

# DETERMINING THE SOURCES OF FAECAL POLLUTION IN URBAN STORMWATER USING CARBON STABLE ISOTOPE RATIOS ( $\delta^{13}\text{C}$ ) OF SELECTED FAECAL STEROLS

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## ABSTRACT

Faecal pollution is a threat to human health and impacts heavily on human usage of coastal ecosystems. Bacterial indicator organisms are typically used to monitoring faecal pollution, and can identify if human health guidelines are exceeded, but are unable to distinguish between sources. Faecal pollution is attributed to a number of sources including leaking and/or overflowing sewage infrastructure, livestock, domestic pets and native animals. Faecal sterol analysis can be used to distinguish between the sources of human, herbivore and other faeces. For example, coprostanol is found in the faeces of humans and higher mammals and comprises approximately 60% of the total sterol fraction of human faeces. However, faecal sterol analysis has limited use in separating different animal sources. This paper will present new research undertaken in the Browns Creek catchment, Lismore, NSW that uses the  $\delta^{13}\text{C}$  of faecal sterols to identify the sources of faecal pollution. Preliminary results suggest that dog and human faeces are introduced to the river via the stormwater sub catchment, and that flying fox and bird faeces are present upstream. This technique has potential to be able to identify the sources of faecal pollution in waterways.

## INTRODUCTION

Due to the health risks of associated pathogens, human faecal pollution of coastal systems has long been recognised as having negative impact on human usages such as recreation and fisheries. Traditionally bacterial assays such as thermotolerant coliform bacteria (faecal coliform), *Escherichia coli* and faecal streptococci have been used to indicate bacterial pollution of receiving waters. Although microbial detection indicates faecal pollution it can be misleading in determining risks to human health due to the presence of external stresses such as exposure to light, industrial toxins, bacterial pathogens, chlorination and grazing by microzooplankton, which deplete faecal bacteria numbers whilst other harmful organisms may survive (Bate et al 1997). Faecal coliform are ubiquitous to the animal kingdom and as such, routine measurements are unable to determine the source of faecal material (Leeming and Nichols 1996).

Faecal sterol analysis is a technique that has been used to identify human faecal contamination in a variety of coastal environments across the globe through the identification of the faecal sterol coprostanol or herbivore contamination through the identification of 24-ethyl coprostanol with *gas chromatography/mass spectrometry* (GCMS). Faecal sterol analysis is limited however as it is only able to differentiate human and herbivore from other sources of contamination, yet birds, dogs and native wildlife are also contributors to faecal coliform within stormwater catchments (Leeming *et al.*, 1998).

Measuring the isotopic ratios of faecal sterols can potentially, further refine source identification. Past studies have measured isotopic ratios of bacteria in natural systems

using bacterial phospholipid fatty acids (Abraham *et al.*, 1998; Boschker *et al.* 2001; Pelz *et al.*,2001; Boschker and Middleberg, 2002; Fang *et al.*, 2004), nucleic acids (Pelz *et al.* 1998) and bacterial amino acids (Pelz *et al.*,1998; Ong and Mann, 2007). Using *gas chromatography/combustion/isotope ratio mass spectrometry* (GC-C-IRMS) it is possible to isolate source specific sterol compounds, which can be used as bacterial biomarkers. The aim of this paper is to investigate the potential of compound specific isotopic analysis to identify the sources of sterol compounds (biomarkers). It is part of a larger study to use these biomarkers to identify and quantify the sources of contamination in Wilsons River, Lismore via the Browns Creek urban stormwater catchment.

## **METHODS**

### **Collection and preparation of source material and samples**

Sources chosen to investigate in this study were human (source 1), dog (source 2), flying fox (source 3) and bird (source 4) and cow (source 5). Three replicates of each source, except human, were collected from within the Browns Creek catchment, Lismore, NSW from obviously fresh material in sterile plastic containers. The human source was collected from the East Lismore sewage treatment plant in 5L polyethylene containers. Influent was filtered through glass fibre filters (nominal pore size 0.7µm), which were frozen at -20°C until preparation. Slurries were made with the other source material by weighing out approximately 2g and vortexing with 10mL mili-Q for 30 seconds then sonicating for 30 minutes at most intense setting. Water column samples were collected in 5L polyethylene containers and filtered through glass fibre filters (nominal pore size 0.7µm). Samples were frozen until preparation at -20°C.

All glassware used was washed with 1:1 dichloromethane (DCM) and methanol (MeOH) prior to use. Total lipids were extracted quantifiably using a modified one-phase Bligh and Dyer method (1959). After phase separation, the lipid solvent layer was drained into a round-bottomed flask. Solvents were evaporated at 35°C under pressure and the elute was transferred to screw top test tube with 2mL of DCM. Sterols and hydrocarbons were extracted from the elution following alkaline saponification of the total lipid extract. Products were extracted three times into Hexane/DCM (4/1 v/v) then reduced to 1mL under nitrogen gas, transferred to GC vial with 500µL DCM wash. The samples were blown to dryness then converted to their trimethylsilyl esters (O-TMSi) by treatment with 100 µL bis(trimethylsilyl)-trifluoroacetamide (BSTFA, 50µL, 60°C, 60min). The vials were then made up to 500µL with DCM.

Whole animal delta<sup>13</sup>C was derived from animal hair or feathers. Source material delta<sup>13</sup>C was derived from dried and pulverised faecal matter. Dried samples were weighed to appropriate weights between 0.200mg and 0.800mg and sealed in tin capsules. Capsulated samples were analysed by Continuous flow - Combustion - Isotope Ratio Mass Spectrometry (CF-C-IRMS).

### **Instrument analysis**

#### *Compound Identification*

Gas chromatographic analyses will be performed with a Agilent 6890 GC equipped with SGE BPX5 50m \* 0.22mm id cross-linked methyl silicone (0.1 mm film thickness) fused-silica capillary column, head pressure 70 kPa, total flow 35 mL min<sup>-1</sup>, purge flow 1 mL min<sup>-1</sup>, injector temperature 300°C, detector temperature 310°C. The temperature

programme was as follows: initial temperature 250°C, held for 3 min then 10° min<sup>-1</sup> to 340°C held for 20min. 5µL injections were standard. Detection was performed by Agilent 5973 MS with source temperature at 230°C. Peak areas were quantified with Enhanced Chem Station G1701BA Version B.01.00 software on a DOS computer. Peak identification was based on comparison of retention times with those obtained for standards. Blank analyses will be performed and should indicate no contamination above the detection limits of ~1 ng g<sup>-1</sup> and ~1 ng L<sup>-1</sup>.

#### *Whole animal delta<sup>13</sup>C*

The CF-C-IRMS system is comprised of a Thermo FlashEA 1112 (combustion column at 1020°C, reduction column at 650°C, GC column at 45°C, He carrier gas at a flow rate of approx. 100ml/min) interfaced through a Thermo Conflo-III to a Thermo Delta V Plus IRMS. Calibrated reference gas (CO<sub>2</sub>) was introduced via Conflo-III interface at the start of each run. Certified reference materials (NBS-19 and NIST 8457) were analysed at start, middle, and end of run as well as laboratory working standard AT-1 (Acetanilide, -1.58 +/- 0.2 delta<sup>15</sup>N; -26.04 delta<sup>13</sup>C(n=20)) calibrated against above certified reference materials). Percentages were based off of area/weight response to pure acetanilide.

#### *Sterol delta<sup>13</sup>C*

Prepared source material or sample was injected into the GC at six microlitres. The inlet was splitless at 300°C and 50psi and the column (30had a constant flow of 1ml/min. The oven started at 65°C, which was held for 4.2 minutes, the temperature was then increased by 10°C/min til 250°C and held for 30 seconds, then increased by 10°C/min to 340°C and held for 20 minutes. GC effluents were converted to CO<sub>2</sub> by combustion furnace then introduced to the IRMS (Thermo) inlet. Carbon isotopic ratios expressed in δ notation were determined by comparison to PeeDee Belmenite (PDB) with the following equation.

$$\text{delta}^{13}\text{C}_{\text{PDB}} = [(R_{\text{sample}}/R_{\text{standard}}) - 1]$$

### **Interpretation**

#### *Corrections to measured delta<sup>13</sup>C*

Chromatograms created on GCMS data for each animal source were overlaid to determine source biomarkers. Derivatising biomarkers caused the addition of three methyl groups which were corrected for with the following equation:

$$\text{delta}^{13}\text{C}_{\text{O-TMSi}} = (x) \text{delta}^{13}\text{C}_{\text{sterol}} + (1-x) \text{delta}^{13}\text{C}_{\text{BSTFA/MeOH}}$$

where delta<sup>13</sup>C<sub>O-TMSi</sub> is the measured isotopic ratios of the O-TMSi esters, δ<sup>13</sup>C<sub>BSTFA/MeOH</sub> is the measured isotopic ratio of derivatised methanol and x is the fractional carbon contribution of the sterol to the ester. In the case of a C<sub>27</sub> sterol x would equal 27/30. Further corrections were made to account for fractionation from whole animal delta<sup>13</sup>C by subtracting the corrected delta<sup>13</sup>C<sub>sterol</sub> from the delta<sup>13</sup>C<sub>whole animal</sub>.

## **RESULTS AND DISCUSSION**

## Compound Identification

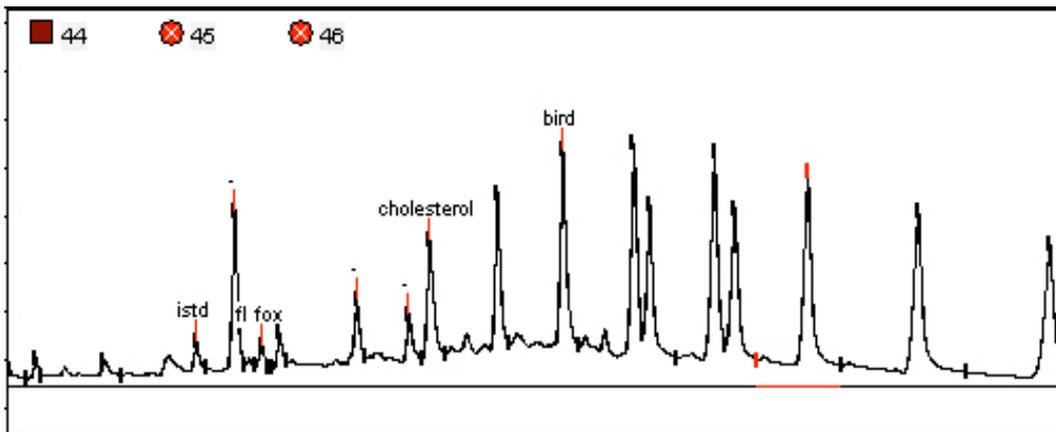
Source specific compounds were isolated and the retention times, associated ions and corrected delta <sup>13</sup>C values are summarised in Table 1.

**Table 1: Retention times, ions, delta <sup>13</sup>C and ratio to cholesterol of biomarkers**

Source	Retention time minutes	Compound Ion	Corrected delta <sup>13</sup> C ‰
sewage	20	370	-30.21
dog	17.79	268	-45.84
flying fox	19	341	-36.28
bird	31.1	400	-31.52
cow	Need to review sample preparation		

## Identification of sources within samples

Selected biomarkers were shown to be present in both the river and in the drain, an example of which is shown in Figure 1.



**Figure 1: Chromatogram from Isodat data for Site 1 (upstream of urban centre), 9/11/06 showing flying fox and bird biomarkers. Istd=internal standard.**

These preliminary findings indicate that dog and human faeces are introduced to the river via the stormwater sub catchment, and that flying fox and bird faeces are present upstream. These preliminary results suggest that this technique has potential to be able to identify the sources of faecal pollution.

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## REFERENCES

Abraham W-R, Hesse C and Pelz O (1998) Ratios of Carbon Isotopes in Microbial Lipids as an Indicator of Substrate Usage. *Applied and Environmental Microbiology*, **64**: 4202-09.

Bate, N. Hewlett, R. Leeming, R. and Reed, J (1997) *Origins of faecal contamination of the Lower Yarra River-An investigation using faecal sterols and bacteria*. Environment Protection Authority, Victoria.

Bligh EG and Dyer WM (1959) A rapid method of total lipid extraction and purification, *Canadian Journal of Biochemistry and Physiology*, **35**: 911-17

Boschker HTS, de Graaf W, Köster M, Meyer-Reil L-A and Cappenberg TE (2001) Bacterial populations and processes involved in acetate and propionate consumption in anoxic brackish sediment. *FEMS Microbiology Ecology*, **35**:97-103.

Boschker HTS and Middelburg JJ (2002) Stable isotopes and biomarkers in microbial ecology. *FEMS Microbiology Ecology*, **40**:85-95.

Fang J, Lovanh N and Alvarez PJJ (2004) The use of isotopic and lipid analysis techniques linking toluene degradation to specific microorganisms: applications and limitations, *Water Research*, **38**: 2529-36.

Leeming R, Bate N, Hewlett R and Nichols PD (1998) Discriminating faecal pollution: a case study of stormwater entering Port Phillip Bay, Australia. *Water Science Technology*, **38**: 15-22.

Leeming R and Nichols PD (1998) Determination of the sources and distribution of sewage and pulp-fibre-derived pollution in the Derwent Estuary, Tasmania, using sterol biomarkers. *Marine and Freshwater Research*, **49**: 7-17.

Ong SE and Mann M (2007) Stable isotope labeling by amino acids in cell culture for quantitative proteomic. *Methods in Molecular Biology*. **359**: 37-52.

Pelz O, Hesse C, Tesar M, Coffin RB and Abraham W-R (1998) Development of Methods to Measure Carbon Isotope Ratios of Bacterial Biomarkers in the Environment. *Isotopes in Environmental and Health Studies*, **34**:131-44.

Pelz O, Chatzinotas A, Zarda-Hess A, Abraham W-R and Zeyer J (2001) Tracing toluene-assimilating sulfate-reducing bacteria using <sup>13</sup>C-incorporation in fatty acids and whole-cell hybridisation. *FEMS Microbiology Ecology*, **38**: 123-131.

Phillips DL and Gregg JW (2003) Source partitioning using stable isotopes: coping with too many sources. *Ecosystems Ecology*, **136**:261-9.