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Evidence of septic system failure determined by a bacterial biochemical fingerprinting method

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ABSTRACT

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Aims: To provide evidence of septic system failure by comparing two faecal indicator bacteria, enterococci and *Escherichia coli*, from defective septic tanks and adjacent creeks.

Methods and Results: A biochemical fingerprinting method was used to type and compare enterococci and *E. coli* strains from 39 septic tanks with creek water samples. Phenotypic diversity of enterococci (0·5 ± 0·3) and *E. coli* (0·5 ± 0·3) in septic tanks were significantly lower than those found in water samples (0·8 ± 0·1, $P < 0{\cdot}0001$ for enterococci and 0·9 ± 0·1, $P < 0{\cdot}0001$ for *E. coli*). Among 1072 enterococci isolates tested from septic tanks, 203 biochemical phenotypes (BPTs) were found of which 98 BPTs from 33 septic tanks were identical to several water samples. Similarly, among 621 *E. coli* isolates tested from septic tanks, 159 BPTs were found of which 53 BPTs from 26 septic tanks were also identical to water samples. The number of the latter bacteria was significantly ($P = 0{\cdot}01$) higher in water samples collected from downstream compared with that of upstream in the study area. A high similarity between the populations of both indicator bacteria was also found between defective septic tanks and downstream water samples further indicating the contamination of both creeks by defective septic systems.

Conclusions: Biochemical fingerprinting of faecal indicator bacteria is a useful and rapid method to provide direct evidence for septic system failure. Combination of both faecal indicator bacteria (enterococci and *E. coli*) provides a better judgement of the performance of a septic system.

Significance and Impact of the Study: This study is the first to provide direct evidence of septic system failure by identifying the presence of specific bacterial types in septic tanks and surface waters. Based on our findings, we suggest that the performance evaluation of a septic system should be accompanied by direct analysis of faecal indicator bacteria.

Keywords: biochemical fingerprinting, *E. coli*, enterococci, septic systems, surface water.

INTRODUCTION

Faecal contamination from humans and animals is believed to be a major cause for increased microbiological and nutrient loads in coastal and inland waterways (Abdullah 1995; Parveen *et al.* 1999; Lipp *et al.* 2001). On-site wastewater treatment systems such as septic systems are designed to accept domestic wastewater and prevent biological and nutrient contaminants from entering surface and ground waters. A septic system consists of a tank that provides preliminary treatment of domestic household wastes, comprising sedimentation of solids and floatation of fats and greases, and a soil absorption field where final treatment including biological stabilization and pathogen removal process take place (Geary 1988). Such systems are common in nonsewered urban and rural residential areas (Geary and Gardner 1998). For instance, in the US more than 25% of people rely on septic systems alone (Scandura and Sobsey 1997; Rubin 2002)

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and for Australia this figures around 12% (Geary 1992; Geary and Gardner 1998).

Septic systems may fail (not work properly), and release nutrients and pathogens into the environment (Geary and Gardner 1998). The failure rate is reported to be considerably high (Schueler 1999) and is believed to be >40% in Australia (Jelliffe 1995). However, the rate may vary in different communities. For instance, failure rates of 55 and 82% for two communities in South Australia have been reported (Geary 1988). Effluents from failed septic systems may deteriorate surface and ground water quality (Yates 1985; Scalf *et al.* 1997; Geary and Gardner 1998), and are thought to be a potential source for contamination of lakes, rivers and coastal waters (Griffin *et al.* 2001). Faecal contamination of surface water has been traditionally assessed by enumerating faecal indicator bacteria, such as coliform, that may come from all warm-blooded and some cold-blooded animals, as well as on-site and municipal wastewater treatment systems. However, the value of coliform as an indicator of faecal contamination has recently been questioned, mainly because these bacteria can also originate from various sources such as soil, agricultural runoff, decaying vegetation and industrial processes (Geldreich 1990; Dombek *et al.* 2001). Instead, it has been suggested that enterococci and *Escherichia coli* are much better indicators of faecal contamination, as these bacteria colonize in the gut of humans and other warm-blooded animals (Leclerc 1993). However, it has also to be noted that the sole presence of these bacteria in surface waters does not provide definitive information regarding their possible source(s) (Kühn *et al.* 1997a,b).

Identification of major contaminating source(s) is necessary for the management of surface and ground water quality. Several studies have identified human vs nonhuman sources of faecal contamination by using different typing methods (Calci *et al.* 1998; Parveen *et al.* 1999; Wiggins *et al.* 1999; Carson *et al.* 2001). These methods, although quite discriminatory, are either laborious and/or expensive, or cannot be used for the large number of isolates involved in ecological studies (Kühn *et al.* 1995; Olive and Bean 1999). A biochemical fingerprinting method (PhPlate system; PhPlate AB, Stockholm, Sweden), for typing of different bacterial species, has been reported (Kühn *et al.* 1995, 1997a,b). 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 analysed with the PhPlate software to calculate the level of similarity between tested isolates. The results are presented in the form of a dendrogram. This system has high reproducibility (Kühn *et al.* 1995), and is shown to be quite discriminatory and comparable with many genotypic methods in comparative studies (Kühn *et al.* 1995, 1997a,b). Its use enables researchers to monitor the spread of bacterial phenotypes among different samples. The PhPlate system is a rapid method and is suitable for studies involving large numbers of isolates, and is therefore an excellent tool for studying the persistence of intestinal bacteria over a long period of time (Möllby *et al.* 1993; Kühn *et al.* 1995). This system has been used to type enterococci and *E. coli* strains with a high degree of discrimination and reproducibility (Kühn *et al.* 1997a,b; Iverson *et al.* 2002; Vilanova *et al.* 2002).

The use of septic systems is common in Australia. For instance, in southeast Qld there are around 16 000 septic systems in service in the Maroochy Shire alone, of which 67% have been reported to be defective or failing (Jelliffe 1995). In this study, we used the PhPlate system to provide evidence of septic system failure by typing and comparing faecal indicator bacteria found in septic tanks with those in adjacent creeks.

MATERIALS AND METHODS

Catchment sampling

A catchment-based approach was applied to this study, in which sampling sites were chosen from affected and nonaffected areas within a catchment. For this study, the Eudlo catchment was selected based on the number of septic systems (*ca* 1534), the population size (*ca* 6000) and because most of the area (i.e. >85%) of the catchment is not serviced by a centralized sewage treatment plant.

Initially, one-off samples were collected at the low flow from five sites (sites I–V) of the Eudlo creek mainstream and from six subcatchments (SC-A to SC-F). From the six SC, an upstream site (U) was sampled in addition to the SC outlet (D), as it is connected to the mainstream (Fig. 1a). These SC reflected a variety of land-use activities designed to capture the variability and potential sources of faecal contamination. Two of the SC (SC-A and SC-B) were classified as urban with a high density of septic systems in close proximity to natural waterways. The remaining four SC contained a rural land-use and peri-urban activities with reduced densities of septic systems.

Based on the preliminary data on the number of faecal indicator bacteria obtained from the six SC, two of these SC (SC-A and SC-B), that yielded much higher numbers of faecal indicator bacteria than others, were selected for further study (Fig. 1b). Both SC initially drain pristine areas but then flow through the Eudlo Township. Twenty-five of 39 (64%) septic tanks were located within 60–70 m distance of the creek of SC-B (Fig. 1b).

From these two selected SC, 30 water samples were collected on a 2-week interval basis between July and December 2003. Samples were collected from the upstream SC-A (U) and downstream SC-A (D) of SC-A, and from

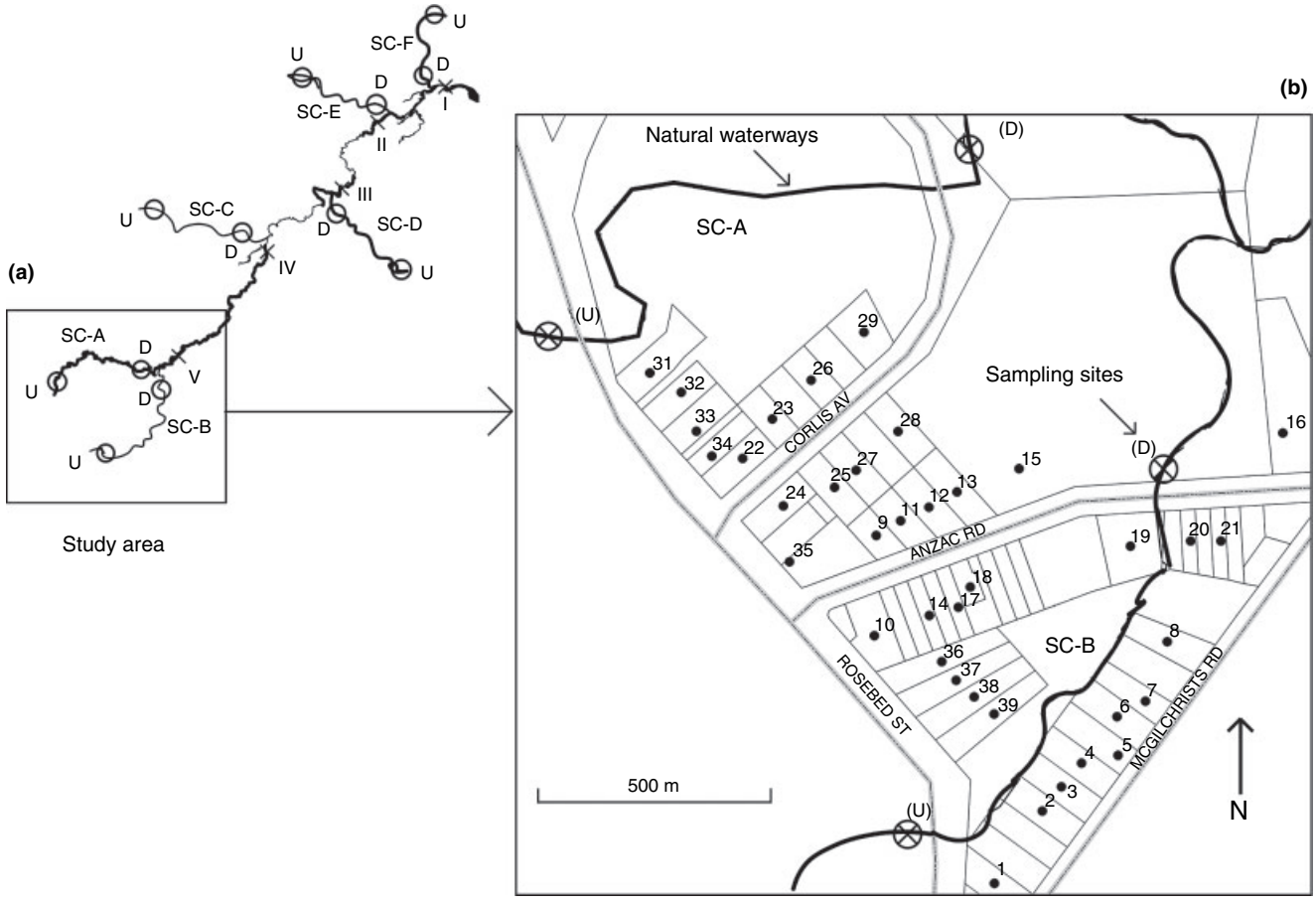

Fig. 1 (a) Sampling sites (I-V) on Eudlo creek mainstream and selected subcatchments (SC-A to SC-F) with an upstream (U) and downstream (D) sampling sites (○). (b) Study area, SC-A and SC-B showing the location of the upstream (U) and downstream (D) sampling sites (⊗), and the location of septic systems (●) in the Eudlo Township

upstream SC-B (U) and downstream SC-B (D) of SC-B (Fig. 1b) on seven to eight occasions and tested for the number of faecal indicator bacteria. An additional site, located 5 km upstream of the study area, was also selected and considered as 'control site' (not shown in Fig. 1). The control site is characterized by low density of septic systems. Furthermore, it receives water mainly from pristine areas, not easily accessible to human and naturally containing low level of faecal indicator bacteria. Altogether, seven samples were collected from the control site for enumeration of faecal indicator bacteria throughout the study. Of these, 21 water samples were further tested (from upstream and downstream of both SC) for biochemical fingerprinting of faecal indicator bacteria (Table 1).

Water samples from all sites were collected in 500 ml sterile bottles from 30 cm below the water surface and transported on ice to the laboratory and tested within 6 h. Different dilutions of water samples were filtered through a 0·45 μM Millipore membrane filter (Millipore, Bedford, MA, USA), and placed on chromogenic *E. coli*/coliform (Oxoid, UK) and m-enterococcus agar plates (Difco, USA); the plates were incubated at 37°C for 24 h (for *E. coli*) and 48 h (for enterococci). After incubation, the number of colonies was enumerated and calculated per 100 ml. All samples were tested in triplicate.

Septic systems sampling

Of the 48 septic systems surveyed in the study area (i.e. Eudlo township), 32 (67%) tanks needed cleaning out during the survey and 23 (72%) of these systems had soggy absorption fields. Four (8%) tanks had structural problems such as broken baffles or lids. Two (4%) systems had technical faults such as the absorption field being located close to a water bore or the tanks were installed below the flood level. Three (6%) tanks had insufficient capacity for the household wastes and only seven (15%) systems were found well-maintained. Eventually, nine septic systems were not included because the properties were vacant during the survey and/or they were located in areas not accessible for sampling.

Table 1 Number of septic and water samples tested for bacterial enumeration and biochemical fingerprinting

Subcatchment (SC)	No. (code) of septic systems sampled in SC	Creek water sampling sites in SC	No. of samples tested for			
			Enumeration		Fingerprinting	
			Ent	*Escherichia coli*	Ent	*E. coli*
SC-A	14 (SEP22–35)*	(U)	7	7	3	3
		(D)	7	7	3	3
SC-B	25 (SEP1–21, 36–39)*	(U)	8	7	3	2
		(D)	8	7	12	4
Control site			7	7		
Total	39		37	35	21	12

Ent, enterococci; U, upstream; D, downstream.
* Based on visual inspection, two septic systems in both subcatchments were found to be well-maintained.

Three samples (where possible) were collected from the remaining 39 septic systems (35 defective and four well-maintained septic systems) at different time intervals between July and December 2003 in conjunction with water samples from the upstream and downstream of adjacent creeks. Samples were collected from the outlet of the septic tanks with a transport sterile swab (Interpath, Melbourne, Australia) and transported to the laboratory on ice, kept at 4°C and cultivated within 24 h. Samples were streaked on chromogenic *E. coli*/coliform (Oxoid) and m-enterococcus (Difco) agar plates and were incubated at 37°C for 24 h (for *E. coli*) and 48 h (for enterococci).

Biochemical fingerprinting with the PhPlate system

A biochemical fingerprinting method (PhPlate system; PhPlate AB) was used to type both faecal indicator bacteria. In this study we used two types of plates, specifically developed for typing of *E. coli* (PhP-RE plates) and enterococci strains (PhP-RF plates). Reagents used in the PhP-RE and RF plates have been described before (Kühn *et al.* 1995; Iverson *et al.* 2002; Vilanova *et al.* 2002). In brief, each micro-plate contains eight parallel sets of 11 dehydrated reagents. Three hundred and fifty micro litres of appropriate growth medium was dispensed into the first well of each row (not containing any dehydrated reagents) and 150 μl of the same growth medium was also dispensed to the rest of the wells. The growth medium for enterococci contains 0·2% (w/v) proteose peptone, 0·05% (w/v) yeast extract, 0·5% (w/v) NaCl and 0·011% (w/v) bromothymol blue, and for *E. coli* 0·1% (w/v) proteose peptone and 0·011% (w/v) bromothymol blue.

From each septic tank, up to 64 enterococci and 32 *E. coli*, and from 21 water samples, up to 40 enterococci and 32 *E. coli* colonies (where possible in all above cases) were picked with sterile toothpick directly from the primary isolating agar plates and suspended into the first well of each row (contained 350 μl of growth medium). Twenty-five micro litres of suspension (aliquots) were transferred into each of the other 11 wells (containing 150 μl growth medium) with the aid of a multi-channel pipette. 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 (Labsystems multiskan, Helsinki, Finland).

After final reading, the mean value for all the three readings was calculated for each isolate (biochemical fingerprint). Similarities between the isolates were calculated as correlation coefficients and clustered according to the unweighted pair group method (UPGMA) with arithmetic averages (Sneath and Sokal 1973). An identity (ID) level (Kühn *et al.* 1997a,b) of 0·975 was established based on the reproducibility of the system after testing 10 isolates in duplicate. Isolates with similarity higher than the ID-level were regarded as identical and assigned to similar biochemical phenotypes (BPTs). BPTs with identical isolates were called common (C-BPT) and those with one isolate were called single (S-BPT) (Möllby *et al.* 1993). Representative isolates of each C-BPT and all single BPTs were tested for esculin hydrolysis on bile esculin agar (Oxoid) to confirm the identification of enterococci. These isolates were saved on tryptic soya broth (Oxoid) with 15% (v/v) glycerol at −80°C.

The phenotypical 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 consisting of different BPTs and is low (minimum 0) if the population consists of few BPTs. The phenotypical similarity between different bacterial populations in two or more samples was calculated as population similarity (Sp) coefficients. The Sp coefficient calculates the proportion of isolates that are identical in two or more compared bacterial populations (Möllby *et al.*

1993). For example, if two populations contain similar dominating BPTs, the Sp-value is high (maximum 1), but if they contain different BPTs, the Sp-value is low (minimum 0). Clustering of Sp coefficients was performed according to the UPGMA method to yield a dendrogram. All data handling, including optical readings, calculations of correlations and coefficients, diversity indexes, Sp-values, as well as clustering and printing dendrogram were performed using the PhPlate software version 4001 (PhPlate AB).

Statistical analysis

Analysis of variance (ANOVA) on ranks (Kruskal–Wallis nonparametric test) was used to compare the significance of difference of bacterial populations among water samples from upstream, downstream and control sites. Mann–Whitney's nonparametric test was used to compare the significance of difference between sites (i.e. upstream vs downstream, upstream vs control and downstream vs control). Mann–Whitney's nonparametric test was also used to compare the significance of difference between the mean number of BPTs and Di found in septic tanks and water samples.

RESULTS

Comparison of bacterial populations in subcatchments

The samples collected from upstream and downstream in SC-A showed that the level of enterococci in these sites did not significantly differ from each other (Fig. 2a). However, there were significant differences ($P = 0{\cdot}001$ for upstream and $P = 0{\cdot}001$ for downstream) between these sites and the control site (Fig. 2a). Similar results were found in SC-B (Fig. 2b). The number of *E. coli* in upstream and downstream sites in SC-A did not differ significantly from each other. However, there were again significant differences ($P = 0{\cdot}001$ for upstream and $P = 0{\cdot}001$ for downstream) between these sites and the control site (Fig. 2c). There was however, a significant difference ($P = 0{\cdot}01$) between the number of *E. coli* in upstream and downstream sites in SC-B. The number of these bacteria in these sites also significantly differed ($P = 0{\cdot}001$ for upstream and $P = 0{\cdot}001$ for downstream) from those of the control (Fig. 2d).

Biochemical phenotypes of indicator bacteria

A total number of 1072 enterococci and 641 *E. coli* isolates were typed from 35 defective and four well-maintained septic tanks. Among enterococci isolates, up to 11 BPTs were found in each septic tank. When BPTs from all septic tanks were compared with each other, a total number of 110 BPTs were found (Table 2). Of these, 31 BPTs were found in more than one septic tank and the remaining 79 BPTs were unique to individual septic tanks. Among the *E. coli* isolates, up to 12 BPTs were found in each septic tank and a total number of 114 BPTs were found in all septic tanks. Of these, 27 BPTs were found in more than one septic tank and the remaining 87 BPTs were unique to individual septic tanks (Table 2).

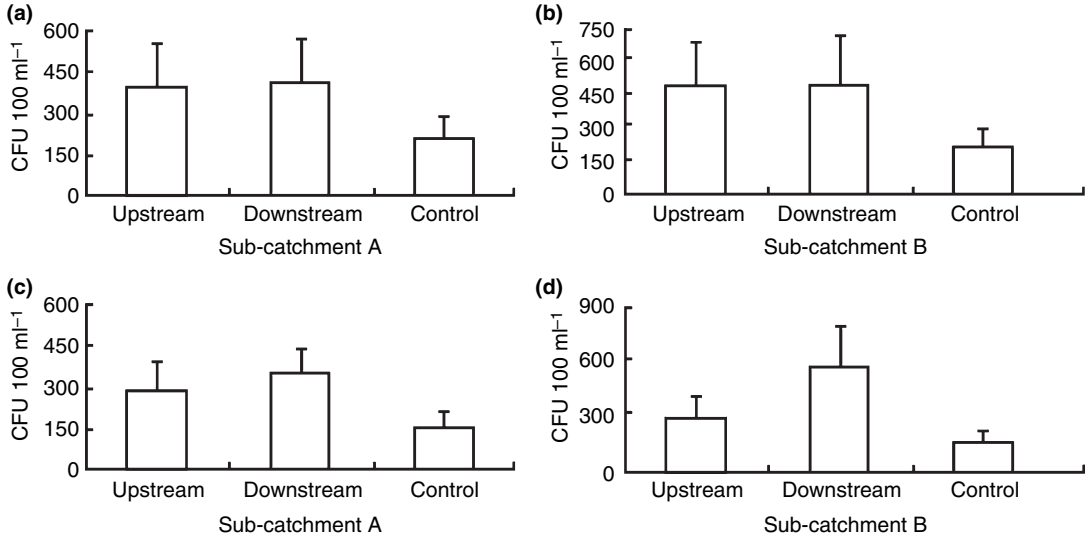

Fig. 2 The mean and standard deviation of enterococci (a and b) and *Escherichia coli* (c and d) isolates at upstream and downstream locations in subcatchments A and B relation to the control site. (a) *P*-value 0·0019 (upstream vs control) and 0·0012 (downstream vs control). (b) *P*-value 0·0044 (upstream vs control) and 0·01 (downstream vs control). (c) *P*-value 0·0092 (upstream vs control) and 0·01 (downstream vs control). (d) *P*-value 0·0023 (upstream vs control), 0·001 (downstream vs control) and 0·0153 (upstream vs downstream)

Table 2 Number of biochemical phenotypes (BPTs) in all septic tanks and water samples

	No. of isolates typed		No. of BPTs found		No. of shared BPTs		No. of Unique BPTs	
Sample	Ent	*Escherichia coli*	Ent	*E. coli*	Ent	*E. coli*	Ent	*E. coli*
Septic tanks	1072	641	110	114	31	27	79	87
Water samples	781	264	108	93	40	21	68	72

Ent, enterococci.

From 21 water samples tested, nine samples did not yield *E. coli* at the dilution rate used. A total of 781 enterococci and 264 *E. coli* isolates were typed. Among enterococci isolates, up to 19 BPTs were found in each water sample and yielding a total number of 108 BPTs in all water samples. Of these, 40 BPTs were found in more than one water sample. Among *E. coli* isolates, up to 18 BPTs were found from each water sample and yielding a total number of 93 BPTs in all water samples. Of these, 21 BPTs were found in more than one water sample (Table 2).

For enterococci, the mean numbers of BPTs (4 ± 2·3) per septic tank were significantly ($P \leq 0{\cdot}0001$) lower than those found in water samples (13·6 ± 2·9). Similar results were found with *E. coli* (Table 3). The mean Di of enterococci and *E. coli* population from septic tanks (0·5 ± 0·3 and 0·5 ± 0·3, respectively), was significantly ($P \leq 0{\cdot}0001$ for both) lower when compared with populations of enterococci and *E. coli* in water samples (0·8 ± 0·1 and 0·9 ± 0·1 respectively) (Table 3).

Comparison of septic BPTs with water samples

Ninety-eight BPTs from 33 septic tanks were found in different water samples (mainly from downstream of both SC) on several occasions (Table 4). Of these, 71 BPTs were found in more than one septic tank (29 defective and four well-maintained). However, 17 unique BPTs were only found in 12 defective septic tanks and were identical to downstream water samples. For *E. coli*, 53 BPTs from 26 septic systems were also found in different water samples (Table 5). Of these, 38 BPTs were found in more than one septic tank (23 defective and three well-maintained) and 17 unique BPTs were only found in 14 defective septic tanks.

Table 3 Phenotypic diversity in septic and water samples

	Mean no. of BPTs/sample		Mean Di	
Sample	*Escherichia coli*	Ent	*E. coli*	Ent
Septic tanks	4 ± 2·3[a]	4 ± 2·4[a]	0·5 ± 0·3[a]	0·5 ± 0·3[a]
Creek water	12·9 ± 2·1[b]	13·6 ± 2·9[b]	0·8 ± 0·1[b]	0·9 ± 0·1[b]

$P \leq 0{\cdot}0001$ for all [a] vs corresponding [b]; Di, diversity index.

Comparison of septic unique BPTs with the databank

Unique BPTs (both enterococci and *E. coli*) from septic tanks were compared with our recently developed databank containing 403 enterococci and 397 *E. coli* BPTs from nine different animal species (i.e. cattle, horse, dog, pig, kangaroo, deer, sheep and chicken). This comparison showed that only one of these unique BPTs was identical to those of the databank.

Population similarities between septic tanks and creek-water samples

Enterococci and *E. coli* populations from each septic tank were also compared with corresponding populations from water samples collected from upstream and downstream of SC-A and SC-B. Different Sp-values were obtained for each comparison of both faecal indicator bacteria. For certain septic tanks, enterococci populations showed high similarities (high Sp-values) with downstream water samples than *E. coli*, while this was quite the reverse for other septic tanks. For instance, while we found high similarities between enterococci populations from septic tank 3 (i.e. SEP3) and those of downstream water samples (Fig. 3a), there was no similarity between *E. coli* populations of this septic tank and the same water samples (Fig. 4a). Similarly, while enterococci populations from septic tank 4 (i.e. SEP4) showed no similarity (Sp-value of 0) to downstream water samples (Fig. 3a), there was a low similarity between *E. coli* populations of this septic tank and water samples (Fig. 4a). However, both enterococci and *E. coli* populations from these two septic tanks showed low Sp-values (if any) to upstream samples (Fig. 3b and 4b).

DISCUSSION

A failing/defective septic system is considered to be the one that allows the discharge of effluent with nutrients and pathogens. The failure of a septic system generally refers to failure of the absorption field. Failure to pump effluents results in excess effluents build up in the tank, which eventually enters into the absorption field. The organic matters present in the effluent cause clogging of the soil in the absorption field (Healy and Laak 1974; Kristiansen 1982), which hinders the movement of effluents. This failure can also be caused by other factors such as the absorption area being too small, unsuitable depth or type of soil, undersized or improperly designed septic tanks, a high water table and physical damages to pipeline and lack of

Table 4 Identical biochemical phenotypes (BPTs) of enterococci in septic tanks and water samples

Septic tank code	No. of occasions tested (no. of isolates tested per occasion)	No. of BPTs found in septic tank	Identical BPTs common to septic tanks and water samples (no. of unique BPTs)	No. of water samples containing identical septic BPTs
SEP1	2 (32, 24)	5	2	2
SEP2*	1 (15)	5	(3)	2
SEP3*	1 (32)	11	9 (2)	7
SEP5	1 (31)	5	2	2
SEP6	1 (40)	2	2	2
SEP7	1 (23)	1	1	1
SEP8*	1 (40)	4	3 (1)	3
SEP9	1 (8)	2	2	2
SEP10*	2 (16, 16)	8	3 (1)	3
SEP11†	2 (24, 8)	5	1	1
SEP12*	1 (24)	6	2 (1)	2
SEP14	3 (32, 24, 24)	9	6	6
SEP15	1 (31)	2	1	1
SEP17†	1 (16)	2	1	1
SEP18	2 (24, 23)	6	5	5
SEP19*	1 (103)	7	3 (2)	3
SEP20	2 (32, 8)	9	2	2
SEP21	1 (31)	9	2	2
SEP22*	2 (31, 16)	11	6 (1)	4
SEP24*	1 (32)	3	(1)	1
SEP25*	1 (24)	7	6 (1)	4
SEP26*	2 (8, 23)	4	4 (1)	3
SEP27	1 (24)	3	3	2
SEP28*	2 (24, 8)	7	3 (2)	2
SEP29†	3 (8, 8, 16)	6	4	3
SEP30	2 (16, 23)	10	4	3
SEP31†	1 (16)	3	3	3
SEP32*	1 (32)	6	4 (1)	3
SEP33	1 (8)	3	2	2
SEP35	1 (24)	6	1	1
SEP36	1 (7)	1	1	1
SEP37	2 (7, 24)	9	3	3
SEP38	1 (32)	4	2	2

*Septic tanks with unique BPTs.
†Well-maintained septic systems.

maintenance (Gordon 1989). While detailed description of failed septic systems is not described in literatures, it appears that clogging of the absorption field, because of failure to pump the tank on time, is the leading cause of septic system failure (Moore 1990). In our study, 32 septic systems needed cleaning out during the survey, of which 23 systems had soggy absorption fields (considered absorption field failure).

In our study, all samples were collected from the outlet of the septic tanks instead of the absorption field. This was carried out to provide a better understanding of the indicator bacteria present in septic tanks rather than absorption field, which may contain indicator bacteria from other sources (i.e. domestic animals). We also investigated the presence and abundances of different types of enterococci and *E. coli* in both the septic tanks and water samples to specifically address the failure of septic systems.

The study area contained 48 septic systems in an area of around 1·2 km^2. Previous studies reported a high failure rate of septic system that occurs in areas generally containing high densities of septic systems (Jelliffe 1995). This was consistent with our finding that 41 septic systems (85%) were classified defective when assessed by standard inspection guidelines adopted by the local government (i.e. Maroochy Shire Council) and, of these, 23 systems showed signs of absorption field failure. However, our results showed a higher rate of septic system failure when we compared enterococci and *E. coli* from septic tanks with

Table 5 Identical biochemical phenotypes (BPTs) of *Escherichia coli* in septic tanks and water samples

Septic tank code	No of occasions tested (no. of isolates tested per occasion)	No of BPTs found in septic tanks	Identical BPTs common to septic tanks and water samples (no. of unique BPTs)	No of water samples containing identical septic BPTs
SEP1*	1 (6)	2	(1)	1
SEP2*	1 (24)	12	5 (1)	2
SEP3	1 (10)	4	1	1
SEP4*	2 (13, 10)	7	5 (1)	2
SEP5*	2 (6, 24)	8	5 (1)	4
SEP7*	1 (3)	2	1 (2)	1
SEP8	1 (23)	4	2	1
SEP9*	1 (15)	4	1 (1)	7
SEP10*	1 (32)	7	2 (1)	3
SEP12*	1 (16)	8	2 (1)	1
SEP13*	1 (24)	2	(2)	1
SEP14*	1 (4)	3	(1)	1
SEP18	1 (16)	5	2	1
SEP19	1 (58)	2	1	1
SEP21	1 (11)	2	1	1
SEP22*	1 (24)	6	(1)	1
SEP23	2 (15, 32)	11	6	5
SEP24	1 (16)	6	1	1
SEP26†	1 (3)	1	1	1
SEP29†	1 (23)	5	1	1
SEP31*,†	1 (7)	1	(1)	1
SEP34*	2 (2, 8)	3	2 (1)	1
SEP35	1 (3)	3	1	1
SEP37	2 (6, 20)	3	1	1
SEP38*	2 (15, 16)	9	(2)	3
SEP39	1 (6)	5	2	2

*Septic tanks with unique BPTs.
†Well-maintained septic systems.

water samples. These results indicate that while an absorption field may not show signs of failure, faecal indicator bacteria can still be released into the nearby creeks. This was evidenced by our finding that identical BPTs of both indicator bacteria, specific to these septic tanks, were found in water samples. Furthermore, the level of both faecal indicator bacteria was significantly higher in downstream than upstream sites, although it was not consistent for both indicator bacteria or in both SC. For instance, the level of *E. coli* was significantly higher in downstream than upstream of SC-B, where 25 septic systems are located within 60–70 m range of the creek. Analysis of water samples showed that the diversity of both faecal indicator bacteria in surface water was significantly higher than septic tanks. This is probably because of the fact that surface water receives bacteria from diffuse sources such as animal farms or industrial processes via surface run-off results in diverse populations. It is also possible that not all indicator bacteria introduced into septic systems through defecation and household wastes survive, leaving only a few types in each septic tank. If the latter is true, then it can be concluded that certain types of indicator bacteria have a better ability to survive in septic tanks than others. Alternatively, it is possible that human faeces in each household contained only few types of enterococci and/or *E. coli*. Nonetheless, the fact that these common types were found in majority of the septic tanks and in high numbers indicates that these specific fingerprints can be used as a sign of human faecal contamination in natural waters.

For each indicator bacteria, identical BPTs were found in both septic tanks and water samples. However, it was more pronounced for enterococci. For instance, 98 BPTs from 33 septic tanks were found in water samples, whereas this figure for *E. coli* was 53 BPTs from 26 septic tanks. This could be partially because of the fact that enterococci strains have a better ability to survive in environment than *E. coli* (Baudisöva 1997). It is also possible that this was merely because of the smaller number of *E. coli* isolates, tested from both septic tanks and water samples, and therefore a lesser chance of finding identical isolates in both samples.

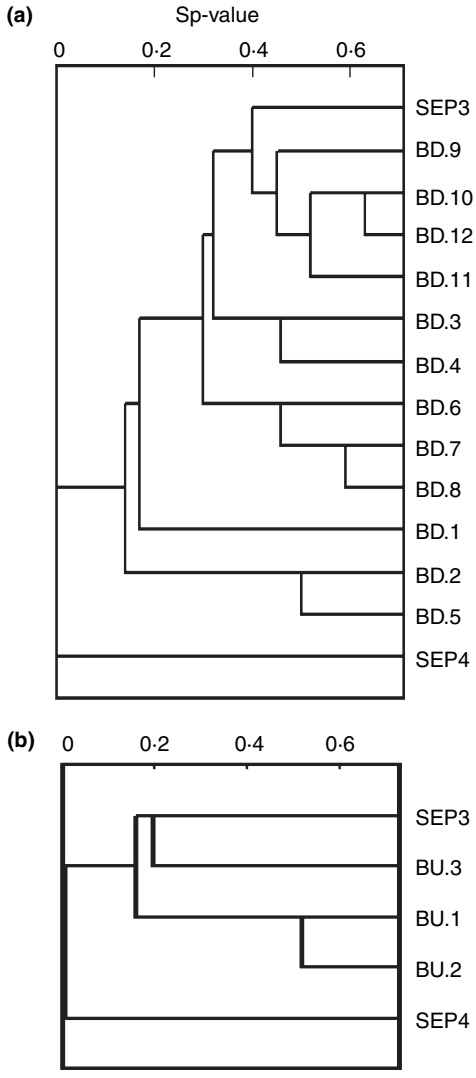

Fig. 3 A representative example of an unweighted pair group method (UPGMA) dendrogram of population similarity coefficients for enterococci isolates between septic tanks (SEP3 and SEP4) and downstream (BD.1 to BD.12) and upstream (BU.1 to BU.3) water samples of the subcatchment B

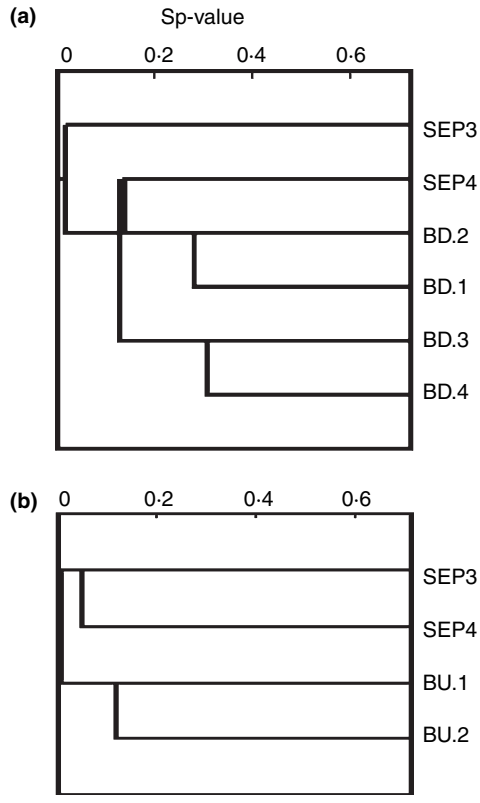

Fig. 4 A representative example of an unweighted pair group method (UPGMA) dendrogram of population similarity coefficients for *Escherichia coli* between septic tanks (SEP3 and SEP4) and downstream (BD.1 to BD.4) and upstream (BU.1 to BU.2) water samples of the subcatchment B

Twenty-six septic tanks contained unique BPTs of either enterococci (12 septic tanks) or *E. coli* (14 septic systems) or both (four septic tanks). These unique BPTs were identical to downstream water samples. Not surprisingly, these septic systems were classified as defective when assessed by standard inspection guidelines. Well-maintained septic tanks also contained unique BPTs, which were not identical to water samples (except one unique BPT from a well-maintained septic tank, which was identical to a BPT in water sample at one occasion), which suggest that well-maintained septic systems do not contribute faecal bacteria to surface waters. On the basis of this finding, we suggest that unique BPTs from septic tanks can be used as specific fingerprints to identify septic systems, which are contributing faecal bacteria to surface waters.

It has been reported that farm and wild animals also contribute to faecal contamination in surface waters (Wiggins *et al.* 1999; Harwood *et al.* 2000). Using the same biochemical fingerprinting method, we recently developed an animal species-specific metabolic fingerprint databank containing 403 enterococci and 397 *E. coli* BPTs from nine animal species (i.e. cattle, horse, duck, dog, pig, kangaroo, deer, sheep and chicken) (W. Ahmed, R. Neller and M. Katouli, in preparation). To rule out the possibility that unique BPTs found in septic tanks and downstream water samples are not driven from farm or wild animals, we compared all unique BPTs from septic tanks with our databank and found that none of these unique BPTs were identical to those of the databank, further supporting our finding. In our study, we also found enterococci and *E. coli* unique BPTs in water samples, which were not identical to BPTs from septic tanks. To identify the source(s) of these BPTs, we compared them with our databank. This

comparison showed that certain unique BPTs from water samples were identical to unique BPTs from certain animal species (data not shown). This comparison not only identified other potential sources of indicator bacteria in the study area, but also explained the reason for high diversity of these bacteria in water samples.

The presence of large number of identical BPTs, including unique BPTs in creeks supports that the presence of identical strains is not just a co-incidental distribution of faecal indicator bacteria. Comparison of populations of enterococci and *E. coli* found in septic tanks and corresponding creeks, also showed a high similarity between individual septic systems and water samples. The Sp-value used in this study compares the proportion of identical BPTs in two or more samples and therefore gives a better understanding of the overall similarity between compared populations. In our study, the mean Sp-value for enterococci populations from septic tanks and water samples was higher than *E. coli* (data not shown). This could again be the result of the fact that the number of *E. coli* isolates tested was smaller than enterococci and therefore less identical BPTs were obtained among these samples. Furthermore, the diversity of *E. coli* in water samples was quite high and therefore the proportion of identical strains in two compared samples was low. We also compared populations of both indicator bacteria from septic tanks in SC-B with water samples from upstream and downstream of SC-A and vice versa, and found a very low Sp-value between these samples (data not shown). These findings further support our results that faecal-indicator bacteria found in the lower reaches of each SC originated mainly from defective septic systems draining into these SC.

It should be noted, however, that in some cases enterococci BPTs from certain septic tanks were found in water samples, while no identical *E. coli* BPTs from the same septic tank was found in water samples or vice versa. For instance, while 33 septic tanks showed identical enterococci strains in water samples, an additional five septic tank showed identical *E. coli* strains in water samples. Similar results were also found when we compared unique BPTs for both faecal-indicator bacteria from septic systems. These findings suggest that combinations of both enterococci and *E. coli* should be used to trace the source(s) of bacterial contamination in such investigations.

Biochemical fingerprinting with the PhPlate system used in this study has been shown to be highly specific in identifying bacterial strains in many epidemiological and ecological studies (Kühn *et al.* 1991, 1997a,b; Iverson *et al.* 2002). We have recently used this system to compare the diversity and occurrence of different BPTs of faecal-indicator bacteria in different animal species and have found that while several animal species carry similar BPTs, each animal species also carries many unique BPTs, which were not found in other animal species (W. Ahmed, R. Neller and M. Katouli, in preparation). Based on these results we conclude that the PhPlate system can be used as a potential tool in identifying the source of faecal contamination in surface waters.

In conclusion, we found identical BPTs of both enterococci and *E. coli* strains in septic tanks and water samples from two adjacent creeks. To our knowledge, this is the first study that provides direct evidence of septic system failure by identifying identical bacterial isolates in water samples. Based on our findings, we suggest that performance evaluation of a septic system in accordance with the established guidelines should be accompanied by direct bacterial analysis of septic tanks and water samples before a final judgment is made on septic systems' performance. We further conclude that the biochemical fingerprinting method used in this study can provide additional information about the percentage and level of faecal indicator bacteria and their persistence in septic systems and water samples.

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