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SOURCING FECAL POLLUTION FROM ONSITE WASTEWATER TREATMENT SYSTEMS IN SURFACE WATERS USING ANTIBIOTIC RESISTANCE ANALYSIS

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ABSTRACT

Aims: To identify the sources of fecal contamination in investigated surface waters and determine the significance of onsite wastewater treatment systems (OWTS) as a major contributor to fecal contamination.

Methods and Results: Antibiotic Resistance Patterns (ARP) were established for a library of 717 known *E. coli* source isolates obtained from human, domesticated animals, livestock and wild sources. Eight commonly used antibiotics, including Amoxicillin, Cephalothin, Erythromycin, Gentamicin, Ofloxacin, Chlortetracycline, Tetracycline and Moxalactam, at four different concentrations were used to obtain ARP's for *E. coli* isolates. Discriminant Analysis (DA) was used to differentiate between the ARP of sources isolates. The developed ARP library was found to be adequate for discriminating human from non-human isolates, and was used to classify 256 enumerated *E. coli* isolates collected from monitored surface water locations.

Conclusions: The resulting ARP DA indicated that a majority of the fecal contamination in more rural areas was non-human, however the percentage of human isolates increased significantly in urbanised areas using OWTS for wastewater treatment.

Significance of Results: This study signifies the feasibility of using antibiotic resistance patterns for source tracking fecal contamination in surface waters, and linking fecal contamination to OWTS. The information will enable regulatory authorities to implement appropriate management practices to reduce the contamination of water resources caused by high densities and failing OWTS.

Keywords: Onsite Systems, *E. coli*, Antibiotic Resistance, Discriminant Analysis

INTRODUCTION

Increased urbanisation and inappropriate site and soil characterisation has led to numerous scenarios of failing onsite wastewater treatment systems (OWTS), resulting in the contamination of ground and surface water by inadequately treated sewage effluent (McNellie et al 1994, Harris 1995, Paul et al 1997, Young and Thackston 1999, Paul et al 2000, Lipp et al 2001, Pang et al 2003). Contamination of ground and surface water resources by effluent discharged from OWTS is of critical concern due to health risks, and the degradation of recreational and drinking water resources due to nutrient inputs (Hagedorn et al 1999, Wiggins et al 1999). In order to effectively manage the inherent risks resulting from the contamination, identification of the different sources of contamination is crucial. The most recent methods for identifying fecal contamination are based on the use of bacterial source tracking (BST) techniques to detect pollution sources.

Fecal bacteria can be emitted from various sources, including agricultural sources, wild and domesticated animals, urban development and effluent treatment facilities such as OWTS (Kelsey et al 2004). Consequently, fecal coliforms are the most commonly used indicators of fecal pollution of water sources. However, the feasibility of adopting fecal coliforms as an indicator of fecal contamination is the subject of debate (Hagedorn et al 1999, Meays et al 2004). Although indicating that fecal contamination is apparent, indicators do not necessarily give an accurate portrayal of the transportation and survival of other pathogenic organisms they are intended to identify. This is compounded by the fact that the fecal indicators may not be from one particular source, but rather from a variety of sources in the localised region. The presence of fecal bacteria in water resources only indicates that fecal contamination has occurred (Meays et al 2004).

Fecal coliform bacteria inhabit the intestinal tract of all warm-blooded animals. Hence fecal coliform counts from a contaminated waterway will not provide information as to the actual source of the contamination. This information is important as fecal pollution resulting from human sources will establish a high public health risk due to the possible presence of pathogenic organisms. Additionally, if the fecal source is known, suitable management actions can be implemented to prevent further contamination and to mitigate the health risks (Harwood et al 2000).

One of the most commonly suspected sources of fecal contamination of water resources are OWTS, particularly septic tank-soil adsorption systems. In the United States, septic tanks have been reported as the second most frequent source of fecal contamination of groundwater (US EPA 1996). However, due to the numerous possible sources of fecal bacteria, it has until recently been difficult to isolate onsite systems as a prominent source of fecal pollution. Several attempts at BST methods have been trialled in recent years with limited success (Hagedorn et al 1999, Meays et al 2004). These include: calculating the ratio of fecal coliform to fecal streptococci (Pourcher et al 1991, Howell et al 1996); determining proportions of thermotolerant coliforms to fecal sterols (coprostanol and 24-ethylcoprostanol) (Leeming et al 1998); and species differentiation of fecal streptococci amongst various animals (Deveries et al 1993). More current BST methods have employed molecular methods such as genetic makeup profiles of specific bacteria isolates, including random amplified polymorphic DNA or rep-PCR DNA extraction methods (Parveen et al 1999, Dombeck et al 2000). Additionally, the physiological characteristics used in biochemical BST techniques, such as Antibiotic Resistance Patterns (ARP) of different sources of fecal bacteria have also been used (Wiggins 1996, Hagedorn et al 1999, Wiggins et al 1999, Harwood et al 2000, Whitlock et al 2002, Booth et al 2003, Wiggins et al 2003). The main advantage of utilising ARP techniques over molecular methods is that ARP profiles can be used on more inclusive taxonomic groups of fecal coliforms and fecal streptococci, with hundreds of fecal isolates able to be analysed within a few days of sample collection at a fraction of the cost of molecular methods (Whitlock et al 2002). However ARP has been criticised with respect to its ability for accurately predicting fecal sources, as the grouping of isolates could be influenced by prior exposure to antibiotics (Dombeck et al 2000). This criticism assumes that antibiotic resistance is solely the result of acquired resistance, following exposure to a particular antibiotic and does not allow for inherent resistance patterns or mutations of analysed isolates. Nevertheless, due to the lower cost and faster turn-around time, ARP will continue to be a widely used method for sourcing fecal contamination.

ARP essentially utilises the resistance of selected fecal bacteria isolates, in this case *Escherichia coli (E. coli),* to several antibiotics at varying concentrations in order to obtain their resistance profiles. The underlying assumption of the ARP technique is that due to the increased use of antibiotics by humans and domesticated animals, isolated *E. coli* bacteria from these host sources will have higher resistance than that of wild animals (Wiggins 1996). The ARP technique requires a library of known *E. coli* isolates, from human and non-human

sources, to be tested for their respective ARP. These are then analysed statistically using multivariate discriminant techniques to separate the respective patterns into source groups. Once the known source library has been developed, *E. coli* from the investigated water samples are tested for their ARP and compared to the known source library and categorised according to the respective grouping of known source isolates with similar ARPs.

The main focus of the study discussed in this paper was to utilise the ARP technique for determining the potential sources of fecal contamination in two mixed landuse catchments, Bonogin Valley and Tallebudgera Creek, in the Gold Coast region, Queensland State, Australia. Both catchments have significant densities of OWTS. Although fecal pollution is evident, no positive confirmation of whether human, and hence onsite wastewater treatment systems, are the major source of fecal contamination has been confirmed. The use of ARP provided a means of identifying the major sources of fecal contamination, and a subsequent assessment of the potential public health risk associated with high densities of onsite systems was undertaken.

MATERIALS AND METHODS

Study Area and Location of Monitoring Sites

The catchments under investigation as part of this study are located in the Gold Coast region, Queensland State, Australia. Gold Coast currently has over 15,000 OWTS with a majority of them being conventional septic tank-soil absorption systems. Large clusters of OWTS exist in various locations, and their cumulative effect has become a major concern for the region's local government. Additionally, Gold Coast is a major tourist destination, and has significant ecosystems such as World Heritage sites, important water resources and Ramsar wetland sites. Monitoring sites for collecting water samples were established in several areas located within two adjacent, small mixed landuse catchments; Bonogin Creek catchment and Tallebudgera Creek catchment. Figure 1 shows the locations of the sampling areas and the corresponding catchments. Routine monitoring of these catchments by the local government indicated that high levels of fecal coliforms were evident within their major waterways.

The Bonogin Creek catchment covers 27.5 km^2 and drains into the Nerang River system, and eventually into Moreton Bay. The catchment consists of rolling undulated terrain with mixed landuse. These include urbanised development, all of which rely on onsite wastewater treatment systems, small agricultural areas mostly for livestock production, and large extents of native eucalyptus bushland in the upper region. Essentially, the main area of concern in relation to human fecal contamination is in the developed part of the catchment, where a large number of OWTS are present. As shown in Figure 1, one monitoring site was installed upstream of the urban development (BOS1), in order to obtain an assessment of the background level of FC and *E. coli* entering the developed region. The remaining monitoring sites were located throughout the urbanised area, with BOS3 and BOS5 downstream of areas where the number of OWTS significantly increases.

Tallebudgera Creek catchment, covering 97.7 km^2, is very similar in setting to Bonogin Creek Catchment, and has similar landuses. The major difference between the catchments is that the Tallebudgera Creek is tidally influenced at monitoring site TA1, which has an influence on the fate and transport of FC and *E. coli* in the downstream end of the creek. TA3 was located upstream of the urbanised areas in order to determine background levels of FC and *E. coli* entering the developed areas, with TA2 located approximately in the middle. A majority of the landuse in both catchments upstream of the developed areas of interest have mixed farmland and native bushland, with an increase in rural properties closer to the urbanised development. As such, a majority of the fecal pollution entering the developed areas would be expected to be caused by wild animals and livestock, with progressively increasing levels of domesticated animal and human contamination through the urban developments.

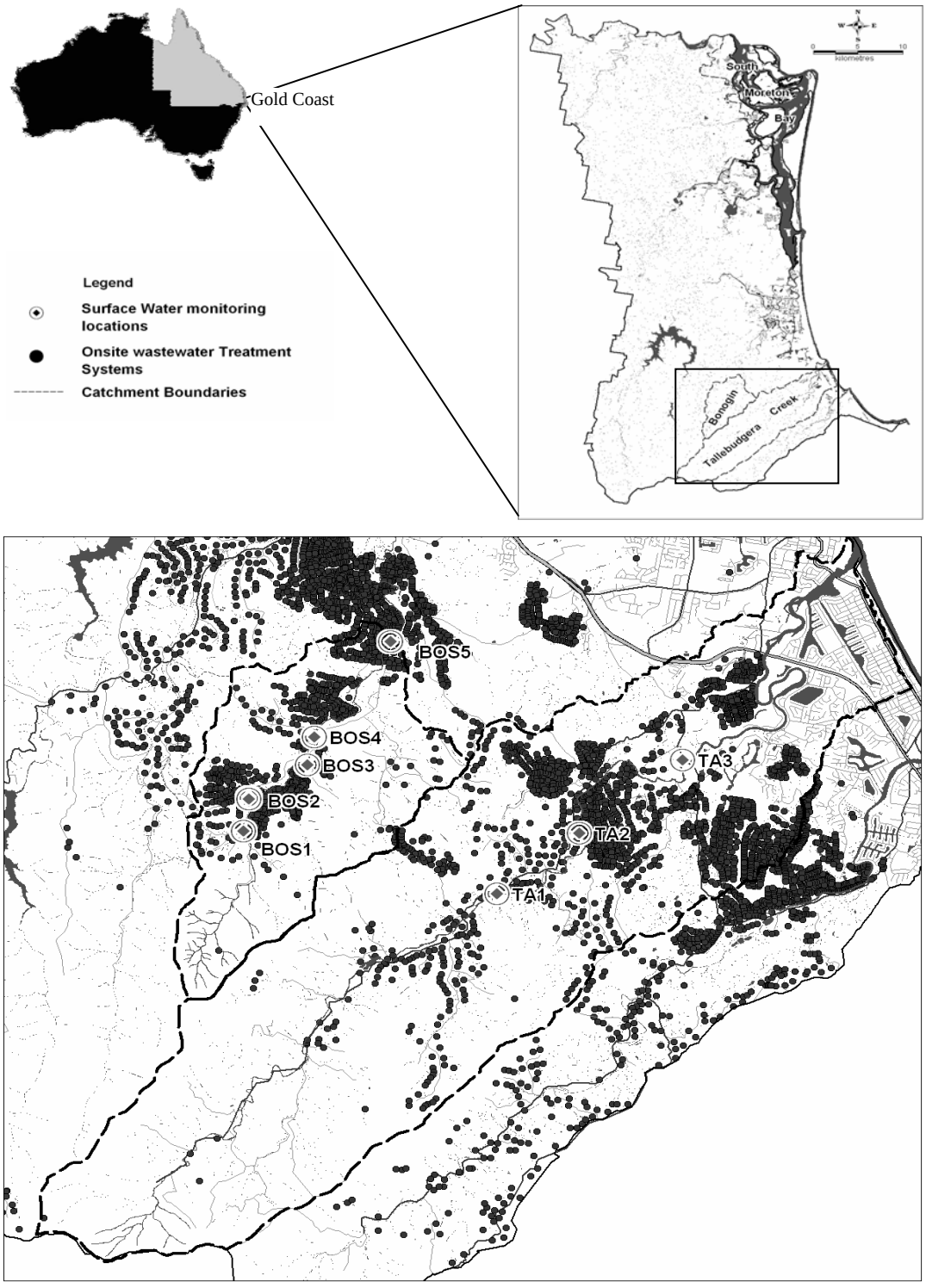

Figure 1: Gold Coast region showing OWTS locations, investigated catchments and monitoring sites.

Sample Collection

A total of 64 surface water samples were collected on a fortnightly basis over a four month period from each of the eight surface water monitoring locations in Bonogin Creek and Tallebudgera Creek catchments. This sampling period was selected to allow the collection of

samples during both the drier winter period following into the spring wet season. Water samples were collected in sterilised glass bottles, stored and transported in crushed ice until analysis could be undertaken. All samples were analysed within 8 hours of collection.

Development of Source Library

To develop the source library of known *E. coli* isolates, fecal samples were collected from human and the primary non-human sources of fecal matter within the catchments. Five fecal samples were collected directly from humans in order to ensure that known human *E. coli* isolates were obtained. Two additional human fecal samples were also collected from public septic tank systems within each catchment, as well as from a local municipal wastewater treatment plant. The main reason for collecting fecal samples directly from humans as well as from wastewater treatment facilities was to compare the accuracy of the predictive capability of samples collected from treatment facilities to that from actual human sources. Even though the majority of *E. coli* isolates collected from these wastewater treatment facilities would be of human origin, there is a possibility of cross-contamination with non-human *E. coli* isolates, such as from birds and rodents. Additionally, obtaining samples from public sewage treatment facilities allows extra diversification between human source isolates in the source library.

Major non-human fecal sources were identified throughout the sampling phase, including livestock, domestic and wild animal sources observed near monitoring locations. Nineteen fecal samples were collected representing the three major sources of domesticated animals in both catchments, including dogs, cats and poultry. Fecal samples from dogs and cats were collected from healthy domestic animals not undergoing antibiotic treatment. Poultry fecal samples were collected from free range poultry farms. Additionally, fourteen livestock fecal samples representing beef and dairy cows, horses and goats were obtained from agricultural farms within both catchments. All livestock animals within these catchments are grass fed, with fecal samples collected from fresh manure piles dispersed throughout the farms grazing pastures. Fifteen fecal samples representing five wild animal sources were collected in each of the catchments to obtain a random representation for the whole of the contributing catchment. Sources included kangaroo, wallaby, koala, possum, and waterfowl. All these sources were observed in the catchments, with fecal samples collected from observed resting or roosting sites.

E. coli Isolate Enumeration

Collected water and fecal samples from known sources for developing the source library were tested using membrane filtration techniques. Isolation of *E. coli* from fecal samples obtained from known sources was achieved by adding 1.0 g of fecal matter or 1.0 ml of effluent sample to 100 ml of sterile buffered dilution water (0.0425 g l^{-1} KH_2PO_4 and 0.4055 g l^{-1} $MgCl_2$ in 100 ml distilled water) and vortexing for one minute (APHA 1999). Serial dilutions of 10^{-2} and 10^{-4} were prepared in buffered dilution water, and 1 ml, 10 ml and 90 ml of the 10^{-4} dilution were filtered for analysis. For collected water samples, volumes ranging from 0.1 ml to 100 ml were filtered to permit isolated colonies on each plate.

Filtration was performed for both fecal and water samples, using 0.45 μm, 47 mm sterile gridded filter membranes (Millipore Corporation, Bedford, MA). Following filtration of each sample, the membranes were aseptically transferred to petri-pads soaked in M-Endo medium (Millipore Corporation, Bedford, MA) and incubated at 30°C for 24 hours. The filter funnel apparatus was treated with 70% Ethanol between uses, and then washed thoroughly with sterile distilled water. Following 18 - 24 hours incubation, plates with isolated colonies were selected for use in isolation of putative *E. coli*. Colonies with a metallic sheen were taken to indicate putative *E. coli.* These colonies were sub-cultured onto Nutrient agar plates, and then further tested for Indole reaction, (Growth in Tryptone water at 37°C for 24 hours followed by addition of Kovac's Indole Reagent) and for growth plus gas production at 44.5°C in Brilliant Green Lactose Bile Broth (BGLBB) (Eijkmann test). In the case of a large number of sheened colonies being present, the number of colonies selected for isolation was taken as equal to the square-root of the number of colonies present. Those isolates with a positive reaction to both tests were recorded as confirmed thermotolerant *E. coli.*

Antibiotic Resistance Pattern Analysis

ARP analysis was used to identify the different sources of fecal contamination in ground and surface water, with the main aim of identifying human from non-human sources. This was to obtain a more accurate picture of the level of *E. coli,* and consequently fecal contamination of water sources from onsite systems. The process used for determining the respective ARP of *E.*

coli followed the procedure outlined by Harwood et al (2000) and Whitlock et al (2002). Antibiotic stock solutions were prepared from available commercial antibiotics (Sigma Chemical Co. St Louis) and applied to sterile trypticase soy agar (TSA) prior to pouring into 150 mm sterile petri dishes. Each petri dish contained one specific concentration of each antibiotic. The antibiotics used and their respective concentrations are as follows; Amoxicillin (5, 10, 15 and 20 μg l^{-1}); Cephalothin (10, 25, 50 and 100 μg l^{-1}); Erythromycin (20, 50, 100 and 200 μg l^{-1}); Gentamicin (20, 40, 60 and 80 μg l^{-1}); Ofloxacin (5, 10, 15, and 20 μg l^{-1}); Chlortetracycline (20, 40, 60 and 80 μg l^{-1}); Tetracycline (20, 40, 60 and 80 μg l^{-1}); and Moxalactam (5, 10, 15 and 20 μg l^{-1}). The choice of antibiotics used in this study took into account the differential response of the various sources of *E. coli* to the eight different antibiotics used (Harwood et al 2000 and Whitlock et al 2002). In some cases this could be attributed to previous antibiotic exposure in human treatment and food sources of both domesticated and livestock animals. However, resistance to antibiotics is a very complex process, and is also reliant on other inherent microbiological properties of the organisms, and not simply acquired as a result of previous exposure to antibiotics. The chance that any two *E. coli* isolate sources would have been exposed to exactly the same antibiotics within their lifetime is minimal. Therefore, the choice of antibiotics utilised in this study were selected due to their common use in human and domesticated animals.

Isolates selected as having sheened colonies on m-Endo, and both Indole and Eijkmann positive, were included for ARP profiling. The isolates were inoculated into nutrient broth and incubated for 18 hours at 37°C. Subsequent broths were diluted to 0.5 MacFarland Standard in fresh nutrient broth. The diluted isolates were placed in multipoint inoculator cups (Denley Multipoint Inoculator A400) for inoculation onto a series of 32 antibiotic plates (8 antibiotics, 4 different concentrations), plus one TSA medium blank. Plates were incubated at 37°C for 24 hours.

After incubation, each plate of isolates was inspected and the relative growth for each antibiotic and concentration was recorded. Four different ratings (1 to 4) were utilised to distinguish respective ARPs. An isolate received a rating of (1) for no growth; (2) for filmous growth; (3) for restricted growth of colonies (growth of a few colonies); and (4) for full growth of colonies. The main reason for using the four ratings was to include more variability into the patterns than would be achieved through the use of two values (for example 1 for no

growth and 2 for full growth). These ARP ratings were utilised for discriminating between the respective source isolates.

Discriminant Analysis of Antibiotic Resistance Patterns

Antibiotic resistance patterns for each of the source and unknown *E. coli* isolates (based on the 1-4 scale for growth) were input into a spreadsheet and analysed using Discriminant Analysis (DA) with StatisiXL ver1.4 software (Roberts and Withers 2004). DA is a multivariate statistical analysis technique where a data set containing *X* variables is separated into a number of pre-defined groups using linear combinations of analysed variables. This allows analysis of their spatial relationships and identification of the respective discriminative variables for each group (Wilson 2002). Objects that retain similar variances in the analysed variables will have similar discriminant scores, and therefore when plotted, will group together. Also relationships between variables can be easily identified by the respective coefficients. Strongly correlated variables will generally have the same magnitude and orientation when plotted, whilst uncorrelated variables are typically orthogonal to each other.

There are two main functions for which DA is commonly employed, and is most beneficial for ARP analysis. Firstly, it can be used to analyse the differences between two or more groups of multivariate data using one or more discriminant functions in order to maximally separate the identified groups. Secondly, DA can be employed to obtain linear mathematical functions which can be used to classify the original data, or new, unclassified data, into the respective groups (Brereton 1990). This classification procedure can be used to calculate the percentages of misclassified isolates and determine the average rate of correct classification (ARCC) of isolates in their respective categories (Wiggins 1996).

To provide a more rigorous predictive ability for the source library, a cross-validation procedure (also referred to as *hold-out analysis* or *jack-knifing*) was undertaken. This procedure randomly removes isolates from the known source library and treats them as an unknown source to test the classification ability of the library (Harwood et al 2000). In order to assess the representativeness of the developed library for accurately classifying isolates, cross-validation of the library isolates was performed. The process utilised in this study followed similar procedures to the pulled-sample cross-validation process described by

Wiggins et al (2003). As multiple isolates from the same sample may have similar resistance profiles, the library may appear to be more representative due to this profile similarity. To overcome this issue, all isolates from the same sample were removed during the pulled-sample cross-validation procedure, and reclassified according to the resistance profiles of the remaining isolates. For the human versus non-human pooled analysis, five random samples from the human category and ten from the non-human category were individually pulled out and reclassified.

As the main aim of the study was to determine the percentage of human versus non-human sources, all non-human sources were initially pooled together into one category. This consisted of pooling the ARP of all wild, livestock and domesticated animal isolates and all human isolates into single individual pooled categories. The pooled category method was expected to provide higher average rates of correct classification for the source library, as has been found in past studies (Wiggins et al 1999, Harwood et al 2000, Booth et al 2003). However, in order to assess the ability of the library to classify between different non-human sources, an additional analysis was performed with pooled categories consisting of human, livestock, domestic and wild animal isolates. Additionally, in order to obtain reasonable discrimination between source isolates at this scale, and to ensure that the developed source library is adequately representative to provide sufficient separation between source groups as well as group isolates, it must contain sufficient isolates to be representative of the organism being classified (Hagedorn et al 1999).

RESULTS

Fecal Coliform and E.coli concentrations

Surface water samples collected from Bonogin and Tallebudgera Creek over a four month monitoring period were analysed for fecal coliform (FC) and *E. coli* isolates. Table 1 provides the monthly averages for FC and *E. coli* isolates for both investigated catchments. Fluctuations in the numbers of FC and *E. coli* are obvious and are primarily related to rainfall (as indicated in Table 1). However, higher numbers of both FC and *E. coli* were obtained from the more undeveloped regions in the upstream segments of Tallebudgera Creek (TA1) catchment, compared to Bonogin Valley Catchment, which retained higher levels in samples

collected from the urbanised regions (BOS3-BOS5). This was the major difference in the results obtained for these similar catchments. Additionally, for both catchments, some counts during low rainfall periods remained high, and it is postulated that this is due to continuous non-point source of contamination such as failing OWTS. Therefore, if this was the case, ARP of isolates collected from the monitoring sites would indicate a higher proportion of human isolates during these high counts, with lower percentages of non-human sources.

Table 1: Rainfall versus counts of FC and *E.coli*

Monitoring Site	June		July		August		September	
Bonogin Valley								
Rainfall[a] mm	52		18		13		1	
	FC (E.coli) cfu/100mL[b]							
BOS1	279	(80)	75	(34)	73	(57)	32	(12)
BOS2	95	(33)	247	(58)	113	(74)	350	(18)
BOS3	351	(36)	238	(50)	203	(96)	15	(8)
BOS4	383	(175)	239	(115)	438	(360)	15	(9)
BOS5	295	(180)	153	(70)	219	(90)	39	(20)
Tallebudgera								
Rainfall[a] mm	64		23		21		3	
	FC (E.coli) cfu/100mL[b]							
TA1	528	(40)	128	(75)	155	(117)	30	(15)
TA2	310	(179)	131	(99)	114	(69)	55	(20)
TA3	140	(128)	86	(30)	89	(54)	30	(25)

[a] Total monthly rainfall
[b] Average Monthly counts

Antibiotic Resistance Patterns

From the 55 fecal samples collected from known sources, a total of 717 *E. coli* isolates were enumerated, and their patterns of antibiotic resistance determined. Analysed ARP for known source isolates indicated distinctive patterns depending on the sources. Table 2 provides the resistances of *E. coli* isolates to the different antibiotics used. From the antibiotic resistances obtained for the library of known sources, no *E. coli* isolates were found to have any significant resistance to Gentamicin and Ofloxacin. However, domestic isolates from one fecal sample (cat) was found to have minor resistance to Gentamicin. Human isolates had a lower resistance to higher concentrations of all antibiotics, although the best separation between human and non-human isolates was Amoxicillin (15 and 20 $\mu g\ L^{-1}$) and

Table 2: ARP of source isolates

		% Resistant isolates from respective sources			
Antibiotic	Conc^{n} (μg/mL)	Human (*n = 134*)	Domestic (*n = 137*)	Livestock (*n = 157*)	Wild (*n = 188*)
Amoxicillin	5	100.00	97.67	96.00	100.00
	10	41.25	95.67	91.67	98.40
	15	11.25	85.33	81.33	75.53
	20	9.19	62.00	54.67	59.57
Cephalothin	10	95.63	94.67	92.33	96.28
	25	23.13	63.00	8.67	45.74
	50	4.06	53.33	1.67	29.26
	100	3.69	42.67	0.00	23.40
Erythromycin	20	100.00	95.67	97.00	99.47
	50	66.88	88.00	95.00	96.81
	100	15.63	66.67	79.67	62.77
	200	5.00	38.33	8.67	23.94
Gentamicin	20	0.00	3.00	0.00	0.00
	40	0.00	3.00	0.00	0.00
	60	0.00	3.00	0.00	0.00
	80	0.00	0.00	0.00	0.00
Ofloxacin	5	0.00	0.00	0.00	0.00
	10	0.00	0.00	0.00	0.00
	15	0.00	0.00	0.00	0.00
	20	0.00	0.00	0.00	0.00
Chlortetracycline	20	46.25	89.67	86.33	88.83
	40	27.50	55.33	13.00	35.11
	60	6.25	42.67	7.00	14.89
	80	2.50	18.00	3.67	12.77
Tetracycline	20	0.00	30.67	7.67	13.83
	40	0.00	15.33	0.00	13.83
	60	0.00	6.33	0.00	0.00
	80	0.00	2.33	0.00	0.00
Moxalactam	5	28.75	29.00	0.00	25.53
	10	11.88	28.00	0.00	25.00
	15	12.50	26.00	0.00	20.21
	20	10.63	23.33	0.00	20.21

Erythromycin (50, 100 and 200 μg L^{-1}), with minor separation for Cephalothin (50 and 100 μg L^{-1}), Chlortetracycline (40, 60 and 80 μg L^{-1}). Contrastingly, livestock sources (beef and dairy cows, horses and goats) had the best separation for Cephalothin (50 and 100 μg L^{-1}) and

Chlortetracycline (40, 60 and 80 μg L^{-1}). Wild isolates did not show any specific relationship between resistances to a certain antibiotic, although a slightly higher resistance was found for Erythromycin. Instead, the wild isolates retained similar patterns to those obtained for other non-human sources, particularly livestock isolates.

Discriminant Analysis (DA) of *E. coli* Antibiotic Resistance Patterns

DA for the pooled human versus non-human isolates performed exceptionally well with an ARCC of 93.8%, as indicated in Table 3. Both human and non-human categories showed clear discrimination between isolates, as shown in Figure 2. The correct classification rates were similar to those derived in other studies which achieved ARCC of >80% for human versus non-human pooled categories (Wiggins et al 1999, Harwood et al 2000, Whitlock et al 2002, Booth et al 2003). Both categories were classified particularly well, with incorrect classification rates of 10% and 2% for human and non-human respectively.

Table 3: Classification rates and ARCC for human vs non-human source isolates

Source	Number and %CC isolates classified as		
	Non-Human	Human	Correctly Classified
Non-Human (*n = 557*)	544	13	97.7%
Human (*n = 160*)	16	144	90.0%
Average Rate Correct Class. (ARCC)			93.8%

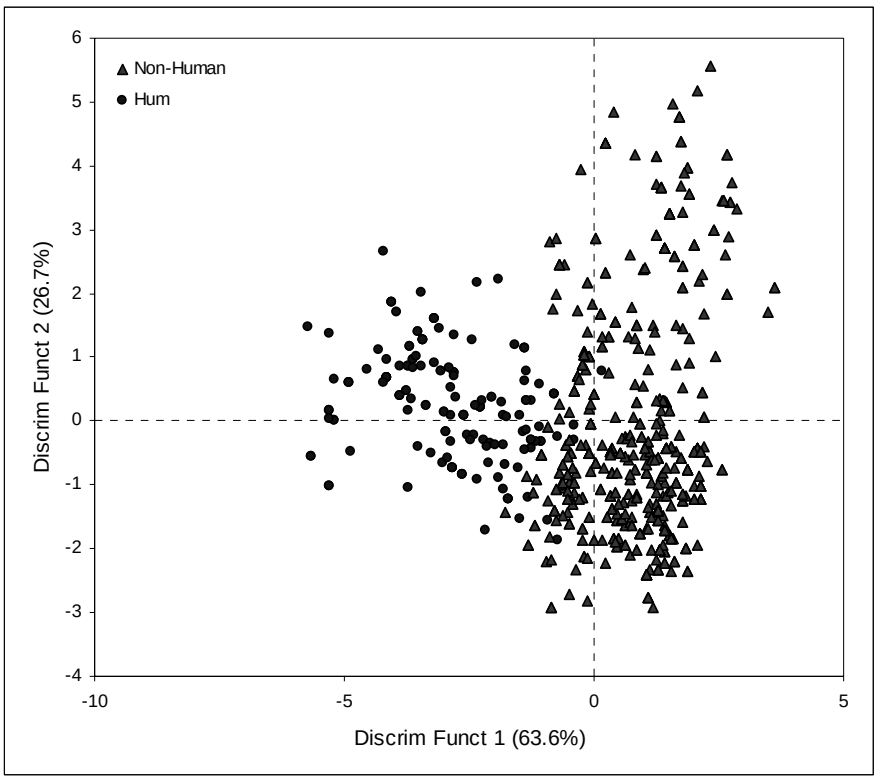

Figure 2: Discriminant analysis plot of source library isolates for pooled human versus non-human categories

To assess whether the source library retained enough isolates to correctly classify the unknown sources, a pulled-sample cross-validation was conducted. The overall ARCC for the libraries used to reclassify randomly pulled human samples was 89.2%. For reclassifying randomly pulled non-human source samples, the ARCC for the sources libraries was 81.3%. These ARCC values were very similar to those obtained for the original source library. Hence, the ARCC's confirmed that the library was sufficiently large enough to provide adequate discrimination between human and non-human sources. Pulled non-human source samples had slightly lower correct classification rates mostly due to the relationship between the wild and livestock categories.

For the pooled categories of human, livestock, domestic and wild isolates, the respective classification rates for categorical discrimination are provided in Table 4. The ARCC for discriminating human, domestic, livestock and wild sources was 83.6%. Compared to the previous human versus non-human analysis, an overall lower ARCC was achieved. This is associated with the lower separation between non-human sources, mostly due to the similar ARP profiles between wild and livestock categories, as depicted in Figure 3. However, the classification rate for human sources was 90.6%, indicating that discrimination between

human, and livestock, domestic and wild sources was still quite high. The main source of misclassification was for domestic isolates, with 15.0% misclassified as livestock and 6.0% misclassified as wild isolates. The remaining categories retained similar rates with 8.8% of human isolates misclassified as wild.

The ARCC's for the pulled-sample cross-validation of the four separated categories indicated lower discrimination potential compared to that for the previous DA using human versus non-human isolates. Lower ARCC's were achieved between livestock, domestic and wild sources (78.7%, 68.8% and 77.4% respectively), although the ARCC for human isolates (87.26%) still provided exceptional discrimination between human and the other three categories. The discrimination of domestic sources was lower, resulting from the misclassification of a majority of these sources as livestock. The average rate of domestic sources misclassified as livestock was 25%.

Table 4: Classification rates and ARCC for human, domestic, livestock and wild source isolates

Source	Number and %CC isolates classified as				
	Domestic	Livestock	Wild	Human	Correctly Classified
Domestic (*n = 179*)	141	27	11	0	78.8%
Livestock (*n = 190*)	5	157	28	0	82.6%
Wild (*n = 188*)	5	25	155	3	82.4%
Human (*n = 160*)	1	0	14	145	90.6%
Average Rate Correct Class. (ARCC)					83.6%

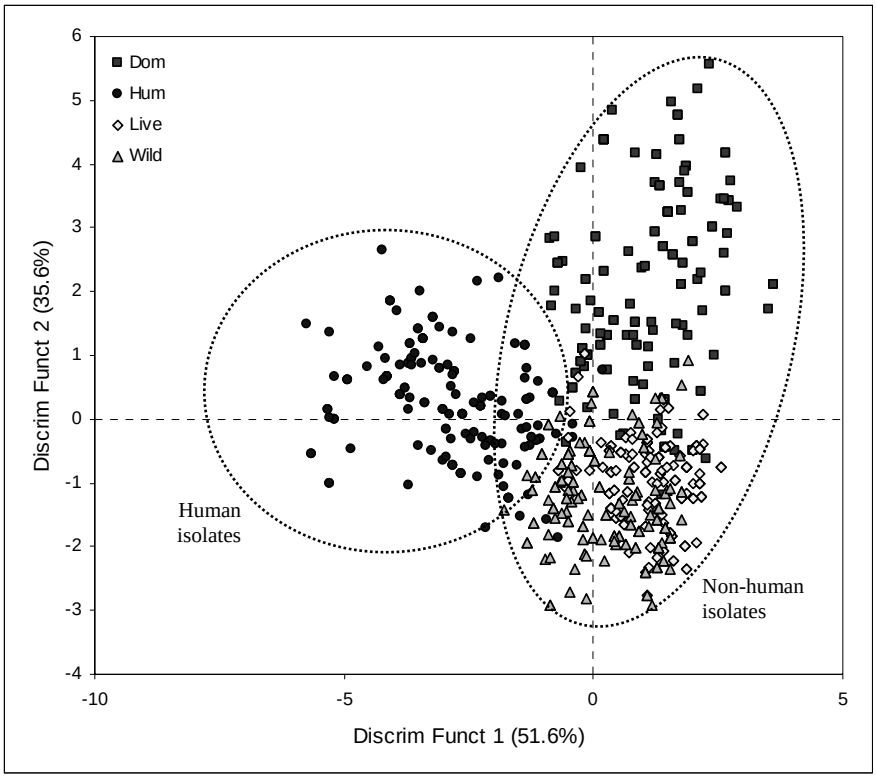

Figure 3: Discriminant analysis plot for pooled human, domestic, livestock and wildlife source categories

Classification of Unknown Source Isolates

From the samples collected from the eight monitored water sample locations, 256 unknown isolates were enumerated from samples collected from the Bonogin Creek catchment, and a further 169 were collected from the Tallebudgera creek catchment over the four months of sampling. Applying DA to the unknown source isolates, utilising the human versus non-human source library, the percentage of human isolates contained in the collected water samples were obtained. Table 5 provides the percentages of human and non-human isolates from the respective catchments. From the DA analysis of samples obtained from Bonogin Creek, 40%, 55%, 10%, 52% and 56% of the isolates from BOS1 to BOS5 respectively were classified as human. For Tallebudgera Creek, 24%, 37% and 47% of isolates obtained from TA1, TA2 and TA3 respectively were identified as human. From the other classified sources (Table 5), it was obvious that in the upper regions of both catchments, the major source of fecal pollution is contributed mostly from non-human or animal sources. As the creeks meander through the rural property and urbanised areas, increases in the percentage of human *E. coli* isolates occurred.

Table 5: Source identification of unknown isolates from monitored sites

Monitoring Site	No. Isolates[a]	Source Identification (%) of unknown source isolates					
		Human[b]	Non-human[b]	Human[c]	Domestic[c]	Livestock[c]	Wild[c]
Bonogin Valley	*(n = 256)*						
BOS1	45	40	60	40	0	0	60
BOS2	48	55	46	33	0	17	50
BOS3	23	10	90	9	18	55	18
BOS4	93	52	48	31	14	7	48
BOS5	46	56	44	56	22	0	22
Tallebudgera Ck	(*n = 169*)						
TA1	51	24	76	24	0	53	24
TA2	74	37	63	37	6	34	23
TA3	43	47	53	47	0	0	53

[a] Unknown isolates from collected from monitored sites over four months sampling period
[b] Pooled source categories for human vs non-human isolate DA
[c] Pooled source categories for human, domestic, livestock and wild isolate DA

Subsequent analysis using the human, domestic, livestock and wild isolate source library showed that the majority of the sources identified in Bonogin Creek catchment in the upper regions originated from wild sources (60% BOS1). The percentage of wild isolates in the water samples decreased as the creek passed through the rural areas, with subsequent increases in domestic and livestock isolates. After passing through the urbanised areas using OWTS, increases in the percentage of human isolates was found. For Tallebudgera Creek catchment, the percentages of non-human isolates from the upper catchment were classified as being wild or livestock. An increase in human isolates was similarly found for Tallebudgera Creek as the creek passed through the more urbanised areas.

DISCUSSIONS

The number of FC and *E. coli* contained in the collected water samples from Bonogin Creek and Tallebudgera Creek catchments indicated varying fluctuations between sampling events, as depicted in Table 1. This is primarily related to the rainfall within each catchment, which typically causes an increase in the number of FC and *E. coli*. Rainfall is generally regarded as having a significant influence on the level of FC in surface water (Ackerman and Weisberg

2003, Muirhead et al. 2004, Noble et al. 2004). However, as shown in Table 1, some counts remained quite high during low rainfall periods. Although rainfall will inevitably cause an overall increase in FC numbers within a catchment, it will also have a dilution effect on continuous sources of contamination. This results in a reduction in FC from point sources to surface water, while other sources of fecal contamination are increased. This would be the case for OWTS adjacent to surface water sources, which will provide a continuous flow of contamination into the water if they are failing. Therefore if this was the case, ARP of isolates collected from the monitoring sites would indicate a higher proportion of human isolates during these lower counts, with lower percentages of non-human sources.

The results of the DA undertaken on the known source *E. coli* isolates indicated that applying ARP for the identification of human vs non-human sources of fecal contamination was successful. Additionally, with the only means of wastewater treatment in the investigated study regions being onsite systems, the high percentages of human *E. coli* isolates found in collected water samples indicates that a majority of these isolates would be from OWTS. To correctly classify the sources of selected isolates, developed libraries must contain enough isolates to ensure they are representative enough to provide adequate discrimination between known source isolates (Wiggins 1996). It is generally recommended that a few hundred isolates for each identified source may be necessary for providing adequate discrimination between source isolates (Hagedorn et al 1999, Wiggins et al 2003). However, in the present study, it was found that a smaller source library was sufficient for obtaining the desired outcomes. The main purpose of this study was to discriminate between human and non-human sources. Due to the distinct discrimination achieved between these two source classifications, the smaller source library was able to provide an ARCC of >90% between human and non-human isolate ARP's. However, to adequately discriminate between the different non-human sources, a larger library would be necessary. Additionally, the source library was developed for discrimination and classification of *E. coli* isolates from relatively small catchments (< 100km^2) in the same geographical location. Consequently, the number of *E. coli* isolates required to provide a representative library for discriminating human versus non-human sources is less than would be necessary for discrimination of sources from large catchments across different geographical and spatial boundaries as observed in other studies (Hagedorn et al 1999, Harwood et al 2000, Wiggins et al 2003).

In assessing the representativeness and ability of the developed source library for correctly classifying human from non-human sources, it was apparent that the library retained adequate source isolates to accurately classify unknown isolates from the catchment studies as either human or non-human, with an ARCC of 93.8%. A pulled sample cross-validation of the developed human versus non-human source library further confirmed that the library was sufficient to provide adequate source classification. An overall ARCC of 89.2% for the pulled sample cross-validation was obtained for randomly pulled human sample isolates.

However, in order to discriminate non-human isolates into appropriate representative sources, the library required more non-human source isolates in order to develop a more representative collection. The ARCC for discriminating between four source groups was lower, achieving 83.6% correct classification of known source isolates. The lower ARCC obtained through this DA was due to similarities between livestock and wild source isolates. As most of the identified wild sources generally co-inhabited the same areas as livestock sources including sharing a common food and water supply, similar ARP profiles would be expected. Collecting more fecal samples for livestock and wild source categories would provide more separation between ARP profiles allowing better discrimination. However, as the main focus for this study was to determine the percentage of human isolates at monitored locations, the DA for the developed library was able to provide an adequate classification.

Classification of the unknown *E. coli* isolates collected from the monitored surface water provided two significant findings. Firstly, during high rainfall events lower human source isolates (classified using the human versus non-human source library) were found in upstream segments of the investigated catchments, with increasing percentages of human sources as the surface water meandered through the urban developments. Secondly, more in-depth investigation of the respective classified isolates indicated that higher percentages of classified human isolates were related to the FC and *E. coli* counts during low rainfall conditions towards the end of the sampling period. This indicated that a continuous source of human fecal contamination is being emitted into the monitored creeks. Therefore, as the only means of wastewater treatment within these catchments are OWTS, a majority of these human isolates can be attributed to poorly performing OWTS. Additionally, higher levels of both FC and *E. coli* were observed downstream of the urbanised areas using OWTS, and subsequent ARP also indicated that the majority of human isolates classified were from downstream of these urban developments. As such, it is apparent that the OWTS have an impact on the water

quality as it passes through the developed regions, with the greatest influence noticeable during drier conditions, when other source contamination would be low.

The increasing use of OWTS in rapidly urbanising areas without centralised sewage treatment facilities can cause detrimental environmental and public health impacts. However, the ability to assess sewage contamination of surface water in areas of high densities of OWTS has been difficult as no feasible means of identifying the various sources of fecal pollution has been available until recently. The use of ARP for identifying the various sources of fecal contamination within surface water catchments has shown promising results, and its use for linking this contamination to OWTS in the investigated area has also been beneficial. From this study, it was found that within the two investigated catchments, the majority of fecal contamination in the upstream segments was a result of non-human, or animal sources. It has tentatively been shown that it is most likely a result of wild animals, although domesticated animals (such as dogs) also contributed substantially in the lower section of the catchment. With regard to human fecal contamination, it was found that the number of human isolates in both catchments increased as the water courses passed through the urbanised regions that utilised OWTS for sewage treatment and disposal. As no other means of sewage disposal is currently available in these catchments, it is predicted that most of the human fecal contamination is the result of poorly performing or failing OWTS. Additionally, the percentage of human *E. coli* isolates versus non-human was found to increase during dry weather, indicating that a continuous source of human fecal isolates was contaminating the surface water. Similarly, this could be attributed to failing OWTS within the catchments.

The use of ARP for identifying the extent of human fecal contamination within the investigated catchments has provided beneficial information regarding fecal contamination issues related to OWTS. The information obtained through this study, has been utilised by the local regulatory authority to implement more appropriate management practices to reduce the contamination of water resources caused by high system densities and failing OWTS.

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