

Article

# Are Sterols Useful for the Identification of Sources of Faecal Contamination in Shellfish? A Case Study

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**Abstract:** This work aimed to identify the major source(s) of faecal pollution impacting Salcott Creek oyster fisheries in the UK through the examination of the sterol profiles. The concentration of the major sewage biomarker, coprostanol, in water overlying the oysters varied between 0.01  $\mu\text{g L}^{-1}$  and 1.20  $\mu\text{g L}^{-1}$ . The coprostanol/epicoprostanol ratio ranged from 1.32 (September) to 33.25 (February), suggesting that human sewage represents the key input of faecal material into the estuary. However, a correlation between the sterol profile of water above the oysters with that of water that enters from Tiptree Sewage Treatment Works ( $r = 0.82$ ), and a sample from a site (Quinces Corner) observed to have a high population of Brent geese ( $r = 0.82$ ), suggests that both sources contribute to the faecal pollution affecting the oysters. In identifying these key faecal inputs, sterol profiling has allowed targeted management practices to be employed to ensure that oyster quality is optimised.

**Keywords:** faecal contamination; oysters; shellfisheries; sterols; coprostanol

## 1. Introduction

The assessment of faecal pollution is of considerable importance for public health, in addition to aesthetic and ecological concerns. Faeces usually contain a variety of pathogenic microorganisms, together with many undesirable chemical pollutants; therefore, contamination of aquatic environments and shellfisheries are an obvious hazard to public health [1]. Bacterial indicators, such as the faecal coliforms (FC)/faecal streptococci (FS) ratio, have been used to quantify the extent of faecal pollution and even to determine the contributions of different sources of faecal pollution entering aquatic systems [2]; however, their use has been questioned due to highly variable survival rates during disinfection and environmental stressors such as salinity, temperature and sunlight [1,3–5]. In addition, speciation of FC and FS to the phenotypic level cannot differentiate between sources of faecal pollution, because there is no single species restricted to a single animal [6–8].

The River Blackwater Estuary in the UK represents a highly productive shellfish cultivation area of the Essex coast (Figure 1). The native oyster breeds naturally throughout the Blackwater Estuary, especially in the outer reaches to the south of Mersea Island. Oysters are filter feeders, and phytoplankton are their main source of food. This ability to retain faecal micro-organisms, especially viruses, makes shellfish potentially susceptible to accumulating faecal pathogens from the overlying water [9]; recent

reductions in the numbers of oysters in the estuary have raised concerns about the possible increased levels of water contamination. The identification of the main sources of faecal pollution contaminating the oysters will provide the basis for an effective management plan.



**Figure 1.** Map of the study site, Salcott Creek Channel, Essex, UK showing the location of the sampling sites.

The use of biomarkers, and in particular faecal sterols, can help distinguish between human and animal sources of faecal contamination, and even between different animal species [10,11]. The potential source specificity of faecal sterols arises from three factors. The first is the animal's diet; omnivorous, carnivorous and herbivorous diets (e.g., geese, dogs and sheep respectively) each produce a distinctive sterol profile and, therefore, the proportions of sterol precursors entering the digestive tract are different [11]. Secondly, irrespective of diet, higher animals can biosynthesise sterols, which are then discharged to the digestive tract [4]. Finally, intestinal flora (anaerobic bacteria) in the digestive tract of some animals biohydrogenate sterols to stanols of various isometric configurations [4,12]. Unfortunately, individual sterols cannot differentiate between sources of faecal pollution because they are not restricted to one organism [13]. For example, coprostanol, although the principal sewage biomarker, has also been found in the faeces of marine mammals (e.g., whales and seals), as well as pigs and cats [10,14,15].

However, it has been suggested that evidence of contamination originating from human waste can be obtained by calculating the ratio of coprostanol to other sterols. The coprostanol/epicoprostanol ratio was proposed [16] as a useful index to distinguish between inputs of domestic, urban sewage and material of non-human origin (epicoprostanol is a minor component in human faeces). They suggested that human-related wastes would result in a ratio close to or larger than one (a greater proportion of coprostanol). Lower values for the ratio would indicate that the dominant inputs were from marine mammals. The ratio of coprostanol/cholesterol has also been suggested as a useful sewage indicator since it "normalises" for the total lipid content, as cholesterol is present in most organisms [17]. The ratio examines the different biogenic sources of sterols and confirms the urban sewage source [18], and has been shown to exhibit a positive correlation with other markers of sewage pollution analysed in oceans [19] and estuarine sediments [20]. Another ratio was proposed by Grimalt, Fernandez [21]. In environments not impacted by faecal pollution, the hydrogenation of cholesterol to cholestanol through cholestanone is favoured compared to hydrogenation of cholesterol to coprostanol, which takes place within the intestine. Grimalt, Fernandez [21] utilised the relative amounts of the coprostanols ( $5\beta$  stanols) to the summation of the coprostanols and cholestanols ( $5\beta + 5\alpha$  stanols) as a means of differentiating urban sewage pollution from natural sources. Another ratio that has been used for faecal source identification is that of coprostanol to 24-ethylcoprostanol. Coprostanol is the principal human faecal sterol, and 24-ethylcoprostanol (the  $C_{29}$  homologue of coprostanol) is the principal faecal sterol excreted by herbivores. It was therefore proposed that it was possible to determine the contribution of faecal matter from these two sources relative to each other by calculating the ratio of coprostanol to 24-ethylcoprostanol in human and herbivore (sheep, cow, etc.) faeces [10] and then compare them to those obtained for environmental samples [10,22]. This ratio was first proposed by Bethell, Goad [23]

and later by Evershed and Bethell [24] to differentiate between human and herbivore sources of faecal contamination. Values greater than 1.5 were considered indicative of human pollution [19].

Recently we identified potential sources of faecal streptococci and *E. coli* affecting oyster quality in the River Blackwater Estuary, UK [2]. The transition from low to high tide significantly decreased the concentration of faecal streptococci in waters overlying the oyster beds, indicative of a freshwater input of faecal pollution in oyster bed waters. However, we were unable to confirm the source of the pollution. Here, we aim to identify the major source(s) of faecal pollution impacting the Blackwater Estuary oyster fisheries in the UK through the examination of the sterol profiles of major suspected inputs of faecal pollution into the oyster shellfisheries and of environmental samples (water, animal and bird faeces and oysters) collected from the area surrounding the oyster beds. In addition, the potential use of the various proposed sterol ratios for identification of the main source of faecal pollution impacting the oysters from environmental samples was investigated.

## 2. Materials and Methods

### 2.1. Description of the Sampling Area

Salcott Creek/Channel is located to the west of West Mersea Island at the mouth of the Blackwater Estuary of the North Sea [2]; it occupies a surface area of approximately 18 km<sup>2</sup> (Figure 1). The different sampling sites used in this study are summarised in Table 1.

**Table 1.** The different sampling sites used in the sterol study.

Site No	Site Name	Source	Comments
1	Quinces Corner input (QC)	Water	Water from (a) sheep grazing farmland and (b) fields where large flocks of Brent geese rest, especially during the winter period, drains into the creek.
2	Salcott STW (outlet pipe)	Water	Secondary treated wastewater from Salcott Sewage treatment work (STW) drains into the creek via an outlet pipe.
3	Virley (Tiptree STW)	Water	Water originates from Tiptree STW. Sewage effluent from Tiptree STW is piped for approximately 3.5 km and then discharged at Virley Brook.
4	Oyster Beds (OB)	Water, oysters	Water samples from above oyster beds.

NB. Sites 1–3 constitute the main suspected inputs of faecal pollution in Salcott Creek.

### 2.2. Sample Collection

Water samples (5 L) were collected monthly (with the exception of August and October) over 12 months in glass containers with Polytetrafluoroethylene (PTFE) lids cleaned using 3% Decon 90, rinsed with distilled water and oven dried. Faecal samples were collected in cleaned glass containers and then covered with aluminium foil. Bird droppings and sheep faeces were collected from fields near Site 1. Oysters were collected from Salcott Creek oyster beds (Site 4) and stored in an insulated box containing ice packs. All samples were kept at 4 °C until analysis. Due to adverse weather conditions, no oysters were collected in January, April, August or October.

### 2.3. Sterol Extraction and Analysis

Water was filtered through pre-combusted (at 450 °C) GFF glass fibre filters (Whatman, nominal pore size = 0.45 µm, diameter = 47 mm). Water samples were quantitatively extracted using the one-phase CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O Bligh and Dyer method [25] with a ratio of 1:2:0.8 (v/v/v). In this study, 30 mL of H<sub>2</sub>O, 75 mL of MeOH and 37.5 mL of CHCl<sub>3</sub> were added to a separation funnel together with 10–20 µg of internal standard (IS, 5α-cholestane). Sterols were derivatised into their corresponding trimethylsilyl (TMS) ethers by the addition of 100 µL of BSTFA (bis(trimethylsilyl)trifluoroacetamide, Sigma Aldrich, St. Louis, MO, USA).

### 2.4. Sterol Extraction from Oyster and Faecal Samples Using Hexane

Faecal samples (40–50 g wet weight, triplicate) were refluxed in 150 mL of 6% KOH in methanol for 4 h. The resulting liquid phase was centrifuged at 2500 rpm for 5 min. The supernatant was then extracted twice with hexane (30 mL). The hexane fractions containing the sterols were combined and reduced to less than 5 mL by rotary evaporation. Oyster flesh (n = 6 for each analysis) was cleaned by scrubbing and washing in cold distilled water and, following removal, the flesh was freeze dried for 24 h, and 10–15 g of the dried flesh was homogenised and extracted using hexane [26]. Samples extracted with hexane were derivatised as described above.

### 2.5. Sterol Analysis

GC analyses were performed with a Carlo Erba 8060 GC, fitted with a Fisons Mass Detector 800 (i.e., a mass spectrometer, MS). The GC conditions were as follows: separation on an HT-5 (=high temperature) column with a 25 m × 0.22 mm ID and 0.1 µm film thickness using a cool on column injector and a temperature program of injection at 60 °C for 2 min; 10 °C min<sup>-1</sup> to 300 °C; 3 °C min<sup>-1</sup> to 320 °C; 10 °C min<sup>-1</sup> to 360 °C for 10 min. Helium was used as the carrier gas at a flow rate of 2 mL min<sup>-1</sup>, and 1 µL injections were carried out on the GC using an auto-injector (AS-800). The MS was used in the EI mode at 70 eV with mass scanning from 45 to 585 m/z over 1 s. The results were analysed using the MassLab 1.4 software package (ThermoQuest, Manchester, UK) provided with the instrument. Identification of sterols was performed through a combination of standards and application to the National Institute of Standards and Technology (NIST) mass spectral library [13]. Peak identification was based on a relative retention time and total ion mass spectral comparison with an external standard. Sterols were quantified by peak area comparison with the internal standard 5α-cholestane. Extraction efficiency was between 85 and 110%; day-to-day variability showed a relative standard error of <10%. The limit of detection was 0.01 µg L<sup>-1</sup>.

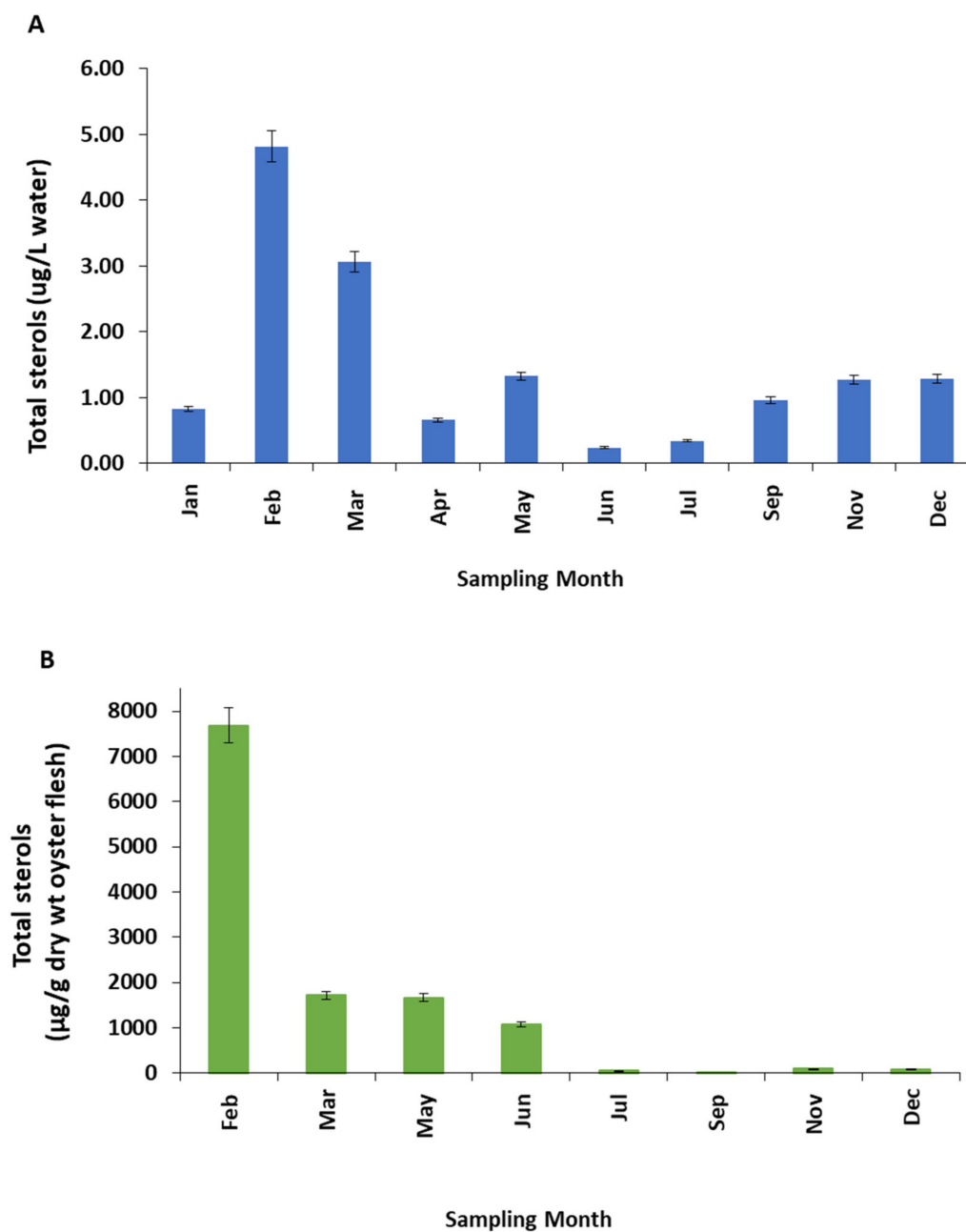
### 2.6. Statistical Analyses

Analysis of variance (ANOVA;  $p = 0.05$ ) was used to determine significant differences among the major sterols and total sterols per month over the 12-month period. Prior to analysis, the homogeneity of variance and normal distribution were assessed. When normality was not fulfilled, data transformation was performed prior to ANOVA (e.g., log transformation) [27]. As ANOVA only identifies significant differences among treatments, to identify treatment effects, the Tukey test ( $p = 0.05$ ) was used. Pearson correlation between sterols in oyster flesh and potential sources of faecal contamination, including both water and animal faecal samples was carried out using SPSS version 24. Both  $p$  values, 0.01 and 0.05, were used for correlation analysis. Results are presented as mean ± standard error unless otherwise stated.

### 3. Results and Discussion

#### 3.1. Sterol Concentrations of Oysters and Overlying Water Sampled throughout the Year

To determine the extent of contamination of faecal sterols, the concentration of faecal sterols in both oyster flesh and the overlying water (Site 4) was assessed over 12 months (Figure 2). In terms of sterol concentrations, as expected, the concentrations found in the water were significantly lower than those found in oyster flesh, with values varying from  $0.25 \mu\text{g L}^{-1}$  in June up to  $4.8 \mu\text{g L}^{-1}$  in February ( $p < 0.05$ , Figure 2). The highest sterol concentrations in the overlying water were observed in February and March ( $4.8 \mu\text{g L}^{-1}$  and  $3.05 \mu\text{g L}^{-1}$ , respectively) (Figure 2a).



**Figure 2.** Total sterol content in (A) water ( $\mu\text{g L}^{-1}$ ) collected from the oyster beds (Site 4), and (B) ( $\mu\text{g g}^{-1}$  oyster flesh) over a 12-month period (mean  $\pm$  standard error). Due to adverse weather conditions, no oysters were collected in January, April, August or October; no water samples were collected in August and October.

The total sterol content of oysters varied significantly, from  $53 \mu\text{g g}^{-1}$  in September to  $7686 \mu\text{g g}^{-1}$  in February, corresponding to the highest concentrations of sterols in waters above the oysters ( $p < 0.05$ ). High total sterol concentrations were also recorded in March, May and June (1720, 1665 and  $1067 \mu\text{g g}^{-1}$ , respectively) (Figure 2b).

### 3.2. Sterol Profile of Oysters and Overlying Water Sampled throughout the Year

The sterol profile of the water collected from above the oyster beds (Site 4) is shown in Table 2. In February, the sterol profile of water was dominated by coprostanol ( $p < 0.05$ ), suggesting human contamination. The absolute concentration of the major sewage biomarker (coprostanol) varied between  $0.01 \mu\text{g L}^{-1}$  on many sampling occasions and  $1.20 \mu\text{g L}^{-1}$  in February, a value significantly higher than all of the other sterols assessed ( $p < 0.05$ ). Leeming, Ball [10] carried out sterol analyses of surface water samples along the Derwent Estuary, Australia; coprostanol was detected in all samples, including those taken at sites remote from the point sources of sewage, with concentrations ranging from  $0.007$  to  $3.465 \mu\text{g L}^{-1}$ . Goodfellow, Cardoso [28] also measured the concentrations of coprostanol in the waters of the Clyde Estuary, Scotland at different sites, with increasing distance from a sewage treatment works. The concentration of coprostanol was reported as  $47.5 \mu\text{g L}^{-1}$  at the point of discharge and decreased as the distance increased. At  $3.6 \text{ km}$  downstream from the discharge point, levels of coprostanol were  $3.8 \mu\text{g L}^{-1}$  and still detectable  $20 \text{ km}$  downstream, though at trace amounts. Gilpin, Gregor [29] carried out sterol analyses of river water samples collected at the point of input of human effluent from oxidation ponds and  $125 \text{ m}$  further downstream. They found that the concentrations of coprostanol, as well as that of other sterols/stanols, decreased significantly even  $100 \text{ m}$  downstream from the effluent. This decrease was due to an increasing dilution effect. In this study, the oyster beds were located approximately  $4.1 \text{ km}$  downstream of the Salcott STW discharge pipe and  $7.7 \text{ km}$  from Tiptree STW (discharge point at Virley). The concentrations of coprostanol in the final effluents of these two sewage treatment works were statistically different,  $12.21 \pm 5.77 \mu\text{g L}^{-1}$  for Salcott and  $0.46 \pm 0.16 \mu\text{g L}^{-1}$  for Tiptree STW ( $p < 0.05$ ).

The sterol profile of oysters was dominated on all sampling occasions by cholesterol. The relative abundance of this sterol ranged from  $51\%$  (July) to  $66\%$  in March (Table 3). Previously, Cathum and Sabik [30] detected high levels of cholesterol in mussels. Cholesterol is a constituent of animal cell membranes and therefore found in most organisms [31]. As a consequence, the use of cholesterol alone as a biomarker for organic matter is somewhat limited due to the diversity of potential source organisms [32,33]. Brassicasterol was the second most abundant sterol present in oysters, except for in July and September, where it was replaced by 24-ethylcoprostanol. The principal herbivore biomarker, 24-ethylcoprostanol, was present in all oyster samples but at a relatively low level, varying between  $0.03 \mu\text{g g}^{-1}$  (September) and  $10.48 \mu\text{g g}^{-1}$  (July); the relative abundance (% of total sterol) of 24-ethylcoprostanol was less than  $1\%$ , except for in July and September, where it was  $19.74\%$  and  $15.59\%$ , respectively (Table 3). Therefore, generally, herbivores (in this study, sheep) did not seem to be the major contributor of faecal pollution in oysters collected from Salcott Creek. Brassicasterol and 22-dehydrocholesterol are the main sterols of phytoplankton [20,32]. The actual concentration of brassicasterol in oysters varied significantly ( $p < 0.05$ ), from  $0.02 \mu\text{g g}^{-1}$  in September to  $1044.90 \mu\text{g g}^{-1}$  in February, with relative abundance varying between  $12\%$  in March and  $24\%$  in November. Oysters are filter feeders, and phytoplankton are their main source of food. Therefore, the presence of algal sterols in oyster samples was expected. The third most abundant sterol found in oysters was  $\beta$ -sitosterol (Table 3). The source specificity of this sterol is diverse. Although it is the principal sterol of terrestrial plants, there is evidence that this sterol can also occur in some species of phytoplankton [32,34].  $\beta$ -Sitosterol is the major sterol in bird faeces and is also found in the faeces of sheep. In February and March, when the high sterol concentrations were found in oysters, high bird populations (wildfowl species) inhabiting the estuary were observed. Since cholesterol and  $\beta$ -sitosterol were found to be the two major sterols in bird faeces in this and other studies [10,35,36], the high cholesterol levels recorded in February and March might originate from birds. Coprostanol, the major sewage (human) biomarker,

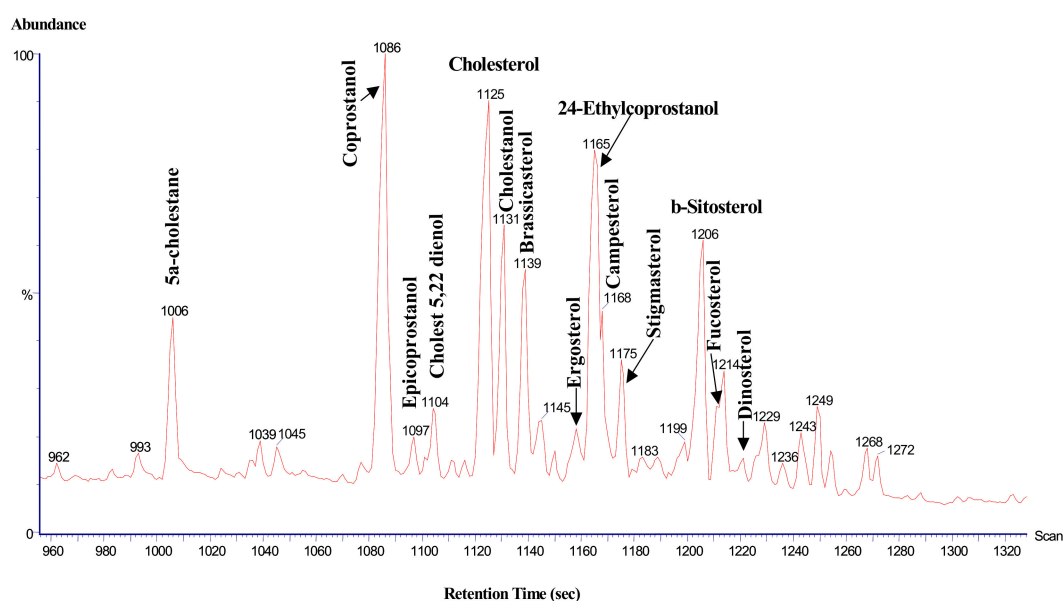
was detected in all of the oyster samples analysed, although the concentration of coprostanol was low, ranging from  $<0.01 \mu\text{g g}^{-1}$  (September) to  $8.67 \mu\text{g g}^{-1}$  (February) (Table 3). Cathum and Sabik [30] carried out sterol analyses of mussels contaminated by sewage, and found coprostanol at a concentration of  $32.25 \mu\text{g g}^{-1}$  dry wt. Gagné et al. [37] also measured coprostanol in freshwater mussels caged 5 km downstream of an effluent plume (primary treated effluent) and found that the sewage biomarker had accumulated in those mussels at a concentration of  $15.50 \pm 6.20 \mu\text{g g}^{-1}$  dry wt.

### 3.3. Sterol Ratio Analysis of Oysters and Overlying Water Sampled throughout the Year

To attempt to determine the origin of the sterol in waters above the oyster beds, the relative concentrations (ratios) of key sterols were examined (Table 4). When examining the coprostanol/epicoprostanol ratio, in all samples, ratios were higher than 1, ranging from 1.32 (September) to 33.25 (February) (Table 4). This ratio is used as a sewage marker, where  $>1$  indicates pollution from human sewage and suggests that human sewage represents a key input of faecal material into Salcott Creek. The ratio is also used to indicate the degree of treatment that sewage received, because the epicoprostanol isomer is principally formed during the treatment of sewage and is only found in trace amounts in human faeces [32]. The coprostanol/cholesterol ratio (another sewage marker) for all samples was significantly lower ( $p < 0.05$ ) than 1, except for February, when it was equal to 1.09 (Table 3). A third ratio commonly used as a sewage marker was the  $5\beta/(5\beta + 5\alpha)$  ratio; it ranged from 0.37 (May) to 0.90 (June). The  $\beta$ -sitosterol/cholesterol ratio was, for most samples, less than 1, except for in September and November, when it was 1.23 and 1.01, respectively. Finally, the coprostanol/24-ethylcoprostanol ratio varied between 0.82 (September) and 21.72 (April). However, for most samples, it was greater than 1.5 (Table 3), again suggesting sewerage effluents as a major contributor to faecal pollution in the waters above the oysters (Site 4).

### 3.4. Sterol Profile Analysis of Potential Faecal Inputs

Previous work identified the upstream nature of the faecal pollution and identified the potential sources of faecal contamination [2] (Table 1). In particular, the Salcott Sewage Treatment outfall was highlighted as a potential major source of faecal pollution. The sterol profile of the water sampled at the outfall (Salcott STW, Figure 1) is shown in Figure 3.



**Figure 3.** Full scan GCMS total ion chromatogram showing sterols detected in a sediment sample collected from Salcott Creek, next to the sewage works outlet pipe.

**Table 2.** Concentrations of 11 major sterols and total sterols ( $\mu\text{g g}^{-1}$  L water) in water collected from above the oyster beds (Site 4) in Salcott Creek over 12 months.

Sterols ( $\mu\text{g g}^{-1}$ L Water)	January	February	March	April	May	June	July	September	November	December
Coprostanol	0.03	1.20	0.11	0.09	0.02	0.02	0.01	0.01	0.04	0.07
Epicoprostanol	<0.01	0.04	0.01	0.01	0.01	<0.01	0	0	0.01	<0.01
22-dehydrocholesterol	0.02	0.02	0.12	0.02	0.13	0.01	0	0	0.03	0.02
Cholesterol	0.38	1.10	1.70	0.34	0.74	0.15	0.19	0.79	0.45	0.50
Cholestanol	0.01	0.54	0.04	0.07	0.03	<0.01	<0.01	0.01	0.02	0.03
Brassicasterol	0.03	0.04	0.18	0.04	0.22	0.03	0.08	0.07	0.07	0.06
Campesterol	0.03	0.05	0.13	0.02	0.04	<0.01	0.01	0.01	0.04	0.04
Ergosterol	<0.01	0.01	<0.01	<0.01	0.01	<0.00	<0.01	<0.01	<0.01	<0.01
Stigmasterol	0.03	0.05	0.05	<0.01	0.01	<0.01	<0.01	0.01	0.05	0.04
$\beta$ -Sitosterol	0.27	0.42	0.36	0.03	0.05	<0.01	0.01	0.02	0.45	0.35
24-Ethylcoprostanol	0.02	0.24	0.06	<0.01	0.01	<0.01	0.04	0.05	0.02	0.03
Total	0.79	2.51	2.65	0.53	1.25	0.19	0.33	0.96	1.14	1.07

Nota bene (N.B.) No water was collected in August and October.

To examine the potential source of the sterols present in the oyster flesh, particularly in the months when high concentrations were observed, sterols were identified and quantified by Gas Chromatography Mass Spectrometry (GCMS) (Table 3). A total of 11 sterols/stanols were detected.



**Table 3.** Concentrations of 11 major sterols and total sterols ( $\mu\text{g g}^{-1}$  dry wt oyster flesh) in oysters collected from Salcott Creek over 12 months.

Sterols ( $\mu\text{g g}^{-1}$ Dry wt Oyster Flesh)	Sampling Months							
	February	March	May	June	July	September	November	December
Coprostanol	8.67	4.80	1.99	1.77	0.13	<0.01	0.09	0.06
Epicoprostanol	0.17	0.15	0.79	0.05	0	0	<0.01	<0.01
22-dehydrocholesterol	461.22	92.37	124.86	50.29	0	0	0	0
Cholesterol	4661.04	1134.92	904.41	671.39	27.29	0.11	52.79	44.82
Cholestanol	62.34	19.66	19.41	11.35	0.47	<0.01	1.29	1.03
Brassicasterol	1044.90	202.53	266.83	155.44	6.78	0.02	23.33	21.09
Campesterol	439.73	89.71	102.83	61.41	2.82	0.01	7.88	6.80
Ergosterol	215.01	42.24	42.18	14.53	1.26	0.01	0.81	1.52
Stigmasterol	97.00	24.20	35.10	14.20	0.70	<0.01	1.90	2.20
B-Sitosterol	693.68	107.96	158.73	85.76	3.15	0.01	9.77	10.56
24-Ethylcoprostanol	1.94	1.76	8.61	0.54	10.48	0.03	0	0
Total	7686	1720	1666	1067	53.07	0.189	97.85	88.09

N.B. Due to adverse weather conditions, no oysters were collected in January, April, August or October.

**Table 4.** Ratios of sterols for water samples collected from above the oyster beds (Site 4) over 12 months.

Sterol Ratios	January	February	March	April	May	June	July	September	November	December
Coprostanol/epicoprostanol	8.07	33.25	7.99	10.86	3.47	5.13	0	1.32	7.03	18.61
Coprostanol/cholesterol	0.08	1.09	0.07	0.25	0.03	0.12	0.04	0.17	0.09	0.15
5 $\beta$ /(5 $\beta$ + 5 $\alpha$ )	0.77	0.69	0.76	0.55	0.37	0.90	0.61	0.81	0.73	0.73
$\beta$ -sitosterol/cholesterol	0.71	0.38	0.21	0.08	0.07	0.03	0.05	1.23	1.01	0.70
Coprostanol/24-ethylcoprostanol	1.70	4.95	1.80	21.72	2.08	4.02	0.20	0.82	2.11	2.48

NB. No water samples were collected in August and October.

The sterol profile of the final effluent collected from Salcott STW was dominated by coprostanol (34.45%) and cholesterol (22.37%). The third most abundant sterol was 24-ethylcoprostanol, accounting for 13.15% of the total sterol (Figure 3). For comparison, sterols were profiled from samples, either water or faecal (sheep and geese), taken at the other sites suspected of contributing faecal contamination into the oyster bed waters (Table 5). Sterol profile analyses were carried out on faecal samples from sheep and birds (Brent geese), as those two groups were identified as possible sources of faecal contamination in Salcott Creek.

**Table 5.** Assessment of sterol profiles of potential inputs of faecal material into waters above the oyster beds (mean  $\pm$  standard error).

Sterol	Sheep N = 3	Brent Geese N = 3	QC Water N = 3	STW Water N = 5	Virley Brook Water N = 4
Coprostanol	58.53 $\pm$ 25.78	1.13 $\pm$ 1.12	5.87 $\pm$ 4.53	12.21 $\pm$ 5.77	0.46 $\pm$ 0.16
Epi-coprostanol	0	0	0.06 $\pm$ 0.03	0.28 $\pm$ 0.09	0.04 $\pm$ 0.01
22-dehydrocholesterol	0	0	0	0.08 $\pm$ 0.05	0
Cholesterol	40.30 $\pm$ 10.81	27.06 $\pm$ 6.67	5.40 $\pm$ 1.88	7.73 $\pm$ 3.66	0.91 $\pm$ 0.23
Cholestanol	10.89 $\pm$ 2.55	0.17 $\pm$ 0.05	0.55 $\pm$ 0.27	1.96 $\pm$ 1.19	0.02 $\pm$ 0.01
Brassicasterol	1.13 $\pm$ 0.52	0.18 $\pm$ 0.10	0.37 $\pm$ 0.17	0.09 $\pm$ 0.03	0.02 $\pm$ 0.01
Campesterol	31.97 $\pm$ 22.38	6.50 $\pm$ 2.85	0.84 $\pm$ 0.23	0.66 $\pm$ 0.33	0.05 $\pm$ 0.02
Ergosterol	9.87 $\pm$ 2.25	2.72 $\pm$ 1.39	0.12 $\pm$ 0.03	0.04 $\pm$ 0.02	0.01 $\pm$ 0.00
Stigmasterol	29.76 $\pm$ 24.29	1.04 $\pm$ 0.24	0.48 $\pm$ 0.25	0.21 $\pm$ 0.12	0.07 $\pm$ 0.03
$\beta$ -Sitosterol	277.33 $\pm$ 248.24	34.31 $\pm$ 16.22	3.03 $\pm$ 1.20	1.55 $\pm$ 0.84	0.35 $\pm$ 0.14
24-Ethylcoprostanol	211.83 $\pm$ 112.30	0.19 $\pm$ 0.17	2.39 $\pm$ 1.22	5.08 $\pm$ 3.21	0.17 $\pm$ 0.04
Total sterols ( $\mu\text{g L}^{-1}$ water/ $\mu\text{g g}^{-1}$ faeces)	671.61 $\pm$ 371.73	73.30 $\pm$ 25.87	19.10 $\pm$ 8.10	29.90 $\pm$ 13.83	2.10 $\pm$ 0.62

Cholesterol (43.57%) and  $\beta$ -sitosterol (37.22%) were the major sterols present in the faeces of Brent geese, while the rest of the sterols detected were present at low levels (Table 5). Coprostanol, the major sterol of human waste, was present at 5.08%, indicating that in bird faeces, anaerobes capable of reducing sterols to 5 $\beta$ -stanols were present in low numbers. Leeming, Ball [10] found that sitosterol, cholesterol, stigmasterol and isofucosterol were the major sterols present in bird faeces; Leeming, Latham [22] confirmed that sitosterol and cholesterol were the two most abundant sterols present in bird faeces. However, Leeming, Ball [10] examined the sterol profiles of various species of birds and reported considerable variation in the amounts of major sterols detected in their faeces.

The sterol profile of sheep faeces was dominated by 24-ethylcoprostanol (31.91  $\pm$  3.02%). Other major sterols included  $\beta$ -sitosterol (25.14  $\pm$  14.95%) and coprostanol (18.11  $\pm$  8.62%). Minor sterols were cholesterol and campesterol, while the rest of the sterols were present at very low abundance (Table 5). The total sterol concentration in sheep faeces was 671.61  $\pm$  371.73  $\mu\text{g g}^{-1}$ . The principal herbivore biomarker 24-ethylcoprostanol is produced in the gut of herbivores by the reduction

of 24-ethylcholesterol ( $\beta$ -sitosterol) [29]. The 24-ethylcholesterol found in sheep faeces originates from the plants they eat. In the digestive system of sheep, the microbial population converts 24-ethylcholesterol into 24-ethylcoprostanol preferentially. This is the same process that occurs in humans, with cholesterol being preferentially converted to the  $5\beta$ -stanol, coprostanol [38].

The results from the current study agreed with other reports [10,22]. Hargan, Stewart [36] also showed that 24-ethylcoprostanol was the predominant sterol in sheep faeces, followed by sitosterol, coprostanol and finally cholesterol. Leeming et al. [10] reported that the total sterol content in sheep faeces was  $1308 \pm 87 \mu\text{g g}^{-1}$ ; this value is two-fold greater than that reported in this study. This was possibly due to the fact that Leeming et al. [10] measured the concentrations of 17 different sterols, while only nine sterols were used/available in this study.

The total sterol concentration in Salcott STW water was significantly greater ( $29.90 \mu\text{g g}^{-1}$ ) than any other water sample ( $p < 0.05$ ), although lower than sterol concentrations in sheep and Brent geese faecal samples (Table 5). In human faeces, coprostanol and 24-ethylcoprostanol are the predominant sterols, but in sewerage effluents, 24-ethylcoprostanol was replaced by cholesterol as the second most abundant sterol because of additional inputs of this sterol from non-faecal sources, such as domestic wastes, food scraps, etc. [10]. The mean coprostanol concentration detected in Salcott STW effluent samples was  $12.21 \pm 5.77 \mu\text{g L}^{-1}$ , which was comparable to that reported previously [30], where coprostanol was measured in the final effluent from a sewage treatment plant (STP) at a concentration of  $14.667 \mu\text{g L}^{-1}$ . Leeming et al. [10] measured the concentrations of coprostanol in effluents from five different sewage treatment plants that underwent secondary treatment. The coprostanol concentration ranged from 8 to  $369 \mu\text{g L}^{-1}$ . The mean coprostanol concentration detected in Salcott STW effluents fell within that range. The sterol profile of water collected from Virley Brook (Tiptree STW discharge point) was dominated by cholesterol. Other major sterols included coprostanol,  $\beta$ -sitosterol and 24-ethylcoprostanol (Table 5). The mean total sterol content of water samples collected from Virley Brook was significantly lower ( $p < 0.05$ ) compared to that of Salcott STW ( $2.10 \pm 0.62$  vs.  $29.20 \pm 13.83 \mu\text{g L}^{-1}$ , respectively, Table 5).

The sterol profile of water collected from the QC input was dominated by cholesterol. Other major sterols included coprostanol and sitosterol, and the fourth most abundant sterol was 24-ethylcoprostanol (Table 5). Water at QC is discharged through a drainage pump and originates from adjacent farmland (sheep) and from surrounding fields where large flocks of Brent geese rest in the winter period. Therefore, cholesterol at QC could have originated from two different sources (sheep and birds). Coprostanol mainly originates from sheep, as it is rarely present in bird faeces;  $\beta$ -sitosterol may originate from three possible sources (birds, sheep and terrestrial plants); 24-ethylcoprostanol mainly originates from sheep, as it is the principal herbivore biomarker (Table 5).

### 3.5. Correlation Analysis of Overlying Water Site 4 with Water Sampled from Sites 1–3

As expected, as oysters are filter feeders, the Pearson correlation analysis (Table 6) confirmed a significant correlation between the sterol profile of the oysters and the sterol profile analysis of the overlying water ( $r = 0.91$ ,  $p < 0.01$ ). However, two other interesting correlations were observed (Table 6). Firstly, there was a correlation between the sterol profile of water above the oysters and the oyster flesh with the sterol profile of water taken from Site 3, Virley Brook, the location where treated water from Tiptree Sewage Treatment Works enters the creek ( $r = 0.82$ ,  $p < 0.01$  and  $0.70$ ,  $p < 0.05$ , respectively); secondly, there was a correlation between water from above the oysters (Site 4) and water sampled from Site 1, Quinces Corner ( $r = 0.71$ ,  $p < 0.05$ ). Quinces Corner was observed to have a high bird population, and the correlation of water from above the oysters with the faecal sterol profile of Brent geese ( $0.82$ ) confirmed the possibility that faecal material from large bird populations may be in part responsible for the faecal load in the water above the oysters (Table 6).

**Table 6.** Pearson correlation between sterols in oyster flesh and potential sources of faecal contamination, including water and animal faecal samples. The \* and \*\* showed significant differences at 0.05 and 0.01, respectively.

	Oyster Flesh	Sheep	Brent Geese	QC (Site 1)	Salcott STW (Site 2)	Virley Brook (Site 3)	Water above the Oyster Bed (Site 4)
Oyster flesh	-	-0.079	0.60	0.50	0.30	0.70 *	0.91 **
Water above oyster bed	0.91 **	0.24	0.82 **	0.71 *	0.438	0.820 **	1

### 3.6. Sterol Ratio Analysis of the Potential Inputs of Faecal Contamination in Salcott Creek

From the sterol profiles, the five sterol ratios were calculated for the different potential inputs of faecal matter into Salcott Creek and were then compared to those values obtained from environmental samples (water, sediments and oysters) (Table 7).

**Table 7.** Values of five sterol ratios for samples constituting the major inputs of faecal pollution in Salcott Creek (mean  $\pm$  standard error).

Sterol Ratios	QC (Site 1)	Salcott STW (Site 2)	Virley Brook (Site 3)	Waters above the Oyster Beds (Site 4)
Coprostanol/ Epicoprostanol	16.29 $\pm$ 11.97	49.52 $\pm$ 20.26	11.92 $\pm$ 6.43	8.90 $\pm$ 2.50
Coprostanol/ Cholesterol	0.76 $\pm$ 0.48	1.43 $\pm$ 0.18	0.46 $\pm$ 0.10	0.17 $\pm$ 0.07
5 $\beta$ /(5 $\beta$ + 5a)	0.82 $\pm$ 0.07	0.76 $\pm$ 0.12	0.96 $\pm$ 0.02	0.65 $\pm$ 0.04
$\beta$ -sitosterol/ cholesterol	0.63 $\pm$ 0.24	0.33 $\pm$ 0.14	0.35 $\pm$ 0.10	0.44 $\pm$ 0.11
Coprostanol/ 24-ethylcoprostanol	2.13 $\pm$ 0.76	6.59 $\pm$ 4.70	2.33 $\pm$ 0.53	3.57 $\pm$ 1.55

The coprostanol/epicoprostanol ratio was calculated for all of the potential sources in Salcott Creek and values (Table 7). This ratio was  $>1$  for all samples and was therefore not useful in identifying the different sources. This ratio must be used with caution, since a value greater than 1 is not only indicative of a sewage source. In addition, reports of coprostanol and epicoprostanol concentrations in effluents, sludges and environmental samples have shown that this ratio is extremely variable [39]. In Virley and Salcott STW samples, this ratio was significantly greater than 1 ( $p < 0.05$ ), as expected for human sewage. However, the value of the ratio was also greater than 1 for QC wastewater, which is polluted by either sheep or birds. According to the results of this and other studies [40], the suggested coprostanol/epicoprostanol ratio is not indicative of human sewage only when higher than 1. Ratio values obtained from low abundances of faecal sterols (e.g., at sites remote from sewage inputs) should also be treated with caution. However, Gilpin, Gregor [29] found that ratios of sterols remained approximately the same with increasing distances from sewage discharge points, suggesting no preferential loss of any of the chemical indicators.

The coprostanol/cholesterol ratio has also been used as a sewage indicator in many studies, since this normalises for the total lipid content as cholesterol is present in most organisms [13,29,32,41]. A ratio  $> 1$  is indicative of a sewage source, and  $<1$  indicates a biogenic source [18]. This ratio ranged from 0.17 in waters above the oyster beds to 1.43 in Salcott STW water (Table 7). Salcott STW samples had a ratio  $> 1$ , which was expected since coprostanol is the major sterol in human sewage. However, for Tiptree STW effluents, it was  $<1$ ; this was probably due to the excessive inputs of cholesterol from non-faecal sources, thus making coprostanol the second most abundant sterol in the effluent. The ratio

was also  $>1$  for sheep faeces (Table 5); however, by calculating the coprostanol/24-ethylcoprostanol ratio at the same time, differentiation between a herbivore and a sewage source can be made, since 24-ethylcoprostanol is present at higher levels in herbivores [10,22,36].

$5\beta$ -Stanols, such as coprostanol, do not occur naturally in fresh or marine waters or fully oxic sediments because only anaerobic bacteria appear capable of hydrogenating  $\Delta^5$ -sterols to  $5\beta$ -stanols. However, under conditions of anoxia, relatively small amounts of  $5\beta$ -stanols can be found in sediments not contaminated by faecal pollution [42]. In this study, the  $5\beta/(5\beta + 5\alpha)$  ratio ranged from 0.65 to 0.96 for all samples (Table 7); this suggests that  $5\beta/(5\beta + 5\alpha)$  ratios of  $>0.7$  may not be indicative of human sewage only. Chou and Liu [1] also found that in sediments contaminated by livestock, the  $5\beta/(5\beta + 5\alpha)$  ratio was  $>0.7$ .

The  $\beta$ -sitosterol/cholesterol ratio has been used to assess terrestrial (vascular plants) inputs of organic material in marine and estuarine environments [32,43,44]. Since  $\beta$ -sitosterol is the major sterol in vascular plants, a high ratio ( $>1$ ) is indicative of terrestrial plant matter inputs. This ratio was used in this study to investigate whether it can be used for faecal source differentiation. There was no significant difference in the ratio values between the various inputs ( $p > 0.05$ ) (Table 7). All sewage samples had a ratio  $< 1$ , probably due to the predominance of C27 compared to C29 sterols in human sewage [10]. The ratio was also lower ( $<1$ ) for QC water (Site 1) and the waters above the oysters (Site 4), and this might be attributed to the mixed origin of faecal matter at this site. According to these results, this ratio is not only indicative of terrestrial inputs, since  $\beta$ -sitosterol is a major sterol in some animals too. However, it could be indicative of an animal source if faecal bacterial indicators are also present in the system under investigation. The ratio should be used with caution when assessing faecal inputs from birds, since there is a variation in the sterol profiles of bird faeces. This variation has been observed both at the intra and inter-species level, and arises from differences in the relative abundances of the major bird sterols ( $\beta$ -sitosterol and cholesterol) [10,36].

The coprostanol to 24-ethylcoprostanol ratio was proposed by Bethell et al. [23] to differentiate between human and herbivore sources of faecal contamination. Values greater than 1.5 were considered indicative of human pollution [19]. In this study, the coprostanol to 24-ethylcoprostanol ratio was  $>1.5$  for all environmental samples (Table 7), suggesting that it may not be useful to differentiate sources of faecal pollution in environmental samples.

#### 4. Conclusions

This work aimed to identify the major source(s) of faecal pollution impacting the Blackwater Estuary oyster fisheries in the UK through examination of the sterol profiles of water samples collected from the area surrounding the oyster beds (Site 4). In oyster flesh, the total sterol content varied from  $53 \mu\text{g g}^{-1}$  to  $7686 \mu\text{g g}^{-1}$ , with cholesterol dominating the sterol profile, although coprostanol was detected in all of the oyster samples analysed at concentrations ranging from  $<0.01 \mu\text{g g}^{-1}$  (September) to  $8.67 \mu\text{g g}^{-1}$  (February), suggesting that human sewage represents the key input of faecal material into Salcott Creek. Examination of the three potential sources of inputs into Salcott Channel showed that sterols in water sampled at the Salcott STW (Site 3) were dominated by coprostanol (34.45%) and cholesterol (22.37%), while cholesterol was the major sterol present at Quinces Corner (Site 2). Pearson correlation analysis identified a correlation between the sterol profile of water above the oysters (Site 4) with the sterol profile of water taken from Site 3, Virley Brook, where Tiptree STW is discharged ( $r = 0.82, p < 0.05$ ). A correlation between water from above the oysters (Site 4) and water sampled from Site 1, Quinces Corner ( $r = 0.7, p < 0.05$ ) was observed, suggesting that faecal material from large bird populations may be in part responsible for the faecal load in the water above the oysters. The use of sterol ratios in profiles from the water samples was found not to be useful in identifying the sources of pollution, perhaps due to the complex and diverse inputs at this site. However, sterol profiling was found to be useful in further identifying the key faecal inputs, allowing targeting of management practices to be employed to ensure that oyster quality can be improved.

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