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# **SOURCING FAECAL CONTAMINATION THROUGH ANTIBIOTIC RESISTANCE PATTERN CLASSIFICATION**

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## **Abstract**

Contamination of groundwater and surface water sources with faecal bacteria due to increased urbanisation and high densities of on-site wastewater treatment systems is of vital concern. Increased faecal contamination of water sources can lead to enhance public health risks. Due to the many possible sources of faecal contamination in catchments, and to effectively manage the inherent risks resulting from contamination, identification of the different sources of contamination is crucial. The most recent methods for identifying microbial contamination are based on the use of bacterial source tracking (BST) techniques to detect pollution sources.

Faecal bacteria can be emitted from various sources, including agricultural practices, wild and domesticated animals and effluent treatment facilities such as on-site wastewater treatment systems (OWTS). This is compounded by the fact that the faecal indicators may not be from one particular source, but rather from a variety of sources. Identification of the various sources is important as faecal contamination resulting from human sources entail a high public health risk due to the possible presence of pathogenic organisms. Additionally, if the faecal source is known, suitable management actions can be implemented to prevent further contamination and to mitigate the health risks.

The main focus of the study discussed in the paper was the use of Antibiotic resistance pattern (ARP) technique for determining the potential sources of faecal contamination of ground and surface waters, in the Gold Coast region. The investigated areas have significant densities of on-site wastewater treatment systems. However, although faecal contamination was evident in investigated water sources, whether human, and hence on-site wastewater treatment systems, are the major source of the contamination entailed the use of reliable methodology. The use of ARP provided a reliable means of identifying the major sources of faecal contamination.

## **1.0 Introduction**

Increased urbanisation and inappropriate site and soil characterisation has led to numerous scenarios of failing on-site wastewater treatment systems (OWTS), resulting in the contamination of ground and surface water by inadequately treated sewage effluent (Harris 1995, 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). In order to effectively manage the environmental and public health risks resulting from contamination of water resources, it is necessary to firstly identify the major sources of faecal contamination. The most recent methods for identifying faecal contamination are based on the use of bacterial source tracking (BST) techniques to detect pollution sources.

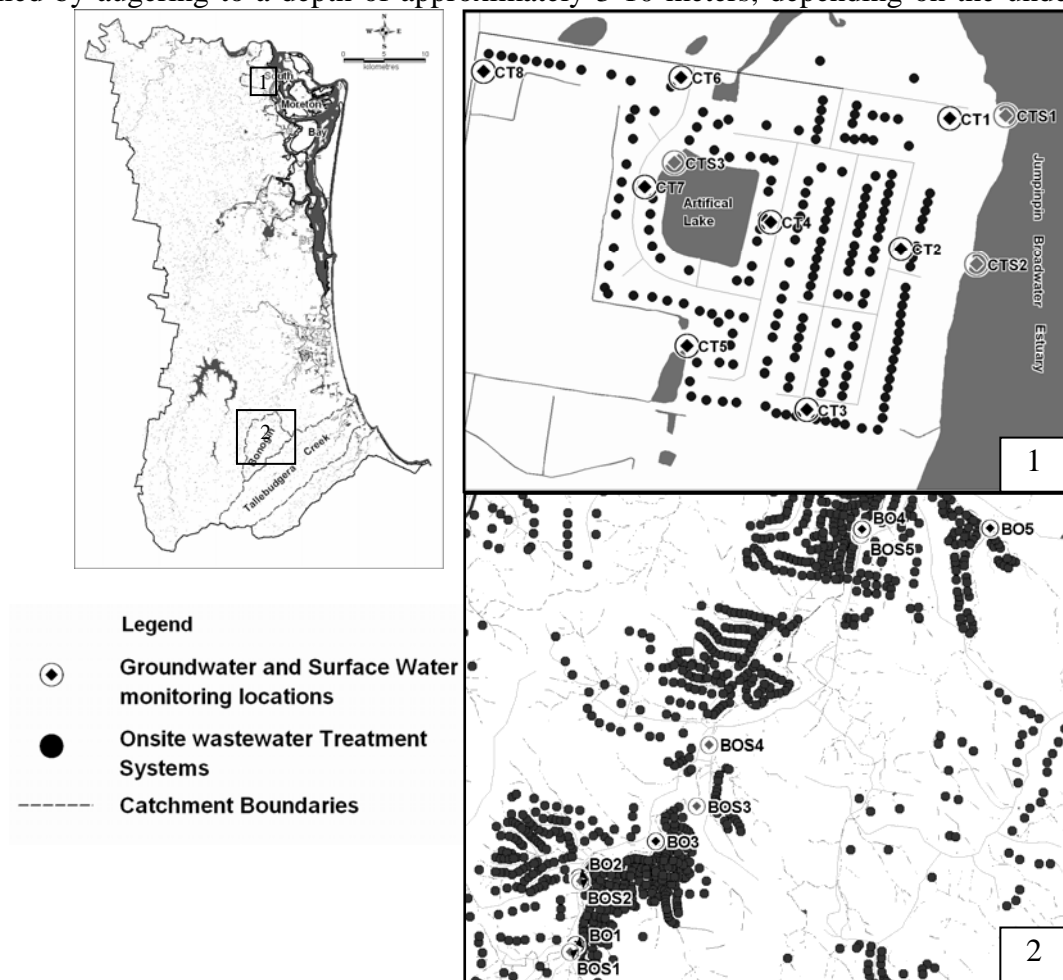
Faecal bacteria can be emitted from various sources, including agricultural sources, wild and domesticated animals and effluent treatment facilities such as OWTS (Wiggins et al 1999, Kelsey et al 2004). However, the feasibility of adopting faecal coliforms as an indicator of faecal contamination is the subject of debate (Meays et al 2004). Although indicating that faecal contamination is apparent, faecal coliform counts do not always provide an accurate indication of the potential source of faecal contamination. One of the most commonly suspected sources of faecal contamination of water resources are OWTS, particularly septic tank-soil adsorption systems (Jelliffe 1995, US EPA 1996). However, due to the numerous possible sources of faecal bacteria, it has until recently been difficult to isolate on-site systems as prominent sources of faecal pollution. Several bacterial source tracking (BST) methods have been trialled in the past with limited success (Meays et al 2004). However, more recent methods have been more successful, including molecular methods such as rep-PCR DNA extraction methods (Dombeck et al 2000) and biochemical methods such as Antibiotic Resistance Patterns (ARP) analysis (Wiggins et al 1999, Hagedorn et al 1999). This study utilised Antibiotic Resistance Patterns (ARP) to identify the proportion of human faecal contamination in ground and surface waters at several monitored locations using OWTS for wastewater treatment. ARP essentially utilises the resistance of selected faecal bacteria isolates and in the case of this study *Escherichia coli* (*E. coli*) isolates, to several antibiotics at varying concentrations in order to determine 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 faecal bacteria from these host sources will have higher resistance than that of wild animals (Wiggins et al 1999). The information obtained through this study has been successfully utilised in assessing the level of public health risk that can be attributed to OWTS in high density areas.

## **2.0 Methods**

### **2.1 Study Areas**

The study areas were located in the Gold Coast region, South-east Queensland. Gold Coast currently has over 15,000 OWTS with a majority of them being conventional septic tank-soil absorption systems. High density areas of OWTS exist in various locations throughout the Gold Coast, and their cumulative impact has become a major concern for the Gold Coast City Council (GCCC). Although the investigated areas do not rely on surface and groundwater as a drinking water source, numerous householders have shallow bores for use for irrigation of gardens. Additionally, these areas are frequented for swimming, fishing and other recreational activities. In order to investigate the impact that high densities of OWTS have on the health of local water sources, bacterial source tracking using ARP analysis was utilised to identify the major contributors of contamination to both groundwater and surface water. Two high density areas were selected to establish monitoring locations; Cabbage tree Point (>1000 systems/km<sup>2</sup>); and Bonogin (>500 systems/km<sup>2</sup>). Figure 1 shows the locations of the monitoring sites. Cabbage Tree Point, located on the Jumpinpin-Broadwater estuary, is situated on Hydrosol soil (Isbell 2002), with an average groundwater depth of 1m below the surface. This is of particular concern as the soil has previously been identified as being unsuitable for attenuating and removing effluent pollutants, and with an average permeability of > 4m/day, the soil effectively allows untreated effluent to pass directly into the shallow groundwater (Carroll et al 2004). At the centre of the community, an artificial lake has been constructed which intercepts a natural drain. Bonogin Valley is located in undulating country in the hinterland. The area selected was a small community surrounding Bonogin Creek. The majority of soils within this region are Kurosol (or highly acidic) soils (Isbell 2002). These soils have been identified as suitable soils for effluent treatment, provided no limiting sublayers prevent percolation of effluent through the subsoil (Carroll et al 2004). Groundwater depth varies throughout the region, with monitoring wells having recorded

depths of 2 to greater than 10m. A previous study identified high levels of faecal contamination in Bonogin Creek (Carroll et al 2005). Groundwater monitoring wells were installed by augering to a depth of approximately 3-10 meters, depending on the underlying



**Figure 1** Study area showing location of surface and groundwater monitoring sites. Legend: Groundwater monitoring sites - CT Cabbage Tree Point; BO Bonogin Valley. Surface water monitoring sites - CTS Cabbage Tree Point; BOS Bonogin Valley.

hydrogeological features of the area. Surface water monitoring locations were located upstream, downstream and throughout the study area, as indicated in Figure 1.

## 2.2 Sample Collection

A total of 168 surface and groundwater samples were collected on a fortnightly basis over a four month period from each of the monitoring locations. Groundwater monitoring wells were purged prior to sampling, to ensure representative samples were obtained. Surface water samples were collected at least 1.5 m from the stream bank to ensure there was minimal disturbance of sediment. All water samples were analysed within 8 hours of collection.

## 2.3 Development of Source Library

To develop the source library of known *E. coli* isolates, faecal samples were collected from human and the primary non-human sources of faecal matter identified within each of the monitored regions. Five faecal samples were collected directly from humans in order to ensure that known human *E. coli* isolates were obtained. Two additional human faecal samples were also collected from public septic tank systems within each catchment, as well as from a local municipal wastewater treatment plant.

Nineteen faecal samples were collected representing the three major sources of domesticated animals in both catchments, including dogs, cats and poultry. Faecal samples from dogs and

cats were collected from healthy domestic animals not undergoing antibiotic treatment. Poultry faecal samples were collected from free range poultry farms. Additionally, fourteen livestock faecal 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 faecal samples collected from fresh manure piles. Fifteen faecal 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 near the sampling locations, with faecal samples collected from observed resting or roosting sites.

## 2.4 *E. coli* Isolate Enumeration

Water and faecal samples were tested using membrane filtration techniques. Isolation of *E. coli* from known sources was achieved by adding 1.0g of faecal matter or 1.0mL of effluent sample to 100mL of sterile buffered dilution water (0.0425gL<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> and 0.4055gL<sup>-1</sup> MgCl<sub>2</sub> in 100ml distilled water) and vortexing for one minute (APHA 1999). 1mL, 10mL and 90mL of a 10<sup>-4</sup> dilution were filtered for analysis. For collected water samples, volumes ranging from 0.1mL to 100mL were filtered to permit isolated colonies on each plate.

Faecal and water samples were filtered using 0.45µm, 47mm sterile gridded filter membranes (Millipore Corporation, Bedford, MA). Following filtration, membranes were aseptically transferred to petri-pads soaked in M-Endo medium (Millipore Corporation, Bedford, MA) and incubated at 30°C for 24 hours. Following incubation, plates with isolated colonies were selected for further analysis of thermotolerant *E. coli*. Colonies with a metallic sheen were taken to indicate putative *E. coli*. These colonies were sub-cultured onto nutrient agar plates, and 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 putative colonies present. Isolates with a positive reaction to both tests were recorded as confirmed thermotolerant *E. coli*.

## 2.5 Antibiotic Resistance Pattern Analysis

ARP analysis was used to identify the different sources of faecal contamination with the main aim of identifying human from non-human sources. This was to obtain a more accurate picture of the level of human *E. coli*, and consequently faecal contamination of water sources from on-site systems. The process used for determining the respective ARP of *E. coli* followed the procedure outlined by 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 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 20µgI<sup>-1</sup>); Cephalothin (10, 25, 50 and 100µgI<sup>-1</sup>); Erythromycin (20, 50, 100 and 200µgI<sup>-1</sup>); Gentamicin (20, 40, 60 and 80µgI<sup>-1</sup>); Ofloxacin (5, 10, 15, and 20µgI<sup>-1</sup>); Chlortetracycline (20, 40, 60 and 80µgI<sup>-1</sup>); Tetracycline (20, 40, 60 and 80µgI<sup>-1</sup>); and Moxalactam (5, 10, 15 and 20µgI<sup>-1</sup>).

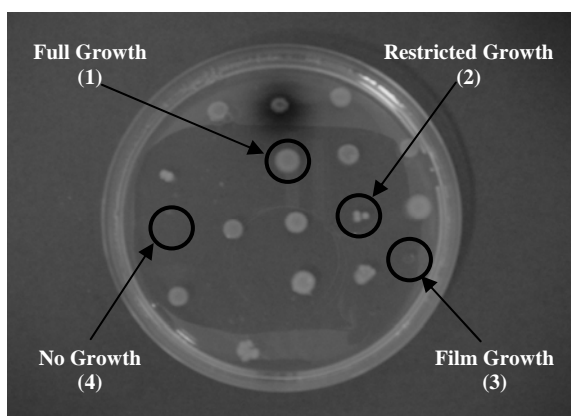


Figure 2 Assigning values representing *E. coli* ARP

For both known and unknown sources isolates, only isolates confirmed as thermotolerant *E. coli* were included for ARP profiling. 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 (Denly 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 of each antibiotic and concentration was recorded. Four different ratings (1 to 4) were utilised to distinguish respective ARPs as illustrated in Figure 2. 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. These ARP ratings were employed for discriminating between the respective source isolates.

## **2.5 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 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 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).

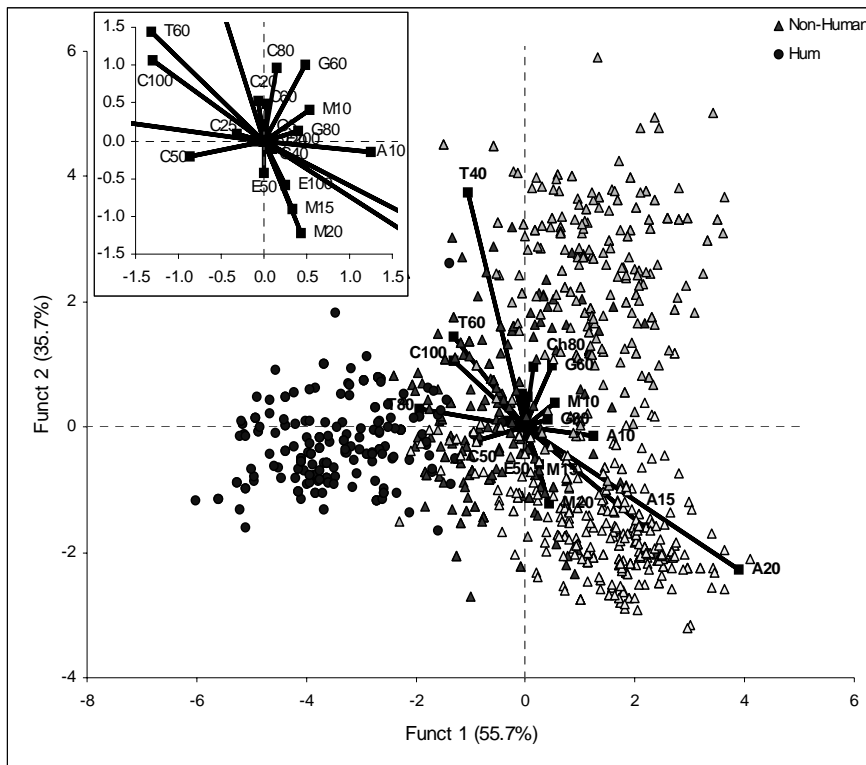
To provide a more rigorous predictive ability for the source library, a cross-validation procedure (also referred to as *hold-out analysis* or *jack-knifing*) was undertaken. This procedure randomly removes isolates from the known source library and treats them as an unknown source to test the classification ability of the library (Harwood et al 2000). The process utilised in this study followed similar procedures to the pulled-sample cross-validation process described by Wiggins et al (1999). 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. As the main aim of the study was to determine the percentage of human versus non-human sources, all non-human source ARP were pooled together into one category, with human sources in a separate category. The pooled category method was expected to provide higher average rates of correct classification for the source library, as has been found in previous studies (Wiggins et al 1999, Harwood et al 2000).

## **3.0 Results and Discussion**

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

From the 55 faecal samples collected from known sources, a total of 926 *E. coli* isolates were enumerated, and their patterns of antibiotic resistance determined. Analysed ARP for known source isolates indicated distinctive patterns depending on the respective sources. 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 20µg/mL) and Erythromycin (50, 100 and 200µg/L), with minor separation for Cephalothin (50 and 100µg/L), Chlortetracycline (40, 60 and 80µg/L) as shown in Figure 3. Some distinct patterns were noticeable between the various non-human sources, as shown in Figure 3. However, as only human versus non-human separation was necessary, these resistance patterns between the non-human sources were not investigated further as part of the study.

Discriminant analysis of the pooled human versus non-human isolates performed exceptionally well with an Average Rate of Correct Classification (ARCC) of 97.2%, as indicated in Table 1. Both categories showed clear discrimination between isolates, as shown



**Figure 3 Discriminant analysis plot of source library isolates for pooled human versus non-human categories. Antibiotics: A-Amoxicillin; C-Cephalothin; Ch-Chlortetracycline; E-Erythromycin; G-Gentamicin; M-Moxalactam; O-Ofloxacin; T-Tetracycline.**

in Figure 3. The rates of correct classification were similar to those derived through other studies which achieved ARCC of >85% for human versus non-human pooled categories (Wiggins et al 1999, Harwood et al 2000, Whitlock et al 2002).

**Table 1: Classification rates and ARCC for human vs non-human source isolates**

Source	Number & %CC isolates classified as		
	Non-Human	Human	Correctly Classified
Non-Human ( <i>n</i> = 766)	744	22	97.2%
Human ( <i>n</i> = 160)	5	155	97.1%
Average Rate Correct Class. (ARCC)			97.2%

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 91.2%. For reclassifying randomly pulled non-human source samples, the ARCC for the source library was 88.3%. These ARCC values remained similar to the classification rate obtained for the original source

library. Therefore, the ARCC's indicated that the library was sufficiently large enough to provide adequate discrimination between human and non-human sources. Pulled non-human

Monitoring Site (No. Isolates <sup>b</sup> )	E. coli (Mean) cfu/100mL	Source Identification (%) of unknown source isolates	
		Human	Non-human
<b>Cabbage Tree Point (n = 186)</b>			
<i>Groundwater</i>			
CT1	12	33.6	66.4
CT2	86	14.5	85.5
CT3	10	49.6	50.4
CT4	19	9.6	90.4
CT5	10	63.2	36.8
CT6	39	54.2	45.8
CT7	864	23.4	76.6
CT8	578	0.0	100.0
<i>Surface Water</i>			
CTS1	64	100.0	0.0
CTS2	169	34.5	65.5
CTS3	330	55.6	44.4
<b>Bonogin Valley (n = 288)</b>			
<i>Groundwater</i>			
BO1	340	50.0	50.0

source samples had slightly lower correct classification rates mostly due to the relationship between the wild and livestock categories.

### 3.4 Classification of Unknown Source Isolates

The use of ARP for determining the potential sources of faecal contamination within the monitored regions was successful in indicating the effect OWTS had on monitored ground and surface waters. From the collected water samples, 474 unknown isolates were enumerated and tested for their ARP. Applying DA to the unknown source isolates using the human versus non-human source library for classification, the percentage of human isolates contained in the water samples were obtained. Table 2 provides the percentages of human and non-human isolates identified from the surface and groundwater monitoring locations. Several monitoring locations were found to have high percentages of human E. coli isolates within

BO3	33	5.6	94.4
BO4	11	33.8	66.2
BO5	940	63.2	36.8
<i>Surface Water</i>			
BOS1	133	40.0	60.0
BOS2	148	54.5	45.5
BOS3	315	10.0	90.0
BOS4	356	51.7	48.3
BOS5	190	55.6	44.4

<sup>a</sup> Mean of *E. coli* counts taken over 4 month period

<sup>b</sup> Unknown isolates from collected from monitored sites analysed with ARP

**Table 2: Source identification of unknown isolates from monitored sites**

collected water samples. From the DA analysis of groundwater samples collected from Cabbage Tree Point, 49.6, 63.2 and 54.2% of the isolates from sites CT3, CT5 and CT6 respectively, were classified as human *E. coli*. Additionally, analysis of surface water samples at Cabbage Tree Point showed that 100.0 and 55.6 % of isolates at CTS1 and CTS3 respectively, were classified as human origin. From these results, it is obvious that any faecal contamination present in both groundwater and surface water at Cabbage

Tree Point is influenced by OWTS.

Samples collected from Bonogin Valley also indicated substantial human faecal contamination. Groundwater monitoring sites at Bonogin Valley had 50 and 63.2% of isolates collected from BO1 and BO5 respectively classified as human *E. coli*. However, of more significance were samples collected from surface water monitoring locations, with 54.5, 51.7 and 55.6% of isolates from sites BOS2, BOS4 and BOS5 respectively classified as human *E. coli*. This is significant as the percentage of human *E. coli* was shown to increase as Bonogin Creek meandered through the community utilising OWTS. This indicates that the on-site systems within this high density area appear to be contaminating the surface water more than the groundwater. This was found to be related to overland flow after a rainfall event (Carroll et al 2005).

The classification of human *E. coli* within the monitored areas was of vital importance for assessing the public health risk as a result of high density of OWTS. Although high levels of *E. coli* may indicate that a public health risk may be present for primary contact (>150cfu/100ml - ANZECC 2002), when assessed in relation to human source isolates, a reduced level of public health risk may be achievable. However, several of the monitoring locations were found to have high levels of human *E. coli*, consequently increasing the public health risk in relation to primary contact.

#### 4.0 Conclusions

High densities of OWTS can cause significant public health risk as a result of faecal contamination caused by poorly performing OWTS. However, although indicating that faecal contamination has occurred, the use of simple faecal coliform counts does not accurately indicate that the contamination is caused by inappropriately treated effluent entering local water resources. Consequently, more appropriate means of identifying the source of contamination needs to be conducted prior to implementing management practices to reduce the contamination. Therefore, in order to investigate the impact of OWTS in Cabbage Tree Point and Bonogin Valley, bacterial source tracking to identify the respective faecal sources was undertaken. The use of ARP for identifying the sources of faecal contamination in surface and groundwater water has shown promising results, and its use for linking this contamination to OWTS in the study areas has also been beneficial. From this study, it was found that the majority of faecal contamination at a number of monitored locations was due to human contamination. The major source of this contamination would be OWTS, being the only form of sewage treatment within these areas. In addition to identifying the various sources of faecal contamination, the information obtained through the analysis of antibiotic resistance patterns

and relative percentages of human source faecal contamination can be employed in establishing more appropriate risk assessments of OWTS. This would allow regulatory authorities to implement robust management practices to reduce the risk to public health.

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