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The Use of Coprostanol, Epi-Coprostanol and Cholesterol in Evaluating Potential Sewage Input from Downstream River towards Marine Environment

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Contamination of sewage is a major concern in the river and marine environments since sewage can cause disease and ecosystem health problems. Worldwide, coprostanol, coprostanol and epi-coprostanol were used in sewage assessment due to their resistance towards environmental stressor. This study assessed the distribution of coprostanol, epi-coprostanol and cholesterol in the particulate of sewage treatment plant (STP) effluents and river water samples. The targeted analytes were extracted using sonication and quantified using gas chromatography-mass spectrometer (GC-MS). The potential sewage pollution in the river and marine environments were assessed using diagnostic approach and linear regression technique. Based on the result, cholesterol (mean = 15.6 mg L^{-1}) was found to be higher than coprostanol (mean = 6.0 mg L^{-1}) and epi coprostanol (mean= 2.3 mg L^{-1}) in STPs samples. Similar to STPs, cholesterol (mean= 4.6 mg L^{-1}) also was detected higher than coprostanol (mean= 2.4 mg L^{-1}) and epi-coprostanol (mean= 2.4 mg L^{-1}) in the river water samples. The double plot ratios of epi-coprostanol/cholesterol versus epi-coprostanol/coprostanol revealed the discharge of treated and untreated sewage into the river water. The river water samples were impacted by treated sewage and non-human sources. The linear regression analysis indicates potential moderate to high increasing trend (p<0.05) of sewage discharge towards downstream of the river. These findings provide an enhanced means in assessing sewage contamination input into the river and its potential effect towards the marine ecosystem.

Keywords: Sewage pollution; coprostanol; epi-coprostanol; diagnostic ratios; linear regression

I. INTRODUCTION

Tropical coastal and marine environments receive the major part of the global annual riverine inputs of freshwater, including dissolved and particulate substances into the ocean. The inputs shelter a number of diverse ecosystems and most of the population, which in turn depend economically on their natural resources (Loh *et. al.,* 2008; Raymond & Bauer, 2001). As a consequence, rivers being the most important transport media for anthropogenic inputs such as sewage from the urban area into the ocean. This incident also contributes towards the steadily declining of the biologically diverse ecosystem in the world (Wear & Thurber, 2015).

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Furthermore, there is evidence to link sewage runoff to coastal eutrophication, harmful algal blooms and toxic substance accumulation in coastal water (Barrington *et. al.,* 2015, 2013; Lapointe *et al.,* 2017). The particular concerns are the ecological consequences of sewage in coastal areas since marine areas are typically among the most productive ecosystems. The ongoing growth in the coastal population and the consequent increase in discharges of faecal sources are also thought to be responsible for the deterioration of the quality of river and seawater (Magam *et. al.,* 2016; Speranza *et al.,* 2018).

Generally, sources of faecal contamination in the water are classified into "nonhuman" faecal contamination (natural sources, e.g., migratory and local wildfowl, sheep and cattle, effluent from dairies, poultry farms, piggeries, and slaughterhouses) and "human/sewage" faecal contamination (Hussain *et al.,* 2010). Globally, chemical markers such as faecal sterols and stanols were analysed to determine the level and the extent of sewage contamination in water bodies. These techniques have environmental stability, ease of detection and can be used to discriminate between human and non-human contamination (Furtula *et. al.,* 2012a; Martins *et. al.,* 2007; Nichols *et. al.,* 1993; Saim *et. al.,* 2009; Tran *et al.,* 2015).

In many sewage assessments studies, cholesterol, coprostanol and epi-coprostanol were investigated to reveal the severity of sewage pollution (Alsalahi *et. al.,* 2015; Mudge & Duce, 2005). Cholesterol is among the abundant sterols exist in sewage and also act as a marker of marine sterol, invertebrates and marine zooplankton (Loh *et. al.,* 2006; Mudge & Bebianno, 1997; Volkman, 1986). Cholesterol alone was not preferred to be used in sewage assessment, considering its ubiquitousness in various faecal animals, sewage and biogenic sources (Antanasijević *et. al.,* 2018; Shah *et al.,* 2007a). Cholesterol was often been utilised with coprostanol and epi-coprostanol in sewage identification studies.

Coprostanol is produced by the metabolism of cholesterol in the human/animal guts and was among the highest concentrations found in sewage effluent between 40-60% percent from total sterols (%) (Leeming *et. al.,* 1996; McCalley *et. al.,* 1981; Nichols *et. al.,* 1996; Shah *et al.,* 2007a) Coprostanol also has been reported in sewage-contaminated surface waters (Furtula *et. al.,* 2012b; Gottschall *et. al.,* 2013; Saim *et al.,* 2009) and sediments (Carreira *et. al.,* 2002; Frena *et. al.,* 2016b; Pratt *et al.,* 2007). In the meantime, epi-coprostanol is derived by coprostanol biosynthesis using microbes in sewage treatment plants (STPs) and is further used to differentiate between treated, partially treated and untreated sources of sewage (Furtula *et. al.,* 2012a; Martins *et. al.,* 2007; Reichwaldt *et al.,* 2017).

The use of chemical markers was often combined with diagnostic ratios and statistical analysis such as linear regression to provide an informative overview of the correlation between chemical markers and trend analysis of the targeted analytes. The double plot ratio of coprostanol/cholesterol against epi-coprostanol/ cholesterol also was demonstrated to indicate more accurate and precise sewage assessment (Kolm *et. al.,* 2018; Lyons *et. al.,* 2015; Mudge & Duce, 2005). The ratio of coprostanol/cholesterol with value >0.5 indicates sewage contamination while < 0.5 was non-human faecal contamination (Quéméneur & Marty, 1992; Carreira *et al.,* 2004) and the ratio of epi-coprostanol/coprostanol indicate sewage treatment efficiencies with values <0.2 implied untreated sewage; 0.2-0.8 is partially treated sewage and > 0.8 is categorised as treated sewage (Mohd Ali *et. al.,* 2015; Mudge & Duce, 2005). The use of statistical analysis such as linear regression also has been successfully implemented in assessing sewage contamination status in rivers and marine areas (Cabral *et. al.,* 2018; Juahir *et. al.,* 2010; Martins *et. al.,* 2014; Nasir *et. al.,* 2011; Tyagi *et al.,* 2007).

This research was conducted at Linggi River, Negeri Sembilan, an area under intense pressure from rapid urban and rural development with a growing population (Abdul Zali *et. al.,* 2021; Aburas *et. al.,* 2017; MAMPU, 2020a, 2020b). Due to that condition, possible sources of pollution of the Linggi River may arise from undermining or old-technology STPs, improper maintenance of individual septic tanks, domestic waste, improper handling of animal farms as well as agricultural runoffs. Furthermore, insufficient treatment of sewage discharge is one of the persistent environmental problems which lead to the degradation of the quality of streams and marine water (Ariffin & Sulaiman, 2015; Nanyan *et. al.,* 2016; Praveena *et al.,* 2015). In recent studies, Linggi River is reported to receive the impact of major pollution

from sewage, domestic, industrial and agricultural activities. (Elias *et. al.,* 2018a, 2018b; Fadhil *et. al.,* 2015; Khalik *et al.,* 2015).

Therefore, a precise pollution assessment using molecular markers must be performed to protect the water sources and marine areas of the desired area. This study aims to determine the sewage sources using diagnostic ratios and potential sewage contamination from upstream towards marine environment by using linear regression technique.

II. MATERIALS AND METHOD

Sampling was conducted in April 2018 at Linggi, Negeri Sembilan. All samples were collected in duplicate from selected STP facilities (STP2, STP4 and STP5) and nearby river water samples (RW1, RW2 and RW3) (Figure 1). The river water samples were collected after the discharge point from nearby STPs. A detailed description of the sampling sites is listed in Table 1. The STPs and river water samples were collected using pre-cleaned bucket. The water samples were filtered using pre-combusted (4 h at 450°C) GF/F Whatman (0.7 µm pore size) to obtain particulate samples by filtering 100 ml STP effluents and 1 L river water samples. The particulate samples were kept in a cooler box during transportation.

In the laboratory, the particulate samples obtained were stored in -20°C and freeze-dried in two weeks prior to analysis. The calibration standards for coprostanol, epi-coprostanol and cholesterol were purchased from Chiron AS, Norway and solvents (dichloromethane (DCM), methanol and iso-octane) were obtained from Fisher Scientific International Inc. The derivatising agent for the sterols and stanol compounds was N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA): trimethylchlorosilane (TMCS), 99:1 (Supelco, USA).

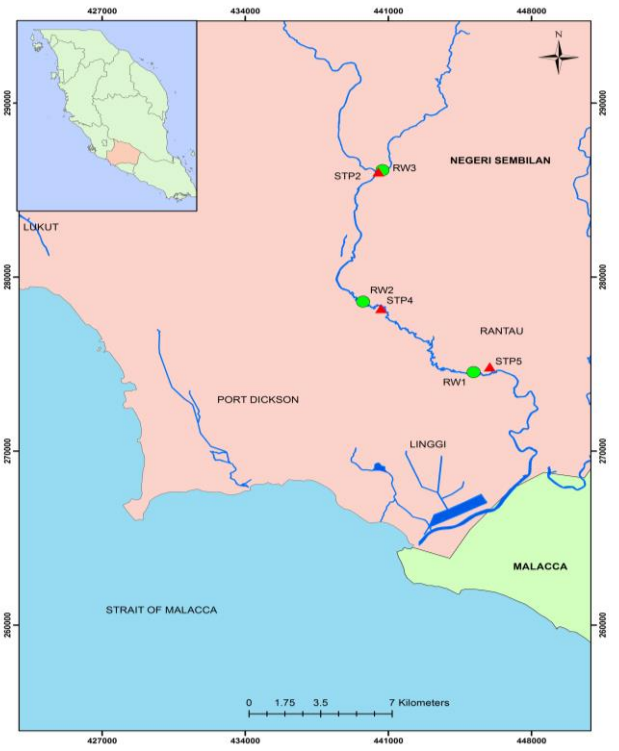

Figure 1. STPs and river water sampling station (▲ = STPs; ● = river water)

Table 1. Description of the sampling site

No	Site	Town/Area	STP type/descriptions
1	STP2	Taman Sri Anggerik	Secondary treatment (oxidation pond)
2	STP4	Taman Desa PD	Secondary treatment (Extended aeration)
3	STP5	Taman Linggi Maju	Secondary treatment (Extended aeration)
4	RW1	Linggi town	Small town, cow farm
5	RW2	Taman Desa PD	Small housing area, STP
6	RW3	Rantau	Palm oil plantations, small town, housing

Detail description on the sample analysis was explained in Abdul Zali *et al.* (2021). Initially, the particulate samples were sonicated using 30 ml methanol and potassium hydroxide solution, followed by 30 ml of 1:1 methanol: DCM and 30 ml of DCM. The extracts from sonication were collected and reduced to 2 ml using a rotary evaporator and solvent exchange using iso-octane. The samples were derivatised using BSTFA-TMSCS at 70°C for an hour for the gas chromatography-mass spectrometer (GC-MS) analysis.

The targeted compounds were determined using Agilent 7890 GC interfaced to an Agilent USA 5975C Mass Selective

Detector (MSD) of using single ion monitoring mode (SIM) (Table 2). The Agilent 7890 GC was equipped with a DB-5ms Ultra Inert (UI)-fused capillary column (30 m x 0.25 mm I.D x 0.25 μm film thickness). Samples were injected in splitless mode at 280 °C with helium as the carrier gas at a flow rate of 1.2 ml/min. The gas chromatography oven was set at 70 ° C and held for 1 minute. The oven temperature was subsequently raised from 30 °C/min to 180 °C and kept for 1 minute, followed by a gradual rise of 5 °C/min to 310 °C, which was then retained for 5 minutes.

Targeted compounds were identified based on the retention time of the reference standards and monitored ions (Table 2). Six calibration points of 0.50 mg L^{-1}, 1.00 mg L^{-1}, 2.50 mg L^{-1}, 5.00 mg L^{-1}, 7.50 mg L^{-1}, and 10.00 mg L^{-1} were established with the correlation coefficient (R^2), presented in Table 2. The method detection limit (MDL) for coprostanol, epi-coprostanol and cholesterol was 0.10 mg L^{-1}, 0.10 mg L^{-1} and 0.05 mg L^{-1}, respectively. Calibration verifications (quality control standards) were run for every twenty samples that were analysed; the values ranged between 70-130%. All quality control and quality assurance were determined and checked during samples analysis (EPA, 2007).

Table 2. GC-MS description of the targeted compounds

Trivial name	Mass	Base peak	Qualifier ion 1 (Q_1)	Qualifier ion 2 (Q_2)	Correlation of coefficient (R^2)
Coprostanol	460	370	75	215	0.998
Epi-coprostanol	460	370	75	215	0.998
Cholesterol	458	129	329	368	0.999

III. RESULTS AND DISCUSSION

A. Distribution of Coprostanol, Epi-Coprostanol and Cholesterol in STPs and River Water Samples

Figure 2 shows the mean and standard error of coprostanol, epi-coprostanol and cholesterol concentrations in the particulate of (a) STP samples and (b) river water samples towards downstream of Linggi River. Based on the result, cholesterol (mean = 15.6 mg L^{-1}) was found to be higher than coprostanol (mean = 6.0 mg L^{-1}) and epi coprostanol (mean= 2.3 mg L^{-1}) in STPs samples. Similar to STPs, cholesterol (mean= 4.6 mg L^{-1}) also was detected higher than coprostanol (mean= 2.4 mg L^{-1}) and epi-coprostanol (mean= 2.4 mg L^{-1}) in the river water samples. Therefore, the most abundant compound observed in both type of samples is cholesterol followed by coprostanol and epi-coprostanol. Cholesterol is highest in STP2 (38.6 mg L^{-1}) followed by STP5 (15.7 mg L^{-1}) and STP4 (8.3 mg L^{-1}). Cholesterol is the most abundant sterols in the human organism, it is also expected to have high concentrations in sewage-contaminated samples (Matić Bujagić *et al.,* 2016)

For STP samples, the order of sterols/stanols abundance for STP2 and STP5 is cholesterol > coprostanol > epi-coprostanol. This is similar to previous research where the abundance of cholesterol than coprostanol and epi-coprostanol was reported in sewage effluents (Furtula *et. al.,* 2012a; Reichwaldt *et. al.,* 2017; Shah *et al.,* 2007a). In contrast, coprostanol was found to be higher than cholesterol in sewage samples (Leeming *et al.,* 1996). The ratio value of epi-coprostanol/ coprostanol in STP2 and STP5 were 0.1 and 0.1, respectively. The ratio value of epi-coprostanol/coprostanol greater than 0.8 has been proposed as treated sewage and ratio value less than 0.2 has been proposed as untreated sewage (Mudge & Seguel, 1999). Meanwhile, the order of sterols/stanols abundance for STP4 is cholesterol > epi-coprostanol > coprostanol with the ratio value of epi-coprostanol/coprostanol in this sample is 1.2. Therefore the effluent from STP2 and STP5 were categorised as untreated sewage and effluent from STP4 was classified as treated sewage. The significant level of epi-coprostanol than coprostanol in STP4 compared to STP2 and STP5 may due to the treatment process efficiencies and possible malfunction of treatment process in STP2 and STP5 (Furtula *et. al.,* 2012a; Mudge & Duce, 2005; Reichwaldt *et al.,* 2017).

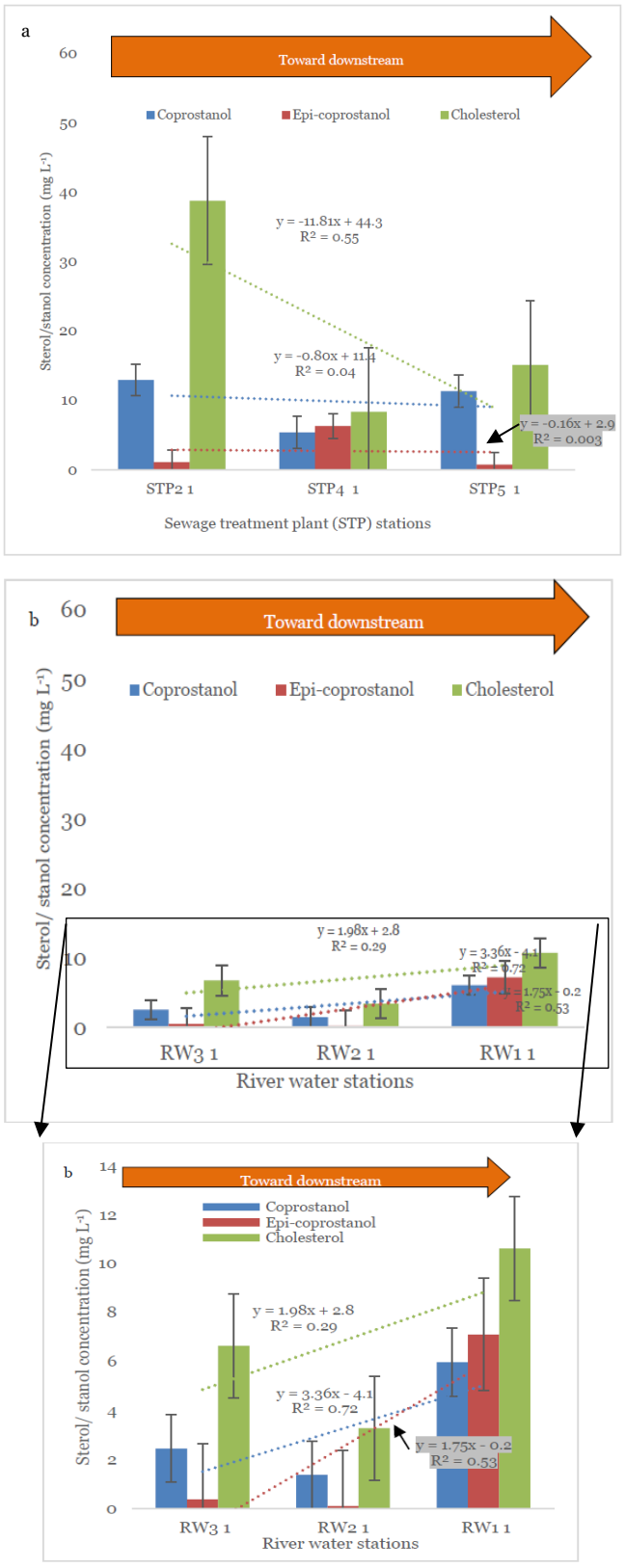

Figure 2. The mean and standard error of coprostanol, epi-coprostanol and cholesterol concentrations in particulate samples and their linear regression trends in (a) STP samples; (b) river water samples; R^2= correlation coefficient

For river water samples, the level of cholesterol in RW1, RW2 and RW3 is 10.6 mg L^{-1}, 3.2 mg L^{-1} and 6.6 mg L^{-1}, respectively. The order of sterols/stanols abundance for RW1 is cholesterol > epi-coprostanol > coprostanol while for RW2 and RW3 are cholesterol > coprostanol > epi-coprostanol. The abundance of cholesterol in river water samples may be derived from various potential sources such as sewage and biogenic sources (Antanasijević *et. al.,* 2018; Bull *et. al.,* 2002; Leeming *et al.,* 1996). However, in this study, we cannot assign the origin of cholesterol to biogenic sources exclusively due to high concentrations of faecal stanols (coprostanol and epi-coprostanol found in the samples (Frena *et al.,* 2016a).

RWI (5.97 mg L^{-1}) has the highest concentration of coprostanol compared to RW2 (1.36 mg L^{-1}) and RW3 (2.44 mg L^{-1}) samples. This occurrence was suspected from the cumulative loading of sewage from the upstream region since coprostanol was lower in RW2 and RW3. The level of coprostanol in this study was slightly lower compared to the study by Isobe *et al.* (2002) which reported coprostanol value between 0.07- 35.43 mg L^{-1} in the major river water samples in Malaysia. The variation of coprostanol, epi-coprostanol and cholesterol in river water samples may be due to several mixing pollution sources such as discharge from STPs, animal farms, agricultural activities and natural abundances (Adnan *et. al.,* 2012; Mohd Ali *et. al.,* 2015; Shah *et al.,* 2007b).

B. Diagnostic Ratios of STPs and River Water Samples

Despite the decrease concentrations of the targeted analytes in river water samples, the ratios between compounds remain distinguishable (Sinton *et al.,* 2010). Thus, accurate sewage assessment can be performed using diagnostic ratios. The cross plot coprostanol/cholesterol ratio versus epi-coprostanol/coprostanol ratio provides rapid visualisation of the STP and river water samples that contain the greatest amount of treated, partially treated and untreated sewage (Figure 3).

Figure 3. The cross plot of coprostanol/cholesterol versus epi-coprostanol/coprostanol (▲ = STPs ; ● = river water)

For STP samples, STP4 and STP5 were classified as treated and untreated sewage, respectively. STP4 has higher amount of epi-coprostanol than coprostanol while STP5 is otherwise.

STP2 was mistakenly categorised as non-human source in the double plot ratios (Figure 3). This STP has the extraordinary amount of cholesterol relative to STP4 and STP5, which can be attributed to the high number of microorganisms used during the treatment process (Aris, 2015; Gupta *et al.,* 2012) and may due to the contribution of cooking oil from domestic waste (Furtula *et. al.,* 2012a; Speranza *et al.,* 2018). Therefore, STP2 also may have potentially high amount of cholesterol been discharged in receiving water.

For river water samples, the double-plot ratios also identified RW2 and RW3 were affected by non-human sources while RW1 was affected by treated sewage from station STP4. The significant non-human faecal sources at RW2 and RW3 may due to the potential agricultural runoff from adjacent rubber and palm oil plantations along Linggi River and animal farms (DOVSM, 2018; Khalik *et al.,* 2015). The correct sewage assessment was challenged in mixed faecal sources environment such as river water (Lim *et. al.,* 2017; Shah *et al.,* 2007a). Therefore, the diverse pattern of coprostanol, epi-coprostanol and cholesterol entering the Linggi River and then consequently marine areas may come from multiple sources of contamination through sewage, household waste, animal farming and natural sources.

C. Investigating Potential Sewage Input into The Marine Environment Using Linear Regression

Figure 2(b) illustrates positive linear regression of coprostanol, epi-coprostanol and cholesterol in river water samples. The river water samples from RW3, RW2 and RW1 (towards downstream) were affected by increasing trend ($p<$ 0.05) of coprostanol, epi-coprostanol and cholesterol (Figure 2(b)). Coprostanol has the highest positive R^2 with 0.72 followed by moderate R^2 for epi-coprostanol (0.53) and low R^2 for cholesterol (0.29) (Zhou *et al.,* 2010). The high R^2 of coprostanol and moderate R^2 for epi-coprostanol denoted that the moderate to high potential sewage pollution towards downstream of the river and consequently might affect nearby coastal and marine environment. Coprostanol is a major compound detected in sewage effluents and epi-coprostanol was produced during the sewage treatment process (McCalley *et. al.,* 1981; Mudge & Duce, 2005). There is also evidences where the municipal wastewater from far upstream of the river reach coastal areas and might potential to the sensitive marine environment (Dsikowitzky *et. al.,* 2018; Zhang, 1999)

The low R^2 value for cholesterol towards marine environments in RW3, RW2 and RW1 suggest its mixing behaviour from anthropogenic and biogenic sources in the river water. Furthermore, the elevated of sewage markers (coprostanol, epi-coprostanol and cholesterol) were identified at RW1 may be derived from untreated STP5 sewage and treated STP4 sewage that reaches RW1 due to river currents and/or other STPs along the Linggi River where untreated domestic and industrial wastewater is continually discharged (Cabral *et. al.,* 2019; Martins *et al.,* 2018). In fact, the similar profile of the double plot diagnostic ratios in RW1 is close to that found in STP4 station, indicating that the river is true conductors of sewage discharge (Carreira *et. al.,* 2015; Costa *et al.,* 2018). As a result, the estuaries and coastal areas of Linggi may be affected by sewage pollution (Elias *et. al.,* 2020; Praveena *et. al.,* 2013; Tan & Lee, 2014).

IV. CONCLUSION

The study of sterols and stanols has been proven to be useful in identifying sewage indicators that can be used to evaluate the level of human faecal pollution in river water and subsequently the marine ecosystem. The results in this study clearly showed that the river water samples received untreated, treated sewage and non-human sources. The possible sources were originated from human faeces/ sewage reaching the river through treated and untreated sewage as well as terrestrial input and/or biogenic sources for non-human sources. The biplot diagnostic ratio of coprostanol/cholesterol versus epi-coprostanol/coprostanol and linear regression analysis further confirmed the discharge of the sewage into the river and subsequently may affect the marine environment. This research will collectively contribute to the knowledge of sterols and stanols biomarkers in sewage assessment studies as well as highlight the need to take measurements for riverine and marine pollution mitigation actions.

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