South East Queensland Healthy Waterways Partnership

Microbial Source Tracking in Non-Sewered Catchments

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Our Vision: "By 2026, our waterways and catchments will be healthy ecosystems supporting the livelihoods and lifestyles of people in South East Queensland, and will be managed through collaboration between community, government and industry."

MICROBIAL SOURCE TRACKING IN NON-SEWERED CATCHMENTS

SUBMITTED TO
NRMSEQ

SUBMITTED BY

Warish Ahmed and Mohammad Katouli

INTRODUCTION

Non-point and point sources such as domestic and wild animal defecation, malfunctioning septic trenches, storm water drainage, urban-runoff and municipal wastes are regarded as contributors of faecal contamination. Various human enteric pathogens such as *Salmonella* spp., *Shigella* spp. (Faruque *et al.* 2002) and hepatitis A (Griffin *et al.* 1999) have been found in receiving water bodies due to human faecal contamination. Wastewater from domestic and/or farm animals such as cattle, horses and poultry may further contribute pathogens such as *Escherichia coli* O157:H7, *Cryptosporidium* spp. and *Giardia* spp. (Martin *et al.* 1986; Ong *et al.* 1996). Identification of major sources of faecal bacteria and potential pathogens is therefore necessary for the improved management of coastal creeks, lakes and rivers. However, the identification and quantification of pathogens from environmental sources can be a cumbersome task (Payment, 1993). Alternatively, the uses of indicators such as *Escherichia coli* and enterococci commonly found in the intestine of warm-blooded animals in relatively high numbers have been considered as ideal faecal indicators (Baudisöva, 1997). However, the presence of such indicator bacteria in surface waters can only be seen as a measure of the quality of the water but does not provide definitive information with respect to possible sources (McLellan, 2004). Genotypic and phenotypic methods have been developed to distinguish the various sources of human and animal faecal contamination (Ahmed *et al.* 2005; Carson *et al.* 2001) in surface waters. Some of these methods (i.e. rep-PCR, biochemical fingerprinting method, antibiotic resistance profiles) require the development of a known source database from host groups, based on the hypothesis that phenotypic or genotypic characteristics of specific bacterial strains are associated with specific animals (Scott *et al.* 2002). The developed database is then used to compare fingerprints from these same indicator bacteria found in receiving waters.

We have recently reported on the development of a large metabolic fingerprint database and used that to identify the sources of faecal contamination in Eudlo Catchment, Qld, Australia (Ahmed *et al.* 2005). This database has also been used in cross-catchment study with great success (Ahmed *et al.* 2006). In this study, the same database has been used to identify the sources of faecal contamination in Four Mile, River Oaks and Bergin Creeks which are exclusively serviced by on-site waste water treatment systems in the Pine River City Council, Qld, Australia.

MATERIALS AND METHODS

Water sampling

In all, 11 water samples were collected from 4 Mile Creek (5 samples), River Oaks Creek (4 samples) and Bergin Creek (2 samples) on 4 occasions. Samples were collected after low to moderate rainfall events and were transported to University of the Sunshine Coast. All samples were tested for the concentration of faecal indicator bacteria (i.e. faecal coliform, enterococci and *Escherichia coli*) within 6 h after collection by using membrane filtration method.

Isolation of faecal coliform, enterococci and Escherichia coli

Serial dilutions were made for all samples and filtered through a 0.45µm pore size (47mm-diameter) nitrocellulose membranes (Advantec, Japan) and placed on m-enterococcus (Difco, UK) and RAPID' *E. coli* 2 (REC 2) with supplement (Bio-rad, USA) agar plates. Plates were then incubated at 37ºC for 48h (for faecal streptococci) and at 44ºC for 24h (for faecal coliform and *E. coli*). The REC 2 medium, used for isolation of *E. coli* is based on the detection of 2 enzyme activities; β-D-glucuronidase (β-gluc) and β-D-galactosidase (β-gal). The hydrolysis of chromogenic substrates results in purple *E. coli* (β-gluc positive/ β-gal positive) and blue faecal coliform colonies (β-gluc negative/ β-gal positive). The supplement added to the medium inhibits interfering Gram-negative flora, which can be found in wastewater and natural waters. Single purple colonies from this medium were streaked on McConkey agar (Oxoid, USA) 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 plates were also tested for esculin hydrolysis

on to Bile Esculin Agar (Oxoid, UK) and incubated at 45°C for 1h to confirm their identification as enterococci (i.e. black coloration) (Manero and Blanch, 1999).

Biochemical fingerprinting

In this study, we used two types of micro plates specifically developed for typing of enterococci (PhP-RF plates) and *E. coli* strains (PhP-RE plates) (PhPlate system, PhPlate AB, Stockholm). The growth medium for PhP-RF and RE was prepared according to the manufacturer instructions. To maximize the metabolic fingerprint, from each sample up to 39 colonies of enterococci and 39 colonies of *E. coli* were picked with sterile toothpicks from the agar plates and tested with the PhPlate system (PhPlate AB, Stockholm) (Kühn *et al.*1995). An identity (ID) level of 0.96 was established based on the reproducibility of the system after testing 20 isolates in duplicates. Isolates with similarity higher than the ID-level were regarded as identical and assigned to the same biochemical phenotype (BPT). The phenotypic diversity among the isolates was measured with Simpson's index of diversity (Di) (Atlas, 1984). Di in the present study depends on isolates distribution into different BPTs. Diversity is high (maximum 1) for a population consists of different BPTs and is low (minimum 0) if the population contains of few dominating BPTs. The phenotypic similarity between different bacterial populations in two or more samples was calculated as population similarity (Sp) coefficient. The Sp coefficient calculates the proportion of isolates that are identical in two or more compared bacterial populations. It is high (maximum of 1) if two populations contain similar BPTs and is low (minimum of 0) if the populations contain different BPTs.

Data analysis

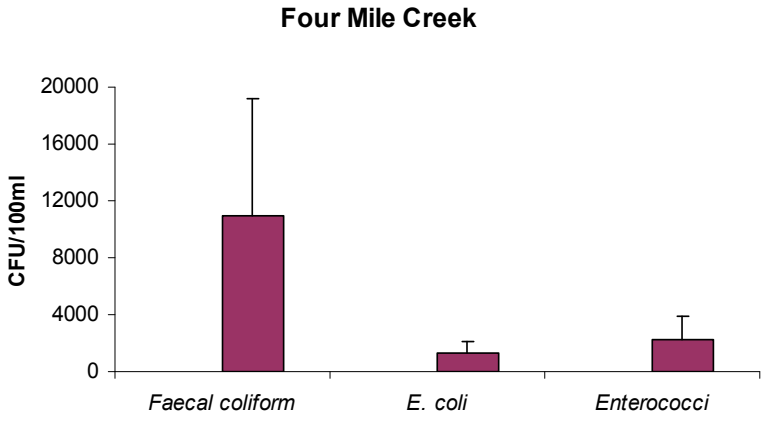
All data handling, including optical readings, calculations of correlations and coefficients, diversity indexes, Sp-values and as well as clustering and printing dendrograms, was performed using the PhPlate software version 4001 (PhPlate system, PhPlate AB, Stockholm).

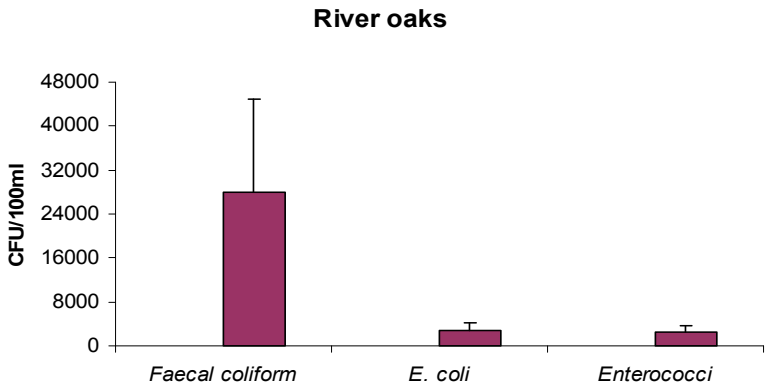
Source tracking database

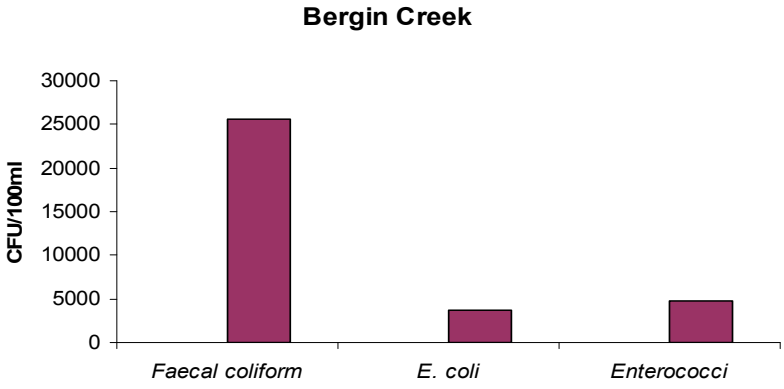
The database used in the study was developed by testing 4,833 enterococci isolates and 4,508 *E. coli* isolates from 10 host groups. These host groups included humans (via septic tanks and STPs), cattle, horses, chickens, dogs, Kangaroos, waterfowl (including ducks and birds), deer, sheep and pigs. The representativeness and stability of the fingerprints were assessed prior its application in a previous cross catchment study and the database was successful to categorize the sources of dominant faecal indicator bacterial contamination in a coastal lake (Ahmed *et al.* 2006). Since then, the database is being regularly updated by adding more bacterial isolates from different farms from different catchments. The most important feature of this database is that it has been developed by using stringent sampling program (Ahmed *et al.* 2005). The fingerprints were categorized on the basis of occurrence in host groups. The database consists of 308 unique (UQ) enterococci BPTs and 303 UQ *E. coli* BPTs. These UQ BPTs were specific to host groups. In contrast, 286 enterococci shared BPTs and 295 *E. coli* SH-BPTs were also found among host groups. These BPTs were shared between either humans or animals or among animal host groups. The latter was also used to identify non-specific animal contribution to the studied creeks.

RESULTS

Abundance of faecal indicators







Diversity of enterococci and E. coli in water samples

A total of 11 water samples were collected from 3 creeks. From each water sample, up to 40 enterococci isolates and up to 39 *E. coli* isolates (where possible) were typed (Table 1) for comparison with the database. The mean diversity of enterococci (Di= 0.84 ± 0.09) and *E. coli* (Di= 0.90 ± 0.04) were high (maximum of 1) in water samples indicating diverse sources of these bacteria (Table 1).

Table 1: Number of enterococci isolates and *Escherichia coli* isolates tested on different occasion and their diversity

Samples	Enterococci					*E. coli*				
	No of isolates tested				Mean Di ± SD	No of isolates tested				Mean di ± SD
	O1	O2	O3	O4		O1	O2	O3	O4	
Four Mile creek	37	39	39	(39 + 40)*	0.85 ± 0.08	37	22	33	(28 + 35)*	0.90 ± 0.05
River oaks creek	38	39	39	39	0.87 ± 0.12	31	28	35	36	0.89 ± 0.04
Bergin creek	39	0	39	0	0.81	39	0	27	0	0.94
Total	**114**	**78**	**117**	**118**	**0.84 ± 0.09**	**107**	**50**	**95**	**99**	**0.90 ± 0.04**

O1: 20-01-06, O2: 22-02-06, O3: 28-02-06, O4: 04-03-06.* Isolates from 2 samples (base flow and rising stage) collected from same creek.

Population similarity analysis of water samples

In all, 426 enterococci isolates and 351 *E. coli* isolates were typed with the biochemical fingerprinting from 3 creeks (Table 2). A population similarity (Sp) analysis was performed on populations (enterococci and *E. coli*) from all 3 creeks. The result indicated a high population similarity (maximum of 1) between Four Mile and River Oaks (0.53 for enterococci and 0.41 for *E. coli*). This data suggest that the faecal inputs for these two creeks may be more similar than that of Bergin (Table 2).

Table 2: Population similarity (Sp-values) among enterococci and *E. coli* populations found in studied creeks.

Creeks	Number of isolates		Population similarity value (Sp-value)					
			Four Mile Creek		River Oaks Creek		Bergin Creek	
	Ent	*E. coli*	Ent	*E. coli*	Ent	*E. coli*	Ent	*E. coli*
Four Mile Creek	194	155	0.00	0.00				
River oaks Creek	155	130	**0.53**	**0.41**	0.00	0.00		
Bergin Creek	78	66	0.20	0.32	0.30	0.31	0.00	0.00

Source identification

A total of 176 enterococci BPTs and 194 *E. coli* BPTs were found among 426 enterococci and 351 *E. coli* isolates tested from all 3 creeks. Of the 75 enterococci BPTs and 89 *E. coli* BPTs found in water samples from Four Mile, 2 (3%) enterococci BPTs and 2 (2%) *E. coli* BPTs were of humans (Table 3). Of the same water samples 45 (60%) enterococci BPTs and 47 (53%) *E. coli* BPTs were of animals (larger group containing specific and non-specific animal-BPTs). These specific animal-BPTs have been further categorized to individual host level in Table 4. The remaining 28 (37%) enterococci BPTs and 40 (45%) *E. coli* BPTs could not be identified as any host group. Of the 68 enterococci BPTs and 68 *E. coli* BPTs found in River Oaks, 8 (12%) enterococci BPTs and 6 (9%) *E. coli* BPTs were of humans (Table 3). Of the same water samples 43 (63%) enterococci BPTs and 34 (50%) *E. coli* BPTs were of animals. These specific animal-BPTs have been further categorized to individual host groups in Table 4. The remaining 17 (25%) enterococci BPTs and 30 (41%) *E. coli* BPTs could not be identified as any host group. Of the 33 enterococci BPTs and 37 *E. coli* BPTs found in River Oaks, 6 (18%) enterococci BPTs and 7 (19%) *E. coli* BPTs were of human (Table 3). Of the same water samples 40 (32%) enterococci BPTs and 12 (32%) *E. coli* BPTs were of animals. These specific animal-BPTs have been further categorized to individual host groups in Table 4. The remaining 17 (51%) enterococci BPTs and 18 (49%) *E. coli* BPTs could not be identified as any host group. 5 water samples tested from the Four Mile Creek only 2 (i.e. 40%) samples were positive for human unique signature. In contrast, all samples (i.e. 100%) from River

Oaks and Bergin contained human unique signature, indicating humans via septic tanks may have contributed these indicator bacteria in these creeks. Among animal host groups, waterfowl contributed more than others (Table 4). Interestingly, a very few BPTs from water samples were identical to kangaroos (1 BPT), sheep (1 BPT), deer (none) and pigs (none) support the findings that the sources are correctly classified.

Table 3: Comparison of biochemical phenotypes (BPTs) from water samples with the database

Creeks	No of BPTs found		Human UQ-BPTs		Animal-BPTs		Unknown-BPTs	
	Ent	*E. coli*	Ent (%)	*E. coli*	Ent (%)	*E. coli*	Ent (%)	*E. coli*
Four mile Creek								
O1 (20-01-06)	18	21	0 (0)	0 (0)	7 (39)	9 (43)	11 (61)	12 (57)
O2 (22-02-06)	12	11	1 (8)	0 (0)	9 (75)	5 (45)	2 (16)	6 (55)
O3 (28-02-06)	6	17	0 (0)	0 (0)	6 (100)	10 (59)	0 (0)	7 (41)
O4 (04-03-06) S1	18	19	0 (0)	0 (0)	10 (55)	12 (63)	8 (45)	7 (37)
S2	21	21	1 (5)	2 (10)	13 (62)	11 (52)	7 (33)	8 (38)
Total	**75**	**89**	**2 (3)**	**2 (2)**	**45 (60)**	**47 (53)**	**28 (37)**	**40 (45)**
River Oaks creek								
O1 (20-01-06)	15	19	2 (13)	1 (5)	5 (33)	10 (53)	8 (53)	8 (32)
O2 (22-02-06)	21	10	3 (14)	2 (20)	15 (71)	7 (70)	3 (14)	1 (10)
O3 (28-02-06)	13	17	0 (0)	2 (12)	11 (84)	9 (53)	2 (16)	8 (35)
O4 (04-03-06)	19	22	3 (16)	1 (5)	12 (63)	8 (36)	4 (21)	13 (59)
Total	**68**	**68**	**8 (12)**	**6 (9)**	**43 (63)**	**34 (50)**	**17 (25)**	**30 (41)**
Bergin Creek								
O1 (20-01-06)	20	23	3 (15)	4 (17)	5 (25)	7 (30)	12 (60)	12 (53)
O3 (28-02-06)	13	14	3 (23)	3 (21)	5 (38)	5 (36)	5 (38)	6 (43)
Total	**33**	**37**	**6 (18)**	**7 (19)**	**10 (30)**	**12 (32)**	**17 (51)**	**18 (49)**

Table 4 Comparison of biochemical phenotypes (BPTs) from water samples with the UQ-animal database

Creeks	Waterfowl BPT (%)		Cattle BPT (%)		Chicken BPT (%)		Horses BPT (%)		Dogs BPT (%)	
	Ent	*E. coli*	Ent	*E. coli*	Ent	*E. coli*	Ent	*E. coli*	*Ent*	*E. coli*
Four mile Creek										
O1 (20-01-06)	2 (11)	3 (14)	2 (11)	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (10)
O2 (22-02-06)	1 (8)	0 (0)	0 (0)	0 (0)	1 (8)	1 (9)	1 (8)	0 (0)	0 (0)	1 (9)
O3 (28-02-06)	1 (17)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (12)
O4(04-03-06) S1	3 (17)	2 (11)	1 (6)	1 (5)	0 (0)	0 (0)	1 (6)	0 (0)	2 (12)	1 (5)
RS	4 (19)	4 (19)	1 (5)	0 (0)	0 (0)	0 (0)	1 (5)	0 (0)	1 (0)	4 (19)
Total	**11 (15)**	**10 (17)**	**3 (4)**	**2 (2)**	**1 (1)**	**1 (1)**	**3 (4)**	**0 (0)**	**3 (4)**	**10 (11)**
River Oaks										
O1 (20-01-06)	1 (7)	2 (11)	1 (7)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5)	0 (0)	0 (0)
O2 (22-02-06)	4 (19)	2 (20)	1 (5)	0 (0)	1 (5)	0 (0)	1 (5)	1 (10)	0 (0)	1 (10)
O3 (28-02-06)	2 (15)	3 (18)	2 (15)	0 (0)	0 (0)	2 (12)	0 (0)	0 (0)	0 (0)	1 (6)
O4 (04-03-06)	3 (16)	0 (0)	0 (0)	0 (0)	2 (11)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	**10 (15)**	**7 (10)**	**4 (6)**	**0 (0)**	**3 (4)**	**2 (3)**	**1 (1)**	**2 (3)**	**0 (0)**	**2 (3)**
Bergin Creek										
O1 (20-01-06)	1 (5)	3 (13)	0 (0)	0 (0)	1 (5)	0 (0)	1 (5)	0 (0)	0 (0)	0 (0)
O3 (28-02-06)	1 (8)	1 (7)	0 (0)	0 (0)	0 (0)	0 (0)	1 (8)	0 (0)	1 (8)	1 (7)
Total	**2 (6)**	**4 (11)**	**0 (0)**	**0 (0)**	**1 (3)**	**0 (0)**	**2 (6)**	**0 (0)**	**1 (3)**	**1 (3)**

Population similarity analysis between septic tanks and water samples

The number of all fingerprints found in each water sample from each creek were pooled and compared with randomly chosen (unbiased) 450 human isolates. The results indicated a low population similarity (Sp) between populations from creeks and humans. This could be due to the fact that the number of isolates from each creek was smaller than the number of humans isolates and therefore comparison

yielded a low population similarity values. However, a better value obtained when populations from all creeks were pooled and compared with the human populations. To obtain better results more bacterial isolates from surface waters should be tested in such analysis. Nevertheless, this data also indicate that there is human signature present in water samples.

Table 5: Indicator bacterial populations (Sp-value) from different creeks with randomly chosen populations from septic tanks.

Creeks	No of isolates		Population similarity value to humans (n=450)	
	Ent	*E. coli*	Ent	*E. coli*
Four mile Creek	194	155	0.14	0.12
River Oaks creek	154	130	0.14	0.10
Bergin Creek	78	66	0.08	0.11
Total	**426**	**351**	**0.14**	**0.16**

CONCLUSIONS

- Abundance of faecal indicators is quite high indicating that there is faecal contamination in the samples from studied creeks.
- High diversity of faecal indicator bacteria in water samples indicating diverse sources of contamination.
- Population similarity analysis of the indicator bacteria among 3 creeks indicating that the sources of contamination in Four Mile and River Oaks may be similar.
- 3% enterococci and 2% *E. coli* from Four Mile Creek were categorized as specific human contamination when compared with the database.
- 12% enterococci and 9% *E. coli* from River Oaks were categorized as specific human contamination.
- 18% enterococci and 19% *E. coli* from Bergin Creek were categorized as specific human contamination.
- The overall percentage contribution from humans was low when compared with the database though the study areas are exclusively serviced by septic systems and some of which may be failing and releasing faecal bacteria. Perhaps a better approach would be to collect septic samples from the studied creeks catchment and upgrade the existing database. It has to be noted that, some of the septic systems in the study area may have their own unique BPTs. Therefore presence of such BPTs in water samples could not be identified with the existing database. This hypothesis should be tested.
- A good agreement observed between both faecal indicator bacteria in terms of source identification increases the confidence level that the sources are correctly identified.
- Around 45% of both faecal indicator bacterial types could not be identified to any host groups. To increase the identification level, more bacterial isolates from various host groups present in the studied creeks should be included in the database.
- The percentage contribution from animal host groups was higher than humans.
- The percentage contribution from humans in Bergin Creek is higher than those of any single animal host group.
- Among animal host groups, waterfowls were consistently found in most of the water samples tested.
- Among 11 samples tested in this study 8 contained human signatures.
- Population similarity analysis further supports the findings that there is specific (identified in this study) and non-specific (could not be identified) human contamination may be present.
- Fingerprints from water samples were not identical to pigs, sheep, deer and kangaroos and therefore management efforts should not focus on these host groups.

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