

Population similarity of enterococci and *Escherichia coli* in surface waters: A predictive tool to trace the sources of fecal contamination

W. Ahmed, R. Neller and M. Katouli

ABSTRACT

A biochemical fingerprinting method (the PhPlate system) was used to compare similarities between *Escherichia coli* and enterococci populations from surface water samples with those found in different animal species during the wet and the dry seasons in order to predict the dominant source(s) of fecal contamination in a local creek. A significant increase in the number and diversity of enterococci was observed in the creek during the wet season. Enterococci population from water samples also showed a higher population similarity with animal species than did *E. coli*. A higher population similarity was found between both indicator bacteria and animal species during the wet season with highest population similarities found in dogs, horses, cows and kangaroos. In contrast, a low population similarity was found for both fecal indicator bacteria from humans with water samples during the wet and the dry seasons, indicating that humans are not a major source of contamination in the studied creek. The results also indicate that the population similarity analysis of enterococci population has an advantage over *E. coli* in tracing the possible source(s) of contamination in the studied creek and that population similarity analysis as used in this study can be used to predict the source(s) of fecal contamination in surface waters.

Key words | biochemical fingerprinting, enterococci, *E. coli*, population similarity, surface water, water quality

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INTRODUCTION

Indicator bacteria have been used for many years to determine the quality and safety of surface and ground waters. The presence of these bacteria in surface waters also indicates that the pathogenic organisms such as *Salmonella* spp., *Shigella* spp. and hepatitis A may be present. Human fecal contamination, via defective septic systems has recently shown to be a major source of such fecal contamination in surface waters (Ahmed *et al.* 2005a). Therefore, identification of this and other major contaminating sources is necessary to implement management plans so as to minimize public health risks.

While fecal coliforms have been historically used as an indicator of the microbiological quality of both surface

and ground waters (Hagedorn *et al.* 1999; Harwood *et al.* 2000; Hartel *et al.* 2002; Scott *et al.* 2002), their value has been questioned, because these groups of bacteria can also derive from various sources other than warm-blooded animals (Kreider 1995; Hagedorn *et al.* 1999; Dombek *et al.* 2000). Instead, it has been proposed that enterococci and *E. coli* be used as substitutes for fecal coliforms since these bacteria are exclusively found in the intestine of warm-blooded animals and their presence in surface waters indicates that recent contamination has occurred by humans or other warm-blooded animals (Godfree *et al.* 1997). However, it must be noted that the presence of such indicator bacteria in surface waters

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does not provide specific information regarding the contributing source(s), and therefore further analysis is required to trace the source(s) of such contamination.

While several genotypic and phenotypic methods have been used to trace the sources of contamination in surface waters (Simmons *et al.* 1995; Parveen *et al.* 1999; Wiggins *et al.* 1999; Bernhard & Field 2000; Harwood *et al.* 2000; Carson *et al.* 2001; Hartel *et al.* 2002; Ahmed *et al.* 2005b), none of these methods are regarded as a gold standard or benchmark to date. For instance, genotypic methods, though highly discriminatory, can be laborious and/or expensive for bacterial source tracking/ecological studies where a large number of isolates need to be tested (Kühn *et al.* 1995; Olive & Bean 1999; Hartel *et al.* 2002).

A highly discriminative but less expensive biochemical fingerprinting method (the PhPlate system; PhPlate AB, Stockholm, Sweden) has been reported and used for testing a large number of indicator bacteria from surface waters with a high degree of discrimination and reproducibility (Kühn *et al.* 1997; Ahmed *et al.* 2005a, b). This method enables one to test and compare populations of indicator bacteria from surface waters and a wide variety of suspected sources. We have recently used this method to develop a metabolic fingerprint database of both enterococci and *E. coli* bacteria specific to host animal species to trace the sources of fecal contamination in surface waters (Ahmed *et al.* 2005b). The database developed by this method was quite specific and was able to trace more than 65% of both indicator bacteria in the studied catchment. We have also used this method to provide evidence of septic system failure by comparing specific/unique enterococci and *E. coli* biochemical phenotypes (BPTs) found in septic tanks with those found in the downstream water samples of nearby creeks (Ahmed *et al.* 2005a).

In this study, we used population similarity analysis to compare the overall similarity between enterococci and *E. coli* populations in surface waters with those found in fecal samples of different animal species, as a rapid predictive tool to trace the sources of fecal contamination in a catchment. The study was undertaken in the Eudlo Creek catchment on the Sunshine Coast in Southeastern Queensland, Australia.

METHODS

Animal species sampling

Ten species of animals were sampled. These species included horses, cattle, sheep, pigs, ducks, chickens, deer, kangaroos, dogs and humans (via septic tanks). For each farm animal species, samples were collected from 20 farms (where possible) as described before (Ahmed *et al.* 2005b). The sampling farms were located within (i.e. horses, cattle, ducks, chickens, dogs and humans) and outside (i.e. sheep, kangaroos, pig and deer) of the studied catchment area. From each of these farms up to 3 animals were sampled and from each sample up to 12 colonies of enterococci and *E. coli* (where possible) were tested to obtain their biochemical fingerprint with the PhPlate system (see below). In total, 326 samples were collected from fresh feces of individual animals with sterile swabs. All samples were inserted into Amies transport medium (Interpath, Melbourne, Australia), transported on ice to the laboratory and were tested within 6 hours.

Human isolates were obtained from 39 septic tanks from a previous study performed simultaneously in the same creek (Ahmed *et al.* 2005a). All septic tanks tested for this study were within 50–100 m distance of the creek. In all, 790 enterococci and 641 *E. coli* isolates from septic tanks were included in this study to be compared with water samples. Fecal samples from septic tanks were collected from the outlet of the septic tanks with the aid of sterile swabs and inserted into Amies transport medium (Interpath), transported on ice to the laboratory and were tested within 6 hours.

Isolation of indicator bacteria

All fecal samples were streaked on m-enterococcus agar plates (Difco, USA) as recommended by the Standard Methods for the Examination of Water and Wastewater (Greenberg *et al.* 1992) and chromogenic *E. coli*/coliform (Oxoid, UK) agar plates and were incubated at 37°C for 24 hours (for *E. coli*) and 48 hours (for enterococci). Chromogenic *E. coli*/coliform medium allows specific detection of *E. coli* through substrate cleavage by the enzyme glucuronidase and formation of purple

colonies, which are different from other fecal coliforms (rose/pink colonies).

Biochemical fingerprinting

The principle of the biochemical fingerprinting with the PhPlate system has been described previously (Möllby *et al.* 1993). This method measures the kinetics of bacterial metabolism in liquid medium in microtitre plates. For each bacterial isolate, it yields a biochemical fingerprint made of several quantitative data, which are analyzed with the PhPlate software to calculate the level of similarity between tested isolates. In this study, we used PhP-RF plates (for enterococci) and PhP-RE plates (for *E. coli*) for typing of bacterial isolates (PhPlate AB, Stockholm, Sweden). Reagents used in the PhP-RF and RE plates have been described before (Kühn *et al.* 1995; Vilanova *et al.* 2002; Iverson *et al.* 2003). The growth medium for PhP-RF contained 0.2% (w/v) proteose peptone (Oxoid), 0.05% (w/v) yeast extract (Oxoid), and 0.5% (w/v) NaCl, and 0.011% (w/v) bromothymol blue, and for *E. coli* it contained 0.1% (w/v) proteose peptone, and 0.011% (w/v) bromothymol blue according to the manufacturer instructions. Isolated bacterial colonies for both indicator bacteria were randomly selected with sterile tooth pick directly from the primary isolating agar plates (see above) and suspended into the first well of each row containing 350 μ l of growth medium. Aliquots of 25 μ l of bacterial suspension were transferred into each of the other 11 wells containing 150 μ l growth medium with the aid of a multi-channel pipette. Plates were then incubated at 37°C and A_{620} was measured at 16, 40 and 64 hours for enterococci and 7, 24 and 48 hours for *E. coli* 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) according to Kühn *et al.* (1991). An identity (ID) level of 0.965 was established for the system based on testing 20 isolates in duplicate. Isolates showing similarity to each other above the ID-level were regarded as identical and were assigned to a biochemical phenotype (BPT). A representative of each *E. coli* BPT was tested for indole production and a representative of each enterococci BPTs was tested for esculin hydrolysis on bile esculin agar

(Oxoid) to confirm their identification (American Public Health Association 1995) before they were saved and their fingerprints were further analyzed.

The phenotypic diversity among the isolates was measured with Simpson's index of diversity (D_i) (Atlas 1984). D_i in the present study depends on isolates distribution into different BPTs. It is high (maximum 1) for a population consisting of different BPTs and is low (minimum 0) if the population consists of few BPTs.

The similarity between different bacterial populations in two or more samples was calculated as a population similarity coefficient. The population similarity coefficient calculates the proportion of isolates that are identical (i.e. having the same BPT) in two or more compared bacterial populations (Kühn *et al.* 1991). It is high (maximum 1) if two populations contain similar BPTs, and is low (minimum 0) if the population contains different BPTs. Clustering of population similarity coefficients was performed according to the unpaired group method with arithmetic averages (UPGMA). All data handling, including optical readings, calculations of population similarity values as well as clustering and printing dendrograms, was performed using the PhPlate software version 4001 (PhPlate system, PhPlate AB, Stockholm).

Water samples

Water samples were collected from the Eudlo Creek mainstream, a sub-catchment of the Maroochy River in the Sunshine Coast region of Queensland, Australia. The creek is approximately 8 km in length and has been reported by the Environmental Protection Agency (EPA) to be contaminated with a high level of fecal indicator bacteria and nitrates. The Eudlo Creek catchment was selected based on the number of septic systems (approx. 1,534), the population size (approx. 6,000) and because most of the area (i.e. >85%) of the catchment is not serviced by a centralized sewage treatment plant (STP). Furthermore, the catchment is characterized by high number of animal farms (approx. 50). Water samples were collected from 3 sites along the mainstream (EC1 to EC3) during November to December 2003 (wet season) and from 5 sites (EC1 to EC5) during August to September 2004 (dry season). In all, 27 water samples were collected from these sites. In addition,

samples were collected from a control site located 5 km upstream of the study area during both wet and dry seasons. In all, 7 samples were collected from this site during the dry (3 samples) and the wet season (4 samples). Table 1 shows that number of samples collected and the number of isolated tested from each sample. The control site is characterized by low density of animal farms and septic systems and receives water mainly from pristine areas not easily accessible to humans and containing naturally low levels of fecal indicator bacteria. All samples were tested in triplicate. Water samples were collected and tested as described in an earlier paper (Ahmed *et al.* 2005b). In brief, 500 ml sterile bottles were filled at 30 cm below the water surface and transported on ice to the laboratory and filtered through a 0.45 µm pore size cellulose membrane filters (Millipore, Bedford, Massachusetts, USA) within 6 hours. Filters were then placed on m-enterococcus (Difco) and chromogenic coliform/*E. coli* agar plates (Oxoid, USA) and incubated at 37°C for 24 hours (for *E. coli*) and 48 hours (for enterococci) as described above. After incubation, colonies were enumerated and calculated as CFU per 100 ml. From each water sample, up to 40 (where

possible) isolates were typed with the PhPlate system as described above. Representative of each *E. coli* BPTs was tested for the production of indole and representative of each enterococci BPT was tested for esculin hydrolysis on bile esculin agar (Oxoid) before further analysis.

Statistical analysis

Analysis of variance (ANOVA) was used to compare the significance of difference between the numbers of fecal indicator bacteria among sampling sites. Mann-Whitney's non-parametric test was used to compare the significance of the difference between the mean numbers and BPTs of fecal indicator bacteria in water samples during the wet and the dry seasons.

RESULTS

Both enterococci and *E. coli* were detected in all water samples throughout the study. The number of enterococci and *E. coli* collected from these sites during wet season ranged from 510 to 921 CFU/100 ml and 340 to

Table 1 | Number of water samples and bacterial isolates tested during the wet (sites EC1 to EC3 only and the control) and the dry season (sites EC1-EC5 and the control)

Sampling sites	No. of samples	Sampling period													
		Wet season (occasion number)						Dry season (occasion number)							
		(1)		(2)		(3)		(1)		(2)		(3)		(4)	
Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>
EC1	7	32	32	29	23	38	19	22	18	23	7	23	21	39	33
EC2	7	38	25	39	22	36	21	23	19	23	14	23	29	39	33
EC3	7	39	14	40	65	39	18	23	20	23	11	23	7	39	37
EC4	3	–	–	–	–	–	–	23	–	23	–	23	–	–	–
EC5	3	–	–	–	–	–	–	23	16	23	13	23	11	–	–
Sub-total	27	109	71	108	110	113	58	114	73	115	45	115	68	117	103
Control	7	7	8	6	3	11	7	4	3	7	9	6	7	8	6
Total	34	116	79	114	113	124	65	118	76	122	54	121	75	125	109

Ent: enterococci.

1,014 CFU/100 ml respectively. During the dry season, these figures ranged from 101 to 700 CFU/100 ml for enterococci and 120 to 600 CFU/100 ml for *E. coli*. One-way ANOVA demonstrated that the number of indicator bacteria at the various sampling sites differed significantly from the control site during the wet season for both enterococci and *E. coli* and during the dry season for enterococci only (Figures 1a and b). The mean number of enterococci in water samples during the wet season was significantly higher than that of the dry season ($P = 0.03$) (Figure 1a insets) For *E. coli*, however, the mean number did not differ significantly (Figure 1b insets).

Similarly, the diversity of enterococci BPTs in water samples during the wet seasons (0.85 ± 0.05) was significantly ($P = 0.03$) different from that of the dry season

(0.72 ± 0.14). For *E. coli*, the mean diversity did not differ between the wet (0.93 ± 0.09) and the dry season (0.89 ± 0.11).

For each season, the indicator bacteria from each sampling site were pooled and similarities between populations at different sites were calculated as the population similarity. It was found that there were high similarities between both indicator bacterial populations at different sampling sites (Figures 2a and b). However, the mean population similarity for enterococci population (i.e. 0.44) was much higher than that of *E. coli* populations (i.e. 0.26) over the entire sampling periods (i.e. both the wet and the dry season). Seasonally, the mean similarity between both bacterial populations was much higher during the wet

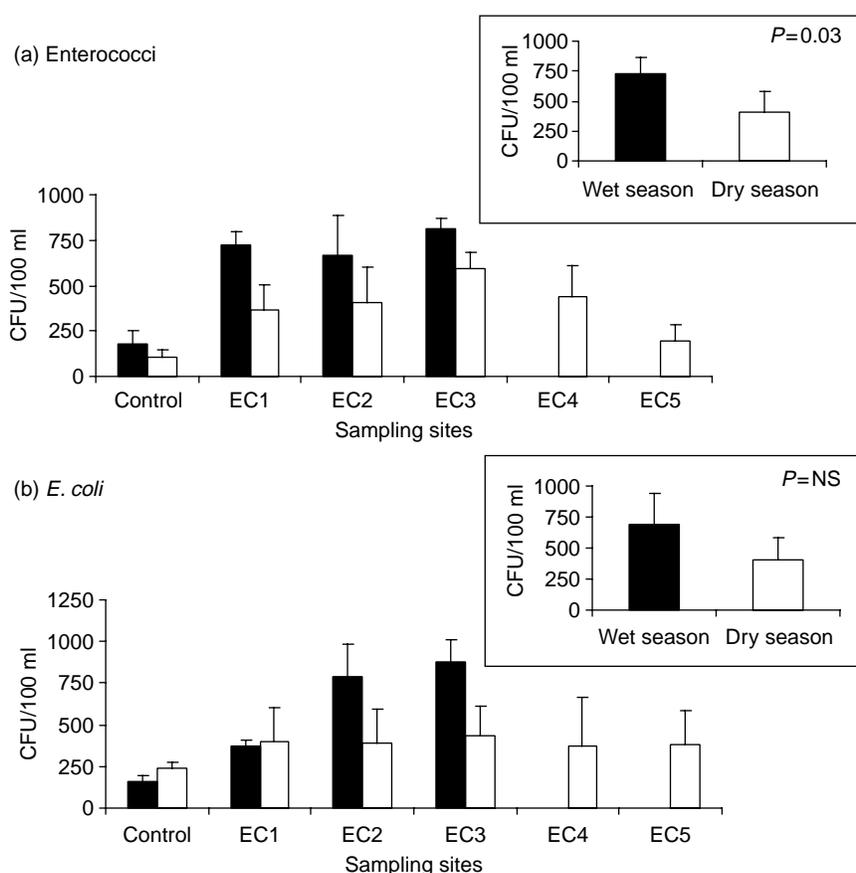


Figure 1 | The mean and standard deviation of enterococci (a) and *Escherichia coli* (b) during the wet (■) and the dry (□) seasons at different sampling sites (EC1 to EC5). No samples from sites EC4 and EC5 were taken during the wet season. For enterococci, significant differences were observed between the sampling sites and the control during both the wet and dry seasons. For *E. coli*, there was a significant difference between the sampling sites and the control only during the wet season. Enterococci during the wet season: EC1, EC2, and EC3 vs. Control = $P < 0.01$. Enterococci during the dry season: EC2, EC4 vs. control = $P < 0.05$, and EC3 vs. control = $P < 0.01$. *E. coli* during the wet season: EC2, EC3 vs. control = $P < 0.001$.

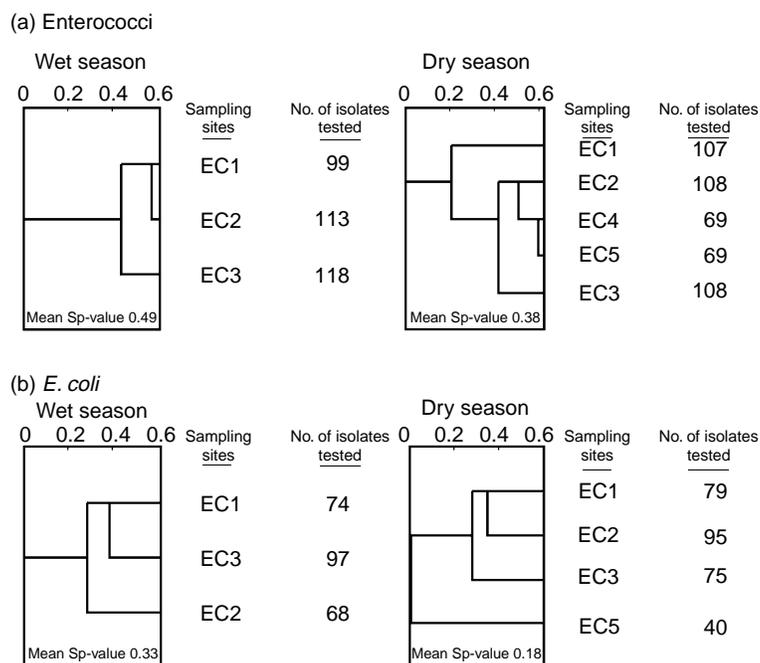


Figure 2 | UPGMA dendrograms of population similarity for (a) enterococci and (b) and *E. coli* at different sampling sites (EC1 to EC5) during both the wet and dry season.

season (0.49 for enterococci and 0.33 for *E. coli*) than the dry season (i.e. 0.38 for enterococci and 0.18 for *E. coli*) (Figures 2a and b).

Enterococci and *E. coli* populations from each animal species were compared with those of water samples (i.e. EC1- EC5) during the wet and dry seasons. Generally enterococci population from animals showed a higher similarity to those of water samples than *E. coli* (Table 2). However, bacterial population from the control site showed very low population similarity to those from all animals tested. Furthermore, the similarity of both indicator bacterial populations was higher for water samples during the wet season than the dry season, with samples from dogs, horses, cows, kangaroos and duck showing the highest similarity (Table 2). To identify whether this similarity between animal groups and water samples are based on the presence of specific bacterial groups, a pair wise comparison between the populations of each indicator bacteria from all animal species was undertaken. These results showed that certain animal species had a higher population similarity to each other than the others (Table 3). For instance, indicator bacteria from cows had high population similarity (i.e. >0.30) with majority of the animals tested

(See Table 3, highlighted figures). Similarly, indicator bacteria from ducks had high similarity to most animal species. In contrast, indicator bacteria from humans had a very low similarity to all animal species tested (Table 3).

DISCUSSION

In this paper, we analyzed population structure of enterococci and *E. coli* from different animal species and compared such similarities with that found in water samples collected from a local creek, to determine whether we could predict such sources of fecal contamination using the PhPlate system. The population similarity co-efficient was used to measure the proportion of identical bacterial isolates in two or more samples and to provide a better understanding of the overall similarity between compared populations. We previously used this system to compare similarity between bacterial populations from a number of septic tanks with those found in water samples collected from downstream of a sub-catchment (Ahmed *et al.* 2005a) and showed that the system was able to identify the failed septic system which contributed to fecal contamination in the creek. However, in

Table 2 | Comparison of population similarity of enterococci and *E. coli* isolated from animal species and water samples (EC1-EC5 and the control)

Animal species	Population similarity value to sites EC1-EC5				Population similarity value to control site			
	Enterococci		<i>E. coli</i>		Enterococci		<i>E. coli</i>	
	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season
Dogs	0.46	0.36	0.36	0.21	0.11	0.13	0.07	0.05
Horses	0.43	0.22	0.33	0.17	0.09	0.08	0.09	0.04
Cows	0.42	0.23	0.37	0.27	0.07	0.11	0.05	0.06
Kangaroos	0.41	0.17	0.24	0.15	0.06	0.08	0.03	0.06
Pigs	0.32	0.19	0.25	0.19	0.04	0.01	0.01	0.0
Sheep	0.30	0.14	0.16	0.16	0.00	0.03	0.0	0.02
Deer	0.29	0.17	0.25	0.19	0.01	0.03	0.0	0.0
Chickens	0.28	0.21	0.26	0.20	0.07	0.04	0.03	0.02
Ducks	0.28	0.22	0.35	0.26	0.04	0.02	0.01	0.03
Human	0.20	0.20	0.17	0.14	0.03	0.05	0.02	0.01

such analysis sampling protocols should be focused on testing a large number of bacterial isolates from both the suspected source(s) and the receiving waters.

In this study, the number of bacterial isolates from different animal species ranged from 148 (kangaroo) to 399 (chicken) for enterococci and from 99 (kangaroo) to 386 (dogs) for *E. coli*. We also tested a large number of bacterial isolates from human (via septic tanks) and water samples. The mean population similarity for both fecal indicator bacteria between different sampling sites showed to be higher in the wet season than those obtained during the dry season. This is probably because during the wet season a large number of bacteria are washed into the creek via surface run-off. Under these conditions, the chance of finding similar BPTs between two compared populations is high. It has to be noted that, when the diversity of two bacterial populations is low, the degree of similarity between these populations is determined by the similarity between the dominant BPTs in these samples. In our study, the diversity of enterococci in water samples during the wet

season was significantly higher than during the dry season. This however, was not the case for *E. coli*, probably because these strains are more diverse in the environment than enterococci (Ahmed *et al.* 2005a, b). Nonetheless, high diversity of both faecal indicator bacteria in water samples indicates non-point sources of fecal contamination in the studied creek.

We also found that the mean population similarity of enterococci during both wet and dry seasons were higher than those of *E. coli*. Similar results have been reported by Vilanova *et al.* (2002), but the reason for this is not fully understood. One possible explanation is that enterococci survive longer in natural waters than *E. coli* (Baudisöva 1997). This may influence their diversity as new BPTs are cumulatively added into the existing BPTs resulting in an overall higher diversity and subsequently higher population similarity.

To determine the dominant source(s) of fecal indicator bacteria in creek water samples, bacterial populations (both enterococci and *E. coli*) from each animal species were

Table 3 | A correlation coefficient matrix showing the degree of similarity between indicator bacterial populations (Sp-value) from different animal species. Populations showing similarity above 0.30 were highlighted.
Ent: enterococci

Animal species	Dogs		Horses		Cows		Kangaroos		Pigs		Sheep		Deer		Chickens		Ducks		Human		
	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>	Ent	<i>E. coli</i>									
Dogs	1.00	1.00																			
Horses	0.12	0.30	1.00	1.00																	
Cows	0.17	0.32	0.27	0.34	1.00	1.00															
Kangaroos	0.36	0.22	0.23	0.31	0.27	0.33	1.00	1.00													
Pigs	0.29	0.27	0.14	0.19	0.35	0.26	0.27	0.20	1.00	1.00											
Sheep	0.12	0.27	0.15	0.17	0.39	0.31	0.16	0.25	0.23	0.25	1.00	1.00									
Deer	0.19	0.35	0.21	0.27	0.36	0.41	0.23	0.25	0.25	0.25	0.28	0.39	1.00	1.00							
Chickens	0.27	0.23	0.08	0.15	0.15	0.26	0.29	0.16	0.26	0.34	0.13	0.30	0.13	0.30	1.00	1.00					
Ducks	0.27	0.35	0.39	0.22	0.34	0.39	0.47	0.19	0.30	0.31	0.26	0.30	0.26	0.38	0.27	0.33	1.00	1.00			
Human	0.11	0.12	0.09	0.08	0.12	0.08	0.10	0.05	0.11	0.06	0.09	0.07	0.09	0.04	0.17	0.07	0.16	0.07	1.00	1.00	

compared with the population from water samples collected in both the wet and dry season. Whilst populations of both fecal indicator bacteria collected from humans showed a low similarity with those collected from water samples, highest population similarities were found between dogs with water samples followed by horses, cows and kangaroos. Interestingly, the bacterial populations from these same animal species showed a low similarity with water samples during the dry season. In contrast, bacterial populations from the control site showed very low similarity to those from animal species and therefore the high similarity between indicator bacteria from animal species and the water samples could be due to indirect contamination of the creek water by animal (both domestic and wild) defecations. Pair-wise comparison of the population of indicator bacteria from different animal species showed that certain animal species shared similar populations with others. This can limit the identification of the main contributing source in the creek. It has to be noted that, in the studied catchment, the number of certain animal species was either very low (e.g. sheep and pigs) or they were not found in the catchment (e.g. deer). The high similarity of indicator bacteria from these animal species with water samples was therefore due to the high similarity of the indicator bacterial populations between these animals and the other dominant species. Based on these findings, we suggest that analysis of bacterial populations to identify the sources of contamination in ecological studies should be performed in conjunction with the local knowledge of the dominant animal species residing in the catchment. The fact, that indicator bacteria from human sources showed a low similarity with animal species and as well as water samples, indicate that humans are not a major source of contamination and that the population similarity as used in this study could serve as a reliable tool for distinguishing between human and animal fecal contamination.

In our study, we also found overall higher population similarity for enterococci than *E. coli* during both seasons, indicating that the population similarity analysis of enterococci could have advantages over *E. coli* during both the wet and dry seasons. Whilst we found that the population similarity analysis of indicator bacteria can provide a rapid means of predicting the possible sources of contamination in surface waters, these data should be interpreted with

caution as some species of animals may share a portion of identical population with other species of animals. To overcome this problem, a high number of bacterial isolates should be tested from as many animals as possible and the data should be analysed in conjunction with the knowledge of dominant species of animals residing in the catchment.

CONCLUSIONS

1. A high population similarity was obtained for both enterococci and *E. coli* from dogs, horses, cows and kangaroos with water samples during the wet and dry seasons. In contrast, a low similarity was found between the human and animal species and water samples, indicating humans are not a major source of contamination in the catchment.
2. The increased number of enterococci and *E. coli* in the creek during the wet season was associated with higher diversity of enterococci and a higher population similarity between both indicator bacteria in water and animal samples, indicating that surface run-off washes a high numbers of fecal bacteria into the studied creek from defecation of domestic and native animals.
3. The population similarity analysis as used in this study can be used to predict the source of fecal indicator bacteria in surface waters. However, such analyses should be done with knowledge of the dominant animal species in the catchment area. In addition, to avoid over interpretation a large number of bacterial isolates, where possible, should be tested from animals and water samples.
4. Of the two indicator bacteria used in this study, enterococci appeared to have an advantage over *E. coli* in predicting the possible sources of fecal contamination in water samples.

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