourced isolates may be missed or be unable to be categorized. Testing for human-specific biomarkers, however, allows screening thousands of bacteria from water sample, potentially increasing the detection sensitivity.

Interestingly, we detected human faecal pollution in one sample (event 6, November) from the ROD sub-catchment by the *E. coli* library but not with the enterococci library or any of the markers. It is possible that our PCR detection method was not stringent enough to detect human faecal pollution. However, we increased the specificity of the assay by optimizing our PCR conditions and retested this sample and we were unable to amplify any products. There could also be a difference in persistence/survival among the different markers. It is difficult to interpret such inconsistency based on the limited number of samples collected and tested, especially when the water sample was highly polluted. This inconsistency warrants more rigorous comparison among these methods.

Human-specific *Bacteroides* and *esp* biomarkers generally showed good agreement in detecting human faecal pollution, with six out of seven samples in agreement (presence/absence). PCR detection of these markers is rapid and sensitive. However, primer sets are not available for all animal species, and some of these methods are not quantitative. The prevalence of toxin gene markers such as *esp* in environmental samples may be low; if so, a cultural enrichment step can be performed prior to testing by PCR, negating the advantage of rapid detection and potentially introducing bias. Another limitation of the *esp* marker is that certain *E. faecalis* from humans contain *esp* gene and therefore, use of this marker could potentially be under estimating human faecal pollution in a water sample. Perhaps, a qPCR method may provide more information regarding the persistence of this particular marker in relation to the total *E. faecalis*/enterococci. The survival rate of these markers in environmental samples and their correlation with faecal coliforms or pathogenic microorganism are not well understood and therefore, it is not yet possible to directly estimate health risks.

5. Conclusions

- Although the number of samples analysed was small, two trends are suggested, which could be tested in further studies. First, the LI (PCR) methods may be more sensitive than the LD (indicator) methods. Second, by combining the two appropriate approaches, more human-positive samples were identified than either approach would have identified alone.
- The concurrent use of four MST methods to identify human faecal pollution in stormwater draining three non-sewered catchments led us to conclude that human faecal pollution is exported from these catchments during storm events.
- The *E. coli* and enterococci metabolic fingerprint libraries used in this study were capable of detecting human faecal pollution although the percentage identification of human faecal pollution was low.
- The presence of host-specific biomarkers further confirmed human faecal pollution, and appears promising as an accurate, sensitive measures of human faecal pollution that can compliment LD methods. In view of this, we suggest that a combination of methods should be used for faecal source tracking to obtain a better understanding regarding the polluting sources.

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