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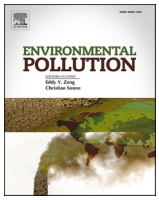

The use of sterol profiles, supported with other faecal source tracking methods, to apportion septic tanks contamination in rural catchments☆

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ABSTRACT

Identifying the origin of faecal pollution in water is needed for effective water management decisions to protect both human health and aquatic ecosystems. Traditionally used indicators of faecal contamination, such as *E. coli*, only indicate pollution from warm-blooded animals and not the specific source of contamination; hence, more source specific tracers are required. The study has focussed on separating the two main sources of contaminants within rural catchments in Ireland, agriculture and on-site wastewater treatment systems (predominantly septic tanks). While human-specific effluent tracers may assist in identifying potential pathways from individual septic tanks to surface waters, it is difficult to quantify the cumulative impact of such systems at a catchment scale. This study has investigated faecal sterols as a method to quantify such an impact on four small catchments in areas of low subsoil permeability with high densities of septic tanks. The results demonstrate the usefulness of faecal sterols which provide a quantitative evaluation of the respective impact between agricultural pasture inputs and on-site effluent showing differences between the four catchments. The study also highlights the need to derive more specific local reference sterol profile databases for specific countries or regions, using local source material of animal faeces and effluent. Two intensive sampling campaigns on the four catchments then used faecal sterols in parallel to fluorescent whitening compounds (FWCs), caffeine, artificial sweeteners and selected pharmaceuticals to gain further insights and confirmation about contamination hotspots as well as providing comparison between the different parameters. The combination of sterols, FWCs, caffeine, acesulfame and cyclamate has proven suitable to provide an estimate of the extent of human contamination in these rural catchments and has yielded additional information about potential pollution pathways and proximity of contamination. Overall, this methodology can help to facilitate a targeted and effective water management in such catchments.

1. Introduction

Identifying the origin of faecal pollution in water is essential to provide evidence for effective water management decisions for the protection of human health and aquatic ecosystems (Verhougstraete et al., 2015). EU legislation, for example, requires a targeted implementation of Programme of Measures as set out within bespoke River Basin Management Plans to mitigate such sources of pollution to meet the objectives of the Water Framework Directive 2000/60/EC. The enumeration of selected micro-organisms such as *E. coli* has traditionally been used as an indicator of faecal contamination but will only indicate the presence or absence of faecal pollution from warm-blooded animals and not the specific source of contamination. Hence, a wide range of microbiological, biochemical and chemical faecal source tracking methods have been investigated for their suitability to identify contamination sources (Ahmed et al., 2005; Digaletos et al., 2023; Fennell et al., 2021; Lapworth et al., 2018; Petrovic et al., 2008; Richards et al., 2017; Scott et al., 2002; Spoelstra et al., 2020; Tanna et al., 2020).

Such tracking methods include fluorescent whitening compounds

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(FWCs), which originate from laundry detergents. The fluorometric detection method represents a cost effective, easy and quick monitoring tool (Hartel et al., 2007a) which have been used successfully to identify pollution from human sources (Cao et al., 2009; Dickerson et al., 2007; Dubber and Gill, 2017; Hagedorn et al., 2005; Hartel et al., 2007a,b). However, interferences from organic matter which increase detection limits and high dilution, both reduce FWC detectability in catchments.

Caffeine has been found by a number of studies to be a reliable indicator of domestic wastewater inputs (Benotti and Brownawell, 2007; Buerge et al., 2009; Glassmeyer et al., 2005; Kolpin et al., 2004; Nakada et al., 2008; Peeler et al., 2006). It is highly source-specific and has a short half-life in the surface water environment which makes it a potentially good indicator of recent DWWTS inputs (Bradley et al., 2007; Buerge et al., 2003). Equally, the usage of artificial sweeteners has been studied with a view to using them as tracers of anthropogenic wastewater in surface waters (Buerge et al., 2009; Liu et al., 2014; Oldfield et al., 2020; Richards et al., 2017) given their significant rise as an ingredient in food and beverages over the last number of decades. Their chemical stability means that they pass through the human digestive system largely unchanged. Whilst there is a wide range of these artificial sweeteners on the market, the five which appear to be most commonly observed compounds in surface water environments appear to be acesulfame, sucralose, saccharin, cyclamate and aspartame (Arbelaez et al., 2015; Gan et al., 2013).

Pharmaceuticals and their metabolites are introduced through effluent discharges into the aquatic environment and can be detected in surface and groundwaters (Cahill et al., 2004; Glassmeyer et al., 2005; Kolpin et al., 2004; Liu et al., 2014). Carbamazepine, an anti-seizure/anti-epileptic drug and the antibiotic sulfamethoxazole are compounds that have been frequently detected in effluents and environmental waters (Al Aukidy et al., 2012; Bu et al., 2013; Glassmeyer et al., 2005; Loos et al., 2009; Noedler et al., 2013; Subedi et al., 2015; Teerlink et al., 2012; Yang et al., 2016). Due to their human specificity, the wide-scale use of antibiotics and their environmental persistence (especially high for carbamazepine), they are considered to have great potential as reliable tracers for wastewater in the environment (Benotti and Brownawell, 2007; Glassmeyer et al., 2005; Nakada et al., 2008).

While source tracking using such human effluent specific tracers may assist in identifying potential pathways from individual DWWTS to surface waters there is a difficulty of scaling up their contribution as a cumulative impact at a catchment scale (Digaletos et al., 2023; Geary and Lucas, 2019; Verhougstraete et al., 2015). Variations in sterol profiles of animal faeces, can be used as a biomarker technique to help distinguish faecal pollution sources (Leeming et al., 1996) and also quantify the relative proportions of contamination from different sources, hence the focus on the technique in this study. These profile differences are caused by the animal's dietary sterol intake (e.g. mainly cholesterol for carnivores and phytosterols for herbivores), its metabolic production of sterols and the gut microbiota which convert the ingested sterols to stanols of various isomeric configurations. Many studies have highlighted the usefulness of faecal sterols in the determination of contamination sources (Adnan et al., 2012; Derrien et al., 2012; Gilpin et al., 2011; Jardé et al., 2007; Nakagawa et al., 2021; Nash et al., 2005); however, issues with the interpretation of results have also been reported (Furtula et al., 2012; Shah et al., 2007) and they proved to be inconclusive in a recent groundwater contamination study in Ireland (Fennell et al., 2021). While coprostanol was usually observed in the highest concentration from human derived faecal samples, it is also present in substantial quantities in a range of herbivores and hence cannot be used as a unique human marker. As a consequence, the whole sterol profile and proportional contributions of certain sterols have to be analysed in order to draw conclusions of potential sources (Bujagić et al., 2016). The interpretation of these, using ratios and/or statistical discriminant analyses, however, might not be as simple and reliable in catchment waters due to mixtures of faecal samples from a range of species, especially where human and different animal faecal contributions overlap (Furtula et al., 2012; Shah et al., 2007). Hence, a multi-metric approach, where faecal sterol analysis is complemented with other suitable indicators, is recommended (Biache and Philp, 2013).

In rural Ireland the main pollution pressures for water quality are agricultural activities, predominantly pasture, and domestic wastewater treatment systems (DWWTSs). Due to a dispersed settlement pattern in these areas a high proportion (28.7%) of households nationally rely on on-site treatment systems, mainly consisting of a septic tank and a percolation area (CSO, 2016). Especially in areas of inadequate soil percolation, water quality can be impacted by failing systems that have been installed in inappropriate locations. Problems arise from surface water ponding and runoff, or due to alterations that allow an illegal direct discharge to water courses (Keegan et al., 2014; Gill et al., 2018).

Hence, the objective of this study is to test the suitability of faecal sterol profiles supported by a combination of other selected chemical faecal source tracking methods (fluorescent whitening compounds, caffeine, artificial sweeteners and selected pharmaceuticals) to quantify the extent of human faecal contamination from DWWTSs in rural catchments, targeting high-risk catchments where it is suspected that surface water quality is being impacted by such malfunctioning om-site systems.

2. Materials and methods

2.1. Study sites and sampling points

Four small catchments with high densities of DWWTSs in areas with a high "Likelihood of Inadequate Percolation To Ground" (LIPTG) were selected using ArcGIS based on a list of priority characteristics, summarised in Table 1. In each catchment, a point on the main stream was selected upstream from the main cluster of DWWTS as the *Upper* monitoring point, and one downstream of the cluster of DWWTS was selected as the *Lower* monitoring point. The number of houses located upstream and downstream of the *Upper* monitoring point are listed in Table 1 as well as the proportion of houses located <100 m from the main river. Further sampling points were used on each catchment to capture mid-stream sites as well as any small tributaries entering the main streams, as shown on Fig. 1(a–d). The grazing livestock present on all catchments were cattle, sheep and horses, with clear evidence their direct access into the streams at some points.

2.2. Routine monitoring parameters

CTD sensors (OTT hydrometry, UK) were installed at the upstream and downstream sites in all study catchments to provide continuous monitoring of water levels as well as temperature and electrical conductivity. Weather stations (Campbell Scientific, UK) were located at the upstream sites to record meteorological data for the catchments. In all catchments, grab samples were taken on a monthly basis across a year (total number of samples = 264) and were then re-visited the following year during the two comprehensive sampling events in April and July when an additional 138 no. grab samples were collected along the rivers (38, 39, 31 and 30 from catchments C1, C2, C3 and C4 respectively). Samples were collected from the stream banks using an extension poles so as not to disturb the sediment. Samples were collected in 500 mL glass bottles that were flushed three times by the river water before taking the sample. Procedural blank samples were also taken for each sampling excursion by bringing an empty bottle into the field which was filled with ultra-pure (18.2MΩ) water to reproduce the field procedures (storage and transport), before analysis in the laboratory. All samples were analysed for ammonium (APHA, 2022) and traditional bacteriological indicators (*E. coli*, total coliforms and enterococci) were quantified using Colilert-18 (IDEXX, USA). The respective mean daily flow at the rivers' downstream monitoring points and calculated soil moisture deficits are given in Supplemental Information Table S1. The

Table 1
Areal and land-use characteristics of the four selected study catchments.

	Area [km^2]	No. of DWWTS		Density [no./km^2]	DWWTS <100 m from river	Very high LIPTG [area coverage]	Land Use
		upstream	downstream				
Catchment 1 (C1) Co. Wicklow	3.07	0	78	25.4	42%	65%	Pasture (46%) Arable (10%) Forestry (33%) Other (11%)
Catchment 2 (C2) Co. Wexford	2.95	19	78	32.9	19%	95%	Pasture (83%) Arable (0%) Forestry (2%) Other (15%)
Catchment 3 (C3) Co. Cavan	3.85	2	58	15.6	10%	100%	Pasture (85%) Forestry (15%) Other (<0.5%)
Catchment 4 (C4) Co. Longford	2.07	4	36	19.3	50%	100%	Pasture (89%) Arable (0%) Other (11%)

DWWTS = Domestic wastewater treatment systems.
LIPTG = Likelihood of inadequate percolation to ground.

hydrological condition of the catchments between the two sampling dates presented different conditions: April was relatively dry following weeks of little rain, leading to higher soil moisture deficits and low flows in the streams compared to July where there were much higher flows in the streams following heavy rainfall with lower soil moisture conditions across the catchment.

2.3. Faecal sterols/stanols

Each of the 138 surface water samples taken during the two intensive sampling events and analysed for the routine parameters (Section 2.2) were also analysed for 10 faecal sterols. These included cholesterol, coprostanol, epi-coprostanol and cholestanol as well as the most important phytosterols (campesterol, stigmasterol and β-sitosterol) and their transformation products 24-ethyl-coprostanol, 24-ethyl-epi-coprostanol and stigmastanol (see Supplemental Table S2). 5α-cholestane was used as internal standard for quantification of extracted sterols and stanols.

Reference wastewater and faecal samples were taken from separate septic tanks (primary effluent, PE) (n = 8) and small-scale packaged treatment systems (secondary effluent, SE) (n = 5) located across all catchments, and fresh sheep (n = 8), horse (n = 8) and cow faeces and slurry (n = 8) and analysed for their respective sterol/stanol profiles. Furthermore, surface water samples from pristine environments were collected from two rivers, the Cloghoge and Glenmacnass River, in the Wicklow Mountains National Park. All these sampling sites were located far upstream of any human settlement or agricultural activities.

2.3.1. Sample preparation and extraction

The sample preparation and extraction method was similar to that described by Shah et al. (2006). Surface water samples (usually 5–10 L; or 20 L for pristine samples) were taken from study sites, filtered through Sartorius MGF grade glass microfiber filter (0.7 μm pore size) and dried over night at 30–40 °C. Similarly, for the analysis of effluent samples 100–300 mL was filtered and dried. Subsamples of 0.1–0.5 g of dried animal faeces were used for subsequent analysis. Dried solids (5α-cholestane added as internal standard) were extracted with 100 mL diethyl ether (≥99.8% for pesticide residue analysis, Sigma Aldrich) in a Soxhlet apparatus, allowing for at least 6 refluxes. After evaporation of the solvent a saponification with 10% KOH in methanol was carried out (2h at 100 °C). The sample was then neutralised with 6M HCl and dried under a gentle flow of N_2 gas. The precipitate was washed with 100% ethanol before being discarded while the supernatant was dried under N_2 before derivatisation using 200 μL BSTFA +1% trimethyl chlorosilane (1h at 100 °C) to form trimethylsilyl (TMS) derivatives.

2.3.2. GC-MS analysis

The derivatised extracts (final volume of 200 μL) were then analysed by a GC-MS system consisting of a Thermo Scientific Trace Ultra gas chromatograph coupled with a Thermo Scientific ITQ 900 GC-Ion Trap MS System with full-scan electron ionization (EI). The GC was fitted with a Thermo Scientific AI/AS 3000 injector systems, inlet temperature 300 °C, with a glass liner and a non-polar TraceGold TG-SQC GC column (30m × 0.25 mm x 0.25 μm). 1 μL of sample was injected using a split ratio of 20:1. Helium was used as carrier gas at a constant flow of 0.5 mL/min. The sterols/stanols were separated on the column using the following temperature programme: 220 °C held for 1 min, increased to 228 °C (held for 3 min) at 5 °C/min, to 230 °C (held for 2 min) at 5 °C/min, to 270 °C (held for 2 min) at 5 °C/min, to 275 °C (held for 1 min) at 0.5 °C/min, to 285 °C (held for 1 min) at 5 °C/min.

EI mode mass spectra were obtained at 70eV and monitored on the full scan range (m/z 50–500). Quantitative analysis was carried out using Selected Ion Monitoring (SIM) as detailed in Table S2. Peak detection via retention times, sterol identification (with m/z ratios of diagnostic ions) (Table S2) and quantification was performed using the Thermo Xcalibur chromatography software. The quantification was based on the internal standard (IS) 5α-cholestane which was added to the sample prior to extraction. The quantification method utilised a nine-point calibration (20–150 μg/mL, R^2 for all sterols/stanols >0.99) with constant IS concentration of 75 μg/mL. The limit of detection (LOD) for all sterols/stanols was <5 μg/mL except for Cholesterol where the LOD was 10 μg/mL. This equates to a detection limit in the environmental samples of 200 and 100 ng/L for 5 and 10 L filtered sample volumes respectively. For pristine waters and for some samples from the C1 upstream site 20 L were filtered so that the detection limit for these environmental samples was 50 ng/L except for cholesterol where it was 100 ng/L.

2.3.3. Principal Component Analysis (PCA)

All data were first tested for normality by graphical means and Shapiro-Wilk tests. A Principal Component Analysis (PCA) was then performed using the proportions of 10 sterols found in septic tank effluents (primary and secondary treated), animal faeces (cow, horse and sheep), pristine waters and surface water samples. The analysis was carried out using IBM SPSS Statistics v22.0 software with a covariance matrix and varimax rotation. The extraction of components was performed based on an Eigenvalue >1. Component loadings for the variables as well as objective scores for each sample were extracted to create component plots and biplots, respectively.

2.3.4. Sterol ratio analysis

The sterol profiles from the water samples were evaluated using

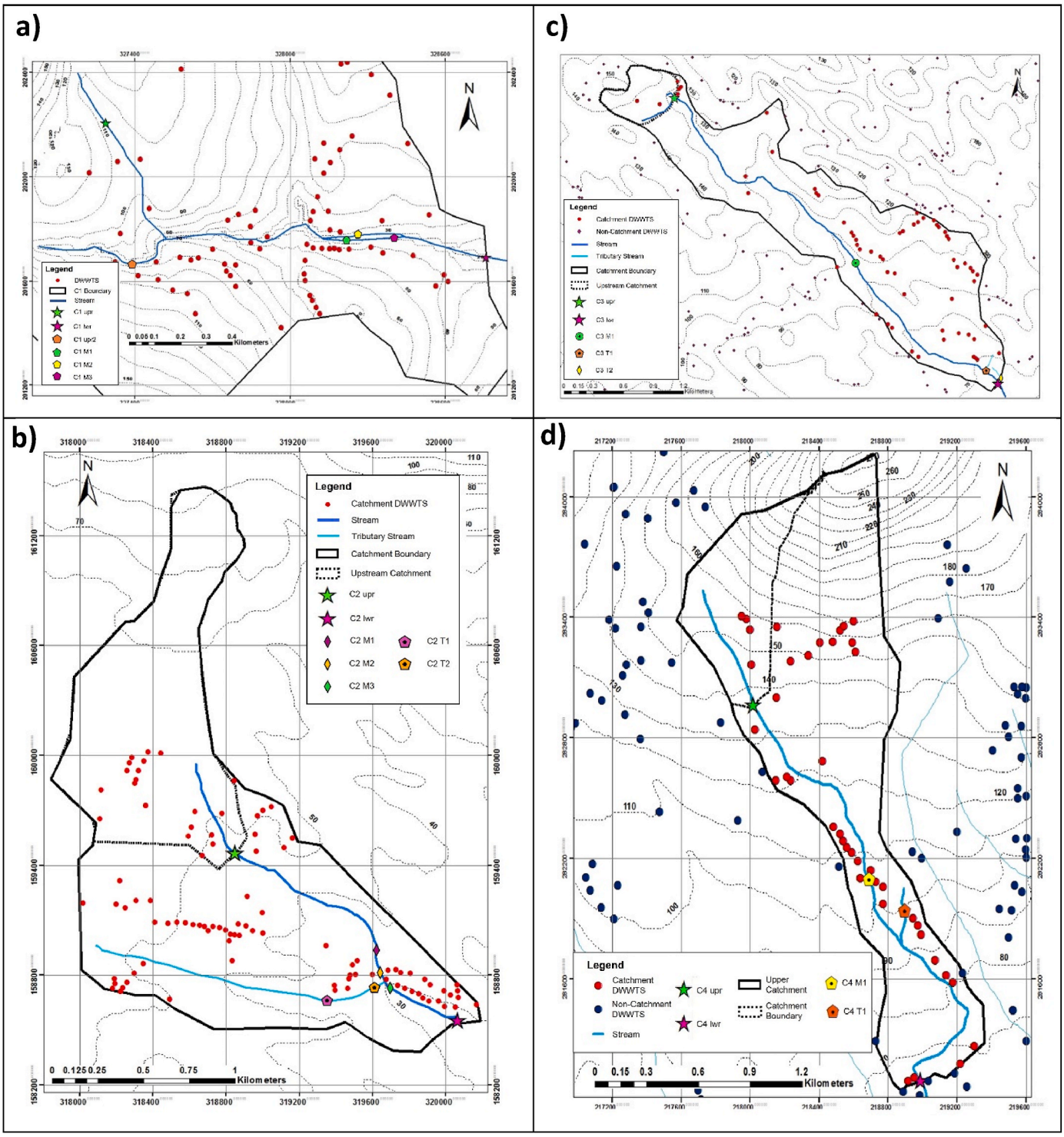

Fig. 1. Maps of the four study catchments showing the DWWTSs and sample locations: (a) C1, (b) C2, (c) C3 and (d) C4.

ratios of different sterol and stanol concentrations, as shown in Table 2. To obtain an indication of the presence of any faecal contamination in the sample, the ratios F1, H1 and R1 were used. If any of those ratios indicated either human or herbivore faecal sources, a further estimation of human and/or herbivore contribution in the sample was calculated by applying ratio Q1. If this ratio value was >60, the faecal pollution was interpreted as 100% human originated and if < 10 it was considered 100% herbivore originated (modified after Gilpin et al., 2011). Values that fell in between these thresholds suggested a mix of sources and the contribution [%] of human vs. herbivore sources was calculated using Q2 in Table 2.

2.4. Fluorescent whitening compounds (FWCs)

Grab samples for FWC analysis were collected and stored in amber glass bottles to protect samples from UV light. A LS55 Fluorescence Spectrometer (PerkinElmer) was used for the fluorescence measurement. Fluorescence PMMA cuvettes with 10 mm optical path length were used for all measurements. The excitation and the emission wavelength were set at 350 and 436 nm, respectively, with a slit width of

Table 2
Sterol ratios and calculations to determine faecal sources and contributions (ESRInstitute of Environmental Science and Research, 2017; Gilpin et al., 2011). The result interpretations and Q2 have been adjusted taking into account findings from the faecal reference materials in this study.

Ratio name	Sterol ratio	Result interpretation
Ratio indicative of unspecified faecal pollution		
F1	β-Sitosterol/24-ethyl-coprostanol	<4 indicates unspecified faecal source >4 suggests plant decay or avian source
Human indicative ratios		
H1	% Coprostanol	>5–6% suggests human source
Herbivore indicative ratios		
R1	% 24-ethyl-coprostanol	>5–6% suggest herbivore source
Contribution quantification		
Q1	Coprostanol/(Coprostanol+24-ethyl-coprostanol)*100	>60% suggests sole human source <10% suggests sole herbivore source
Q2	(Q1-10%) x 2	Human contribution [%] in a mixed source
Avian indicative ratios		
A1	Stigmastanol/(Stigmastonl+24-ethyl-coprostanol+24-ethyl-epicoprostanol)	A1 ratio >0.3–0.4 AND
A2	Cholestanol/(Cholestanol + Coprostanol + Epicoprostanol)	A2 ratio >0.5 suggest avian source

either 5 or 10 nm. The presence or absence of FWCs in surface waters was determined using the photodecay method as described by Dubber and Gill (2017). The limit of detection of the used fluorometer was measured using distilled water as blank in 20 replicates and defined as the average signal strength plus three times the standard deviation of the blank measurements. The photodecay of the samples was measured in triplicates (occasional verification with further 2 replicates) by recording the fluorescence signal after 0-, 1-, and 10-min of exposure to UV light. UV exposure was conducted using a sun lamp with 4 Philips Cleo 15W UV tubes. The ratio of the reduction after 1 min to the reduction after 10 min of UV exposure was determined and samples with a ratio (1/10 min) > 0.25 are considered to contain FWCs.

2.5. Caffeine, artificial sweeteners and pharmaceutical compounds

The surface water samples taken during the two intensive sampling events in April and July were also analysed for caffeine, acesulfame (ACE), sucralose (SUC), saccharin (SAC), cyclamate (CYC), aspartame (ASP), carbamazepine (CBZ) and sulfamethoxazole (SFZ) using High Performance Liquid Chromatography (HPLC) coupled with electrospray ionization tandem mass spectroscopy (ESI-MS/MS), based on a method developed by Tran et al. (2013).

2.5.1. Sample preparation and solid phase extraction

Samples were filtered through Sartorius MGC filters to remove suspended solids (particle retention 1.2 μm) and then pH-adjusted prior to SPE extraction using a 1M hydrochloric acid solution. Water samples were spiked with seven ^{2}H isotope labelled internal/surrogate standards (ILIS), to correct for losses during the extraction process and during analysis in the ESI-MS/MS unit. Individual stock solutions of each target analyte and the ILIS solution were prepared in MeOH/H_2O (50/50, v/v) at 2.0 g/L and 0.05 g/L and stored in the dark at −18 °C. The SPE cartridges (Chromabond HR-X Cartridges, Macherey-Nagel) were preconditioned with 6 mL MeOH for 15 min, followed by 6 mL of MQ water (pH 2) at a flow rate of 3 mL/min. After the conditioning step the surface water samples (0.5 L per sample) were passed through the wet cartridges (8 mL/min) after which the reservoirs and cartridges were rinsed with 15 mL MeOH/H_2O/(10/90, v/v). Following this rinsing, the sides of the cartridges were pushed into the cartridge membrane with a strong stream of N_2 and dried for 40 min under high vacuum. The cartridges were eluted using 2 × 5 mL of MeOH at a flow rate of 1 mL/min. The resulting extracts were dried under a gentle stream of nitrogen at 50 °C and finally dissolved again in a MeOH/H_2O (75/25, v/v) solution to a final volume of 500 μL. This reconstituted solution was vortexed for 30 s prior to filtering through 0.2 μm agilent captivia© filters into the HPLC glass vials. Recoveries for surface water samples were higher than 70% for most of the analytes when the SPE procedure involved HR-X cartridges under pH 2. The RSD was below 10% for most analytes in various matrices.

2.5.2. HPLC-MS analysis

The column used was an Agilent ZORBAX SB-C18 (9150 × 2.1 mm id; 3.5 μm particle size) for separation and quantification of target analytes. Tandem MS was carried out on a triple quadrupole MS (LCMS-8030, Shimadzu, Japan). Ion acquired in MRM modes with a 7 s dwell time. Collision induced dissociation (CID) was performed using argon at approx. 230 kPa. Electrospray source and desolvation temperature were set at 300 and 250 °C. The drying and nebulizing gas flow rates were set at 15 and 3 L/min. Interface voltage and interface current were set at 3.5 KV and 0.2 μA. After choosing the precursor ions, product ions were obtained and optimized with three key parameters: entrance potential (EP), collision energy (CE) and collision exit potential (CXP). Further details regarding the analytical method and validation are given in the Supplemental Information. The limits of quantification (LOQ) for caffeine and ACE were 25 ng/L, for ASP, SFZ and CYC 5 ng/L and for CBZ 1 ng/L. SUC and SAC had a LOQ of 10 ng/L but could not be quantified accurately due to interferences from matrix effects.

3. Results

3.1. Presence of faecal contamination

Traditional bacteriological indicators (total coliforms, *E. coli* and enterococci) and ammonium concentrations were used to indicate areas in the study catchments where faecal contamination was present. In all catchments, grab samples were taken on a monthly basis across a year and were then re-visited the following year during the two comprehensive sampling events in April and July.

E. coli concentrations at C1_lwr were over one magnitude higher than at the upstream site C1_upr, indicating impairment of water quality downstream of the DWWTSs. Two of the midstream sites, C1_M1 and C1_M2, also showed very high *E. coli* concentrations with averages of 560 ± 486 (n = 12) and 533 ± 378 (n = 12) MPN/100 mL respectively (Fig. 2a). The enterococci concentrations show a similar, but more damped pattern, as the *E. coli* results. However, these faecal indicator organisms (FIO) alone are inconclusive regarding the potential source of the faecal matter. Throughout C2 generally high *E. coli* concentrations were detected, with three sites having average concentrations of >1000 *E. coli*/mL throughout the monitoring period (Fig. 2b) - the upstream and two midstream sites (C2_M2 and C2_M3). *E. coli* concentrations in C3 were generally lower compared to the other catchments, however there was a slight deterioration of water quality noticeable in the lower catchment area. Equally, in C4, there approximately four times the *E.coli* on average at the downstream location and almost 25 times the number of enterococci, indicating the presence of some faecal pollution, as well as higher levels in a mid-stream sampling point (C4-M1) close to the septic tanks around these locations.

Similar inferences of pollution in the four study catchments could be made according to the ammonium results (see Supplemental Fig. S1). The EU Surface Waters regulations define a mean concentration of ≤0.065 mg/L NH_3–N (or 95%ile ≤0.14 mg/L NH_3–N) to achieve good water quality status. In C3 and C4 all sites had average concentrations <0.06 mg/L NH_3–N, however, similar to the high *E. coli* concentrations found in C2, concentrations of ammonium were also generally higher

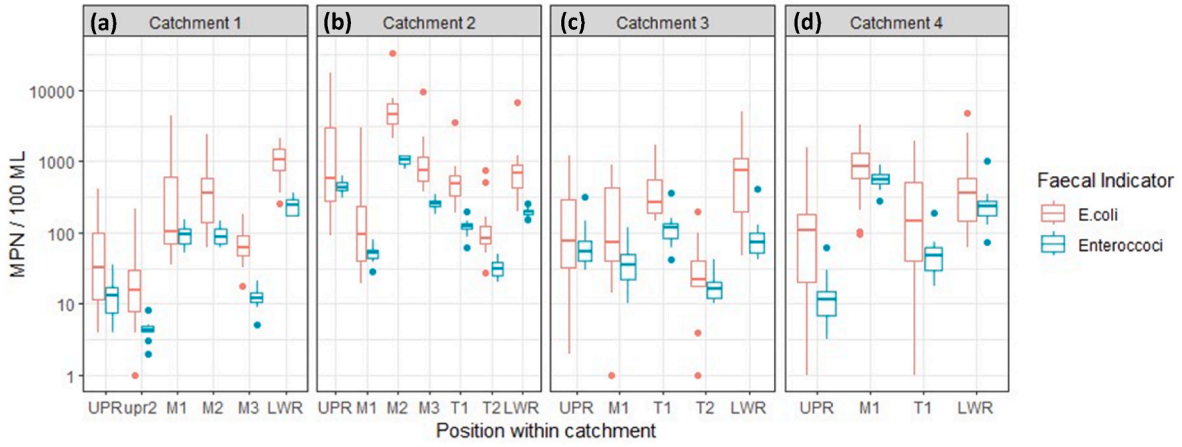

Fig. 2. *E. coli* and enterococci concentrations in all four study catchments, (a) C1, (b) C2, (c) C3, (d) C4, at sampling locations upstream (UPR) and downstream (LWR) of the main cluster of DWWTSs, as well as midpoint locations (M1, M2) and tributaries (T1, T2) of each stream.

with many exceedances of the good quality status (see Fig. S1b). The midstream site M1 in C1 was also not meeting good water quality status requirements (Fig. S1a).

In general, these three indicators do show some pattern of increasing faecal pollution downstream of the main clusters of DWWTS, as well as some hot spots in the mid catchment sampling points, with catchment C2 revealing much higher levels of contamination that the other three. However, these parameters do not allow conclusion to be drawn regarding the sources of contamination and also reveal a wide fluctuation in values between the two sampling events, the first event in April being under baseflow conditions in these low permeability catchments, the second event in July following a period of relatively heavy rainfall. A statistical analysis of all the results in this study shows significant correlations all three indicators *E. coli*, enterococci and ammonium (all $p < 0.05$, $n = 402$) with the strongest correlation between *E.coli* and enterococci ($r = 0.91$) compared to between ammonium and *E. coli* ($r = 0.69$) and ammonium and enterococci ($r = 0.82$).

3.2. Sterols

The sterol profiles from the different reference faecal source material and from the pristine river water samples are shown in Table 3. The sterol content of primary effluent (PE), secondary effluent (SE) and pristine samples, originally in μg/L, were converted to μg/g (dry weight) using the suspended solid content for ease of comparison to the other faecal samples. Suspended solids (SS) for PE samples ranged from 81 to 241 mg/L (ave. 146 ± 59 mg/L) and for SE from 30 to 120 mg/L (ave. 78 ± 45 mg/L). The pristine water samples had SS contents of between only 0.7–1.13 mg/L.

In PE and SE reference samples C_{27} sterols and stanols contributed on average 83–85% to the total sterol content while for cow, horse and sheep faeces they only contributed 13–23%. The herbivores faeces are instead dominated by C_{28} and C_{29} sterols and stanols (81%). Both PE and SE showed very similar 24-ethyl-coprostanol to coprostanol ratios.

The average contribution of coprostanol in PE (12.1 ± 6.5%) was higher than in all other faecal source materials: in comparison the coprostanol in SE (4.5 ± 0.5%) was not significantly different to herbivore faeces. For example, sheep faeces contained slightly higher proportions of coprostanol (8.3 ± 1.2 %), however, the ratio to 24-ethyl-coprostanol makes herbivore faeces distinguishable from SE. The largest contributor to the sterol profile (39–57%) for all herbivores faeces was 24-ethyl-coprostanol.

The pristine waters were characterised by large proportions of phytosterols (>60%) incl. campesterol, stigmasterol and β-sitosterol with 8%, 10.5% and 44.4%. Cholesterol was found to contribute 15% and no coprostanol was detected. Small concentrations of 24-ethyl-coprostanol were found, which could originate from wild animals (e.g. deer) or, as demonstrated by Nash et al. (2005), from plant decay.

The sterol results from the grab samples collected in the rivers across the four catchments reveal the total sterol concentrations varied significantly between and within catchments as well as throughout the year with values ranging from 2 to 162 μg/L. While the sterol content in most of the samples in catchments C1, C3 and C4 were below 8 μg/L with only certain sampling points yielding higher values, concentrations in C2 were generally higher throughout the whole catchment area with most samples >30 μg/L. In comparison, the total sterol content found in the pristine water samples were between 1 and 2 μg/L. Although a high total sterol content does not necessarily imply the presence of faecal contamination it can be indicative of additional contributions from faecal sources to the natural sterol profile.

The PCA revealed that two components together explain 91% of the variation observed in the sterol profiles dataset. Component 1 includes

Table 3
Sterol profiles for analysed faecal source material.

	Primary effluent [μg/g] (n = 8)		Secondary effluent [μg/g] (n = 5)		Cow [μg/g] (n = 8)		Horse [μg/g] (n = 8)		Sheep [μg/g] (n = 8)		Pristine river [μg/g] (n = 6)	
Coprostanol	2315	±1763	474	±75	218	±84	162	±19	517	±103	0	±0
Epi-coprostanol	0	±0	0	±0	82	±28	96	±8	6	±14	0	±0
Cholesterol	12,616	±3270	8684	±2686	778	±347	242	±49	821	±209	281	±3
Cholestanol	193	±80	149	±135	55	±21	53	±9	85	±25	24	±34
24-ethyl-coprostanol	1575	±1155	319	±74	2022	±734	1822	±207	3591	±960	74	±7
24-ethyl-epicoprostanol	0.8	±1.8	0.5	±0.9	827	±421	463	±16	0	±0	43	±61
Campesterol	176	±55	124	±53	40	±8	85	±29	33	±18	149	±50
Stigmasterol	74	±43	94	±44	28	±3	45	±17	17	±6	200	±104
Sitosterol	1269	±333	1139	±609	434	±76	724	±293	427	±99	830	±273
Stigmastanol	72	±34	32	±31	602	±146	367	±38	779	±222	243	±24
Total	18,291	±5244	11,015	±3420	5087	±1760	4059	±104	6275	±1287	1843	±352

the phytosterols β-sitosterol, stigmastanol and campesterol with a positive loading as well as coprostanol, cholesterol and 24-ethyl-coprostanol with a negative loading (Fig. 3). While the phytosterols are important constituents of higher plants (Huang and Meinschein, 1976) and represent a non-faecal source of sterols, coprostanol, cholesterol and 24-ethyl-coprostanol are all major constituents of domestic wastewater effluents and herbivore faecal matter. Even though cholesterol is part of animal tissue and not necessarily of faecal origin, (i.e. it can occur naturally from animal tissues such as from macro-invertebrates and insects), where cholesterol is found in high concentrations it is usually associated either with effluents (see above) or faecal matter from dogs and certain bird species (Leeming et al., 1996). Component 2 includes faecal stanols 24-ethyl-coprostanol, 24-ethyl-epicoprostanol and stigmastanol, all of which are major constituents of herbivore faeces, with a high positive loading and cholesterol with a negative loading (Fig. 3).

Hence, Component 1 separates samples based on faecal and non-faecal sources of their sterol content while Component 2 distinguishes between the origin of faecal contamination (here human vs herbivore). The variation explained by Components 1 and 2 is 52.4% and 38.6%, respectively.

The PCA biplot for all of the source materials and surface water samples (Fig. 4) show the effluent samples, with coprostanol and cholesterol as characteristic sterols, form a large cluster in the third quadrant. The SE samples conglomerate at the bottom of that cluster because of their lower coprostanol and relatively higher cholesterol content. The horse and cow faeces samples, characterised by 24-ethyl-epicoprostanol and 24-ethyl-coprostanol and stigmastanol, all cluster in the second quadrant with no clear separation between them. The sheep cluster positions itself slightly to the left which is explained by the higher coprostanol content and the predominance of 24-ethyl-coprostanol (due to the absence of 24-ethyl-epicoprostanol). The pristine stream water samples, which are characterised by the phytosterols, are located at relatively high values near the x-axis in the first quadrant of the PCA biplot (Fig. 4).

Two surface water samples from C1 appear in the same area of the PCA biplot as the sampled effluents (Fig. 4a). Both of these samples were taken from the downstream site in low flow periods during summer months and had high cholesterol contributions (68% and 73%) as well as a significantly higher total sterol (52 and 55 μg/L) and coprostanol concentrations (9.17 and 4.73 μg/L), clearly indicating faecal contamination from a human source. The PCA results further suggest possibly a mixed (effluent and herbivores) faecal source of sterols found in two samples from the midstream site C1_M1. Also, three samples from the upstream locations seem to be dominated by phytosterols with some influence of herbivore faecal matter, possibly from wild deer that have been reported to have access to the upper catchment area.

In C2 many surface water samples plot in the third quadrant and only very few near the pristine reference samples (Fig. 4b) suggesting that this catchment is more impacted by DWWTSs compared to the other study areas. Sites that appear largely influenced by effluent inputs are C2_upr, the midstream location M3 and the downstream location C2_lwr. Only one sample from C2_M1 seems to be predominantly impacted by herbivore faecal matter.

The sterol profiles in C3 were more similar to pristine water samples - i.e. most are dominated by phytosterols. Samples that appear to have influence of some human faecal sources were C3_upr and lwr from March and July and C3_T1 from July.

Similarly, in C4 most samples plot near the pristine samples along the positive part of the Component 1 x-axis, especially three samples taken from the upstream site. The plot does suggest that two samples taken from the midstream site C4_M1 are impacted by effluents.

3.2.1. *Application of ratios*

Based on the faecal sterol profiles established by Leeming et al. (1996) a range of source indicative sterol ratios have been established in order to differentiate between human and herbivore faecal sources (Gilpin et al., 2011). Once there is an indication of faecal pollution the ratio Q1 in Table 2 is used to estimate the contribution from either

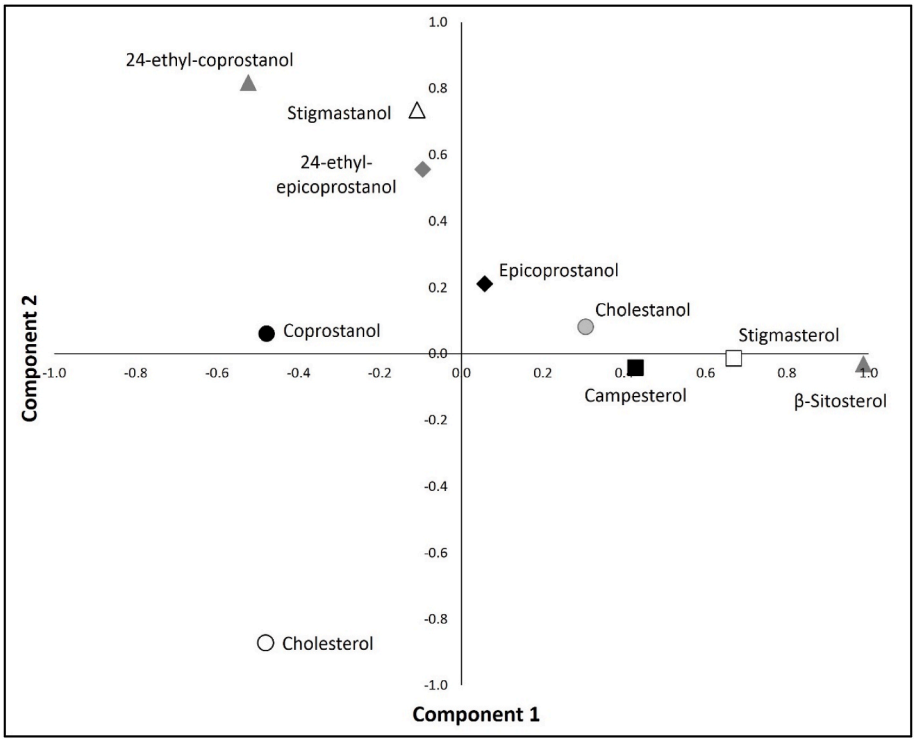

Fig. 3. Component loading for the variables from the PCA performed for source materials and surface water samples. Component 1 explains 52.4% of the overall variation and separates samples based on faecal and non-faecal sources; Component 2 explains 38.6% of the variation and distinguishes between human vs. herbivore sterol sources.

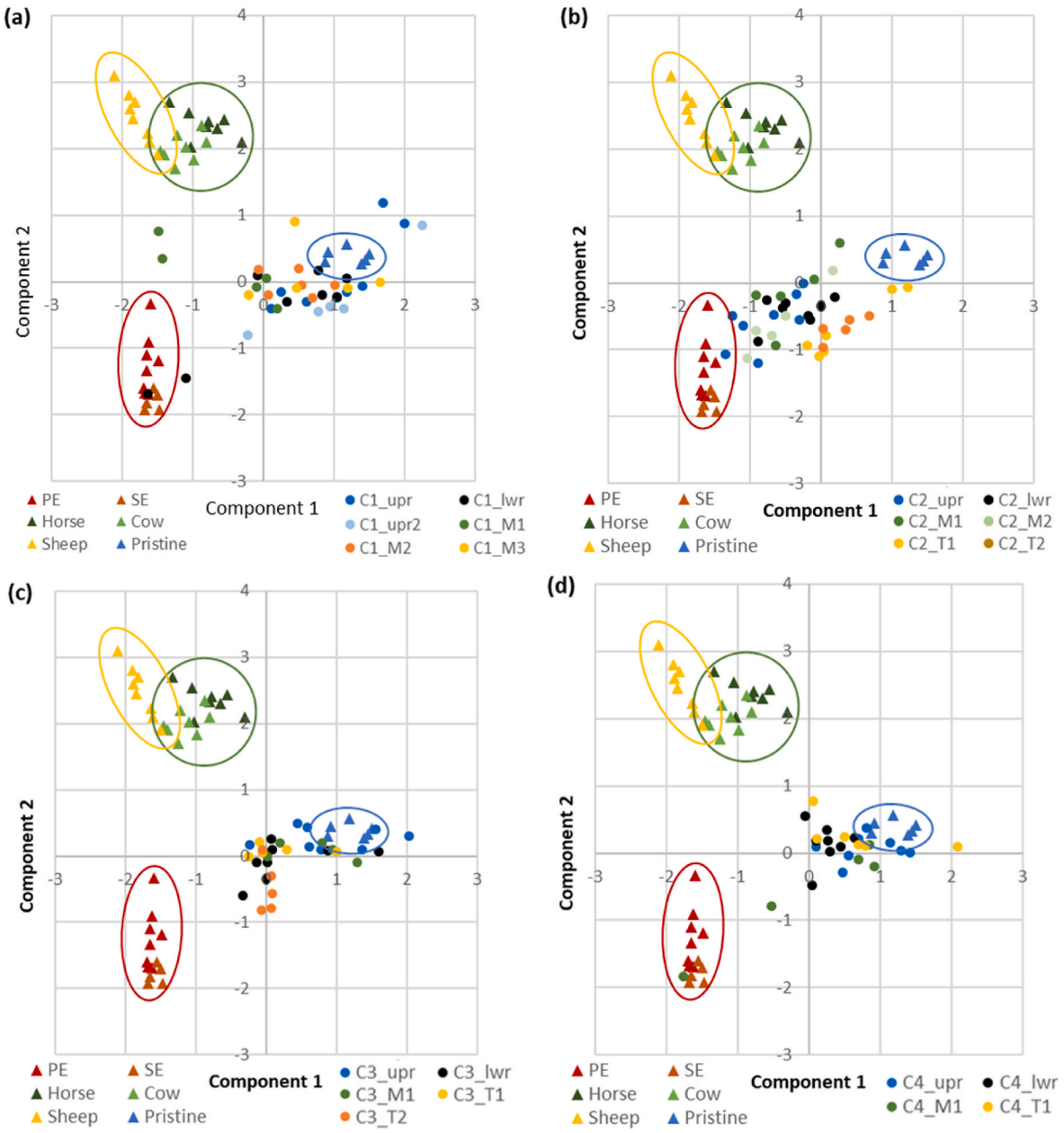

Fig. 4. Biplot for source materials and surface water samples using the relative proportion of all 10 sterols/stanols as variables for the statistical analysis. The individual plots show the water samples from each catchment C1 (a) to C4 (d) with source materials from primary and secondary effluent (PE, SE) as well as from animal faeces and pristine river locations as comparison. Component 1 explains 52.4% of the overall variation (separating samples based on faecal and non-faecal sources); Component 2 explains 38.6% of the variation (distinguishing between human vs. herbivore sterol sources).

source. In this study the average ratio Q1 was 60.4% ± 3.8% and 10.6% ± 2.6% for effluents (PE and SE) and herbivore faeces, respectively. According to predefined thresholds (>75% for human, <30% for herbivore sources) (ESRInstitute of Environmental Science and Research, 2017; Gilpin et al., 2011) the effluents in this study would not be considered to originate 100% from humans but only to 66%, with a 34% contribution from herbivores. This is due to the significant differences of the faecal sterol profiles found in the source materials, as discussed earlier. As a consequence, for the application in this study the thresholds for 100% human and 100% herbivore contribution were chosen based on the average Q1 ratios for effluent and herbivore faeces obtained in this study as opposed to those determined in Australia. The equation Q2 was also modified accordingly (see Table 2).

Fig. 5 summarises the results from the sterol ratio analysis and shows the estimated fraction of faecal pollution that is contributed by human faecal sources compared with herbivores at the monitoring sites. It should be noted that these contributions are only determined for samples where the sterol profile indicated the presence of any faecal contamination. From the results it appears that faecal contamination in C1 and C2 is predominantly coming from human sources. Only at the midstream site C2_M1, where the stream runs through a field with grazing horses, was a significant contribution from herbivore sources found. Generally, there was a higher contribution of herbivore sources found in C3 and C4 than in C1 and C2. However, at C4_M1 a cluster of houses is located in close proximity to the stream, which possibly resulted in higher human contributions to the faecal sterol profile at this sampling site.

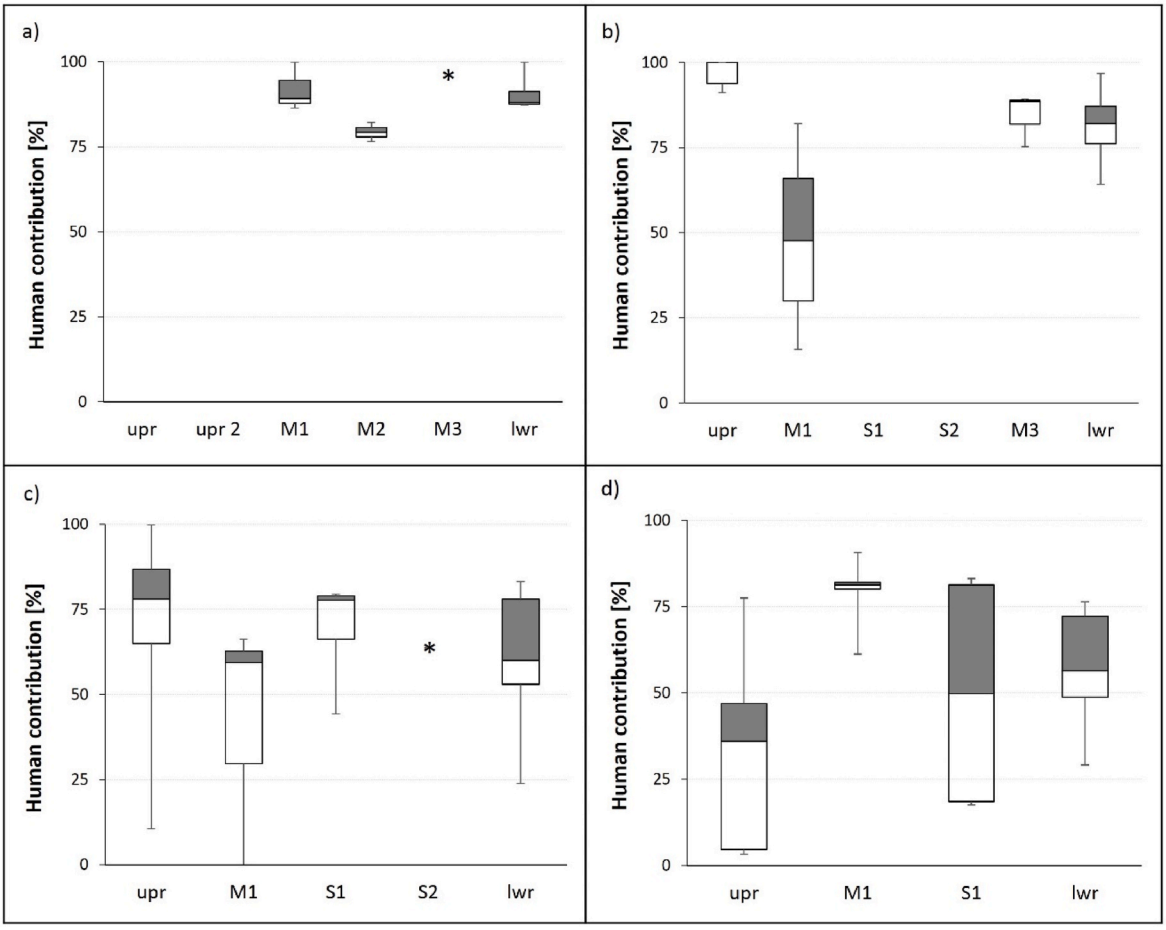

Fig. 5. Boxplot of the human contribution [%] vs. herbivores towards the faecal sterol profile in the four study catchments (a) C1, (b) C2, (c) C3 and (d) C4 throughout across all sampling periods.

3.3. Catchment sampling events using faecal sterols supported with other human specific chemical tracers

In order to overcome the element of uncertainty related to the interpretation of faecal sterols discussed in Section 3.2, the sterol analysis was complemented with other chemical source indicators of human effluent (FWCs, caffeine, artificial sweeteners and pharmaceuticals) during two sampling events in April and July 2016, the results of which are presented in Table 4. Again, it should be note that the hydrological condition of the catchments between the two sampling dates were very different: April being relatively dry following weeks of little rain and low baseflows in the streams compared to July with much higher flows in the streams following heavy rainfall and lower soil moisture conditions across the catchment (see Supplemental Information Table S1).

3.3.1. Chemical faecal source tracking methods

3.3.1.1. FWCs. FWCs are highly human specific and mainly associated with domestic wastewater. The photodecay method ensures that their fluorescent signal can be separated from that of organic matter or from other anthropogenic fluorescent organic compounds (e.g. car fluids and car care products) (Hartel et al., 2007a). Across the four catchments for the two sampling events a total of 21 samples had an average photodecay ratio of >0.25 but only 10 were deemed to be statistically significant (see Table S3), with most of these in catchments C1 and C2.

3.3.1.2. Caffeine. Concentrations of caffeine during the two sampling events ranged from 25 to 478 ng/L with an exceptionally high concentrations of 2 μg/L at the upstream and midstream site of C2 (C2_upr, C2_M3) and 28.6 μg/L obtained for C1_M1 in the July high flow event (Table 4). However, no caffeine was detected at the tributary and downstream sites in C2.

3.3.1.3. Artificial sweeteners. Of the five target sweetener compounds (acesulfame (ACE), sucralose (SUC), saccharin (SAC), cyclamate (CYC), aspartame (ASP)), ASP and SUC were not detected. SAC and CYC were detected on several occasions but mostly at concentrations that were below the quantification limit. The largest concentrations of CYC were found in C2 in July. ACE however appeared to be rather ubiquitous and was detected at all sites except for the two upstream sites in C1.

3.3.1.4. Pharmaceuticals. The two pharmaceuticals selected for this study, carbamazepine (CBZ) and sulfamethoxazole (SFZ), were mainly found in C2 where concentrations of up to 583 and 340 ng/L for CBZ and SFZ respectively were observed. Neither of these compounds were found in C3 and only CBZ was detected in very low concentrations (6 ng/L) at the downstream site in C4 (Table 4).

4. Discussion

4.1. Sterols as a faecal source tracking method

For the faecal sterols, the analysis of the reference samples of human wastewater effluent (PE and SE) showed much higher contributions of C_{27} sterols and stanols compared to the herbivore (cow, horse and sheep) faeces which were dominated by C_{28} and C_{29} sterols and stanols.

Table 4
Overview of the results from all faecal source tracking analytes in the four catchments during the sampling events in April and July 2016.

	Sampling event April 28, 2016							Sampling event July 14, 2016						
Catchment 1	upr	upr2	M1	M2	M3	lwr		upr	upr2	M1	M2	M3	lwr	
NH_3–N [mg/L]	0.0	0.0	0.34	0.0	0.0	0.0		0.0	0.0	3.91	0.0	0.0	0.0	
Total coliform [No./100 mL]	525	734	>24,196	>48,392	4352	15,531		6867	602	>48,392	48,392	9222	12,976	
E. coli [No./100 mL]	10	67	776	10,950	52	2143		2143	<1	22,398	148	104	2153	
% Coprostanol	1.3	0.9	24.8	8.4	3.8	4.4		0.8	0.0	37.1	8.9	4.5	6.2	
% human contribution	0.0	0.0	89.2	76.5	0.0	0.0		0.0	0.0	100	82.1	0.0	87.7	
% herbivore contribution	0.0	0.0	10.8	23.5	0.0	0.0		0.0	0.0	0.0	17.9	0.0	12.3	
FWC	–	–	+	+	–	–		–	–	+	–	–	+	
Caffeine [ng/L]	NF	NF	55	306	NF	145		NF	NF	28,556	NF	NF	478	
Acesulfame (ACE) [ng/L]	NF	NF	485	5885	133	77		NF	NF	417	61	349	245	
Cyclamate (CYC) [ng/L]	NF	NF	+	+	NF	NF		NF	NF	NF	NF	NF	26	
Saccharin (SAC) [ng/L]	NF	NF	NF	NF	NF	NF		NF	+	+	NF	NF	NF	
Carbamazepine (CBZ) [ng/L]	NF	NF	19.3	141	NF	NF		NF	NF	NF	NF	NF	19	
Sulfamethoxazole (SFZ) [ng/L]	NF	NF	NF	NF	NF	NF		NF	NF	NF	NF	NF	NF	

	Sampling event April 28, 2016							**Sampling event July 14, 2016**						
Catchment 2	**upr**	**M1**	**T1**	**T2**	**M2**	**M3**	**lwr**	**upr**	**M1**	**T1**	**T2**	**M2**	**M3**	**lwr**
NH_3–N [mg/L]	0.61	0.13	0.0	0.01	1.34	0.35	0.19	0.24	1.70	0.16	0.72	3.92	2.13	0.00
Total coliform [No./100 mL]	>48,392	31,062	12,976	39,726	>48,392	>48,392	>48,392	>241,960	36,540	26,130	30,760	241,960	29,090	8820
E. coli [No./100 mL]	16,328	2666	196	82	7746	490	8704	27,550	5810	410	740	41,060	10,460	200
% Coprostanol	10.7	4.6	3.8	3.0	n/a	6.8	7.8	18.5	3.5	1.1	0.8	n/a	7.2	4.1
% human contribution	93.9	60.6	0.0	0.0	n/a	89.2	84.0	100	15.8	0.0	0.0	n/a	75.3	0.0
% herbivore contribution	6.1	39.4	0.0	0.0	n/a	10.8	16.0	0.0	84.2	0.0	0.0	n/a	24.7	0.0
FWC	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Caffeine [ng/L]	NF	228.0	NF	NF	NF	49.3	NF	3107.0	33.9	NF	NF	n/a	2343	NF
Acesulfame (ACE) [ng/L]	5011	4365	5332	4587	14,252	10,825	5491	6959	5049	8267	7037	n/a	10,270	2483
Cyclamate (CYC) [ng/L]	NF	+	NF	NF	+	+	+	134	NF	225	NF	n/a	195	196
Saccharin (SAC) [ng/L]	NF	NF	NF	NF	+	NF	NF	+	+	+	+	n/a	+	+
Carbamazepine (CBZ) [ng/L]	NF	535	583	425	335	308	181	NF	507	328	203	n/a	424	170
Sulfamethoxazole (SFZ) [ng/L]	NF	NF	340	253	NF	85	46	NF	NF	172	93	n/a	NF	13

	Sampling event April 22, 2016					Sampling event July 11, 2016				
Catchment 3	**upr**	**M1**	**T1**	**T2**	**lwr**	**upr**	**M1**	**T1**	**T2**	**lwr**
NH_3–N [mg/L]	0.40	0.00	0.00	0.00	0.13	0.00	0.00	0.05	n/a	0.01
Total coliform [No./100 mL]	22,398	5510	11,588	4092	11,600	10,950	28,272	48,392	n/a	39,726
E. coli [No./100 mL]	2100	40	146	20	268	240	1008	1678	n/a	1508
% Coprostanol	22.5	0.6	5.2	1.0	7.6	0.9	1.6	6.6	n/a	3.2
% human contribution	100	0.0	77.7	0.0	78.2	10.6	0.0	44.3	n/a	24.0
% herbivore contribution	0.0	0.0	22.3	0.0	21.8	89.4	100	55.7	n/a	76.0
FWC	–	–	–	–	–	–	–	–	n/a	–
Caffeine [ng/L]	NF	NF	74	NF	99	NF	NF	40.2	n/a	NF
Acesulfame (ACE) [ng/L]	485	115	223	NF	240	242	176	167	n/a	201
Cyclamate (CYC) [ng/L]	NF	NF	NF	NF	+	NF	NF	NF	n/a	NF
Saccharin (SAC) [ng/L]	+	NF	NF	NF	NF	NF	+	NF	n/a	+
Carbamazepine (CBZ) [ng/L]	NF	NF	NF	NF	NF	NF	NF	NF	n/a	NF
Sulfamethoxazole (SFZ) [ng/L]	NF	NF	NF	NF	NF	NF	NF	NF	n/a	NF

	Sampling event April 22, 2016					**Sampling event July 11, 2016**				
Catchment 4	**upr**	**M1**	**T1**	**lwr**		**upr**	**M1**	**T1**	**lwr**	
NH_3–N [mg/L]	0.0	0.01	0.0	0.0		0.0	0.01	0.0	0.0	
Total coliform [No./100 mL]	2595	7746	6896	6510		24,196	48,392	25,994	48,392	
E. coli [No./100 mL]	20	584	0	126		801	1684	590	1008	
% Coprostanol	1.0	5.3	0.4	2.6		0.8	6.2	3.2	6.1	
% human contribution	4.7	90.6	0.0	47.0		0.0	61.2	18.8	50.7	
% herbivore contribution	95.3	9.4	0.0	53.0		0.0	38.8	81.2	49.3	
FWC	–	–	–	–		–	–	–	–	
Caffeine [ng/L]	29.1	152	NF	25.1		NF	32.6	n/a	48.2	
Acesulfame (ACE) [ng/L]	146	968	895	2822		56	289	n/a	230	
Cyclamate (CYC) [ng/L]	NF	NF	NF	NF		NF	NF	n/a	NF	
Saccharin (SAC) [ng/L]	NF	NF	NF	NF		NF	+	n/a	NF	
Carbamazepine (CBZ) [ng/L]	NF	NF	NF	6		NF	NF	n/a	NF	
Sulfamethoxazole (SFZ) [ng/L]	NF	NF	NF	NF		NF	NF	n/a	NF	

This is generally in accordance with earlier findings by Leeming et al. (1996) but the ratio they found between coprostanol to cholesterol in human faeces is significantly different to the effluent samples in this study: they found 61% coprostanol with 24-ethyl-coprostanol the second most abundant sterol, compared to this study which only found contributions of up to 23% for coprostanol in the septic tank effluent (PE) samples, with cholesterol the most abundant sterol at 52–78%. Leeming et al. (1996) acknowledged that changes in the composition will occur when mixed with non-faecal sources (i.e., domestic wastes, food scraps, algae) in sewage effluent. The lower fraction of epi-coprostanol to coprastanol in the PE compared to the more highly treated SE matches findings by Zali et al. (2021) on wastewater treatment plants in Malaysia across a range of different processes.

24-ethyl-coprostanol was the largest contributor to the sterol profile for all herbivores faeces which concurs with Leeming et al. (1996), although they found it only contributed 13% and 19% to the sterol profile in cow/horse and sheep faeces, respectively in Australia. The corresponding epimer, 24-ethyl-epicoprostanol, was only found in horse and cow faeces but not in any of the sheep faeces analysed in this study. This is in direct contrast to findings by Leeming et al. (1996) where this stanol was found exclusively in sheep faeces, but agrees with a later study from Australia which found 24-ethyl-coprostanol, stigmastanol and 24-ethyl-epicoprostanol to be the most abundant sterols in cow faeces (Nash et al., 2005) - sheep faeces were not analysed in that study. These discrepancies show the variance in faecal sterol profiles due to different genetic pools as well as animal diets brought about by different climate, vegetation and management, highlighting the importance for the establishment and use of a local reference material database.

No coprostanaol was detected in the pristine river waters which is consistent with the fact that only anaerobic bacteria can hydrogenate cholesterol to coprostanol and so it is generally not found in unpolluted waters or in fully oxic sediments. However, under anoxic and anaerobic conditions small amounts of coprostanol can be found in environmental samples without contamination by faecal matter (Gilpin et al., 2011). The pristine waters were characterised by large proportions of phytosterols.

In general, the sterol PCA results have detected two key components with Component 1 separating samples based on faecal and non-faecal sources while Component 2 distinguishes between the origin of faecal contamination (here human vs herbivore). The PCA results are similar to those obtained by Biache and Philp (2013) who used sterol data from faecal source material published by Leeming et al. (1996) and complemented them with some of their own (chicken and cow manure, WWTP influent and effluent). While the PCA results can provide information about similarities between sterol profiles and indicate possible faecal sources (Zali et al., 2021), it should be noted that domestic wastewater and bird faeces are both characterised by high cholesterol contributions and so it may not be possible to separate these two sources out based on this type of PCA plot alone. It also appears that no water samples plot as close to the herbivores faecal reference samples as observed for the effluents cluster. Derrien et al. (2012) found that diluted wastewater samples would lie within the “human group” but runoff samples from a plot treated with bovine manure were significantly outside the bovine faeces cluster. This was due to a decrease of the relative proportion of 24-ethyl-epicoprostanol. This, together with mixture effects from other contributing faecal sources, might be the reason why water samples did not separate out that far towards the herbivore clusters. Hence, runoff samples might be more appropriate as reference samples to obtain a better discrimination with the PCA.

As hydrophobic compounds, sterols bind to solids and are incorporated into sediments where they can persist (Bujagić et al., 2016). They have been reported to have some resistance against microbial degradation and are stable in anaerobic conditions (especially in sediment cores) but high temperatures and aerobic conditions can lead to degradation (Korosi et al., 2015). The half-life of coprostanol in aerobic conditions above 20 °C can be less than 10 days and >400 days under anaerobic conditions (Ogura, 1983). Hence, during sediment disturbances a re-suspension of sterols into the water column would affect the results. In previous studies the highest adsorption of faecal sterols has been observed in clay soils (Adnan et al., 2012; Froehner et al., 2010). All of the catchments in this study are characterised by clay-rich soils and so a high removal might be expected en route to the water body. As a consequence, where high human originated faecal sterol concentrations are found in the water, this may be indicative of a direct effluent input via a pipe or overland flow or short preferential flowpaths as a result of inadequate percolation.

The application of source indicative sterol ratios has showed the importance on basing the threshold criteria to distinguish between human wastewater effluent and herbivore faeces on the results of sterols analysis of local reference sources. The use of these sterol ratios on the catchments reveals that faecal contamination in C1 and C2 does seem to be coming predominantly from human sources (see Section 4.3 later).

4.2. Other faecal contamination indicators

4.2.1. E. coli, enterococci and ammonium

The analysis of three more conventional indicators of faecal pollution (*E.coli*, enterococci and ammonium) on these four low permeability catchments has shown some pattern of increasing faecal pollution downstream of the main clusters of DWWTS, as well as some hot spots in the mid catchment sampling points. However, these parameters do not allow conclusions to be drawn regarding the sources of contamination and also reveal a wide fluctuation in values between prevailing hydrological conditions. In general, the impact of septic tanks on streams is more likely to be picked up during baseflow conditions using such indicators of faecal pollution, as shown on a study of 24 different catchments in the USA by Sowah et al. (2014) with a range of different densities of septic tanks. In this study, baseflow conditions predominated during the April sampling event.

4.2.2. Fluorescent whitening compounds

The analysis of other chemical source indicators of human effluent (FWCs, caffeine, artificial sweeteners and pharmaceuticals) was carried out in parallel during the two intensive sampling campaigns (under very different hydrological conditions) to verify and support the interpretation received from the faecal sterol analysis and help to identify human faecal contamination from DWWTSs. Positive FWC results using the photodecay method were predominantly picked up in catchments C1 and C2 (which supports the findings from the sterol analysis). However there was a generally low detection frequency of FWCs in this study, particularly in catchments C3 and C4, can be attributed to the following environmental factors: high dilution, removal through adsorption to soil, photodecay of the fluorescence signal and actual photo-degradation of the compounds in the water column (Dubber and Gill, 2017; Hartel et al., 2007b; Kramer et al., 1996; Poiger et al., 1998; Stoll et al., 1998). However, as a consequence, where detected, FWCs are considered a reliable indicator of recent input of domestic effluents (possibly via direct discharge) relatively close to the sampling site (Dubber and Gill, 2017) and have proved useful for groundwater studies on private wells in Ireland (Fennell et al., 2021) where the contaminant pathway excludes any photodecay process.

4.2.3. Caffeine

Caffeine was picked up in all catchments but with particularly high concentrations at mid point locations in catchments C1 and C2. Other studies have reported caffeine concentrations in DWWTS effluent to vary considerably with concentrations reaching up to 391 μg/L (Chalew and Wienburg, 2007; Richards et al., 2016; Sauve et al., 2012; Seiler et al., 1999; Subedi et al., 2015; Yang et al., 2016). These results generally agree with the findings made by Busse and Natoda (2015) who found caffeine concentrations to vary from 0.029 to 1.19 μg/L at stream sampling sites with known DWWTSs located nearby. Seiler et al. (1999)

observed caffeine concentrations ranging from 1.3 to 2.4 μg/L in surface water downstream of a WWTP outfall, supporting the conclusion that the faecal contamination observed at C2_upr and C2_M3 (Table 4) is of human origin. Due to its high solubility caffeine is very mobile in drains and rivers while the loss due to in-stream sediment sorption is negligible (Buerge et al., 2003). However, high caffeine removal (85%) has been reported via microbial degradation in aerobic conditions of unsaturated soils in DWWTS percolation areas (Gill et al., 2009; Yang et al., 2016). Equally, in the receiving water body, removal by in-stream microbes leads to short half-lives for caffeine which have been estimated to range from 5 days (Buerge et al., 2003) down to less than a day (Bradley et al., 2007). Hence, caffeine can be used as an indicator of recent wastewater contamination events, especially in the context of DWWTSs where its detection may suggest malfunctioning percolation areas either due to saturated soil conditions or due to more direct discharge routes into the water body.

4.2.4. Artificial sweeteners

For the artificial sweeteners, acesulfame (ACE) was found in all catchments, whilst saccharin (SAC) and cyclamate (CYC) were detected but at very low levels. Aspartame (ASP) and sucralose (SUC) were not detected. The literature suggests that ASP is not as frequently detected in surface water due to its high level of instability and biodegradability (Berset and Ochsenbein, 2012; Lange et al., 2012) which corroborates its non-detection in this study. In contrast, SUC and ACE are characterised by a high environmental persistence with very low adsorption and removal rates, thereby presenting as the most prevalent of the artificial sweeteners in surface waters (Al Aukidy et al., 2012; Buerge et al., 2009; Digaletos et al., 2023; Gan et al., 2013; Lange et al., 2012), as reflected in the frequent detection and high concentrations of ACE found in the study catchments. ACE has previously been described as an ideal marker for the detection of domestic wastewater in natural waters due to its observed persistence in septic system plumes and other subterranean matrices (Robertson et al., 2013). SUC, however, was not detected at all, which might indicate that this particular sweetener is not commonly used in products sold on the Irish market. SAC and CYC exhibit low persistence in the environment and high attenuation rates in unsaturated soils as well as high removal rates in wastewater treatment plants have been reported (Lange et al., 2012; Robertson et al., 2013). This may explain the lower detection rate in this study compared to ACE. However, where these sweeteners are detected, they indicate a recent and/or closely located discharge of poorly/partly treated domestic wastewater, whereas ACE would also be detected from fully treated effluents in the river system. It should be noted that whilst artificial sweeteners are mainly associated with domestic wastewater, some can also be used in a number of animal feeds. An additional source of these compounds in surface waters may also originate in rural catchments from the spreading of sewage sludge as a fertilizer on land. This might be particularly relevant for chemically stable compounds, such as ACE, which are not only persistent in the environment but also exhibit a high solubility in water so that they are transported to water courses with minimum losses due to degradation or adsorption to soil (Schaffer et al., 2015).

4.2.5. Pharmaceuticals

The concentrations of the two target pharmaceuticals, carbamazepine (CBZ) and sulfamethoxazole (SFZ), found in catchment C2 are comparable with average CBZ concentrations reported in the literature for centralised WWTP effluents range from 320 to 660 ng/L (Noedler et al., 2013; Teerlink et al., 2012) but as a prescription drug it is not as widely used and average concentrations in DWWTS effluents are usually much lower, varying from 0.61 to 16 ng/L (Oppenheimer et al., 2012; Subedi et al., 2015). The 95 percentile concentration of CBZ in rivers across Europe has been measured at 310 ng/L; however, again due to the variation in usage patterns for this compound, stream concentrations vary widely with a maximum of 11.5 μg/L reported in the same study (Loos et al., 2009). Equally, Noedler et al. (2013) measured CBZ in surface waters across Germany obtaining a median concentration of 58 ng/L, with a maximum of 762 ng/L but its detection was not ubiquitous. CBZ is reported to have a high persistence and to behave very conservatively in the environment; hence, the relatively high concentrations of CBZ found in C2 indicates high loadings from DWWTS effluents to surface water in this catchment. Although SFZ is considered to be more widely used than CBZ and has been reported to have higher detection rates (68%) and higher concentrations (avg. 1.9 μg/L) (Subedi et al., 2015) in DWWTS effluents, SFZ was detected less frequently and with lower concentrations in this study. This observation agrees with findings from other catchment studies (Loos et al., 2009; Subedi et al., 2015) and might be attributable to its higher susceptibility to microbial degradation and sorption processes in soils (Schmidt et al., 2004; Yang et al., 2016) as well as photodecay in the surface water itself (Bahnmuller et al., 2014). In comparison to the concentrations recorded in surface water by Loos et al. (2009) across the EU (avg. 76 ng/L) the SFZ concentrations found in C2 are either comparable with or higher than the average concentration range. Finally, the use of medically prescribed pharmaceuticals, such as CBZ and SFZ, is not as widespread as caffeine or artificial sweeteners. Hence, their value as a tracer in more sparsely populated, rural catchments, especially where pollution "hotspots" caused by single or only a few DWWTSs are suspected, is limited (Digaletos et al., 2023).

4.3. Synthesis of faecal contamination sources in study catchments

According to the faecal sterol analysis, the upstream site in C1 has been classified as un-impacted which appears to be corroborated by the fact that none of the other human effluent indicators were detected at this site. Occasional high faecal contamination, as for example indicated by high *E. coli* concentrations in July 2016, could be attributed to avian sources using the sterol ratios (A1 >0.4 and A2 >0.5). At the midstream site C1_M1 a human sterol contribution of >80% (86–100%) was found at several occasions throughout the monitoring period, indicating faecal contamination from DWWTSs which are located particularly close to the stream in this section (<30 m). These observations usually coincided with the detection of FWCs and were further confirmed by detection of CYC, SAC and CBZ as well as exceptionally high caffeine and elevated ACE concentrations. C1_M2 and the downstream site C1_lwr also showed indication of occasional human faecal contamination during the monitoring period. Here at least 6 indicators tested positive for human faecal sources.

Results from the sterol analysis and most other indicators suggest that C2, the study catchment with the highest number and highest density of DWWTSs, was correspondingly experiencing the highest impact of human faecal sources on water quality; the highest detection rates and highest concentrations of most human source indicators were found in this catchment. The upstream site C2_upr generally had very high human sterol contribution (>90%) with several positive detections for FWCs throughout the monitoring period. The detection of FWCs, caffeine, CYC and SAC in July 2016 confirms proximal pollution by poorly treated effluent. Similar results were obtained for the midstream section around the sampling points C2_M2 and C2_M3, with significantly higher concentrations of ACE than observed in the other study catchments indicating a significant and continuous contribution from human source, suggesting the existence of another effluent discharge point nearby. These results indicate that the microbiological indicator of faecal pollution detected in this section (avg. *E. coli* at M2 and M3 ≥ 3000 MPN/100 mL) must come from a human source. Both the *E. coli* count and the sterol analysis gave no indication of faecal contamination at the tributary locations C2_T1 and C2_T2, but the detection of ACE, CBZ, SFZ, CYC and SAC all suggest the presence of human faecal sources in this area of the catchment. However, due to the persistent nature of some of these tracers and the fact that concentrations for the others were around the detection limit, these results alone are not considered

sufficient to draw clear conclusions.

The water quality in catchments C3 and C4 appeared to be generally less impacted compared to C1 and C2. Furthermore, the human faecal sterol signal was not as dominant, with more apparent contributions from herbivores. This is also reflected in a lower detection frequency and lower concentrations of most of the other human tracers. This may be due to the lower DWWTSs density (see Table 1) and a larger distances on average between houses and the stream. While higher human faecal contributions were still detected occasionally at different sites in these catchments, only one potential "hotspot" for persistent pollution from DWWTSs could be identified at C4_M1 where a couple of houses are located very close to the stream (<30 m). This indicates that the PCA results for the first sterol sample taken at this location was not caused by bird faeces (as discussed previously), with the sterol ratios indicating a significant human contribution (81% human vs. herbivore contributions) and that the stream was impacted by nearby DWWTSs. This was also confirmed by the detection of caffeine, ACE and SAC as further indicators of human faecal contamination.

Finally, the detection of FWCs, caffeine, CYC, SAC and SFZ suggest the existence of preferential flow paths and/or direct discharges of DWWTS effluent to surface waters. Their frequent detection across the four catchments and especially at those sites where significant contamination from human sources was found, highlights the importance of these pollution pathways in catchments with inadequate percolation.

5. Conclusions

The results in this study demonstrate how faecal sterol profiles can be used to identify and estimate the relative impact of DWWTSs as pollution sources in rural catchments. However, it has also been demonstrated that it is necessary to adapt the existing faecal sterol methods more distinctly to specific countries/regions by establishing and using a local reference material database. The study also highlights the benefit of a multi-metric approach with a selection of chemical human-specific markers. The combination of tracers should be chosen to cover different properties, i.e., include persistent and biodegradable compounds as well as tracers of higher and lower water solubility. This will not only cover different detectability chances but can also provide additional information about pollution pathways as well as the timeframe and proximity of contamination.

In this study of rural catchments in Ireland - a maritime temperate country with low permeability glaciated soils - a combination of sterols, FWCs, caffeine, ACE and CYC was found to be most suitable in order to be able to classify different catchments as highly or only mildly impacted by pollution from DWWTSs. The results further highlight the importance of preferential flow paths in the study catchments and suggest the presence of unregulated direct effluent discharges. Moreover, it was possible to identify hotspots of human faecal pollution which can help to concentrate remediation efforts in areas where they will be most effective.

CRediT authorship contribution statement

Donata Dubber: Data curation, Formal analysis, Investigation, Writing – original draft. **Laura Brophy:** Investigation. **David O'Connell:** Formal analysis. **Patrice Behan:** Methodology. **Martin Danaher:** Methodology. **Craig Evans:** Methodology. **Phillip Geary:** Methodology, Conceptualization. **Bruce Misstear:** Conceptualization, Supervision. **Laurence Gill:** Conceptualization, Project administration, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Laurence Gill reports financial support was provided by Environmental Protection Agency.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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