Faecal sterols analysis for the identification of human faecal pollution in a non-sewered catchment

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Abstract
In this study, faecal sterols were used to identify human faecal pollution in a non-sewered catchment in Southeast Queensland, Australia. In all, 36 water samples were collected from six sites on six occasions and the concentration of sterols were determined using gas chromatography and mass spectrometry. The stanols concentration in water samples generally increased with increased catchment runoff. After moderate rainfall, high coprostanols levels found in water samples indicated human faecal pollution via defective septic systems. In contrast, it appears that during dry weather human faecal pollution is not occurring in the study catchment. Sterol profiles also pointed to a cattle farm polluting during modest catchment runoff. The method used in this study was able to identify the sources of faecal pollution to the catchment due to rainfall.

Key words
Faecal pollution, faecal source tracking, faecal sterols, coprostanol, septic systems

Introduction

Septic systems are designed to accept domestic wastewater and prevent microbial and chemical pollutants from entering surface and ground waters. However, these systems may fail and could release nutrients and pathogenic microorganisms into the environment (Ahmed *et al.,* 2005, Geary and Gardner, 1998; Griffin *et al.,* 2001). There are approximately 102,000 septic systems in service in Southeast Queensland, of which 60-80% are thought to be failing (Ahmed *et al.,* 2005; Jelliffe, 1995). While detailed description of failed septic systems is not described in the literature, it appears that clogging of the absorption field is the leading cause of septic system failure (Moore, 1990).

Faecal pollution from point and non-point sources has traditionally been assessed by enumerating faecal indicator bacteria such as *Escherichia coli* and enterococci commonly found in the intestine of warm-blooded animals (Baudiŝová, 1997). However, while the presence of such indicator bacteria in surface waters can be seen as a measure of the quality of the water, it does not provide definitive information with respect to possible sources. In view of this, faecal source tracking (FST) methods have been developed to identify the most

likely source(s) of faecal pollution in surface waters (Field and Samadpour, 2007). These methods could be broadly categorized as microbial methods and chemical methods. In recent years, a number of microbial methods such as biochemical fingerprinting (Ahmed et al., 2005), Antibiotic resistance analysis (Parveen *et al.,*1997), Human- and cattle-specific Bacteroides markers (Bernhard and Field, 2000), human- and bovine-specific viruses (Fong *et al.,* 2005; Hundesa *et al.,* 2006) have been used to identify human and animal faecal pollution in environmental waters.

However, the performance of some microbial methods has not been fully evaluated, or are still under evaluation and to date none of these methods are considered as 'pioneer' or 'gold standard' in terms of identifying the sources of faecal pollution (Field and Samadpour, 2007). On the other hand, chemical methods such as faecal sterols and stanols have also been used extensively for FST (Leeming *et al.* 1996; Suprihatin *et al.,* 2003). Coprostanol is the major sterol in human faeces, generally comprising about 40-60% of the total sterol content (Leeming *et al.,* 1994). Coprostanol is considered a biomarker of human faecal pollution (Leeming, 1997; Murtaugh and Bunch, 1967). However, the use of coprostanol alone as a biomarker can lead to false indication of results as coprostanol is also present in the faeces of some other animals. In addition, small amounts can be generated from cholesterol in anaerobic sediments (Mudge and Gwyn Lintern, 1999). In addition to coprostanol, various other sterols can be found in humans and animals as a result of different diets, variation in digestive tracts and the diversity of gut microflora. The ratio of coprostanol with other faecal sterols has been proposed as an improved chemical method to identify the sources of human and herbivore faecal pollution (Bull *et al.,* 2003; Leeming, 1997). Animals such as dogs and birds generally do not have faecal sterols in their faeces or it could be present in low concentration (Leeming *et al.,* 1996). However, faecal matters from these animals can be distinguished from humans and herbivores by comparing the concentration of *E. coli* and enterococci commonly found in the faeces of these animals with the concentration of the same indicators found in the faeces of human and herbivores based on the faecal sterol concentrations. Such analysis may provide additional information to identify the sources of dog and bird faecal pollution.

In this study, we used faecal sterol analysis to determine whether failing septic systems are contributing faecal pollution into the environment. Surface water samples were collected from a non-sewered catchment (i.e. North Maroochy Catchment, Southeast Queensland, Australia) that contained a high density of septic systems and were tested for the presence of faecal sterols. Sterol ratios were then used to provide further evidence, or to identify the most likely sources of faecal pollution in the study catchment.

Methods

Study catchment and sampling sites. North Maroochy Catchment was chosen for this study because the entire catchment is serviced by septic systems. These systems are not currently being monitored, and they have the potential to fail and transport pollutants into the environment. Three creeks (i.e. Bunya, Fairhill and Davidson) were chosen for water sampling (Figure 1). Samples were collected from three sites (i.e. site BC1, BC2 and BC3) in the Bunya Creek, two sites (i.e. FC1 and FC2) in the Fairhill Creek and one control site (DC1) in the Davidson Creek. Bunya Creek is a small first order stream which receives water mainly from a natural spring located upstream of the creek. Sample site BC1 was located close to the spring and site BC2 was located 600 m downstream and is characterized by nearby residential blocks with septic systems. The BC3 site is a further 800 m downstream and below the residential development and is characterized by cattle grazing. Site FC1 was

located in close proximity to a cluster of older residential blocks in the Fairhill Creek. Site FC2 was located 1.2 km downstream from the older residential blocks. The water flow at this site is primarily associated with storm events. The control site was located in the upstream region of the Davidson Creek, headwaters of the Fairhill Creek. The surrounding area of the control site consisted mostly of rainforest with minimal anthropogenic impacts (< 3 septic systems).

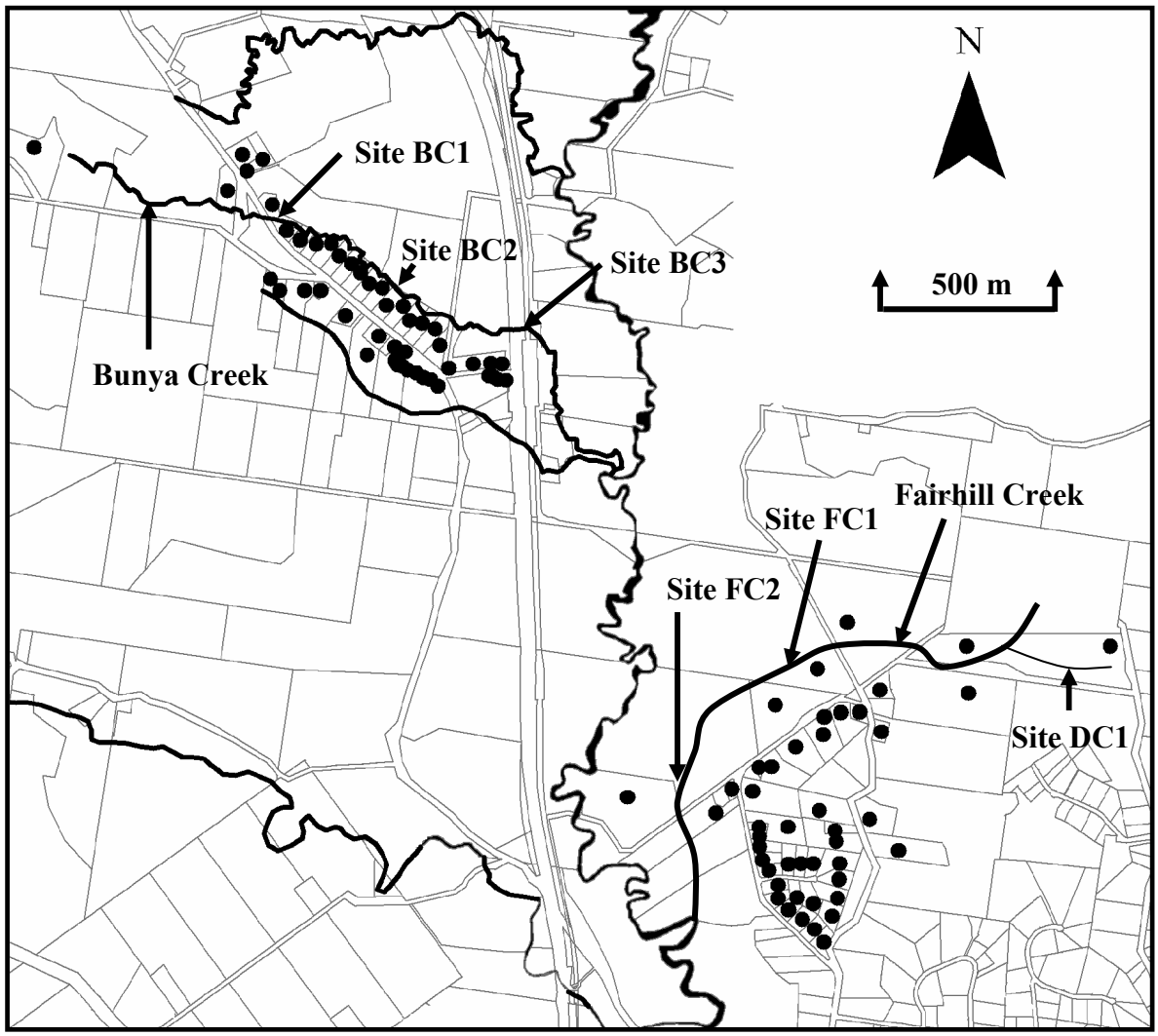

Figure 1. Map showing sampling sites and location of septic systems (●) in North Maroochy Catchment.

Analytical standards. The sterol standards – coprostanol (5β-cholestan-3β-ol), cholestane (5α-cholestane), cholesterol (cholest-5-en-3β-ol), stigmasterol (24-ethylcholesta-5, 22-E-dien-3β-ol), sitosterol (24-ethylcholest-5-en-3β-ol) and sitostanol (24-ethyl-5α-cholestan-3β-ol) were purchased from Sigma (Australia). Epicoprostanol was purchased from Steraloids (USA). The derivatizing agent trimethylsilylimidazole (TMSI) was also purchased from Sigma (Australia).

Sample collection and preparation. Water samples were collected in 10 L pre-cleaned polyethylene containers from the six sites on six occasions using aseptic techniques. The samples were then transported to the laboratory, kept at 4˚C and processed within 24 h. Appropriate volume (i.e. 5-10 L) of each water sample was filtered through 142 mm glass

fibre filters (Advantec, Tokyo, Japan). For lipid extraction of the particulate material, two-phase extraction (utilizing methanol and hexane) was performed using a modification of the one-phase $CHCl_3$-MeOH-H_2O Bligh and Dyer method (1959). The filter discs (containing particulates) were transferred into 250 mL bottles containing methanol (30 mL), hexane (30 mL), sodium carbonate (1 gm) and internal standard (10 µg cholestane in 10 µl dichloromethane) and tumbled for 24 h. The samples were further filtered through 110 mm filter papers (Whatman, Grade number 2) and transferred into liquid separating funnels. The hexane phase was retained and washed with 15 mL Milli-Q water, dried with anhydrous sodium sulphate and then concentrated using nitrogen. The residue was dried and reconstituted in hexane (1 mL) and trimethylsilylimidazole (TMSI) (20 µl) in 1.5 mL vials. The samples were then allowed to stand for 3 h at room temperature before being analysed. Samples from the septic systems near the creeks, were collected directly from the outlet of the septic system. The samples were weighed to determine the septic content. Extraction was performed according to the methanol-hexane method described above.

Analysis of sterol based lipid by gas chromatography and mass spectrometry. Extracts were analysed using a Varian 3900 Gas Chromatograph (GC) (Hansen Way, Palo Alto, USA). The injector temperature was 320°C, and the split was shut for 0.5 min then opened to 50:1. The oven temperature was programmed at 200ºC on injection and increased at 20ºC/min to 240ºC, then increased at 3ºC/min to 320ºC and held for 5 min. The GC was coupled (transfer line 280°C) to a Varian Saturn 2100T Mass Spectrometer (MS) with compound ionization by electron impact energy at 70 eV. The positive fragment ions were analysed over a mass range of 200-550 m/z. Sterols were quantified by reference to standard solutions and expressed as ng/L of water. For interpretation of the faecal sterol profiles found in water samples, and to identify possible human and animal faecal pollution, C27:C29 and 5β:5α ratio comparisons were used (Leeming *et al.,* 1996; Leeming *et al.,* 1998).

Results and discussions

Concentrations of sterols in environmental samples. Low levels of coprostanol and epicoprostanol (ranging from 0-4.0 X 10^0 ng/L) were found in water samples collected on occasion 1 except the sample from site FC1 which had a coprostanol level of 2.3 X 10^1 ng/L (see Table 1). The water sample from this site also had high levels of cholesterol (6.5 X 10^3 ng/L) and sitosterol (1.1 X 10^4 ng/L). Increased levels of coprostanol and epicoprostanol were found in water samples collected on occasion 2 with the highest found in site BC1 (1.2 X 10^3 ng/L and 20 X 10^1 ng/L respectively). The high level of coprostanol suggesting human sourced faecal pollution. The concentrations of other sterols were also high in this site. Prior to this occasion, the catchment experienced moderate rainfall (36 mm) and as a result surface and sub-surface runoff from agricultural areas and failing septic trenches may have increased the levels of sterols in receiving waters. In contrast, the sample from site BC2 had lower levels of coprostanol (2.2 X 10^2 ng/L) and epicoprostanol (3.7 X 10^1 ng/L), although, the levels of cholesterol, sitosterol and sitostanol were high. Site BC3 showed similar patterns except the coprostanol and epicoprostanol concentrations were higher than that of site BC2. The lower levels of sterols in downstream sites (i.e. BC2 and BC3) could be due to the dilution associated with more water flow, which may have masked certain sterols levels. It is also possible that sterols bound particles may have settled in to the sediments in these sites. The sterols are highly hydrophobic molecules which are found to be absent (insoluble) in the clear water column. This is consistent with the research literature (Leeming *et al.,* 1996). However, this could not be confirmed in this study as samples were not collected or tested from the sediments.

The concentrations of stanols were relatively higher in site FC1 compared to site FC2 and the upstream control site DC1. All sites had lower levels of coprostanol (ranging from 1.0×10^0 - 2.3×10^1 ng/L) and epicoprostanol (up to 1.0×10^0 ng/L) with the lowest found in site DC1 on occasion 3. The concentrations of other sterols were also lower on this occasion. This is probably because during this occasion the catchment did not experience any significant rainfall. Higher coprostanol level was again found in all samples on occasion 4 when the catchment had just received 14 mm of rainfall. However, no epicoprostanol was detected in any of these samples. On occasion 5, site BC1 had higher levels of coprostanol (2.0×10^2 ng/L) and epicoprostanol (3.4×10^1 ng/L) compared to sites BC2 and BC3. In contrast, sites FC1, FC2 and the control had lower stanols level. On occasion 6, site BC1 had

Table 1: Sterol profile (ng/L) of water samples collected from six sites on six occasions.

Sampling sites	Sampling events (rainfall)*	Sterols (ng/L)						
		Coprostanol	Epicoprostanol	Cholesterol	5 α Cholestanol	24 Et-coprostanol	Sitosterol	Sitostanol
BC1	Event 1 (6 mm)	6.0×10^{0}	2.0×10^{0}	1.7×10^{3}	1.4×10^{2}	0.0×10^{0}	3.8×10^{2}	1.5×10^{2}
	Event 2 (36 mm)	1.2×10^{3}	2.1×10^{2}	7.6×10^{2}	3.7×10^{2}	4.8×10^{2}	3.8×10^{2}	9.1×10^{1}
	Event 3 (1 mm)	2.3×10^{1}	0.0×10^{0}	9.0×10^{2}	2.3×10^{1}	3.0×10^{0}	9.7×10^{2}	4.5×10^{0}
	Event 4 (14 mm)	2.0×10^{1}	0.0×10^{0}	8.7×10^{2}	6.9×10^{1}	0.0×10^{0}	4.3×10^{2}	1.1×10^{2}
	Event 5 (28 mm)	2.0×10^{2}	3.4×10^{1}	3.8×10^{3}	4.2×10^{2}	7.4×10^{1}	1.3×10^{3}	2.0×10^{2}
	Event 6 (30 mm)	3.0×10^{4}	1.0×10^{3}	3.6×10^{3}	3.6×10^{3}	1.2×10^{3}	9.9×10^{2}	1.1×10^{3}
BC2	Event 1 (6 mm)	4.0×10^{0}	0.0×10^{0}	5.7×10^{2}	4.6×10^{1}	1.2×10^{0}	1.2×10^{2}	4.5×10^{1}
	Event 2 (36 mm)	2.2×10^{2}	3.7×10^{1}	1.2×10^{3}	2.8×10^{2}	2.2×10^{2}	1.5×10^{3}	3.4×10^{2}
	Event 3 (1 mm)	5.0×10^{0}	0.0×10^{0}	2.6×10^{2}	2.9×10^{1}	8.0×10^{0}	2.0×10^{2}	6.9×10^{1}
	Event 4 (14 mm)	5.1×10^{1}	0.0×10^{0}	1.1×10^{3}	1.3×10^{2}	0.0×10^{0}	2.8×10^{2}	1.1×10^{2}
	Event 5 (28 mm)	9.2×10^{1}	1.2×10^{1}	1.2×10^{3}	1.4×10^{2}	8.0×10^{1}	7.7×10^{2}	1.9×10^{2}
	Event 6 (30 mm)	7.0×10^{1}	1.1×10^{1}	1.2×10^{3}	1.3×10^{2}	1.0×10^{2}	3.2×10^{2}	1.3×10^{1}
BC3	Event 1 (6 mm)	4.0×10^{0}	0.0×10^{0}	3.9×10^{2}	3.4×10^{1}	2.3×10^{1}	8.0×10^{1}	2.9×10^{1}
	Event 2 (36 mm)	4.3×10^{2}	9.3×10^{1}	1.5×10^{3}	3.3×10^{1}	6.3×10^{2}	1.5×10^{3}	3.3×10^{2}
	Event 3 (1 mm)	2.4×10^{1}	1.0×10^{0}	5.2×10^{2}	5.3×10^{1}	3.7×10^{1}	5.3×10^{2}	9.2×10^{1}
	Event 4 (14 mm)	6.3×10^{1}	0.0×10^{0}	8.3×10^{2}	8.2×10^{1}	4.5×10^{1}	3.1×10^{2}	5.4×10^{1}
	Event 5 (28 mm)	4.0×10^{1}	0.0×10^{0}	6.1×10^{2}	8.4×10^{1}	2.0×10^{1}	1.7×10^{2}	4.4×10^{1}
	Event 6 (30 mm)	4.0×10^{1}	0.0×10^{0}	5.1×10^{2}	5.1×10^{1}	8.0×10^{1}	9.6×10^{1}	5.8×10^{1}
FC1	Event 1 (6 mm)	2.3×10^{1}	0.0×10^{0}	6.5×10^{3}	3.5×10^{1}	4.3×10^{1}	1.1×10^{4}	4.5×10^{2}
	Event 2 (36 mm)	3.4×10^{2}	1.7×10^{2}	3.7×10^{3}	9.3×10^{2}	6.0×10^{2}	2.2×10^{3}	7.0×10^{2}
	Event 3 (1 mm)	5.0×10^{0}	0.0×10^{0}	6.3×10^{2}	4.1×10^{1}	7.0×10^{0}	5.6×10^{2}	5.3×10^{1}
	Event 4 (14 mm)	4.5×10^{1}	0.0×10^{0}	1.9×10^{3}	3.5×10^{1}	5.6×10^{1}	6.6×10^{2}	1.5×10^{2}
	Event 5 (28 mm)	8.0×10^{0}	0.0×10^{0}	1.1×10^{3}	1.8×10^{1}	9.0×10^{0}	1.7×10^{2}	1.9×10^{1}
	Event 6 (30 mm)	3.4×10^{2}	1.0×10^{1}	3.0×10^{3}	2.0×10^{2}	1.4×10^{2}	1.0×10^{3}	1.3×10^{2}
FC2	Event 1 (6 mm)	0.0×10^{0}	0.0×10^{0}	7.9×10^{2}	4.5×10^{1}	9.0×10^{0}	2.8×10^{2}	2.7×10^{1}
	Event 2 (36 mm)	2.9×10^{1}	0.0×10^{0}	1.3×10^{3}	9.1×10^{1}	7.1×10^{1}	1.0×10^{3}	7.4×10^{1}
	Event 3 (1 mm)	1.6×10^{1}	0.0×10^{0}	4.8×10^{2}	2.6×10^{1}	1.0×10^{1}	8.2×10^{2}	0.0×10^{0}
	Event 4 (14 mm)	2.1×10^{1}	0.0×10^{0}	1.1×10^{3}	1.6×10^{1}	0.0×10^{0}	7.8×10^{2}	1.1×10^{1}
	Event 5 (28 mm)	4.7×10^{1}	1.7×10^{1}	2.6×10^{3}	1.6×10^{2}	1.3×10^{2}	1.5×10^{3}	5.6×10^{2}
	Event 6 (30 mm)	6.5×10^{1}	9.0×10^{0}	8.2×10^{2}	6.5×10^{1}	8.0×10^{1}	5.3×10^{2}	1.0×10^{2}
DC1	Event 1 (6 mm)	1.0×10^{0}	0.0×10^{0}	4.5×10^{1}	3.0×10^{0}	0.0×10^{0}	1.7×10^{1}	3.0×10^{0}
	Event 2 (36 mm)	8.3×10^{1}	2.8×10^{1}	1.6×10^{3}	1.5×10^{2}	6.0×10^{1}	2.7×10^{3}	2.0×10^{2}
	Event 3 (1 mm)	1.0×10^{0}	0.0×10^{0}	1.1×10^{2}	1.0×10^{1}	0.0×10^{0}	5.6×10^{1}	1.0×10^{0}
	Event 4 (14 mm)	1.5×10^{1}	0.0×10^{0}	6.6×10^{2}	6.7×10^{1}	1.2×10^{1}	2.9×10^{2}	1.6×10^{2}
	Event 5 (28 mm)	0.0×10^{0}	0.0×10^{0}	3.6×10^{2}	9.0×10^{0}	0.0×10^{0}	9.0×10^{1}	1.2×10^{1}
	Event 6 (30 mm)	2.2×10^{3}	1.6×10^{2}	5.5×10^{2}	4.6×10^{2}	8.0×10^{2}	2.7×10^{2}	1.3×10^{2}

* Rainfall occurring two days prior to sampling

very high levels of coprostanol (approximately 3.0 X 10^4 ng/L) and epicoprostanol (1.0 X 10^3 ng/L) (this was following 30 mm of rainfall). The presence of high levels of stanols at this site after rainfall events were followed by performing a sanitary inspection. A defective septic system nearby was identified as the most likely source. The trench was located within 5 m distance from the creek and was seeping. For confirmation, samples were collected from the tank and the trench (i.e. soil sample). The sample from the tank had higher levels of coprostanol (8.6 X 10^6 ng/L) while the sample from the trench also had coprostanol but the level was 14 fold lower than that of the tank. This was expected given soil filtering and catchment flushing during rainfall. The concentrations of other sterols were also high at site BC1. However, lower levels of stanols were found in downstream sites (BC2 and BC3). Site FC1 had higher coprostanol level than site FC2. Interestingly, site DC1 also had higher coprostanol (2.2 X 10^3 ng/L) and epicoprostanol (1.6 X 10^2 ng/L). This was surprising as the control site was located in a relatively pristine area with minimal human impacts (< 3 septic tanks). It is possible that seepage from these septic systems could be considered as a contributing factor. This could not be confirmed due to access restriction to these septic systems.

It must be noted that, coprostanol can be found in other animal species such as pigs, cattle, sheep and cats however the concentration is 10 times lower than human faeces (Leeming *et al.,* 1996). Based on our data, it appears likely that the high levels of coprostanols in water samples have originated from failing septic trenches. High levels of coprostanols were also detected in Port Philip Bay, Australia near a sewage treatment plant indicating sewage contamination (O'Leary *et al.,* 1999; Leeming *et al.,* 1998). A recent study also reported the evidence of septic system failure by matching unique bacterial patterns from septic tanks with those found in water samples collected from adjacent creeks (Ahmed *et al.,* 2005).

Sterols ratios for source tracking. The presence of coprostanol alone (lower concentration) may not be sufficient enough to provide the evidence of human faecal pollution as other low level inputs into the aquatic environment are possible. To overcome this, the knowledge of the ratios of particular sterols and stanols in faeces has led to ratio analysis. In recent studies, ratios across a range of C_{27}:C_{29} sterols and 5β:5α stanols have given a more specific measure of pollution (Bull *et al.,* 2003; Leeming *et al.,* 1996; Nash *et al.,* 2005). When C27:C29 and 5β:5α ratios are both greater than 1, the faecal source is likely to be of human origin. Ratios (C27:C29 and 5β:5α) < 1, are indicative of mixed faecal pollution and C27:C29 <1 and 5β:5α > 1 ratios are indicative of herbivore faecal pollution. The ratio analysis was also used as confirmation of the presence of human faecal pollution or others (i.e. herbivores and/or mixed). The ratio of C27:C29 and 5β:5α in samples from site BC1 ranged between 0.93 to 3.41 and 0.02 to 8.91 respectively (see Figure 2). Both ratios were above 1 for two samples (occasions 1 and 6) indicated human sourced faecal pollution at site BC1. The ratios of 0.93 and 0.38 (occasion 3) at this site suggesting mixed faecal pollution. The ratios in samples from site BC2 ranged between 0.79 to 3.43 and 0.18 to 0.70 suggesting that human faecal pollution is not the dominant source. The ratios of 0.79 and 0.70 (occasion 2) at site BC2 suggested mixed faecal pollution. The sterol ratios of 0.92 and 1.61 at site BC3 on occasion 2, strongly suggest herbivores as the major sources of faecal pollution. This is not surprising as site BC3 is characterized by cattle farming. The ratios of 2.57 and 1.10 at this site on occasion 6 are indicative of human faecal pollution. The sterol ratios of C_{27}:C_{29} >1 and 5β:5α <1 at site BC3 on occasions 4 and 5 suggesting humans (C_{27}:C_{29} >1, 5β:5α>1) and cattle (C_{27}:C_{29} <1, 5β:5α>1) are not the dominant sources. Based on our data, it appears that during dry events, septic systems may not contribute faecal pollution into the creeks. The sterol ratios of C_{27}:C_{29} >1 and 5β:5α <1 in sites FC1 on occasions 1 to 5 suggest humans (C_{27}:C_{29}

>1, 5β:5α>1) and cattle (C_{27}:C_{29} <1, 5β:5α>1) are not the dominant sources of faecal pollution. However, the C_{27}:C_{29} and 5β:5α ratios of 2.74 and 1.45 on occasion 6 suggesting the presence of human faecal pollution. The sterol ratios of C_{27}:C_{29} >1 and 5β:5α <1 in site FC2 suggest again that humans and cattle are not the dominant sources of faecal pollution. The ratios at this site suggested mixed faecal pollution. Mixed faecal pollution was also observed in samples from the control site with the exception that a sample (occasion 6) indicated the presence of human faecal pollution.

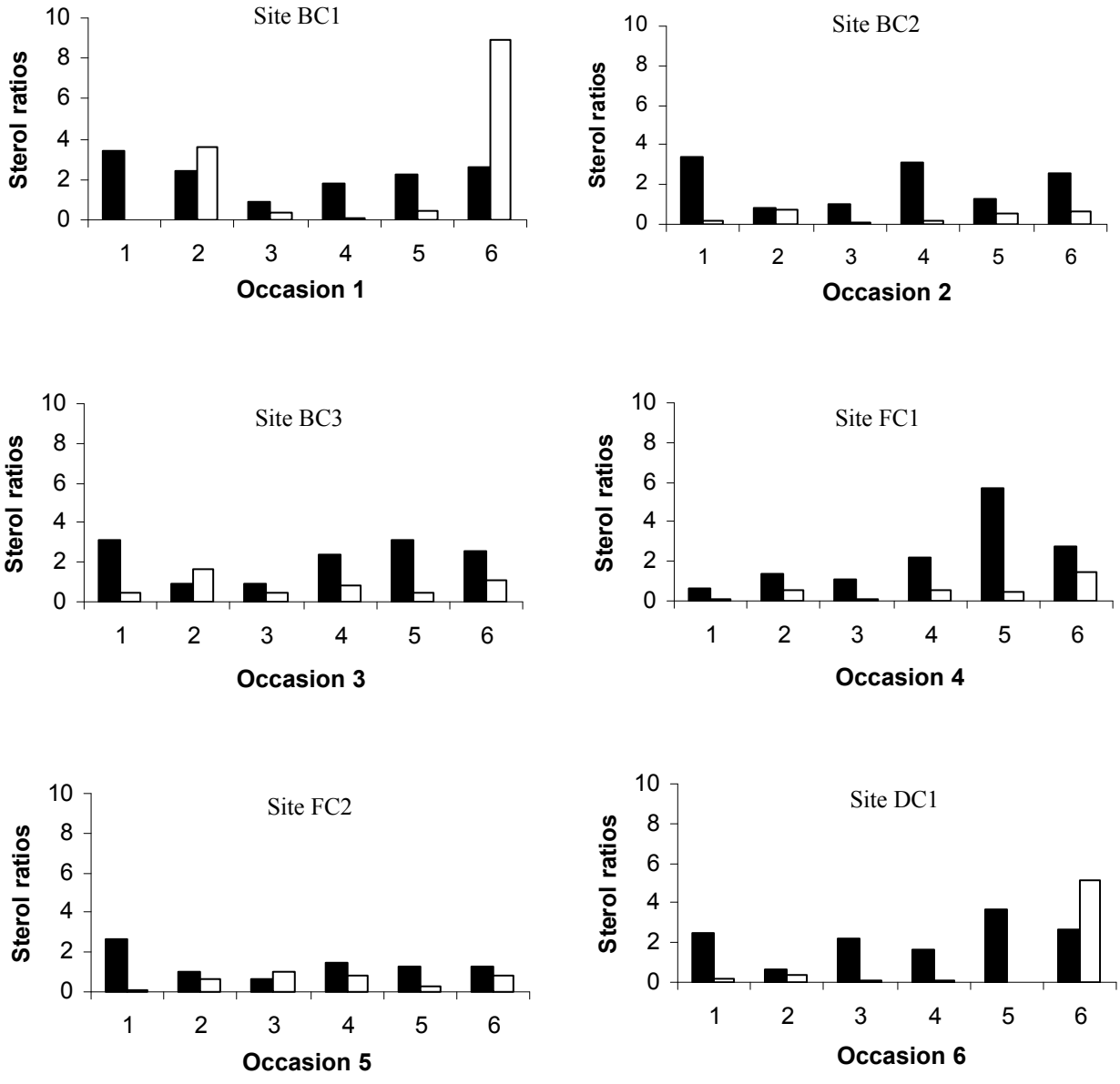

Figure 2: C27:C29 (■) and 5β:5α (□) ratios in water samples collected from six sites in North Maroochy Catchment. (C27:C29 and 5β:5α) >1 indicate human faecal pollution; (C27:C29 and 5β:5α) < 1, indicate mixed faecal pollution; and (C27:C29 <1 and 5β:5α > 1) indicate herbivore faecal pollution.

Conclusions

In this study, faecal sterol analysis was used to identify the presence of human sourced faecal pollution or others (non-point sources) in two adjacent creeks of North Maroochy Catchment. It appears that stanols concentrations generally increased with increased catchment runoff. After moderate rainfall, high coprostanols levels found in water samples indicated human faecal pollution and defective septic systems are the most likely sources of pollution. The human signal was traced on one occasion to a defective septic system. In contrast, it appears that during dry weather human faecal pollution is not occurring in the study catchment. The advantages of faecal sterol analysis are that this method is cheaper and can be performed rapidly compared to faecal source tracking methods that require the development of a database of faecal indicator bacteria such as antibiotic resistance analysis. One major limitation of the method is that the sources could not always be identified due to dilution and mixing of several sources. Another limitation is that the faecal sterols analysis does not provide any information regarding public health risks associated with faecal pollution. Nonetheless, the presence of human faecal pollution in the environment is alarming and such data could be valuable to water quality managers who are charged with protecting water quality and public health.

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