Article

Faecal Sterols and Fluorescent Whiteners as Indicators of the Source of Faecal Contamination

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Human health risks due to infectious diseases in potable or recreational water are traditionally measured by assays for indicator organisms such as faecal coliforms, *Escherichia coli* and enterococci. These groups of organisms are common in most faecal material and their detection, which is relatively easy to establish, is usually a good indicator that water is contaminated by faecal material. However, the ubiquitous nature of these indicators in faecal material from humans, farmed animals, wild animals, and birds can make it difficult to identify the actual source of faecal pollution, particularly when there are multiple potential inputs. Replication of faecal coliforms in aquatic environments has also been reported and this could provide misleading results indicating a faecal source when the biological indicator is persisting in the environment long after the pollution event.[1,2]

Chemicals associated with faecal material have long been attractive candidates for assisting with identification of the source of faecal pollution. Human faecal pollution is often the primary concern and caffeine, bile acids, human hormones, faecal sterols, and components of washing powders have all been utilised to varying degrees of success. Our experiences with two of these chemical indicators – fluorescent whitening agents and faecal sterols is surveyed below.

Fluorescent Whitening Agents

Fluorescent whitening agents (FWAs), also called *optical brighteners*, are fluorescent organic compounds that absorb ultraviolet light and re-emit most of the absorbed energy as blue light. They are used in manufacturing textiles and paper to improve whiteness, and are also added to most washing powders to replace those FWAs lost from clothing during wear and washing where they adsorb to the fabric and brighten clothing. Laundry detergents contain approximately 0.10-0.15% (w/w) FWA between 20-95% of which binds to the fabric during washing with the remainder being discharged with the washing liquor.[3,4] Most household plumbing mixes effluent from toilets with this *grey water* from the washing machine. As a consequence, in both septic tanks and community wastewater systems, FWAs are usually associated with human faecal contamination.

There exits a range of FWAs, but only one, 4,4'-bis(2-
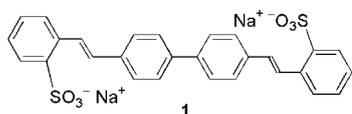
sulfostyryl)biphenyl (**1**) is used in NZ.[5] The key features of FWAs include:

- They are not known to occur as in nature.
- They are highly polar adsorbing strongly to the polysaccharides of paper and clothing.
- Irradiation by sunlight causes them to bind irreversibly to cellulose of protein, enabling binding to cotton and nylon fabrics.
- They are highly water soluble.
- They undergo photochemical degradation; $\tau\frac{1}{2}$ is several hours under summer noon sun.
- They adsorb to soil but only photo-degrade in topsoil; they are assumed *persistent* below the photic zone.
- They are not readily biodegradable.
- They accumulate on sewage sludge with removal rates of 55-98% in sewage treatment plants.
- There are no known health effects of FWAs at levels seen in effluent or water.

Analytical Methodology

FWA **1** was determined in water samples using HPLC with fluorescence detection after extraction from water (100 mL - and centrifuging to remove particulates when necessary) using a C-18 Sep-pak (an extract-clean column) (300 mg) pre-wet with methanol followed by deionised water. FWA **1** was eluted by the mobile phase (5 mL) and then injected (100 μl) on to the HPLC column.[6] Samples obtained from a local fresh water stream were spiked with the equivalent of 0.5 μg/L of **1** and sub-samples stored frozen at -20°C, refrigerated at 4°C in the dark, and at room temperature in ambient light, prior to analysis; none of **1** could be detected following storage at room temperature in ambient light for 48 hours. Samples stored at 4°C in the dark or frozen were found to be stable for at least a month.

Analysis of FWAs in septic tank and community wastewater consistently identified levels between 10 and 70 μg/L; with a detection limit of 0.01 μg/L, this allows for dilution by a factor of perhaps 1,000.

In surveys of streams and stormwater drains, we have detected low levels (<0.01 to 0.06 μg/L) of FWAs in many samples without any clear supporting evidence of human pollution. This probably reflects the low levels of human effluent which may or may not have health implications. Low levels of FWAs may also reflect upstream events with the FWAs surviving transport over large distances. Levels greater than 0.1 μg/L suggest a more significant degree of human sewage input. As a general recommendation, a level >0.2 μg/L is a strong indication of human sewage. Higher levels of FWAs also generally contained high levels of *E. coli* but a direct linear relationship between the two is not always evident.

In our hands, FWAs are the best indicator of potential hu-

man effluent, and provide a focal point for attention. They do not, however, indicate whether human pathogenic (or even indicator bacteria such as *E. coli*) are also transported with the FWAs and contribute to the microbial population in the river. Currently, FWAs are the most practical indicator of human faecal pollution, and studies of their movement and degradation, relative to microbial pathogens and indicators, are required.
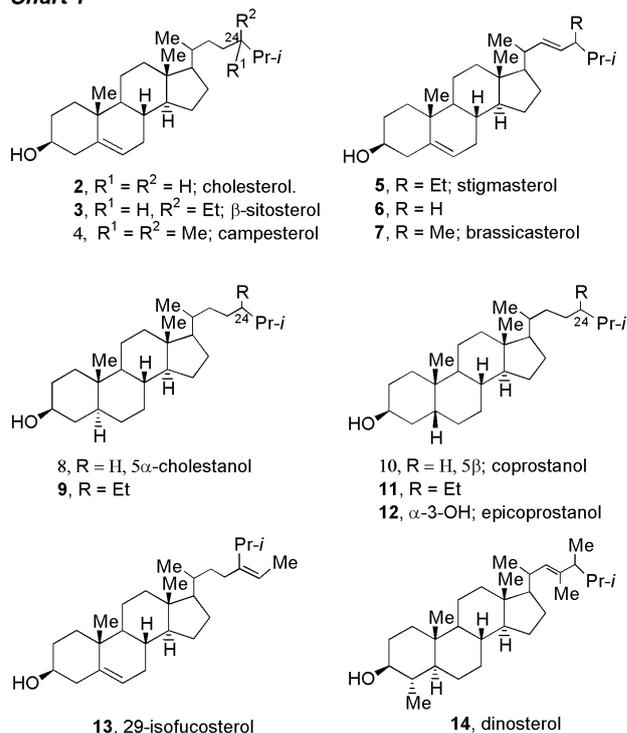
Faecal Sterols

Sterols are neutral lipids that have important biological functions in plants and animals, including cell membrane structures. Faecal sterols are C24-functionalized cholestane-based sterols found in animal faeces. The sterols present in an animal's faeces are determined by three factors, diet; synthesis by the animal (humans synthesize cholesterol), and transformations mediated by micro-organisms resident in the animal's digestive tract. Sterols eaten by animals include cholesterol (**2**) (an important membrane component in animals), and a range of plant-derived compounds that include 24-ethylcholesterol (β-sitosterol) (**3**) and stigmasterol (**5**) (Chart 1).[7] Upon entering the digestive tract some sterols are hydrogenated to stanols of various isomeric configurations by anaerobic bacteria. For example, cholesterol is the C_{27} precursor to 5α- and 5β-C_{27} stanols such as 5α-cholestanol (**8**) and 5β-coprostanol (**10**), hereafter referred to as cholestanol and coprostanol, respectively.

Coprostanol (**10**) is of particular interest in the detection of human faecal pollution as it is the principal sterol identified in human faeces and comprises *ca.* 60% of the total sterol concentration.[8] However, it and other sterols are also found in faeces from a range of other animals, and also in phytoplankton, zooplankton, aquatic plants, and protozoa.[9] Fortunately, they are generally at lower levels and, most importantly, are present in different ratios thereby allowing sterols from these sources to be distinguished. The major plant sterols are β-sitosterol (**3**), campesterol (**4**) and stigmasterol (**5**) (Chart 1), with **3** reported to be the most abundant sterol in plants.[10] The sterols found in higher concentrations in algae are 22-dehydrocholesterol (**6**), brassicasterol (**7**), isofucosterol (**13**), and dinosterol (**14**) (Chart 1).[7] Differentiation of human from herbivore faecal pollution relies on the high production of C_{29} stanols, such as 24-ethylcoprostanol (**11**) in herbivore faeces compared to human faeces. This is the result of herbivore consumption of plant material, which contains predominantly C_{29} sterol precursors. In comparison to **10**, which requires transformation via anaerobic bacteria residing in an animal intestine, **8** is the thermodynamically more stable isomer and commonly occurs in pristine environments where it is biosynthesized by phytoplankton, zooplankton and aquatic plants.

Analytical Method

Faecal sterol analysis is performed by filtering two or more litres of water through glass fibre filters that are then stored frozen until analysed. An internal standard is added and solvent extraction performed prior to hydrolysis, which is followed by back-extraction into hexane. The sterol fraction is eluted into methanol and silylated prior to analysis by GC-MS.[5] Each sterol and stanol result is expressed as parts per trillion (ppt).

Chart 1

2, R[1] = R[2] = H; cholesterol.
3, R[1] = H, R[2] = Et; β-sitosterol
4, R[1] = R[2] = Me; campesterol

5, R = Et; stigmasterol
6, R = H
7, R = Me; brassicasterol

8, R = H, 5α-cholestanol
9, R = Et

10, R = H, 5β; coprostanol
11, R = Et
12, α-3-OH; epicoprostanol

13, 29-isofucosterol

14, dinosterol

With the advent of GC-MS technology there has been a resurgence in the analysis of faecal sterols because of lower detection limits and the ability to identify and quantify a wider range of sterols that co-elute and are inseparable by gas chromatography alone. Comparative analyses have indicated that sterols in samples collected from surface waters begin to degrade after 24 hours at 4°C, however, three cycles of freezing and thawing the samples could be performed without detection of sterol degradation. The recommendation, therefore, is to freeze samples after collection, prior to analysis as they are stable to freeze/thaw cycles.[11]

Practical Examples of the Use of FWAs and Faecal Sterol Data Analysis to Evaluate Water

Table 1 provides analytical results from three different water samples. Samples 1 and 3 are urban streams or stormwater drains, sample 2 is a rural stream, and sample 4 is a duck pond. Interpretation is only undertaken where >2000 ppt of total sterols is identified in a sample. The first ratios considered are coprostanol:cholestanol (**10:8**) and 24-ethylcoprostanol:24-ethylcholestanol (**11:9**), which when greater than 0.5 suggest faecal contamination (preferential reduction of sterol by gut microbiota), whereas a ratio less than 0.3 may suggest environmental reduction by, *e.g.* anaerobic bacteria in sediments. Higher ratios in the range 6-10 are more likely to be due to human pollution as reports on undiluted human faeces give ratios of >10, whereas undiluted sheep and cow faeces have values ranging from 1.5 to 3.0.[12] Sample 3 contains low levels of key sterols thereby preventing further analysis. Interpretation of the results is for faecal sterols not to support a faecal source of the *E. coli* present in the water. This contrasts with the duck pond sample (4) where significant levels of all sterols are present, but low ratios recorded do not support a human, and only marginally support a ruminant faecal source. In contrast, samples 1 and 2 are clearly above 0.5 and support a faecal source.

Table 1. Microbial and chemical analyses of water samples.

Faecal Indicator	Sample			
	1	2	3	4
Total coliforms[a]	65,000	2800	>2,400	11,000
E. coli[a]	6,200	2800	>2,400	2,200
FWA	0.31	<0.01	0.03	<0.01
Sterol/stanol (ppt)[b]				
Coprostanol (**10**)	1285	362	<70	1110
Epicoprostanol (**12**)	45	63	<70	630
Cholesterol (**2**)	8775	1914	5250	14890
24-Ethylcoprostanol (**11**)	405	1425	90	4380
Cholestanol (**8**)	960	296	760	5640
24-Ethylcholestanol (**9**)	160	1095	<70	8490
β-Sitosterol (**3**)	5510	1712	2000	31920
Ratios				
Total sterols	21090	8056	9330	81870
10:8	1.34	1.22	NA	0.20
11:9	2.53	1.30	NA	0.52
% 10:total sterols	6%	4%	NA	1%
10/(8+10)	0.57	0.55	NA	0.16
10:12	28.56	5.75	NA	1.76
10:11	3.17	0.25	NA	0.25
Estimate % human sterols	100%	0%	NA	0%

[a]MPN/100mL. [b]Sterol in parts per trillion.
Note: Ratios for site 3 not calculated as **10** falls below detection limit.

The ratio coprostanol:24-ethylcoprostanol (**10:11**) (**11** is the main herbivore sterol) is the primary indicator of the amount of pollution contributed by human faecal sources compared with the herbivores that have lower levels of coprostanol present. The ratio was >1.0 in site 1 (3.17) suggesting a human source of sterols, and «1.0 for site 2 (0.25), suggesting herbivore faecal contamination was most likely. Further support can be inferred from the ratios of **10**:total sterols and **10**/(**8 + 10**). If coprostanol (**10**) comprises greater than 5-6% of the total sterols, then it suggests human sewage as the source of the pollution.[13,14] Only sample 1 fulfils this criterion. Grimault *et al*.[15] suggested that the if the ratio **10**/(**8 + 10**) is >0.7 it indicates urban sewage, <0.3 unpolluted sediments, and 0.4-0.6 a complex intermediary. Of the samples examined, the duck pond ratios suggest non-human/herbivore faecal sources, while the others complex intermediaries.

Epicoprostanol (**12**) is found in low levels in fresh sewage and at higher levels in treated sewage due to the microbial conversion **2** → **10** → **12** during the treatment. Therefore, a high ratio of **10** to **12**, as shown by sample 1, suggests untreated faecal pollution.

Leeming *et al*.[12,16] have estimated the contribution from human sterols where a mixed pollution event with humans and herbivores is suspected using the formula:

10/(10 + 11) x 100%.

A ratio >75% indicates a solely (100%) human source (sample 1, Table 1) while a ratio <30% indicates a solely herbivore source. A ratio between 75% and 30% with herbivore and human contamination suspected requires further calculation to take into account the 45% difference between % **10** and % **11**. This equates to a factor of 2.22 (100/45). Therefore, for every 1% of *coprostanol* that is below 75%, the proportion of faecal contamination due to humans is 2.2% less, as shown in the calculation below:

[% coprostanol-30] x 2.22 = % human contribution

For example, a sample containing 45% **10** and 55% **11** has as a contribution from humans: *(45 – 30) x 2.22 = 81.5%.* This implies, therefore, an 18.5% (100-81.5%) contribution from herbivores. Sample 1 has a ratio of 76% suggesting that the faecal pollution is 100% human derived, whereas sample 2 with 20% faecal contribution indicates 100% herbivore contamination.

Taken together with FWA analysis (Table 1), sample 1 supports the conclusion that the *E. coli* detected in this water sample are likely to have a human source. The water for this sample was derived from an open concrete-bottomed stormwater drain that flowed through an area surrounded by residential housing. Sample 2 contains significant sterols consistent with faecal origin, but clearly not human derived; together with the absence of FWAs herbivore inputs are suggested. This sample was collected from a stream that ran through farmland where cows and sheep were grazing.

Significant levels of *E. coli* in sample 3 are not supported by the presence of sterols, which for most are at the very low levels indicative of non-faecal sources. The low level of FWAs (0.03 ppt) may indicate events considerably further upstream making the source of the high faecal coliform concentrations unknown. Explanations include the controversial possibility that faecally-derived bacteria harboured by sediments are replicating in the environment,[17-19] or that they come from contribution by wild birds or dogs as the faeces of these species contain either none or only low levels of coprostanol and 24-ethylcoprostanol.[8]

Sample 4, from a duck pond, contains significant sterols highlighting the danger of detecting just coprostanol – the primary human sterol. Analysis of the sterol ratios is inconsistent with either a human or herbivore source, suggesting duck inputs will not, on the basis of sterols, be confused with human or herbivore sources.

Conclusion

The sources of faecal pollution can be difficult to ascertain requiring alternative methodologies. Analytical chemistry has much to offer biology on issues of microbial water quality, with faecal sterols and FWAs able to assist in identifying the source of the contamination. Where sufficient sterols are present, analysis can identify the faecal material in a water sample and indicate whether it is consistent with a human or herbivore source. Some assessment of proportionality is also possible providing ratio analyses of faecal sterols collate all ratios rather than be based on a single result. Careful site assessments are also essential.

In addition to source potential, chemical markers can of-

fer advantages over microbial indicators. Toxic pollutants, *e.g.* chemical disinfectants and heat, may lead to the death of biological indicators, *e.g.* faecal coliforms, while some pathogens may still be present. Faecal sterols are relatively resistant to physical and chemical degradation and can, therefore, be useful indicators where levels of microbes are low but faecal contamination is still suspected. Furthermore, the persistence of sterols in anaerobic sediments makes them candidates for a long-term signature of faecal contamination.

Future study on extending faecal sterol analyses to contributions from dog, cat, avian, and feral animal sources, and to evaluation of survival and transport of chemical indicators relative to both indicators and pathogens is to be undertaken.

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6. HPLC conditions: Column: Applied Biosystems 100 x 4.6 mm RP-18 equipped with a 15 x 3.2 mm RP-18 guard cartridge. Mobile: 1:1 methanol, 0.1M ammonium acetate. Flow rate: 1.5 mL/min. Injection volume: 100 µL. Detection: fluorescence: λ_{ex}=350 nm, λ_{em} = 430 nm. Limit of detection: 0.01 µg/L.

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