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RESEARCH ARTICLE

Tracing sewage contamination based on sterols and stanols markers within the mainland aquatic ecosystem: a case study of Linggi catchment, Malaysia

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Abstract

Sewage contamination is a principal concern in water quality management as pathogens in sewage can cause diseases and lead to detrimental health effects in humans. This study examines the distribution of seven sterol compounds, namely coprostanol, epi-coprostanol, cholesterol, cholestanol, stigmasterol, campesterol, and β-sitosterol in filtered and particulate phases of sewage treatment plants (STPs), groundwater, and river water. For filtered samples, solid-phase extraction (SPE) was employed while for particulate samples were sonicated. Quantification was done by using gas chromatography-mass spectrometer (GC-MS). Faecal stanols (coprostanol and epi-coprostanol) and β-sitosterol were dominant in most STP samples. Groundwater samples were influenced by natural/biogenic sterol, while river water samples were characterized by a mixture of sources. Factor loadings from principal component analysis (PCA) defined fresh input of biogenic sterol and vascular plants (positive varimax factor (VF)1), aged/treated sewage sources (negative VF1), fresh- and less-treated sewage and domestic sources (positive VF2), biological sewage effluents (negative VF2), and fresh-treated sewage sources (VF3) in the samples. Association of VF loadings and factor score values illustrated the correlation of STP effluents and the input of biogenic and plant sterol sources in river and groundwater samples of Linggi. This study focuses on sterol distribution and its potential sources; these findings will aid in sewage assessment in the aquatic environment.

Keywords Sterol · Sewage treatment plant · River water · Groundwater · Sewage input · Principal component analysis

Introduction

Human interferences in the aquatic environment in the form of sewage and domestic waste discharge have become a growing concern in recent years. The assessment of sewage contamination is great attention in both environment and human health, and it can enhance water quality and eliminate the potential risk of infectious diseases. Effluents from sewage treatment plants (STPs) are recognized as being among the main point discharge sources in the environment (Andreozzi et al. 2003). Insufficient treatment of sewage discharge is one of the persistent environmental problems that lead to the degradation of river water, groundwater, and coastal water quality (Saim et al. 2009; Gottschall et al. 2013; Ariffin and Sulaiman 2015; Praveena et al. 2015; Nanyan et al. 2016).

Methods to characterize the sources of sewage contamination in water bodies are identified as microbial source tracking (MST) methods. These are based on the detection of a 'tracer' to achieve extensive characterization of the contaminant (Tran

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et al. 2015; Jardé et al. 2018). The tracer can either be faecal microorganisms or chemical compounds discharged along with faecal matter (Cimenti et al. 2007; Gourmelon et al. 2010; Devane et al. 2019). However, these microorganisms or pathogens and chemicals that are chosen as tracers must fulfil the following criteria: (a) they should reproduce only in the intestinal tract, and (b) chemical markers should be discharged only with faecal wastes of the specific hosts (Standley et al. 2000; Tran et al. 2015; Lu et al. 2016; Geary and Lucas 2019). For instance, chemical markers in MST showed sterols and stanols compounds (Mudge and Duce 2005; Saim et al. 2009), caffeine, pharmaceutical products, and human/animal antibiotic residues in assessing sewage pollution in the aquatic ecosystem (Al-odaini et al. 2010; Ho et al. 2012; Omar et al. 2017; Praveena et al. 2018). Together, all these approaches have provided an enhanced capacity to evaluate sewage contamination, with sterols and stanols being the most common compounds used as chemical markers for faecal pollution and urban wastewater (Carreira et al. 2004; Adnan et al. 2012; Frena et al. 2016a).

Sterols and stanols have been used as indicators of sewage pollution in many aquatic environments due to their recalcitrant and source-specific properties compared to microbial and chemical markers indicators (Nichols et al. 1993; de Martins et al. 2007; Saim et al. 2009; Furtula et al. 2012b; Tran et al. 2015). Coprostanol is produced in the guts of animals by the metabolism of cholesterol (Macdonald et al. 1983). The metabolic end-products vary in concentration in different animal groups based on their diet and intestinal flora (Leeming 1996; Hagedorn and Weisberg 2009). Coprostanol is commonly detected in sewage-contaminated surface waters (Saim et al. 2009; Furtula et al. 2012c; Gottschall et al. 2013) and sediments (Carreira et al. 2002; Pratt et al. 2007; Frena et al. 2016b). Elevated levels of coprostanol have also been detected in groundwaters adjacent to extensive animal farm and wastewater contamination (Focazio et al. 2008; Balderacchi et al. 2013; Derrien et al. 2015). Furthermore, epi-coprostanol is produced by the biosynthesis of coprostanol using microbes in STPs and is further used to discriminate among treated and untreated sewage sources (de Martins et al. 2007; Furtula et al. 2012b; Reichwaldt et al. 2017). Concerning the degree of sewage treatment, values lower than 0.2 for epi coprostanol/coprostanol are indicative of untreated sewage input, whereas values higher than 0.8 are categorized as treated sewage input (Mudge and Gwyn Lintern 1999; Gilpin 2011).

Cholesterol, cholestanol, campesterol, stigmasterol, and β-sitosterol have been investigated in numerous studies that tracked sewage contamination based on sterols and stanols sources. In sewage assessment, cholesterol acts as a precursor of coprostanol and also found in sewage and manure of higher animals. Cholesterol is also the main marker of marine sterol and is dominant in invertebrates and marine zooplankton (Volkman 1986; Mudge and Bebianno 1997; Loh et al. 2006). Campesterol, stigmasterol, and β-sitosterol have been frequently used as markers of terrigenous organic matter as they are present in high amounts in terrestrial higher plants (Volkman 1986; Martins et al. 2011). These markers also were detected in sewage effluents which derived from biogenic sources such as bacteria and microorganisms during aerobic/anaerobic treatment process of the sewage (Fernandez et al. 2007; Reichwaldt et al. 2017). Recent studies have discussed β-sitosterol, which is a marker for phytosterols and has been described as one of the domestic input biomarkers (Froehner and Souza 2010; Antanasijevi et al. 2018). This compound is associated with waste oil and cooking/vegetable oil that are dumped and channelled into STPs in the form of domestic/household waste (Furtula et al. 2012b; Speranza et al. 2018). Due to the aforementioned criteria, multiple sterols and stanols determination can provide a comprehensive sewage assessment into receiving water bodies. The summary description of sterols and stanol in this study was summarized in Table 1.

Table 1 Trivial, systematic name, molecular weight (Mw), and source of sterols and stanols in this study

No	Trivial name	Systematic name	M_w	Source
1	Coprostanol	5β-Cholestan-3β-ol	388	Human/animal stanol Most abundant stanol in human faeces, a precursor of epicoprostanol (soil)
2	Epi-coprostanol	5β-Cholestan-3α-ol	388	Present in sewage sludge from wastewater treatment; high relative amounts suggest older faecal contamination and/or anaerobic sediments
3	Cholesterol	Cholest-5-en-3β-ol	386	Most abundant in animals, traces in plants, fungi and other eukaryotes, terrestrial biomarker Precursor of coprostanol (intestine) and 5a-cholestanol (soil)
4	Cholestanol	5α-Cholestan-3β-ol	388	Reduction product cholesterol (soil), traces in animal tissues, faeces, plants
5	Campesterol	24-Methylcholest-5- en-3β-ol	400	Plant sterol, present in vascular higher plants, abundant in herbivorous diet
6	Stigmasterol	24-Ethylcholest-5,22-dien-3β-ol	412	Plant sterol; present in vascular higher plants
7	β-sitosterol	24-Ethylcholest-5-en-3β-ol	414	Plant sterol; present in vascular higher plants, abundant in herbivorous diet; recently categorized as domestic waste marker

Source: Leeming et al. 2014; Antanasijevi et al. 2018; von der Lühe et al. 2018 and the references herein

Sterols and stanols are the among the key components of organic matter; they are present in a wide range of ecological environments in term of solubility, bioavailability, and mobility, and are influenced by the various biogeochemical process (Carreira et al. 2016; Koolen et al. 2018; Derrien et al. 2019). Numerous researchers estimated the age of the organic matters in the water column where the average organic matters in particulate phase are much older than co-occurring filtered phase (Masiello and Druffel 2001; Raymond and Bauer 2001; Loh et al. 2006). Generally, organic matter are divided into two phases based on pore sizes: (a) filtered or dissolved phase usually the organic matters having particulate size less than 0.2/0.45/0.7 μm and (b) particulate phase is organic matter having particulate size more than 0.2/0.45/0.7 μm (Derrien et al. 2019). These two phases are easily interchangeable and controlled by mechanisms, such as aggregation/dissolution, adsorption/desorption, and dissolution/precipitation in biological processes and photochemical reactions (Zimmermann-Timm 2002; Perdue and Ritchie 2003; He et al. 2016).

Due to their hydrophobic nature, most sterols can be easily associated with particulate phase (Marty and Saliot 1981; Berdié et al. 1995; Fattore et al. 1996; Astel et al. 2007) but at the same time, they also present in the dissolved phase (McCalley et al. 1981; Standley et al. 2000; Isobe et al. 2002; Noblet et al. 2004). Previous studies showed a higher percentage of sterols in particulate phase ranging from 70 to 95% while in dissolved phase ranged from 13 to 66% (Quéméneur and Marty 1994; Isobe et al. 2002; Daughton 2012; Kim et al. 2016). The fate and the behaviour of these compounds in both phases need to be examined extensively especially in tropical aquatic mainland to provide comprehensive sewage pollution assessment. The stable high temperature, high humidity, and frequent rainfall in the tropical region may contribute to the frequent pollution runoff into the aquatic environment and favour pollution solubility in water (Isobe et al. 2004; Zgheib et al. 2011; Sarria-Villa et al. 2016).

The Linggi catchment is situated in Negeri Sembilan, Malaysia. It is an area under intense pressure from rapid urban and rural development with growing number of inhabitants (Aburas et al. 2017; MAMPU 2020a, b). Linggi River which flows through the Linggi region is categorized as a semi-polluted river based on Water Quality Index (WQI) criterion for 2 years (2016 to 2017) (DOE 2018). The potential sources of pollution of Linggi River might be originating from undermining or old-technologies STPs, improper maintenance of individual septic tanks, domestic wastes, improper handling of animal farms as well as agricultural runoffs. This is supported by recent studies which revealed that Linggi River is experiencing major pollution from sewage, and domestic, industries, and agricultural activities (Fadhil et al. 2015; Khalik et al. 2015; Elias et al. 2018a, b). The current knowledge about potential sewage pollution in the river and groundwater in the tropical region of Linggi requires deeper insight. This study aims to determine the distribution of sterols, stanols and their sources in dissolved and particulate phases from selected STPs; groundwater and river waters; and the influence of STPs effluents to river water and groundwater. The findings of this investigation can be applied to sewage evaluation studies in both engineered and aquatic mainland in order to guide the investments towards environmental protection improvement.

Materials and methods

Study area and sampling stations

Sampling was conducted in November 2017 at Linggi, Negeri Sembilan, Malaysia (Fig. 1). Linggi River is situated at Negeri Sembilan state which covers approximately 1530 km^2 and flows through main cities such as Seremban and Senawang towards Strait of Malacca. Negeri Sembilan is one of the warmest regions in Malaysia with an average daily high temperature of between 25 and 35 °C. This state receives between 2 and 12 mm day^{-1} of average monthly rainfall throughout the year. The total populations in Negeri Sembilan are 1.13 million and continuously growing every year. The catchment was occupied with residential and industrial zone in upstream while major agricultural activities (rubber and palm oil plantations), small residential areas, and drinking water source are situated in the middle towards downstream.

All samples were collected in duplicate from six river waters (RWs), six groundwaters (GWs), and five STPs. STP effluents were collected near river and groundwater stations to enhance the correlation of the suspected sources and samples from the site observation (Fig. 1). A detailed description of the sampling sites is listed in Table 2. In this study, sterols and stanols will be analyzed in both filtered and particulate phases. The STP effluents and river water samples were collected in pre-cleaned buckets. Groundwater samples were collected from the wells after the boreholes were purged to a sufficient volume, that is three to ten volume of the wells, in order to ensure that the samples represented groundwater (APHA 2012). The water samples were filtered using pre-combusted GF/F Whatman (0.7 μm pore size) to obtain particulate and filtered samples by filtering 100 ml STP effluents, 1 L river water, and 1 L groundwater at the sampling location. The filtered samples were preserved with 6 M sodium hydroxide (NaOH) until their pH ranged between 5 and 9 and stored in a cooler box during transportation (EPA 2007). In the laboratory, filtered water samples were stored in a chiller at 4 °C under dark condition and analyzed within a week. Meanwhile, the particulate samples were kept at − 20 °C and freeze-dried 2 weeks prior to extraction analysis.

Chemicals and reagents

The calibration standard for this research was purchased from Chiron AS, Norway, and consisted of 50 μg ml^{-1} of a sterol

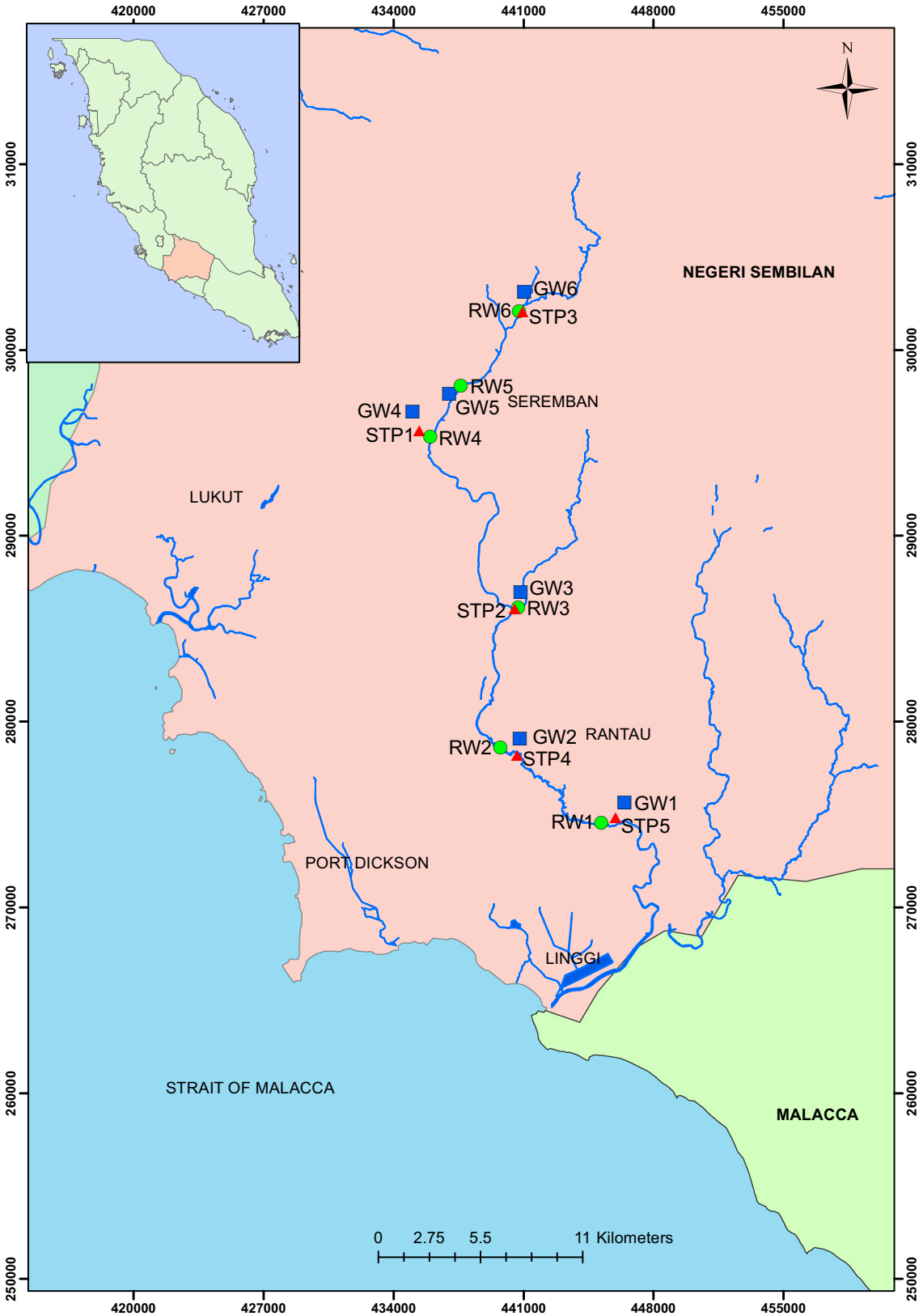


Fig. 1 Map showing the sampling locations along the Linggi River, Negeri Sembilan, Malaysia (GW = groundwater ■; RW = river water ●; STP = sewage treatment plant ▲; DWTP (SAINS) ⬟= Drinking Water Treatment Plant (Syarikat Air Negeri Sembilan (SAINS)))

Table 2 Detailed description of the sampling locations

No	Sampling sites	Town/area	Description	Population equivalent (PE) for STP
1	GW1 and RW1	Linggi town	Small town, cow farm	-
2	GW2 and RW2	Taman Desa PD	Small housing area, STP	-
3	GW3 and RW3	Rantau	Palm oil plantations, small town, housing	-
4	GW4 and RW4	Sungai Ara	Small housing area, urban area	-
5	GW5 and RW5	Mantau	Small housing area, urban area	-
6	GW6 and RW6	Ampangan	Large housing area, goat farm, urban area	-
7	STP1	Taman Mambau Jaya	Imhoff tank	300
8	STP2	Taman Sri Anggerik	Oxidation pond	1625
9	STP3	Kg Ismail	Hi-Kleen (mechanical system)	175
10	STP4	Taman Desa PD	Extended aeration	6425
11	STP5	Taman Linggi Maju	Extended aeration	90

mixture containing coprostanol, epi-coprostanol, cholesterol, cholestanol, campesterol, and β-sitosterol. Stigmasterol standard was purchased from Sigma-Aldrich Corporation. The deuterated standard for surrogates (cholestane-2,3,3,3,4,5-d6) (CHLN- d6) and internal standard (cholesterol-2,2,3,4,4,6-d6) (CHL- d6) were obtained from Toronto Research Chemical Inc. All solvents (methanol, acetone, dichloromethane (DCM), and iso-octane) used in this study were pesticide grade/HPLC grade and purchased from Fisher Scientific International, Inc. The derivatising agent for the sterol compound was N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA): trimethylchlorosilane (TMCS), 99:1 (Supelco, USA).

Sample preparation and sterol analysis

The glassware was rinsed with ultrapure water, methanol, acetone, and DCM for the analyses. They were covered with aluminium foil, placed in an oven overnight at 105 °C, and baked in a furnace at 450 °C for 4 h to remove the organic contaminants. The filtered samples were spiked with 1 ml of 1 μg ml^{-1} of CHLN-d6 and left for 10 min prior to homogenisation. The filtered samples were stirred at 500 rpm for 2 h and passed through the solid-phase extraction (SPE) using C_{18} cartridges (1 g, 6 ml; Dikma Technologies). Initially, the cartridges were pre-conditioned using 10 ml of methanol and 6 ml of ultrapure water using gravity flow. The filtered water samples were loaded into the SPE column at 5 ml/min and dried in vacuum for 30 min. The sterol fractions were eluted using 2 × 3 ml of DCM under gravity flow and stored in the freezer at − 20 °C prior to the quantification analysis.

The particulate samples were added with surrogate CHLN-d6 to obtain a final concentration at 5 μg ml^{-1}. This was followed by 30 ml of methanol and 1 ml of 0.5 M potassium hydroxide (KOH) and the samples were sonicated for 30 min using a sonicator (Brand: JAC ultrasonic, 200 watts). The extracts were filtered and collected in tall vials. The sonication process was repeated using 30 ml of 1:1 (DCM: methanol) and 30 ml DCM. The collected extracts were poured into separating funnel, with 15 ml of ultrapure water added to them to wash off excess KOH. The organic and the water layer of the extracts were allowed to separate, after which the organic was decanted in a round bottom flask.

The extracts from sonication and SPE were reduced to 2 ml per extract using a rotary evaporator and further reduced using gentle nitrogen blower prior to the mark-up using 120 μl of iso-octane. Then, for the gas chromatography-mass spectrometer (GC-MS) injection, internal standard (ISTD) CHL-d6 and derivatising agent BSTFA-TMCS were added to the extracts in the vials to obtain a final volume of 100 μl per vial. The samples were derivatized in an oven at 70 °C for 1 h and were ready for the GC-MS analysis. The sterols and stanols in filtered and particulate samples were determined by an Agilent 7890 gas chromatography (GC) interfaced to an Agilent USA 5975C mass selective detector (MSD) using the single ion monitoring (SIM) mode. The ions monitored for each compound are presented in Table 3. The 7890 GC was equipped with a DB-5ms Ultra Inert (UI)-fused capillary column (30 m × 0.25 mm I.D × 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 programmed at 70 °C and held for 1 min. Subsequently, the oven temperature was increased from 30 °C/min to 180 °C and was held for 1 min, followed by a slower increase of 5 °C/min to 310 °C, which was then held for 5 min.

Compounds were identified based on retention time (R_t) of the reference standards and monitored ions (Table 3). 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 3. The samples with exceeded calibration range were re-diluted to certain factor necessary to bring the concentration within the calibration range. Calibration verification (quality control standards) was done for every twenty samples that were analyzed; the values ranged between 70 and 130%.

Table 3 GC-MS description for sterols and stanols analysis

Number	Trivial name	MW after derivatization	Base peak	Qualifier ion 1 (Q_1)	Qualifier ion 2 ($Q2$)	Correlation coefficient (R^2)
1	CHLN-d6 (surrogate)	378	223	155	363	0.997
2	Coprostanol	460	370	75	215	0.998
3	Epi-coprostanol	460	370	75	215	0.998
4	CHL-d6 (ISTD)	464	333	374	-	
5	Cholesterol	458	129	329	368	0.999
6	Cholestanol	460	215	355	445	0.999
7	Campesterol	474	129	83	343	0.998
8	Stigmasterol	484	129	83	394	0.999
9	β-sitosterol	486	129	357	396	0.998

Base peak, Qualifier ions 1 and 2 were based on Biache and Philp, (2013)

The limit of detection (LOD) was determined by spiking the standards in filtered and particulate samples for each matrix samples (STP effluents, groundwater and river) and calculated based on three times the standard deviation of the mean concentrations of the spiked samples. Limit of quantification (LOQ) was calculated based on ten times the standard deviation of the mean concentrations of the spiked samples. The LOQ ranged from 0.05 to 0.50 mg L^{-1} for all sterols and stanols compounds in STPs, groundwater and river water sample matrix. The samples were analyzed in triplicates for repeatability check and the relative standard deviation (RSD) was less than 20%. Method blanks and spiked samples were analyzed to ensure method performance of every batch of the samples. The recovery of surrogate (CHLN-d6) was between 60% and 120%. The recovery of the spiked filtered and spiked particulate samples was between 45 to 143% and 67 to 150% respectively. Individual sterols and stanols standard recovery and GC-MS chromatogram results were included in Supplementary Information. The quality control standards and spiked samples were repeated in cases where the results were outside the desired range.

Sterol source estimation and the correlation between samples using principal component analysis (PCA)

Variations in environmental data are frequently investigated using an advanced statistical technique, namely principal component analysis (PCA) (de Martins et al. 2007; Fadhil et al. 2015; Osman and Saim 2016). This technique linearly transforms data into several new uncorrelated principal components (PCs), thereby accurately interpreting the variations in the original data (Saim et al. 2009; Jolliffe and Cadima 2016; Osman and Saim 2016). Varimax rotation was performed to increase the participation of variables with higher loading and to simultaneously reduce the redundant variables with low-factor loading values. The rotation was based on the number of the eigenvalues larger than one, which represented high variance and variability (%) in the PCs generated (Kowalkowski et al. 2006; Osman et al. 2012). Before conducting PCA, it is recommended to perform dataset transformation, such as log-ratio transformation. This increases data interpretation in term of outliers and reduces the closure effect of elements for enrichment factors in spatial data evaluation (Aitchison and Greenacre 2002; Antanasijevi et al. 2018; Darabi-Golestan and Hezarkhani 2018; Speranza et al. 2018).

Data were arranged using a total of seven variables in filtered samples (sterols and stanols) and seven variables in particulates samples (sterols and stanols) × 34 observations. They were analyzed to comprehend the patterns and correlations of the sterols and stanols phases in river water and groundwater of nearby STPs. Centered log-ratio transformation was performed on the dataset using CodaPack Version 2 freeware (Faith 2015). PCA was employed using XLSTAT 2016 software. The generated principal components that had eigenvalues greater than one were chosen to justify the number of varimax rotations (Kim and Mueller 1987; Osman et al. 2012). Generally, factor loading greater than 0.5 was included while interpreting the information of the generated varimax factor (VF) (Wu and Liang 2009). Thus, sterols and stanol sources and loading scores represented the correlation between variables and observations. A detailed explanation of PCA, compositional data, and method for centered log-ratio (CLR) transformation was provided by Antanasijevi et al. (2018).

Results and discussion

Level and spatial distribution of sterols in STPs, river water, and groundwater samples

The individual concentrations and distribution (%) of sterols and stanols in filtered and particulate phases are illustrated in Fig. 2 (a(i) and a(ii)) STPs; (b(i) and b(ii)) groundwater; (c(i) and c(ii)) river water, respectively. The concentrations of

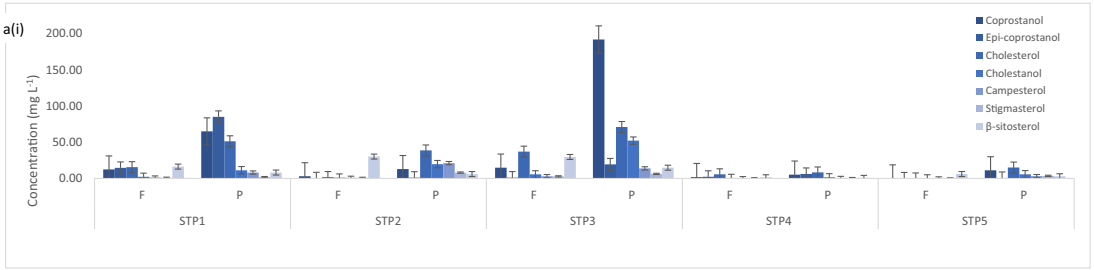
sterols and stanols in the STP samples were between undetected and 192.03 ± 3.25 mg L^{-1}, followed by river water samples, wherein the concentrations were between undetected and 23.57 ± 0.39 mg L^{-1}, and groundwater samples wherein the concentrations were between undetected and 2.05 ± 0.01 mg L^{-1}. The concentrations of sterols and stanols in STPs, river water, and groundwater samples in this study were higher than those in similar studies on STPs (McCalley et al. 1981; Isobe et al. 2002; Shah et al. 2007a; Furtula et al. 2012b), river water (Noblet et al. 2004; Shah et al. 2007a; Saim et al. 2009), and groundwater (Furtula et al. 2012a; Izbicki et al. 2012; Gottschall et al. 2013). The higher concentration of sterols and stanols observed in this study compared to previous research may be due to the abundance of these compounds in STP samples as potential sewage sources, which further contribute to their elevated levels in nearby rivers and groundwater.

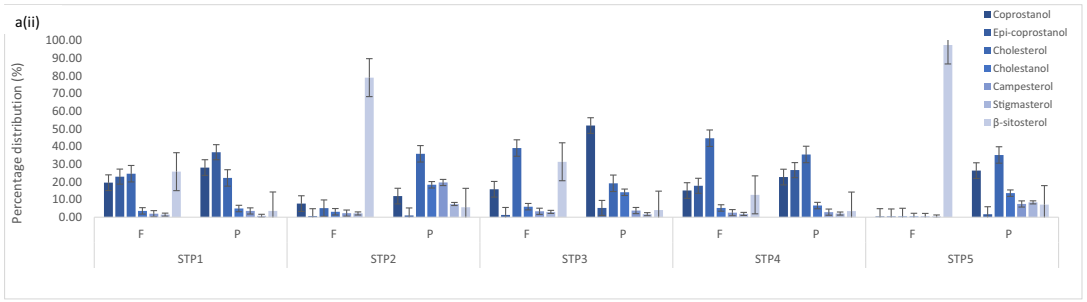
Generally, based on STP, river water, and groundwater samples, the concentrations of sterols and stanols were higher in particulate samples than in filtered samples in all sample types, with some exceptions for coprostanol, epi-coprostanol, cholesterol, and β-sitosterol. This contrasts with the findings of Quemeneur and Marty (1992) and Isobe et al. (2002) wherein the concentrations of all sterols and stanols in their analyzed samples were higher in particulates than in filtered samples. Furthermore, based on all sample types, coprostanol and cholesterol were the most abundant compounds detected in particulate and filtered samples, respectively. Coprostanol and cholesterol have been reported to be among the major compounds detected in sewage effluents (Marvin et al. 2001; Shah et al. 2007b; Saim et al. 2009; Furtula et al. 2012b), river water (Elhmmali et al. 2000; Isobe et al. 2002; Osman et al. 2012), and groundwater (Izbicki et al. 2012; Nakagawa et al. 2016). The presence of coprostanol also was correlated with the sewage impacted sites (Carreira et al. 2004; Froehner et al. 2009; Puerari et al. 2012; Nakagawa et al. 2019). Meanwhile, the abundance of cholesterol is due to its ubiquitous nature as the main constituent of the cell membrane and is dominant in invertebrates and marine zooplankton (Volkman 1986; Hudson et al. 2001; Loh et al. 2008).

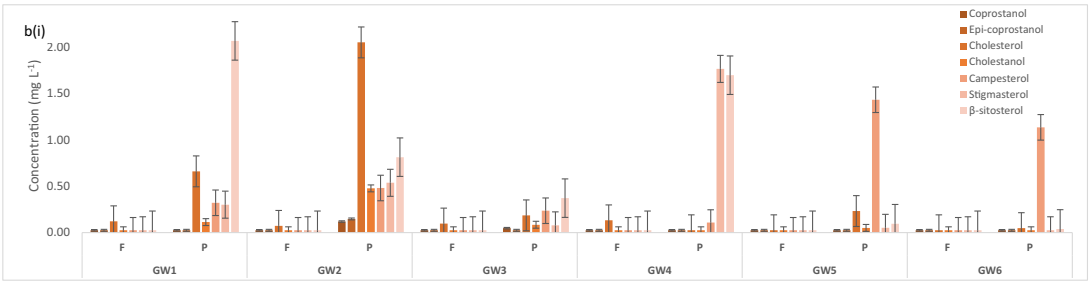
For STP samples, the range of sterols and stanols in filtered samples and particulate sample is undetected–15.60 ± 0.05 mg L^{-1} and 0.48 ± 0.01 mg L^{-1}–192.03 ± 3.24 mg L^{-1}, respectively. The most abundant compounds in the filtered and particulate STP samples were cholesterol and coprostanol, respectively. The elevated levels of cholesterol in filtered samples, which is often detected as a major sterol in sewage, were attributable to the diet intake of humans (Leeming et al. 1996; Shah et al. 2007b) and were not limited to its solubility and adsorption in particulate matter (Walker et al. 1982). Coprostanol is one of the products of cholesterol degradation and acts as a major steroid marker for sewage contamination (Carreira et al. 2002; de Martins et al. 2007; Adnan et al. 2012; Devane et al. 2018). The concentration of this compound was one of the highest among the steroids in sewage effluents, comprising between 40 and 60% of the total (McCalley et al. 1981; Leeming et al. 1996; Nichols et al. 1996; Shah et al. 2007b). In contrast, less than 5% of coprostanol was detected in the filtered sewage effluent samples measured by Reichwaldt et al. (2017). However, in this analysis, coprostanol was between 0 and 14.55% in filtered STP samples and between 11.96 and 51.93% in particulate STP samples (Fig. 3a(ii)). Such differences may be due to the fate of coprostanol in filtered and particulate samples based on the sewage treatment process as well as treatment technologies involved in STPs from this study area and those involved in the STPs from the aforementioned studies.

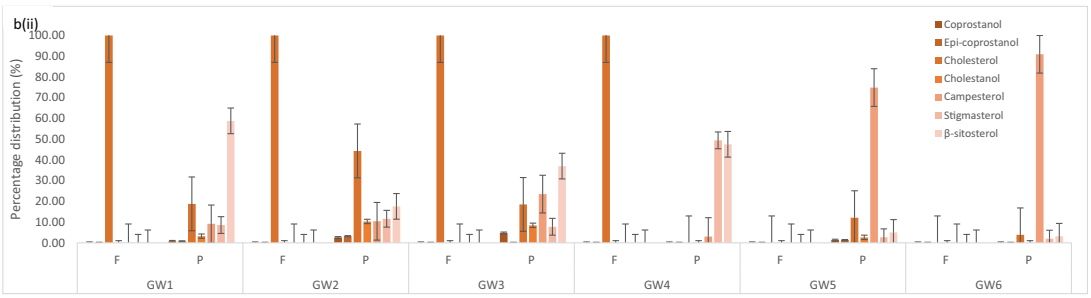
In the STP samples, β-sitosterol was the only compound present in higher concentrations in filtered samples compared to the particulate samples. This compound was identified in the filtered sewage effluent samples (Shah et al. 2007b; Saim et al. 2009), and was linked to the dumped cooking/vegetable oil wastes that are typical domestic waste inputs into the STPs (Furtula et al. 2012b; Speranza et al. 2018). Compared to particulate samples of STPs, the higher amount of β-sitosterol in filtered samples may be attributed to the increased and/or continuous influx of domestic waste, resulting in a longer partitioning period of the filtered phase of β-sitosterol into the particulate phase. The removal of this compound also relies on particulate matter sorption and biodegradation throughout the sewage treatment process (Sin et al. 2015; Xiao et al. 2020). β-sitosterol was also found in sewage effluent and may be a cause for concern in sewage assessment, as the source of this compound is primarily terrestrial input rather than sewage/domestic input (Mudge and Duce 2005; Saim et al. 2009; Adnan et al. 2012; Reichwaldt et al. 2017).

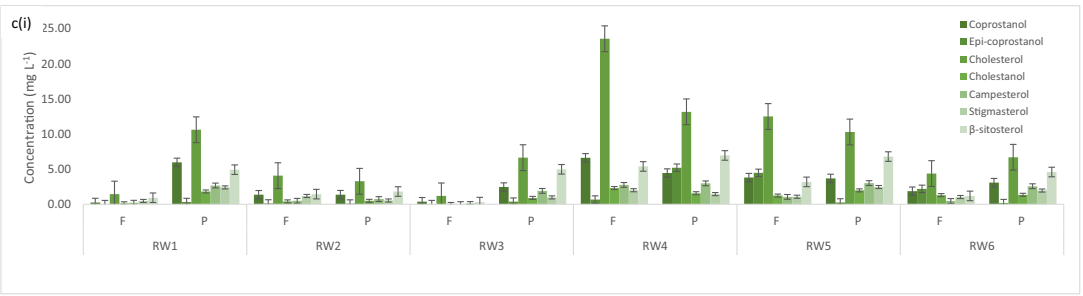
In the STP samples, higher levels of sterol (cholesterol) and faecal stanols (coprostanol and/or epi-coprostanol) were observed in STP1, STP2, and STP3 compared to STP4 and STP5 (Fig. 2a(i)). STP1 uses an Imhoff tank type of technology with an anaerobic treatment process. STP1 also has the highest concentration of epi-coprostanol (85.03 ± 2.83 mg L^{-1}) in the particulate phase compared with other particulate samples. Epi-coprostanol (36.84%) was greater in abundance than coprostanol (28.13%) in the particulate sample of STP1, which may indicate the discharge of treated/aged sewage into the river water. STP2 has a lower amount of filtered and particulate faecal stanols (coprostanol and epi-coprostanol) in its final discharge compared to STP1, which may be attributable to the biological treatment process (aerobic) in STP2 compared to anaerobic treatment of STP1 (Fernandez et al. 2007; Furtula et al. 2012b). The effluent of STP2 contained the highest amount of campesterol (21.18 ± 0.01 mg L^{-1}) and stigmasterol (8.07 ± 0.01 mg L^{-1}) in particulate samples compared with other particulate STP samples. These sterols were


a(i)
Concentration (mg L-1)
200.00
150.00
100.00
50.00
0.00
Coprostanol
Epi-coprostanol
Cholesterol
Cholestanol
Campesterol
Stigmasterol
β-sitosterol
F
P
STP1
STP2
STP3
STP4
STP5


a(ii)
Percentage distribution (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Coprostanol
Epi-coprostanol
Cholesterol
Cholestanol
Campesterol
Stigmasterol
β-sitosterol
F
P
STP1
STP2
STP3
STP4
STP5


b(i)
Concentration (mg L-1)
2.00
1.50
1.00
0.50
0.00
Coprostanol
Epi-coprostanol
Cholesterol
Cholestanol
Campesterol
Stigmasterol
β-sitosterol
F
P
GW1
GW2
GW3
GW4
GW5
GW6


b(ii)
Percentage distribution (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Coprostanol
Epi-coprostanol
Cholesterol
Cholestanol
Campesterol
Stigmasterol
β-sitosterol
F
P
GW1
GW2
GW3
GW4
GW5
GW6


c(i)
Concentration (mg L-1)
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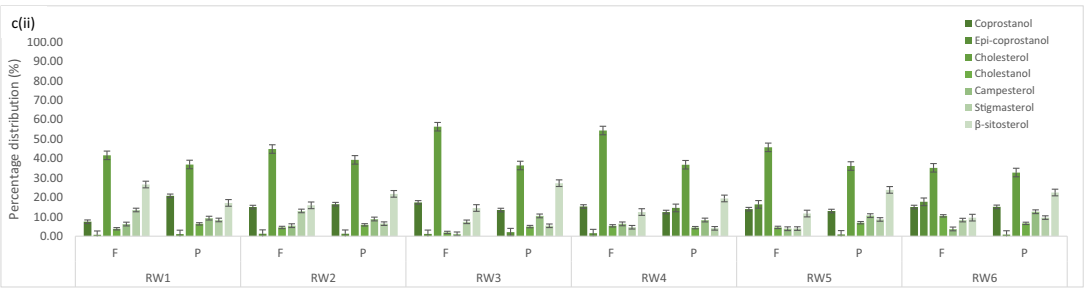
20.00
15.00
10.00
5.00
0.00
Coprostanol
Epi-coprostanol
Cholesterol
Cholestanol
Campesterol
Stigmasterol
β-sitosterol
F
P
RW1
RW2
RW3
RW4
RW5
RW6


c(ii)
Percentage distribution (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Coprostanol
Epi-coprostanol
Cholesterol
Cholestanol
Campesterol
Stigmasterol
β-sitosterol
F
P
RW1
RW2
RW3
RW4
RW5
RW6

◀ **Fig. 2** (a(i)) Individual sterols and stanols concentrations in the filtered and particulate samples of STP samples. (a(ii)) Percentage distribution (%) of sterols and stanols in the filtered and particulate samples of STP samples. (b(i)) Individual sterols and stanols concentrations in the filtered and particulate samples of groundwater samples. (b(ii)) Percentage distribution of sterols and stanols in the filtered and particulate samples of groundwater samples. (c(i)) Individual sterols and stanols concentrations in the filtered and particulate samples of river water samples. (c(ii)) Percentage distribution of sterols and stanols in the filtered and particulate samples of river water samples (F = filtered samples; P = particulate samples)

derived from the microbial community to stabilize and treat sewage (Fernandez et al. 2007; Reichwaldt et al. 2017).

Even though STP3, STP4, and STP5 utilized mechanized systems, their sterols and stanol levels varied significantly. STP3 uses Hi-Kleen technology for treating sewage anaerobically. The results show that the concentration of coprostanol was highest in the particulate (192.03 ± 3.25 mg L^{-1}) and filtered (14.95 ± 1.56 mg L^{-1}) samples. The high concentration of this compound may be due to the relatively low epimerisation of coprostanol to epi-coprostanol in the digested sewage sludge, which could be due to the malfunctioning of STP3 during the treatment process at the time of sampling (McCalley et al. 1981; Mudge and Duce 2005; Furtula et al. 2012b). This finding is similar to the study by Furtula et al. (2012b), who reported lower levels of stanols removal in sewage effluents from anaerobic treatment process STPs compared to sewage effluents from aerobic treatment process STPs.

In STP4 and STP5, coprostanol and epi-coprostanol concentrations were relatively lower in filtered and particulate phases compared to filtered and particulate samples of STP1, STP2, and STP3. STP4 and STP5 implement aerobic treatment processes, and the effluents from these two STPs were superior to those from the other STPs. Coprostanol and epi-coprostanol were undetected in the filtered samples of STP5. Meanwhile, STP4 had the lowest coprostanol level (5.31 ± 0.04 mg L^{-1}) in particulate samples compared to the other STPs. However, the values of coprostanol and epi-coprostanol in this study were still considerably higher than those in studies that used similar treatment systems (McCalley et al. 1981; Shah et al. 2007b; Reichwaldt et al. 2017).

Discrepancy was reported between the conventional and underperforming sewage treatment plants (STP1, STP2, and STP3) and the operational/functional sewage treatment plants (STP4 and STP5) in the filtered and particulate phase, based on treatment technologies and treatment processes. STP1, STP2, and STP3 were fully operational during 1994–1995, whereas STP4 and STP5 were built later in 2006 and 2016, respectively. The variations in sterols and stanols in filtered and particulate samples of STP1, STP2, STP3, STP4, and STP5 were highly influenced by the treatment process and technology in the respective STPs. Occasional malfunctioning of STPs, use of outdated treatment technologies, and an increase in the population in the area may have enhanced the concentrations of sterols and stanols in the wastewater generated, and in turn, may significantly decrease the quality of sewage effluent entering the Linggi River (Pratt et al. 2008; DOS 2019; MAMPU 2020a, b).

In groundwater, sterols and stanols concentration in the filtered and particulate samples ranged between undetected to 0.13 ± 0.01 mg L^{-1}, and undetected to 2.05 ± 0.01 mg L^{-1}, respectively. Cholesterol (0.13 ± 0.01 mg L^{-1}) and β-sitosterol (2.05 ± 0.01 mg L^{-1}) were detected in highest concentrations in the filtered samples and particulate samples of groundwater, respectively (Fig. 2b(i)). The cholesterol concentration in this study was approximately 80 times higher than that reported in previous studies (Barnes et al. 2008; Balderacchi et al. 2013); such elevated levels may be due to their higher levels in the source samples (i.e. STP samples). This further contributes to the higher cholesterol levels in river water, and in turn, in the aquifers that are supplied by this river water (Neal et al. 2005; Wang et al. 2020). In this study, cholesterol and β-sitosterol distribution (%) in particulate samples of groundwater was between 0–44.34% and 3.17–58.83%, respectively (Fig. 2b(ii)). These compounds were also reported as frequently detected sterols in groundwater compared to the other measured sterols and stanols compounds (Focazio et al. 2008; Duong et al. 2015).

GW1 had a β-sitosterol concentration of 2.05 ± 0.01 mg L^{-1} and a cholesterol concentration of 0.13 ± 0.01 mg L^{-1} in the particulate sample. The abundance of β-sitosterol suggests infiltration of domestic waste, such as cooking oil, from onsite wastewater treatment plants (Conn et al. 2006; Lu et al. 2016; Lim et al. 2017) and cholesterol levels were influenced by sewage, terrestrial, and/or marine input (Mudge and Duce 2005; Adnan et al. 2012). Furthermore, the abundance of cholesterol may also be due to its ubiquity in flora and fauna and multiple organic matter sources in groundwater (Volkman 1986; Mudge and Gwyn Lintern 1999; Barnes et al. 2008; Izbicki et al. 2012; Balderacchi et al. 2013; Gottschall et al. 2013).

Coprostanol was only detected in the particulate samples of GW2 at a concentration of 0.12 ± 0.01 mg L^{-1}. In addition, epi-coprostanol was only observed in particulate samples of GW2 at a concentration of 0.15 ± 0.01 mg L^{-1}. The occurrence of faecal stanols (coprostanol and epi-coprostanol) in groundwater samples may be attributable to the percolation and migration of these faecal stanols from possible sewer leakage in the filtered and particulate phases that occurred from the sewage source and deposited in both phases of the river–groundwater distribution system (Fig. 1) (Pang et al. 2004; Carroll et al. 2009). Cholesterol, cholestenol, campesterol, stigmasterol, and β-sitosterol were also dominant in the particulate samples of GW2. Markers for sewage and domestic waste have been frequently detected in groundwater wells near onsite wastewater treatment plants, where cholesterol is the most abundant compound followed by

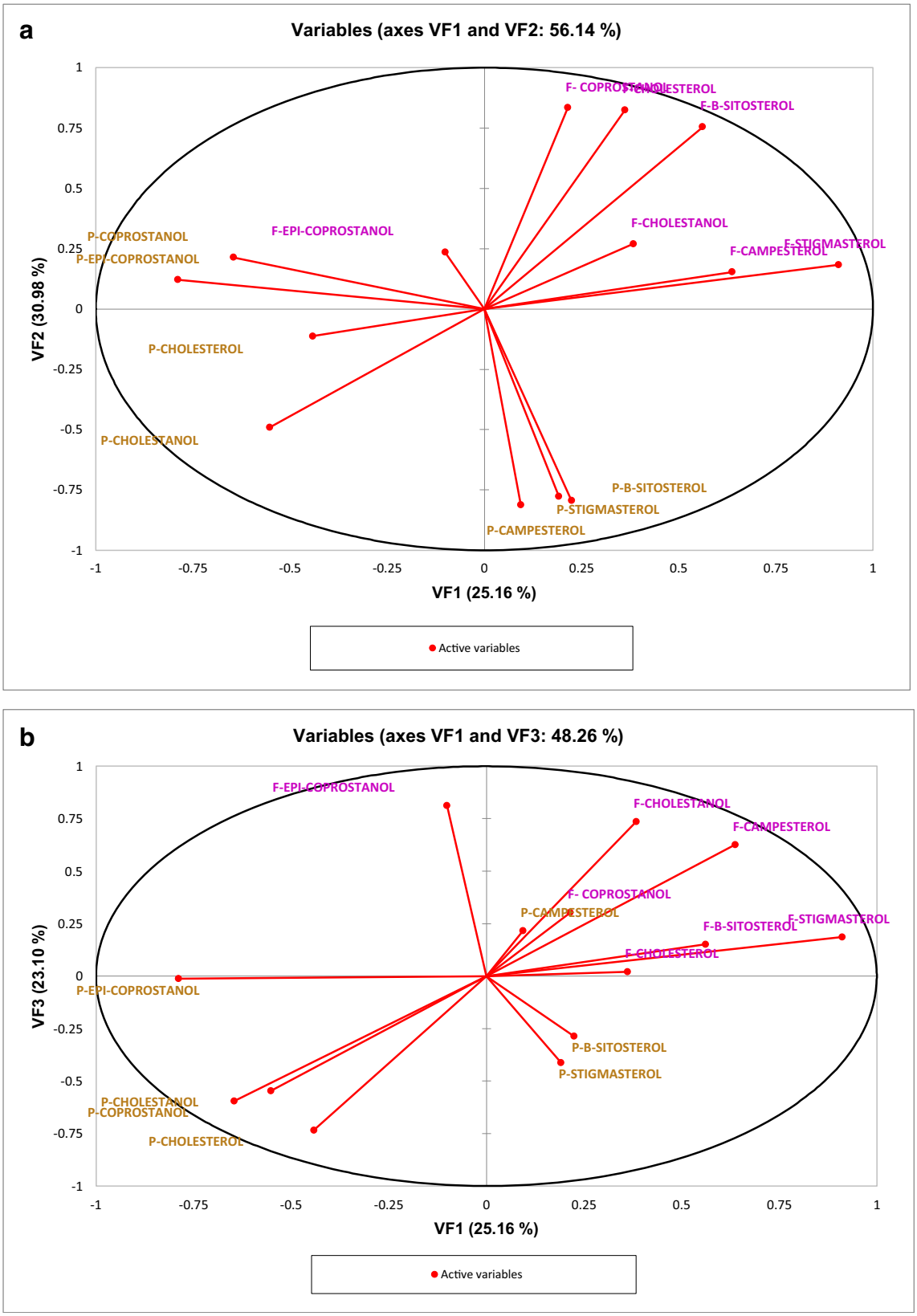


Fig. 3 (a) Loading plot of VF1 versus VF2. (b) Loading plot of VF1 versus VF3. (F = filtered; P = particulate)

coprostanol, β-sitosterol, and stigmastanol (Furtula et al. 2012b; Izbicki et al. 2012). This further suggests that the presence of the aforementioned compounds in both filtered and particulate samples of GW2 and GW3 might be due to a mixture of sources of biogenic and/or anthropogenic origin, such as domestic waste (Froehner et al. 2009; Adnan et al. 2012; Antanasijevi et al. 2018; Pang et al. 2020).

GW4 was characterized by high concentrations of stigmasterol and β-sitosterol, and high concentrations of campesterol were detected in the particulate phase of GW5 and GW6 samples. The variations in cholesterol, campesterol, and stigmasterol levels in groundwater samples were attributable to the bacterial community in the groundwater wells, type of plantation in the vicinity, and distance from the sterol sources (Volkman 1986; Froehner and Sánez 2013; Gottschall et al. 2013; Arcega-Cabrera et al. 2014).

In river water samples, the individual concentration and distribution (%) of sterols and stanols in filtered and particulate samples are displayed in Fig. 2c(i) and c(ii), respectively. All sterols and stanols were identified in all the particulate as well as filtered samples, with the exception of epi-coprostanol in the latter. The range of sterols and stanols in filtered and particulate phases was undetected to 23.57 ± 0.04 mg L^{-1} and undetected to13.17 ± 0.03 mg L^{-1}, respectively. Cholesterol was the most dominant compound in both particulate and filtered phases of the river water samples; however, interestingly, cholesterol was higher in some filtered samples than the particulate samples. The range of cholesterol distribution (%) in filtered and particulate samples was between 35.21–56.38% and 32.79–39.31% (Fig. 2c(ii)). In contrast, as reported in several previous studies, cholesterol was higher in the particulate samples than in filtered samples of river water (Standley et al. 2000; Kim et al. 2016). Cholesterol was also the most dominant compound discovered in other river water samples in Malaysia (Osman et al. 2012; Alsalahi et al. 2015). Cholesterol is synthesized by freshwater organisms such as algae, phytoplankton, and macrophytes, and these are the major sources of this compound in river water (Nishimura and Koyama 1977; Volkman 2005). Anthropogenic sources such as industrial wastewater, agricultural runoff, and sewage effluents are also likely contributors of cholesterol in river water (Mudge and Duce 2005; Shah et al. 2007b; Saim et al. 2009; Furtula et al. 2012c).

In river water samples, coprostanol was detected in all filtered and particulate samples with the highest concentration in filtered and particulate samples being 6.62 ± 0.42 mg L^{-1} and 5.90 ± 0.02 mg L^{-1}, respectively. The higher coprostanol level in filtered samples than in the particulate samples of river water was attributed to the high inflow of the compound from sources such as sewage, leading to a longer partitioning time from the filtered phase into the particulate phase, which may depend on the hydrological conditions of the river (i.e. suspended solids, runoff, and current). The higher levels of coprostanol in filtered samples than particulate samples may also arise from organic matter aggregation/dissolution, resulting in slow exchange from dissolved form to particulate form (Stordal et al. 1996; He et al. 2016). Epi-coprostanol was identified in all particulate samples, and its concentration was slightly higher in filtered samples than in particulate samples in some river water samples. The occurrence and the variation of coprostanol and epi-coprostanol in river water samples may indicate potential inputs from untreated, partially treated and treated sewage discharges (Mudge and Duce 2005; Adnan et al. 2012).

RW1 contained high concentrations of coprostanol, cholesterol, and β-sitosterol in the particulate phase. The elevated levels of coprostanol, cholesterol, and β-sitosterol in the particulate phase of RW1 may be attributed to the direct discharge of cow faeces during surface runoff or rainfall events and effluent discharge of nearby STPs into the Linggi River (Leeming et al. 1996; Shah et al. 2007b). The abundance of coprostanol in the particulate phase of RW1 also may be attributed to the accumulative loading of sewage from the upstream region and the coastal environment (Hussain et al. 2010). RW2 has concentrations of coprostanol, cholesterol, and β-sitosterol ranging from 1.36 ± 0.07 to 4.07 ± 0.01 mg L^{-1} in filtered samples, and from 1.30 ± 0.07 to 3.27 ± 0.01 mg L^{-1} in particulate samples. This location was near STP4, suggesting that the abundance of coprostanol, cholesterol, and β-sitosterol was from its discharge and/or from the cumulative loading of these compounds from farther upstream. Such differences in the concentration and distribution of sterols and stanols in both phases in STP4 (sources) and RW2 (receiving water) may also be due to their mixing with other sterols and stanol compounds, or them being subjected to various biogeochemical processes. The possible dilution of the STP4 effluents resulted in generally lower concentrations of cholesterol and β-sitosterol in RW2 (Wen-Yen and Meinschein 1976; Carreira et al. 2004; He et al. 2018). It was expected that RW3 would receive STP2 effluent containing large amounts of cholesterol, cholestenol, campesterol, and stigmasterol in the particulate phase of the river water (Fig. 2c(ii)). However, only cholesterol was found to be the most dominant sterol in the particulate phase of RW3. This implies a dilution of the river water from various industrial and/or agricultural runoff, which contributed to a depletion in the amount of sterols and stanol in both phases compared to the sewage source (STP2) (Devane et al. 2018; Geary and Lucas 2019).

RW4 had the highest epi-coprostanol concentration of 5.21 ± 0.03 mg L^{-1} compared to other particulate river water samples. A significant amount of epi-coprostanol in STP1 was released into RW4, resulting in a large amount of particulate epi-coprostanol at the RW4 station. All the measured sterols and stanol compounds were detected in the filtered and particulate phases of RW5 and RW6, except for epi-coprostanol in the particulate phase of both samples. RW5 and RW6 exhibited a similar pattern of coprostanol, cholesterol, and β-sitosterol in filtered and particulate phases, which may have been derived from the effluents of STP1 and STP3. The input of faecal

stanols at RW4, RW5, and RW6 could be due to the densely populated areas of Seremban city that has improper sewage treatment facilities (Aburas et al. 2017; Majid et al. 2019). This significantly contributed to the coprostanol and epi-coprostanol (in both phases) in the Linggi River. The diverse pattern of sterol inputs into the Linggi River in particulate and filtered forms may have originated from multiple sources of pollution via sewage, domestic waste, animal farms, and natural sources. Although the presence of faecal stanols in water acts as a chemical indicator of STP efficiency and receiving water quality, it is correlated with bacteriological pathogenic viruses and bacteria, such as *E. coli* (Isobe et al. 2004; Pratt et al. 2008; Gilpin 2011; Reichwaldt et al. 2017), *Enterococcus* spp. (Furtula et al. 2012a), and disruptor hormones (alkyl phenols, bisphneol A, estriol, estrone)((Jeannot et al. 2002; Matić et al. 2014). Furthermore, based on the level and distribution pattern of sterols and stanols, the findings of the current study could not provide a clear understanding of the impact of STPs on river and groundwater ecosystems. Further studies should be undertaken on investigating these aspects with the aim of optimizing sewage treatment techniques and processes to ensure the sustainability of the aquatic ecosystems.

Assessment of sewage contamination based on sterols and stanol sources and its correlation within the mainland aquatic ecosystem using principal component analysis (PCA)

Although some river and groundwater sampling stations had a similar and/or non-similar pattern with their nearby STPs, it is challenging to prove the contribution of sewage in the receiving waterbody. Besides, by relying on distribution pattern only, such interpretation may lead to ambiguity in the fingerprinting of sterol sources and samples. The correlation between sterol sources and samples was often elucidated with the aid of multivariate analysis such as PCA (Saim et al. 2009; Adnan et al. 2012; Frena et al. 2016b). In this study, PCA was performed to enable the identification of potential sterols and stanols sources in filtered and particulate phases of STPs, river water, and groundwater samples. Prior to PCA, CLR transformation was used to perform data pre-treatment from the original dataset (Buccianti et al. 2014; Faith 2015; Antanasijevi et al. 2018). The first three VFs in PCA with eigenvalue more than one were depicted in Table 4. This explained 79.242% of the total variance in all sterols, representing a few linear component combinations of most sterol variabilities from filtered and particulate phases. VF1 was responsible for 46.728% of the total variance in sterols in STPs, and river water and groundwater samples, while VF2 and VF3 accounted for 22.194% and 10.321% of the total variance in sterols, respectively (Fig. 3(a) and (b)).

VF1 was positively dominated by filtered (F)-campesterol (0.638), F-stigmasterol (0.912), and F-β-sitosterol (0.562), and it was highly correlated with fresh inputs from biogenic sterols and vascular plants (Volkman 1986; Alsalahi et al. 2015; Kim et al. 2016) (Fig. 3(a) and Table 4). In contrast, the negative loading of VF1 was highly correlated with particulate (P)-epi-coprostanol (-0.789), P-cholestanol (-0.552), and P-coprostanol (-0.646), thus indicating the source of stanols from an ageing/treated sewage source from nearby STPs in the Linggi river basin (McCalley et al. 1981; Seguel et al. 2001). Several studies on sterols and stanols, focusing on aquatic environments, discussed the dissolved and particulate organics settling in the river sediment and estuaries ranging from recent to a thousand years in age (Masiello and Druffel 2001; Raymond and Bauer 2001; McCallister et al. 2004). Prominently, dissolved organic matter consists of biological reactive components that are cycle-based on the timescale of days to weeks, whereas particulate organic matter can exist longer in the environment (Amon and Benner 1994; McCallister et al. 2004; Loh et al. 2006).

The distinct profile between negative and positive loadings of VF1 demonstrates the difference in sterols and stanols composition present in filtered and particulate samples during the sampling period (Loh et al. 2006). The significant variation in sterols in the particulate and filtered samples may be derived from the type of sterols and stanols sources in the investigated samples as well as their preferred tendency in filtered and/or particulate phases during biogeochemical and/or physical changes. This also further helps to distinguish the degradative state of these STPs, abiotic and biotic exchange, removal

Table 4 Factor loading values of sterols and stanols after varimax rotation in PCA. (*F*, filtered; *P*, particulate)

Compound	VF1	VF2	VF3
F- Coprostanol	0.215	*0.834*	0.303
F-Epi-coprostanol	− 0.101	0.235	*0.812*
F-Cholesterol	0.362	*0.824*	0.020
F-Cholestanol	0.384	0.270	*0.736*
F-Campesterol	*0.638*	0.153	0.626
F-Stigmasterol	*0.912*	0.182	0.186
F-β-Sitosterol	*0.562*	*0.754*	0.151
P-Coprostanol	*− 0.646*	0.214	*− 0.596*
P-Epi-coprostanol	*− 0.789*	0.122	− 0.012
P-Cholesterol	− 0.442	− 0.113	**− 0.734**
P-Cholestanol	*− 0.552*	− 0.491	**− 0.546**
P-Campesterol	0.094	*− 0.813*	0.217
P-Stigmasterol	0.191	*− 0.777*	− 0.412
P-β-Sitosterol	0.224	*− 0.794*	− 0.286
Eigenvalue	6.54	3.11	1.45
Variability (%)	46.73	22.19	10.32
Cumulative (%)	46.72	68.92	79.24

Notes: The bold and italic numbers indicate moderate/strong loading

process in STPs, and natural attenuation (McCallister et al. 2006; de Martins et al. 2007).

The most significant positive factor loading of VF2 (total variability is 22.194%) was due to the high factor loading values of F-coprostanol (0.834), F-cholesterol (0.824), and F-β-sitosterol (0.754) (Table 4). VF2 was influenced by fresh and less treated sewage and domestic sources dominated by filtered coprostanol and β-sitosterol. Although β-sitosterol is always related to plant sterols and to animals with a herbivore diet, this compound has also been detected in STPs samples that are influenced by domestic wastes (Leeming and Nichols 1996; Saim et al. 2009; Furtula et al. 2012b). This finding is also supported by the negative correlation of P-coprostanol, P-stigmasterol, and P-β-sitosterol. The negative loading of VF2 explains the particulate of biogenic sterol and higher vascular plants with campesterol, stigmasterol, and β-sitosterol as dominant biomarkers in negative loading of VF2. VF3, with a total variability of 10.321% (Fig. 3(b)), revealed positive loading, attributable to F-epi-coprostanol (0.812) and F-cholestanol (0.736). Furthermore, VF3 was correlated with the filtered phase of epi-coprostanol and cholestanol, influenced by the fresh-treated sewage source from STPs in Linggi. The presence of epi-coprostanol and cholestanol was related to the degradation of coprostanol by microbes in the sewage treatment process (McCalley et al. 1981; Fernandes et al. 1999; de Martins et al. 2007).

Figure 4(a) and (b) exhibits the factor score loadings of VF1 vs. VF2 and VF1 vs. VF3, respectively. These factor loadings defined the fresh input from biogenic sterol and vascular plant (positive VF1), aged/treated sewage sources (negative VF1), fresh- and less-treated sewage and domestic sources (positive VF2), particulate of biogenic sterol and higher sterols of vascular plants (negative VF2), and fresh-treated sewage sources (VF3). To understand the correlation of sterol and stanols sources with river and groundwater samples from PCA in clear, factor scores values were plotted against VF1, VF2, and VF3 (Fig. 5). The boxes of VF1 (blue), VF2 (orange), and VF3 (grey) with red border line indicate significant correlation of observations (sampling sites) with the respective VFs ($p < 0.05$). This plot also aids in better visualization of samples corresponding to the respective VFs.

Figure 5 illustrates the significant positive loadings of GW4, RW1, RW2, and RW6 for fresh input from biogenic sterol and vascular plants (positive VF1). RW1 and RW2 locations were influenced by higher vascular plant sterol markers, such as campesterol and stigmasterol in the surrounding areas (Volkman 1986; Mudge and Gwyn Lintern 1999). These two locations were surrounded by massive rubber and palm oil plantations. RW6 was situated in the middle of the Seremban town and upstream region of the Linggi river. A significant contribution of biogenic and vascular plant sterols in RW6 was expected from the tropical and reserved forest upstream of the Linggi river basin, compared to anthropogenic sewage sources. Figure 4(a) also verifies the negative correlation of STP effluents in this study with GW4, RW1, RW2, and RW6 sampling stations.

Negative loadings of VF1 demonstrated the association of the aged/treated effluent discharge with STP1, STP4, and STP5. STP1 station had the highest concentration of epi-coprostanol in particulate phase and low level of sterols and faecal stanols in STP4 and STP5. This was attributed to treated sewage effluents from these stations (Shah et al. 2007b). Although STP1 was characterized as a conventional treatment plant releasing high amount of sterols and stanols, epi-coprostanol was dominant in the particulate phase. The negative correlation between natural/biogenic sterols and faecal stanols in the PCA was also reported in other studies (Leeming et al. 1996; Mudge and Duce 2005; Aucélio et al. 2007). These suggested that the contribution of faecal stanols in GW2 station, which was suspected from STP4 (as discussed in the "Level and spatial distribution of sterols in STPs, river water, and groundwater samples" section) was insignificant. This observation was based on PCA biplot and factor score values. Fresh- and less-treated sewage containing domestic sources from STP3 were impacted RW4 and RW5 stations (positive VF2). These two stations were loaded with coprostanol, cholesterol, and β-sitosterol in both phases; however, PCA demonstrated significant loading of filtered sterols from less-treated sewage and domestic sources of STP3 compared to the particulate form. This STP probably underwent mechanical failures in one or more steps of the treatment process during the sampling period. Domestic source contribution also showed high concentrations of β-sitosterol in the dissolved phase of STP3, which may have affected the water quality at RW4 and RW5 stations (Fig. 2c(i) and c(ii)).

The negative loadings of VF2 indicated the presence of particulate biogenic sterols and higher vascular plants, correlating with STP2, GW1, GW2, and GW3 stations. In previous discussions on sterol distribution, negative VF2 was equated with particulate biogenic sterols and higher vascular plants. However, STP2 having significant inputs of campesterol, stigmasterol, and β-sitosterol in sewage effluents led to the misjudgement of VF2 elucidation. Thus, negative VF2 was interpreted as a biological sewage effluent that influenced GW1, GW2, and GW3 stations. These three stations were located further downstream and received sewage effluent from STP2 located near the river water. The migration of sterols into river water and groundwater was affected by their solubilities, travel time, and adsorption in organic matter present in the water (Kihumba et al. 2015; Frena et al. 2016b; Geary and Lucas 2019). Fresh-treated sewage source was demonstrated in VF3 plot. It positively influenced GW5 and GW6 sampling stations and negatively impacted RW3 station (Fig. 4b and Fig. 5). These stations were not correlated with any STPs in this study, suggesting that the input of sterols and stanols from

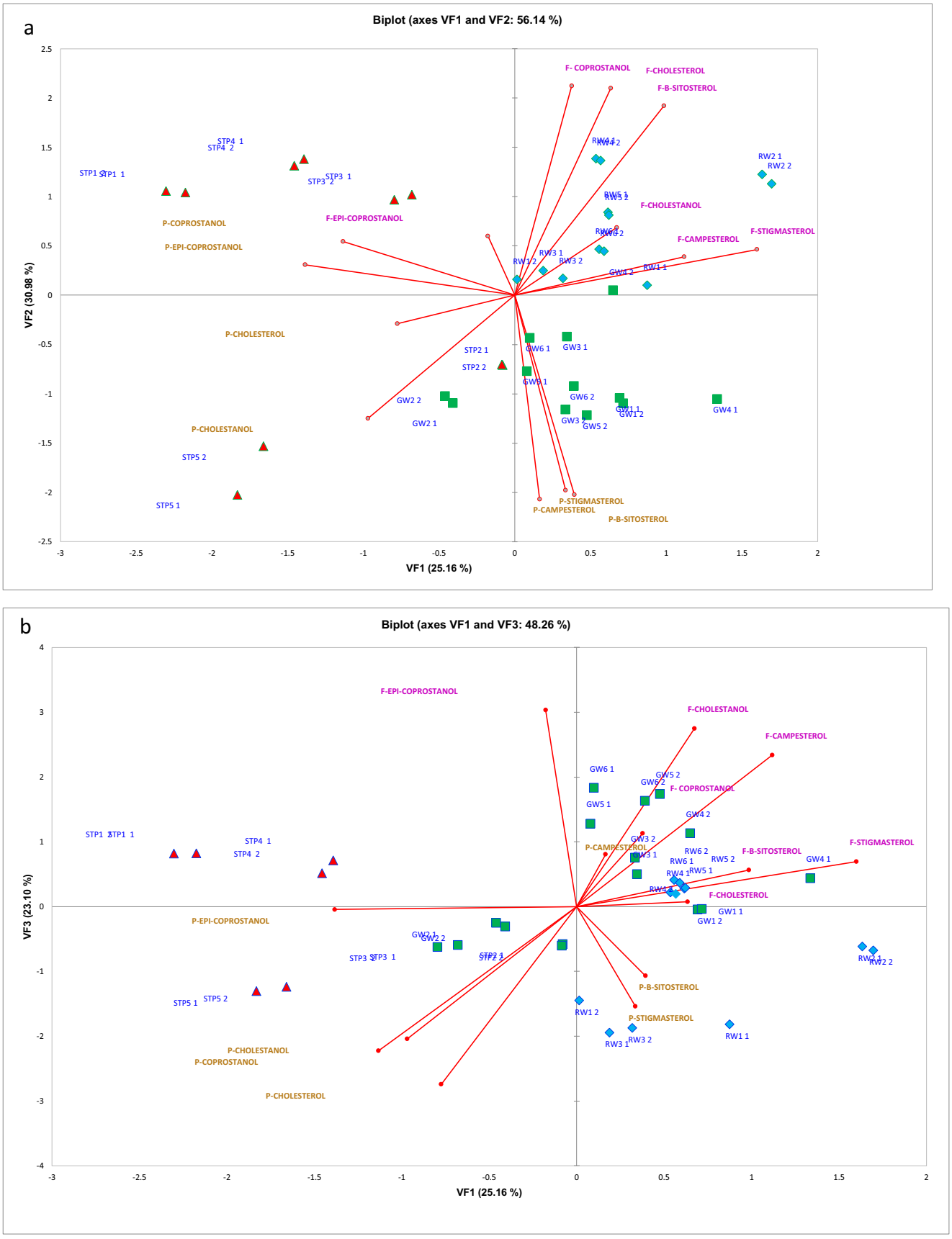


Fig. 4 (a) Score plots of varimax factors (VF1 and VF2). (b) Score plot of varimax factors (VF1 and VF3) of sterols generation from the mixture sources of wastewater treatment plants on groundwater and river water

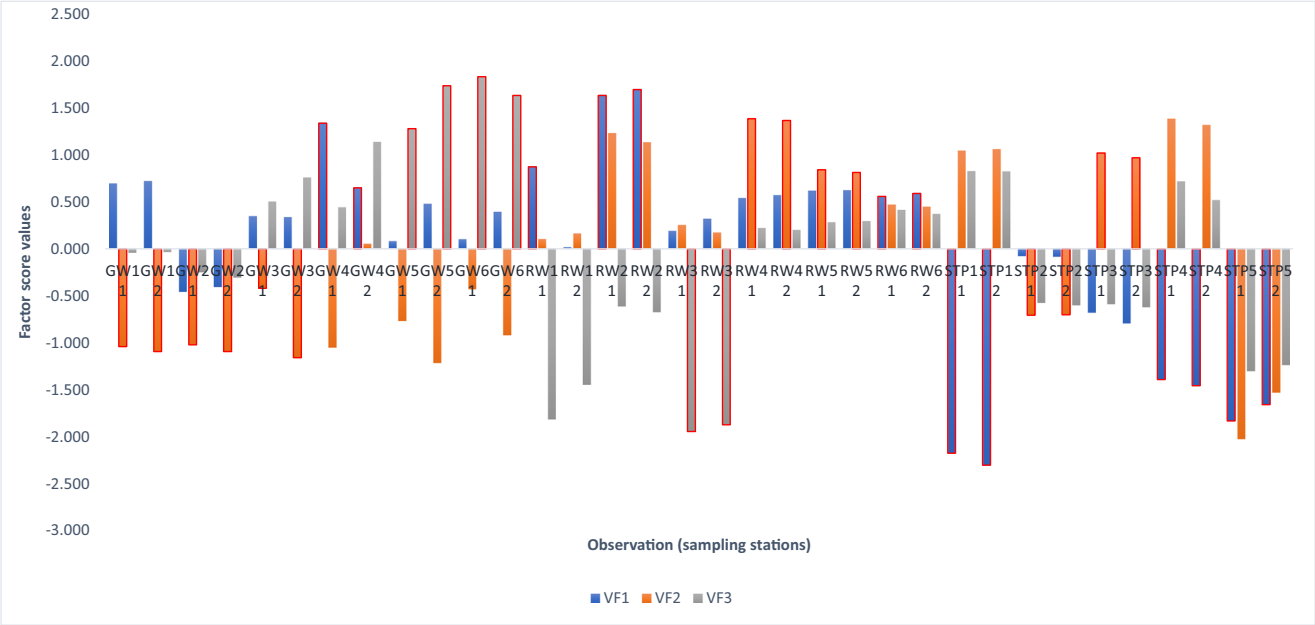


Fig. 5 Factor score values of VF1, V2, and VF3

this loading into GW5 and GW6 originated from other treatment types of STP and/or sterols and stanols sources in Linggi. PCA along with factor score values provided a better interpretation of sterols and stanols sources by examining the distribution and factor loading values. Source interpretation of sterols and stanols, based on factor loading and factor scores values, provides novel insights into sewage pollution characterization and fingerprinting in the aquatic ecosystem.

Conclusions

This study investigated sewage contamination in the river and groundwater of Linggi using the distribution of sterols and stanols markers based on the distribution and PCA. Particulate sterols were more abundant in all the samples, with some exemption for β-sitosterol and epi-coprostanol in STPs and river water samples, respectively. The variation in sterols and stanols in STP samples was influenced by treatment processes and technologies. A significant amount of β-sitosterol in the filtered phase of STPs originated from domestic source contributions. Based on PCA, STP1, STP4, and STP5, effluents were of better quality, compared to STP2 and STP3. The final discharge of STP2 released large amount of biogenic sterol from algae and microorganisms and the least amount of faecal stanol. The elevated level of sterols and stanols in STP3 was due to malfunctioning during the treatment process.

Groundwater samples were altered by biogenic and terrestrial input or higher vascular plants, while river water samples were influenced by the mixture of sterols and stanols sources in filtered and particulate phases. PCA extracted useful information, thus differentiating the biogenic and sewage sources in the filtered and particulate phases within the mainland aquatic ecosystem. In the case of sterol sources that were interpreted using factor loading values, negative VF2 was considered to be particulates of biogenic sterols and higher vascular plants. However, factor loadings and factor scores values explained biological sewage effluent as the new sterol source in negative loading of VF2. PCA also provided a statistically significant correlation between the sterols and stanols sources and sampling sites. The distribution of sterols by visual investigation using individual sterols markers may lead to uncertain judgements. Factor score values of PCA revealed insignificant correlation of STP4 with nearby river and groundwater stations (RW2 and GW2). Furthermore, STP1 was assumed to affect RW4 station; however, PCA showed the significant contribution of STP3 sewage effluent in RW4 station ($p < 0.05$). The migration of sterol and stanols needs to be further studied to understand the sewage contamination pathways in river and groundwater distribution. The use of sterol markers, especially those that are plant and biogenic based, needs to be revised as sterols can also be derived from sewage effluents using biological treatment processes. Further research needs to sample and study all possible sterol sources that influence the water ecosystem at the regional area for better determination of the sources of sterol and stanol at a broader scale across natural and engineered systems.

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Data Availability The datasets analyzed during this current study are available from the corresponding author on reasonable request

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