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Steroid-based tracing of sewage-sourced pollution of river water and wastewater treatment efficiency: Dissolved and suspended water phase distribution

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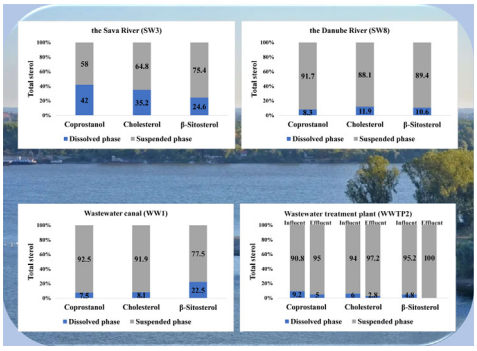
HIGHLIGHTS

- Steroids in river and wastewater in the confluence area of two rivers were analyzed.
- The high-flow Danube was more contaminated than the low-flow Sava River.
- It is necessary to consider both water phases in tracing of sewage-sourced pollution.
- Sterols partition to suspended material in the dissolved/suspended phase distribution.
- Ratio copro/(copro + cholesta) is affected by quality improvement of treated wastewater.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, the environmental distribution of steroid compounds and the level of sewage-derived contamination were assessed using sterol ratios in the confluence area of two major rivers in the Serbian capital, where raw sewage is discharged without any treatment. Special attention was paid to steroids partitioning between the dissolved and suspended phases of river and wastewater samples, since steroids tend to easily bind to particulate matter. The efficiency of sterol removal in two wastewater treatment plants in Serbia was also evaluated. Human/animal sterols coprostanol and cholesterol, and phytosterol β-sitosterol were the dominant compounds in all water samples. The sterol abundance pattern in river water was different from that in raw sewage, indicating a more pronounced biogenic input, as well as greater impact of wastewater discharges on the composition of the suspended phase. Severe contamination of the investigated area was determined, with the Danube being more contaminated than the Sava River due to different hydrodynamic conditions leading to significantly higher sterol levels in the suspended particulate matter. It was also shown that the greater part of human/animal sterols and phytosterols present in river water samples (83.0 ± 11.9 % and 87.1 ± 15.2 %) and wastewater samples (92.1 ± 6.8 % and 95.0 ± 5.7 %) was bound to suspended material compared to the dissolved phase, emphasizing the need to consider and analyze both water phases in the tracing of steroid-based environmental pollution in order to obtain a realistic picture of steroid contamination and their fate in the aquatic environment. A high removal rate (>98 %) of coprostanol and cholesterol during wastewater treatment was determined and only the coprostanol/(coprostanol + cholestanol) ratio was found to be sensitive enough to be affected by an improvement in the quality of treated wastewater.

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1. Introduction

The presence of steroid compounds in natural and wastewaters and their detrimental impact on the aquatic ecosystem have been recognized in the scientific community for more than a few decades. Ecotoxicity research has shown that trace amounts of sterols (MacLatchy et al., 1997; Gagné et al., 2001; Orrego et al., 2010) and steroid hormones (Liu et al., 2011; Jarošová et al., 2015; Pratush et al., 2020) in environmental waters can induce a variety of adverse effects on aquatic organisms, such as abnormal reproductive function, impaired growth and endocrine disruption. Steroids have been extensively investigated in various environmental matrices, including surface and groundwater (Furtula et al., 2012a; Nakagawa et al., 2019; Zali et al., 2021), river sediments (Matić Bujagić et al., 2016; Cabral et al., 2020), marine (Readman et al., 2005; Lyons et al., 2015) and estuary (Frena et al., 2016) sediments, as well as in wastewater (Jeanneau et al., 2011; Furtula et al., 2012b; Andrási et al., 2013). Although some of the steroids occur naturally, they usually enter the environment in great quantities via discharges of raw sewage and treated urban wastewater, as well as via the run-off from agricultural land treated with manure or sludge (Stuart et al., 2012; Jarošová et al., 2015).

Assessment of sewage-derived environmental contamination set by legislation and regulatory agencies for routine monitoring implies the use of bacterial indicators, such as fecal coliforms, total coliforms, *Escherichia coli*, among others. However, these traditional markers can be unreliable and can underestimate health risk even when they meet regulatory standards (Rodrigues and Cunha, 2017; Holcomb and Stewart, 2020). Chemical markers widely used for tracing sewage contamination support and complement conventional indicators. In addition to pharmaceuticals, personal care products, artificial sweeteners and fluorescent whitening agents, sterols are commonly used to determine sewage pollution and to provide essential information on water quality or the performance of wastewater treatment plants (WWTPs) (Lim et al., 2017; Reichwaldt et al., 2017). The most important chemical marker among sewage-derived sterols is coprostanol which accounts for up to 60 % of total sterols in human fecal waste (Leeming et al., 1996). It has been proven to be a useful indicator in sewage-contaminated environments (Adnan et al., 2012; Albuquerque de Assis Costa et al., 2018; Kolm et al., 2018), usually evaluated in relation to other sterols. Coprostanol-dominated sterol ratios indicate human sewage as the source of pollution (Reichwaldt et al., 2017; Fennell et al., 2021). Besides coprostanol, major sterols present in human and animal feces are epicoprostanol and cholestanol (Leeming et al., 1996), which are crucial for determining fecal contamination levels and for differentiating between sewage-sourced and non-human biogenic input, using sterol ratios.

An overview of studies on steroid-based tracing of sewage contamination shows that the majority of them thoroughly investigate the dissolved phase of water samples, while only a few consider the suspended phase as well (Wang et al., 2010; Andrási et al., 2013; Zali et al., 2021). Suspended particulate matter (SPM) is an integral part of the water sample, and the analysis of particle-bound contaminants contributes to a comprehensive water-quality assessment (Schubert et al., 2012). As many steroids exhibit hydrophobic properties and tend to easily bind to particulate matter (Bull et al., 2002), investigation of both dissolved and suspended phases is essential for a complete overview of the extent of sewage-sourced water pollution. Moreover, some studies have suggested that the SPM evaluation indicates the status of environmental contamination on a larger spatial and short-time scale (Cardoso et al., 2016; Cabral and Martins, 2018). The local hydrodynamic pattern promotes water homogenization, dispersion and dilution of suspended particles, leading to a more uniform composition on a larger spatial scale. SPM analysis provides information on recent contamination input, while sediment analysis provides historical and chronic input information on contamination and can indicate hotspot areas and final deposition of sewage particles (Cabral and Martins, 2018).

The key source of water pollution in the Republic of Serbia (RS) is wastewater discharged into the receiving waters without any treatment (either mechanical, biological or chemical). Wastewater in the RS is generated by agriculture, forestry and fishing (54 %), households (28 %), industry (9 %) and other sources (9 %), of which 8.4 % is treated. About 65 % of households are connected to the public sewer system, while the rest dispose wastewater in septic tanks or directly into rivers or streams. Only 18 % of wastewater from the public sewage system is treated prior to discharge, and the most common type of treatment is secondary, which includes biological treatment. About 66 % of the treated sewage wastewater undergoes secondary treatment, 27 % goes through tertiary treatment, while 7 % undergoes primary mechanical treatment (Statistical Office of the Republic of Serbia, 2020, 2021). At present, only 42 municipalities have operational WWTPs of which only a small number of them operate according to the designed criteria, while most of them operate with efficiency far lower than projected. Reconstruction or construction of 18 WWTPs is currently in progress (Environmental Protection Agency (EPA RS), 2019). However, the capital of the RS, Belgrade and some large cities do not have WWTP.

The objectives of the present study were to (i) assess the environmental distribution of the selected steroids in the confluence area of two major rivers in the RS capital with many raw sewage discharges; (ii) determine the level of sewage contamination of river water using sterol ratios; (iii) examine steroids partitioning behavior between the dissolved and the suspended water phases, and (iv) determine WWTP efficiency in steroid removal. Such a comprehensive approach will provide a more realistic picture of steroid contamination and its subsequent fate in the aquatic environment. For this purpose, we used previously developed and optimized liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for analysis of twenty selected human/animal sterols, phytosterols and hormones.

2. Materials and methods

2.1. Chemicals and reagents

Twenty steroids, including six human/animal sterols (cholesterol, coprostanol, epicoprostanol, epicholestanol, cholestanol, cholestanone), five phytosterols (β-sitosterol, stigmasterol, campesterol, desmosterol, sitostanol) and nine hormones (estriol, estrone, 17β-estradiol, 17α-estradiol, equilin, 17α-ethinylestradiol, norethindrone, levonorgestrel, mestranol), were selected for the study based on the frequency of their use and detection in environmental samples. Human/animal sterols were chosen primarily taking into account the major sterols present in human and animal feces (Leeming et al., 1996). High-purity analytical standards (>99 %) of chosen steroids were purchased from Steraloids Inc. (Newport, USA). Common and IUPAC names, CAS numbers, molecular weights, chemical structures, and properties such as water solubility and octanol-water partition coefficient (K_{ow}) of twenty selected steroids are presented in Table S1 (Supplementary Material). A stock standard solution of each steroid was prepared in methanol at a concentration of 100 μg mL^{-1}. Working standard solutions were prepared by mixing appropriate amounts of the stock standard solutions and diluting them with methanol. All solutions were preserved at −4 °C. All solvents and reagents used were HPLC or analytical grade from J.T. Baker or Sigma-Aldrich. Deionized water was obtained by passing the distilled water through the GenPure ultrapure water system (TKA, Niederelbert, Germany).

2.2. Sample collection and sampling sites description

Both surface water and raw wastewater samples were collected in the area of the Serbian capital, the city of Belgrade, around the confluence of the Danube and the Sava River (Fig. 1, Table S2). River water samples were collected at nine sampling sites in a heavily populated area under the influence of sewage discharges nearby, with the exception of SW1 sample taken in a small suburban settlement on the Danube. Three samples were collected from the Sava (before the confluence, samples SW2–SW4), five samples from the Danube (two before the confluence –SW1 and SW5 samples, and three after the confluence, samples SW7–SW9) and one sample was taken at the confluence of two rivers (sample SW6). The sampling site of the SW9 sample was located in a small bay, away from the river

Fig. 1. Map of the Danube River flow and the confluence with the Sava River in Belgrade (Serbia) with the surface water (SW) and wastewater (WW) sampling sites.

mainstream, with limited water flow. River water samples were collected in June 2020 by direct sampling, from a boat, in the middle of the river flow at a depth of about 50 cm. Raw wastewater samples were collected from six sewage discharges (WW1–WW6), as grab samples, in a densely populated area of Belgrade, with corresponding samples of receiving river water taken downstream. Samples WW1 and WW5 were taken from two large sewage canals with 518,224 and 164,653 inhabitants connected to each wastewater canal, respectively. There was no precipitation on the day of sampling, and in the previous two days there was 1.2 mm of precipitation that was described as light drizzle.

Since Belgrade does not have a WWTP, influent and effluent samples were collected from two WWTPs in the small municipalities of Zuce (WWTP1) and Topola (WWTP2) in the RS. For WWTP1, with a capacity of 1000 population equivalent (PE), the mean annual inflow is 128 m^3 day^{-1}, while the mean annual effluent flow is 116 m^3 day^{-1}. The treatment process consists of mechanical pretreatment of wastewater and biological treatment based on activated sludge. Only households and catering facilities are connected to the sewage network. The capacity of WWTP2 is 8000 PE, with the mean annual influent flow of 1089 m^3 day^{-1}, and 978 m^3 day^{-1} flow for the effluent. Similar to WWTP1, households and catering facilities in the city and some rural settlements of the municipality are connected to the sewage system, and mechanical and biological treatments are applied in the plant. The secondary treatment process is based on activated sludge. Composite 24-h samples of influent and effluent wastewater from each WWTP were collected by automatic sampling devices.

All water samples were collected in 1 L PVC bottles and stored in a freezer without preservatives until preparation for analysis, a few days after sampling.

2.3. Sample preparation

To separate the dissolved and suspended phases, water samples (1 L) were thawed and filtered using glass microfiber filters (Whatman GmbH, Dassel, Germany), first with 1–3 μm and then with a 0.7 μm pore size filter. All filters used were previously weighed with analytical precision. The filters with suspended material were stored in the dark for 24 h at room temperature to dry. The filtrate of each sample was immediately extracted for further analysis.

The average amount of suspended solids obtained by filtering nine river water samples was 83 ± 12 mg (ranging from 70 mg to 105 mg). For six samples of raw wastewater from canals with a wide range of inhabitants connected to canals (4620–518,224, Table S2), a wide range of suspended solids masses was obtained (32–149 mg, average 98 ± 42 mg). As for the two WWTPs, with PE 1000 and 8000, 102 mg and 190 mg of suspended solids, respectively, were obtained for the influents. After treatment, the amount of suspended material in WWTP2 effluent was significantly reduced to 7 mg, while in WWTP1 effluent it was reduced to 65 mg.

2.3.1. SPE of dissolved phase of water samples

For the preparation of river and wastewater samples, a previously developed and optimized solid-phase extraction (SPE) procedure was used for determination and reliable confirmation of twenty selected steroids (human/animal sterols, phytosterols, and steroid hormones) in environmental and wastewater (Jauković et al., 2017). Briefly, the extraction was performed using an Oasis HLB cartridge (200 mg/6 mL; Waters, Milford, USA) for preconcentration of 200 mL of surface water samples or 100 mL of wastewater samples, without pH adjustment (or with adjustment if pH is not ~7.5) and elution with 15 mL of methanol. Several calibration solutions were prepared by spiking the water samples prior to the SPE procedure and extracting them using the optimized method. Sample extracts and calibration solutions were evaporated to 0.5 mL, filtered through a 0.45 μm polyvinylidene difluoride (PVDF) filters, from Roth (Karlsruhe, Germany), and analyzed.

2.3.2. USE of suspended phase of water samples

Ultrasound solvent extraction (USE) of the suspended material was performed using an adapted procedure for the extraction of steroid compounds from river sediments (Matić et al., 2014). Dry glass microfiber filters were extracted in plastic tubes (50 mL) using 25 mL of methanol and sonicated for 10 min. The procedure was repeated and 50 mL of the obtained extract was centrifugated for 10 min at 4000 rpm, decanted and evaporated in a gentle nitrogen stream to a volume of 4 mL. The extract was divided into

four portions, two of which were evaporated to dryness and spiked with standard solutions at two calibration levels, while the remaining two were not spiked (samples, $n = 2$). Extracts and standards were filtered using 0.45 μm PVDF filters and analyzed. To compare with the levels of steroids in the dissolved phase, the obtained concentrations for SPM in ng g^{-1} (knowing the weight of SPM) were converted to ng L^{-1} (taking into account the volume of the water sample).

2.4. LC-APCI-MS/MS analysis

LC and MS operating parameters for the determination of 20 selected steroids were developed and optimized in a previous study (Jauković et al., 2017). Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) was used to separate the analytes on a Zorbax Eclipse® XDB-C8 reverse phase column, 150 mm × 3.0 mm i.d. and 3.5 μm particle size (Agilent Technologies, Santa Clara, CA, USA). A corresponding Zorbax Eclipse® XDB C8 precolumn was also installed, 12.5 mm × 4.6 mm i.d. and 5 μm particle size. The mobile phase consisted of deionized water, acetonitrile and 10 % (*v*/v) acetic acid. Mass spectrometric analysis was performed using LTQ Advantage (Thermo Fisher Scientific, USA) linear ion trap mass spectrometer with atmospheric pressure chemical ionization (APCI). LC and MS operating parameters for selected steroids, including LC mobile-phase gradient, MS parameters for data acquisition, and fragmentation reactions for quantification and conformation purposes, are presented in Table S3 in the Supplementary Material.

2.5. Quality assurance and quality control

Extraction blanks were analyzed in each sample batch. For the dissolved phase, the blank was 200 mL of deionized water extracted according to the SPE procedure. For the suspended phase, the blank was glass microfiber filter with pore sizes of 0.7 μm and 1–3 μm extracted using the USE procedure. Selected steroids were not detected in the blanks. The recoveries of analytes from the dissolved phase of water samples (Table S4, Supplementary Material) were in the range 77 %–103 % (for river water) and 82 %–101 % (for wastewater). As for the suspended material, the recoveries ranged from 71 % to 109 % (for river water) and from 73 % to 99 % (for wastewater). The relative standard deviations were lower than 20 %.

The limits of detection (LOD) and quantification (LOQ) of the investigated analytes were calculated using the signal-to-noise (S/N) ratios. LOD and LOQ were determined as the lowest concentrations of analytes in spiked samples with S/N ratio of 3 and 10, respectively (Table S4). Low LODs and LOQs were achieved for the investigated compounds in river water samples, both in the dissolved phase (2.6–8.4 ng L^{-1} and 8.7–28.0 ng L^{-1}, respectively) and in SPM (3.1–10.7 ng L^{-1} and 10.3–35.7 ng L^{-1}, respectively). For wastewater samples, LODs and LOQs were in the range of 3.9–10.9 μg L^{-1} and 13.0–36.3 μg L^{-1} (for the dissolved phase), and 3.5–14.3 μg L^{-1} and 11.7–47.7 μg L^{-1} (for the suspended phase), respectively.

The standard addition method was used as a calibration method in the analysis of the dissolved phase of water samples. After filtration, each water sample was divided into five 200 mL portions (river water) or five 100 mL portions (wastewater). Calibration solutions were prepared by spiking with standard solutions at 10–1000 ng L^{-1} (for river water samples) and at 0.1–10 μg L^{-1} (for wastewater samples). Due to the small amount of suspended material, quantification of analytes in the suspended phase of water samples was performed by dividing the obtained extract (4 mL) into four portions, two of which were evaporated to dryness and spiked with 1 mL of standard solutions at calibration levels of 0.1 and 1 μg mL^{-1} (for river water SPM) or 5 and 25 μg mL^{-1} (for wastewater SPM), while the remaining two were not spiked and were analyzed as samples.

2.6. Sterol ratios

Sterol ratios are commonly applied to determine anthropogenic input (especially fecal contamination) in various environmental compartments. The set of six sterol ratios most reliable for the identification of human fecal contamination and differentiation from natural sterol input were compiled from the literature (Table S5, Supplementary Material). They were calculated for both the dissolved and suspended phases of all surface and wastewater samples. Concentrations of four human/animal sterols (cholesterol, coprostanol, epicoprostanol and cholestanol) detected in the analyzed samples were used in selected sterol ratios. Cholesterol is one of the most abundant sterols, both in human/higher vertebrate organism and in the environment, and is mainly reduced to coprostanol in the digestive tract, while typically transformed into cholestanol in the environment (Devane et al., 2006; Martins et al., 2007). As cholesterol can be present in human and animal waste, as well as in plant material, it cannot be used as a source-specific marker, but in relation to other sterols it can clarify the source of pollution. High amounts of coprostanol indicate human fecal pollution, as it comprises about 60 % of total sterols in human waste (Leeming et al., 1996). Cholestanol is a marker of non-human biogenic input because it is usually present in environments that are not contaminated with fecal material. Epicoprostanol is a coprostanol isomer present in human waste in trace amounts, while it is more abundant in the feces of other mammals (Leeming et al., 1996). It is also formed by anaerobic microbial digestion during the sewage treatment or the aging of fecal matter. Therefore, it is usually used as an indicator of the level of treatment or age of the fecal material (Martins et al., 2007; Adnan et al., 2012; Reichwaldt et al., 2017).

Ratio No. 1 (coprostanol/(coprostanol + cholestanol)) is the most frequently used and one of the most reliable sterol ratios, with values higher than 0.7 indicating certain human fecal contamination (Table S5, Supplementary Material). Values between 0.3 and 0.7 point to mixed sewage and biogenic input, whereas values below 0.3 are characteristic of uncontaminated samples (Grimalt et al., 1990). Ratio No. 2 (coprostanol + epicoprostanol/(coprostanol + epicoprostanol + cholestanol)) compensates for the microbial conversion of coprostanol to epicoprostanol and the influence of the age of fecal pollution (Bull et al., 2002). Namely, epicoprostanol is found in trace amounts in human feces, and is formed by microbial conversion of coprostanol, for example, by digestion of sewage sludge during wastewater treatment (McCalley et al., 1981). Therefore, it can be used as an indicator of treatment level or age of fecal matter (Mudge and Seguel, 1999; Martins et al., 2007). Ratio No. 2 has the same reference values as ratio No. 1. The coprostanol/cholesterol ratio (ratio No. 3) is considered to be a very reliable ratio for distinction between different sterol sources, with a threshold value >1 indicating an anthropogenic source and a value <1 typical of high input from natural sources (Zhang et al., 2008). Ratio No. 4 (coprostanol/(cholesterol + cholestanol)) was proposed by Writer et al. (1995) for identification of the sewage impact in environmental samples, with a reference value of 0.06 employed for differentiation between sewage and non-sewage sterol sources. The coprostanol/epicoprostanol ratio (ratio No. 5) can be used to differentiate human waste input from the fecal inputs of other mammals (Zhang et al., 2008). A ratio value >1.5 indicates human-derived fecal pollution. Ratio No. 6 (epicoprostanol/coprostanol) is regarded as reliable for estimating the degree of wastewater treatment and distinguishing between treated and untreated sewage inputs (Martins et al., 2007), as well as for determining the age of fecal matter (Adnan et al., 2012; Reichwaldt et al., 2017). A ratio value below 0.2 points to a recent input of raw sewage, while a value above 0.8 suggests that treated wastewater or untreated sewage discharged a while ago is a source of pollution. Since there is no WWTPs in the investigated area, only untreated sewage can be considered as the source of sterol input.

In addition to the sterol ratios used to determine sewage-sourced pollution, a range of ratios has been proposed to distinguish between human and different animal sources of fecal pollution in the environment (Table S6, Supplementary Material). Namely, the quality of river water can also be affected by animal fecal pollution, especially in agricultural areas with the practice of land-spreading cattle or pig manure (Jardé et al., 2007; Derrien et al., 2011; Jaffrezic et al., 2011). Ratio No. 7 ((coprostanol + epicoprostanol)/cholesterol) was proposed by Jardé et al. (2007) by analysis of pig, cow and poultry manure. It can clearly distinguish pig

fecal matter from other pollution sources using a reference value higher than 3.7. A ratio value below 0.7 points to bovine pollution source, whereas a value much higher than 3.7 indicates human-sourced contamination. Ratio No. 8 (sitostanol/coprostanol) can be used to differentiate between bovine (value >1) and porcine (<1) pollution in the analyzed samples (Derrien et al., 2011; Jaffrezic et al., 2011). Gourmelon et al. (2010) suggested that a value lower than 1 also indicates human-induced pollution, in addition to the set of markers used to determine the origin of pollution. As a general fecal contamination ratio, coprostanol/cholestanol (ratio No. 9) can determine human/herbivore mammal fecal pollution (value >0.5) and potentially indicate contamination from avian source (<0.5, Devane et al., 2015). Also, avian and wildlife fecal pollution has been shown to be the cause of elevated bacterial indicators in the aquatic environment and two novel sterol ratios have been proposed by Devane et al. (2015) as specific for avian fecal pollution (ratios No. 10 and No. 15, Table S6). However, most of these ratios (Table S6) include two animal-specific markers, 24-ethylcoprostanol (24-ethyl-5β-cholestan-3β-ol) and 24-ethylepicoprostanol (24-ethyl-5β-cholestan-3α-ol). These two sterols are major fecal biomarkers of herbivores (Leeming et al., 1996; Devane et al., 2015; Nash et al., 2005) that can help to discriminate between human and herbivore mammal fecal contamination (Devane et al., 2015) or between bovine and porcine fecal pollution sources (Derrien et al., 2011; Jaffrezic et al., 2011). Nevertheless, the evaluation of the extent of animal fecal contribution in the study was limited due to the lack of these two animal-specific markers and the current set of 11 sterols should be expanded to include 24-ethylcoprostanol and 24-ethylepicoprostanol.

3. Results and discussion

3.1. Steroids detected in river water

The results obtained for both the dissolved and suspended phase of the river water samples (Table 1) show that human/animal sterol cholestanone and nine monitored steroid hormones were not detected in any of the analyzed water samples. Steroid hormones are usually present in the aquatic environment at very low concentrations (Ojoghoro et al., 2021). For example, in the Danube in Hungary (Andrási et al., 2013), low levels of 17β-estradiol (up to 0.7 ng L^{-1}) and 17α-ethinylestradiol (up to 1.2 ng L^{-1}) in the dissolved phase of water samples were found, while only 17α-ethinylestradiol (up to 0.5 ng L^{-1}) was detected in the suspended phase. The inability to detect steroid hormones in river water samples can be explained by the reported LODs and LOQs of the applied analytical methods (Table S4). The applied methods seem to show limited performance for the detection of analytes present in low concentrations close to LODs. As for cholestanone, since it is predominantly formed by the degradation of cholesterol under aerobic conditions, its absence can be attributed to the lack of aerobic microbial activity (Cordeiro et al., 2008; Prost et al., 2018). Five human/animal sterols (cholesterol, coprostanol, epicoprostanol, epicholestanol and cholestanol) were identified in the particulate matter of all investigated samples, while an exception was found for the dissolved phase of the sample SW6 containing only cholesterol, which indicates that it is the least contaminated sample. Cholesterol was the dominant compound in both phases of river water samples (11.9–49.6 % for dissolved phase, 22.0–39.9 % for SPM, Table 1). As expected, cholesterol levels were much higher in SPM (842–38,199 ng L^{-1}) than in the dissolved phase (158–1938 ng L^{-1}) of surface water samples, given its low water solubility and high log K_{ow} (Table S1, Supplementary Material). In fact, all detected sterols have very low solubility in water ($3.8{\cdot}10^{-5}$–$9.5{\cdot}10^{-2}$ mg L^{-1}) and very high log K_{ow} values (8.31–9.73) which explains their preference to partition to SPM in the dissolved/suspended phase distribution. Using the results presented in Table 1, it was determined that 57.4–97.5 % of human/animal sterols and 46.9–94.6 % of phytosterols present in the river water sample were bound to particulate matter compared to the dissolved phase. In rare studies on river water quality involving sterols, another study conducted in northern Serbia showed similar levels in the dissolved phase of water samples from the Danube, Tisza and Begej rivers, in the range of 288–1950 ng L^{-1} (Škrbić et al., 2018). However, much lower concentrations were found in the Danube in Hungary (88–170 ng L^{-1}, Andrási et al., 2013) and the Prut River in the Danube River basin at the Romanian-Moldavian border (22–150 ng L^{-1}, Moldovan et al., 2018). On the other hand, monitoring of surface water quality in three watersheds in China, surrounded by densely populated urban areas, showed higher cholesterol levels in the range 1325–6378 ng L^{-1} (Kong et al., 2015). The cholesterol levels found in the suspended phase were also significantly higher than those in the Danube in Hungary (160–534 ng L^{-1}, Andrási et al., 2013), and similar to those detected in the eight rivers of the largest metropolitan region of Brazil (30–34,800 ng L^{-1}, Albuquerque de Assis Costa et al., 2018).

Although sewage effluents, industrial wastewater, and agricultural runoff are considered major contributors to cholesterol in river water, it can

Table 1
Detected sterol concentrations in dissolved phase* and suspended phase** of surface water (SW) samples.

	Concentration ± SD, ng L^{-1}								
	SW1	SW2	SW3	SW4	SW5	SW6	SW7	SW8	SW9
Cholesterol	195 ± 16* (3092 ± 356)**	332 ± 54 (842 ± 224)	853 ± 182 (1568 ± 149)	158 ± 22 (896 ± 103)	271 ± 15 (6758 ± 626)	261 ± 51 (873 ± 153)	330 ± 54 (3074 ± 375)	529 ± 16 (3931 ± 991)	1938 ± 198 (38199 ± 5944)
Coprostanol	71 ± 5 (951 ± 93)	113 ± 7 (306 ± 57)	384 ± 71 (531 ± 108)	65 ± 6 (487 ± 56)	197 ± 26 (7553 ± 1415)	– (572 ± 52)	109 ± 20 (1593 ± 201)	232 ± 25 (2558 ± 192)	1814 ± 331 (44472 ± 3682)
Epicoprostanol	33 ± 5 (243 ± 32)	92 ± 8 (124 ± 7)	35 ± 5 (134 ± 6)	63 ± 4 (249 ± 47)	45 ± 1 (224 ± 30)	– (203 ± 30)	89 ± 11 (197 ± 39)	65 ± 9 (211 ± 18)	239 ± 43 (1957 ± 136)
Epicholestanol	26 ± 5 (117 ± 6)	30 ± 1 (58 ± 11)	14 ± 2 (80 ± 7)	17 ± 3 (80 ± 6)	19 ± 1 (72 ± 11)	– (73 ± 14)	19 ± 2 (48 ± 8)	32 ± 3 (54 ± 9)	85 ± 8 (258 ± 31)
Cholestanol	39 ± 3 (613 ± 125)	68 ± 8 (136 ± 22)	37 ± 2 (168 ± 28)	61 ± 10 (212 ± 45)	77 ± 7 (800 ± 161)	– (206 ± 12)	135 ± 14 (339 ± 36)	85 ± 13 (347 ± 21)	419 ± 63 (4459 ± 251)
β-Sitosterol	295 ± 23 (2180 ± 414)	– (646 ± 95)	296 ± 14 (906 ± 188)	811 ± 78 (795 ± 92)	– (1137 ± 148)	– (699 ± 100)	681 ± 78 (1200 ± 223)	193 ± 13 (1636 ± 143)	– (15001 ± 1730)
Stigmasterol	131 ± 13 (1052 ± 206)	35 ± 4 (252 ± 30)	36 ± 1 (268 ± 38)	104 ± 6 (243 ± 22)	– (349 ± 25)	118 ± 5 (250 ± 34)	128 ± 19 (400 ± 34)	55 ± 2 (601 ± 63)	488 ± 104 (8557 ± 710)
Campesterol	– (253 ± 35)	(98 ± 14)	25 ± 1 (94 ± 16)	– (44 ± 4)	– (114 ± 12)	– (98 ± 11)	– (132 ± 14)	– (209 ± 32)	163 ± 19 (1508 ± 137)
Desmosterol	47 ± 6 (533 ± 65)	– (82 ± 8)	34 ± 2 (86 ± 9)	45 ± 4 (91 ± 16)	– (85 ± 3)	61 ± 3 (93 ± 12)	35 ± 4 (159 ± 22)	45 ± 1 (219 ± 39)	114 ± 9 (361 ± 23)
Sitostanol	– (2315 ± 214)	– (251 ± 25)	133 ± 21 (391 ± 45)	– (972 ± 198)	– (1272 ± 104)	273 ± 35 (514 ± 72)	– (567 ± 76)	476 ± 35 (420 ± 32)	– (4605 ± 733)
%Cholesterol	23.3 (27.3)	49.6 (30.1)	46.2 (37.1)	11.9 (22.0)	44.5 (36.8)	36.6 (24.4)	21.6 (39.9)	30.9 (38.6)	36.8 (32.0)
%Coprostanol	8.5 (8.4)	16.9 (10.9)	20.8 (12.6)	4.9 (12.0)	32.3 (41.2)	0 (16.0)	7.1 (20.7)	13.6 (25.1)	34.5 (37.3)

also originate from natural sources and even be synthesized by freshwater organisms such as algae, phytoplankton, and macrophytes (Volkman, 2005; Alsalahi et al., 2015). Therefore, it cannot be considered a specific marker of anthropogenic contamination of the aquatic environment.

Another human/animal sterol highly abundant in the studied river water samples was coprostanol (up to 34.5 % in dissolved phase, and 8.4–41.2 % in SPM). If coprostanol comprises >5–6 % of total sterols, it indicates sewage as the source of pollution (Devane et al., 2006). According to this criterion, all samples show municipal wastewater contamination, and the greatest one is detected in samples SW5 and SW9. It has also been suggested that coprostanol values in the range of 60–400 ng L^{-1} in water column (Leeming and Nichols, 1996) and 1000–2230 ng L^{-1} in SPM (Cabral and Martins, 2018) indicate sewage contamination and correspond to the existing bacterial limits (*Escherichia coli* and Enterococci) which indicates poor water quality and water potentially unusable for recreation. Coprostanol was detected in the dissolved phase at levels within the range of 65–1814 ng L^{-1}, with the exception of sample SW6, identified as the least polluted. Regarding the suspended phase of river water samples, coprostanol was found in concentrations ranging from 306 ng L^{-1} to 44,472 ng L^{-1}, the latter being detected in the most contaminated sample SW9. These levels are much higher compared to studies involving the Danube water quality in Hungary (19–42 ng L^{-1} for dissolved phase, 99–266 ng L^{-1} for SPM, Andrási et al., 2013) and the less populated northern part of Serbia (32–430 ng L^{-1} for dissolved phase, Škrbić et al., 2018). However, the detected concentrations of coprostanol are lower than those reported for heavily populated areas of China (up to 4450 ng L^{-1} for dissolved phase, Kong et al., 2015) and Brazil (30–205,000 ng L^{-1} for SPM, de Assis Costa et al., 2018). The authors suggested that insufficient sewage treatment efficiency and inadequate existing management systems for the control of contaminant discharge are the main causes of highly polluted surface waters (Kong et al., 2015), along with the extreme lack of basic sanitation networks (Albuquerque de Assis Costa et al., 2018).

Of the five investigated phytosterols, the most prominent was β-sitosterol, detected at levels up to 811 ng L^{-1} in the dissolved phase of the water samples, and in the range of 646–15,001 ng L^{-1} in the suspended phase. It is a sterol that occurs naturally in terrigenous vascular plants and accounts for up to 95 % of total sterols in plants (Rontani et al., 2014). It is also a major sterol in herbivore waste (Leeming et al., 1996). On the other hand, β-sitosterol is the main phytosterol of many refined vegetable oils (Piironen et al., 2000) and is well known for lowering serum cholesterol levels during dietary treatment (Rondanelli et al., 2013). Therefore, higher vascular plants and terrestrial organic matter, as well as urban wastewaters can be sources of this phytosterol in the environment. Accordingly, it is not easy to compare and explain the detected levels of β-sitosterol with other available studies. Lower concentrations were reported in Hungary (128–379 ng L^{-1} in dissolved phase, 22–1796 ng L^{-1} in SPM, Andrási et al., 2013) and Brazil (110–7380 ng L^{-1}, Albuquerque de Assis Costa et al., 2018), and higher levels were found in northern Serbia (180–1470 ng L^{-1} in dissolved phase, Škrbić et al., 2018) and China (665–3270 ng L^{-1} in dissolved phase, Kong et al., 2015).

The sterols distribution between the dissolved and suspended phases of water samples was also evaluated. Considering the high log K_{ow} values (8.31–8.82 for human/animal sterols and 8.65–9.73 for phytosterols, Table S1) and low water solubility (Table S1), sterols are expected to predominantly partition to SPM. Based on the detected concentrations in both phases of river water samples (Table 1), it was determined that 83.0 ± 11.9 % of human/animal sterols and 87.1 ± 15.2 % of phytosterols partition to SPM in the dissolved/suspended phase distribution (compared to 17.0 % and 12.9 % in the dissolved phase, respectively).

3.2. Steroids detected in sewage wastewater

The results obtained for both the dissolved phase and the SPM of the raw sewage wastewater samples (Table 2) show that cholestanone and nine steroid hormones were not found, and the detected steroids were recorded in much higher concentrations (expressed in μg L^{-1}), compared to river water. Steroid hormones are usually found in wastewater at low μg L^{-1} levels. For instance, in influents of two WWTPs in Hungary (Andrási et al., 2013), 17β-estradiol and estriol were found in concentrations up to 0.03 μg L^{-1} and 0.4 μg L^{-1}, respectively, in the dissolved phase. Estriol was not found in the suspended phase, and 17β-estradiol was detected at levels up to 0.03 μg L^{-1}. The inability to detect steroid hormones in wastewater samples can be explained by the calculated LODs and LOQs of the applied analytical methods (Table S4). The applied methods seem to show limited performance for the detection of analytes present in low concentrations close to LODs, especially in the complex wastewater matrix. The absence of cholestanone can be attributed to a lack of aerobic microbial activity, since cholestanone is predominantly formed by the degradation of cholesterol under aerobic conditions (Cordeiro et al., 2008; Prost et al., 2018). Sewage wastewater samples contained five human/

Table 2
Detected sterol concentrations in dissolved phase* and suspended phase** of wastewater (WW) samples.

	Concentration ± SD, μg L^{-1}					
	WW1	WW2	WW3	WW4	WW5	WW6
Cholesterol	5.9 ± 0.2* (67 ± 16)**	5.1 ± 0.4 (54 ± 5)	3.1 ± 0.5 (97 ± 12)	1.9 ± 0.1 (70 ± 14)	4.0 ± 0.4 (40 ± 11)	4.4 ± 0.4 (86 ± 20)
Coprostanol	12 ± 2 (148 ± 54)	6.9 ± 1.1 (116 ± 14)	3.4 ± 0.1 (229 ± 80)	3.8 ± 0.2 (110 ± 22)	11 ± 1 (152 ± 21)	7.9 ± 0.5 (171 ± 37)
Epicoprostanol	0.25 ± 0.04 (3.0 ± 0.4)	0.24 ± 0.01 (2.3 ± 0.1)	0.24 ± 0.01 (4.1 ± 0.9)	0.32 ± 0.06 (7.0 ± 0.5)	0.38 ± 0.04 (2.4 ± 0.3)	– (3.1 ± 0.4)
Epicholestanol	0.12 ± 0.04 (0.83 ± 0.02)	0.11 ± 0.01 (0.50 ± 0.04)	0.05 ± 0.01 (1.2 ± 0.1)	0.13 ± 0.02 (0.44 ± 0.02)	0.07 ± 0.01 (0.53 ± 0.03)	0.29 ± 0.06 (0.63 ± 0.03)
Cholestanol	0.86 ± 0.22 (8.4 ± 0.9)	0.63 ± 0.08 (8.1 ± 0.9)	0.30 ± 0.01 (11 ± 2)	0.27 ± 0.08 (17 ± 4)	1.3 ± 0.4 (11 ± 2)	– (15 ± 3)
β-Sitosterol	3.2 ± 0.1 (11 ± 5)	3.6 ± 0.3 (32 ± 8)	2.0 ± 0.2 (70 ± 18)	0.97 ± 0.29 (27 ± 7)	2.7 ± 0.1 (23 ± 5)	2.3 ± 0.1 (56 ± 19)
Stigmasterol	0.53 ± 0.08 (5.9 ± 0.9)	0.80 ± 0.18 (7.9 ± 0.4)	0.32 ± 0.04 (10 ± 1)	– (28 ± 4)	0.50 ± 0.02 (4.3 ± 0.3)	1.2 ± 0.1 (12 ± 1)
Campesterol	0.21 ± 0.02 (1.9 ± 0.2)	0.26 ± 0.02 (2.1 ± 0.4)	0.15 ± 0.02 (2.9 ± 0.2)	0.06 ± 0.02 (0.88 ± 0.09)	0.18 ± 0.02 (1.1 ± 0.2)	0.22 ± 0.01 (1.9 ± 0.2)
Desmosterol	– (0.45 ± 0.06)	– (0.46 ± 0.02)	– (0.56 ± 0.01)	– (1.1 ± 0.1)	– (0.29 ± 0.03)	– (2.0 ± 0.1)
Sitostanol	– (4.3 ± 0.8)	– (3.1 ± 0.3)	– (5.9 ± 0.6)	– (1.9 ± 0.2)	– (4.2 ± 0.4)	– (5.1 ± 0.8)
%Cholesterol	25.6 (26.7)	28.9 (23.8)	32.4 (22.5)	25.5 (26.6)	19.9 (16.8)	27.0 (24.4)
%Coprostanol	52.0 (59.0)	39.1 (51.2)	35.6 (53.1)	51.0 (41.8)	54.6 (63.6)	48.4 (48.5)

animal sterols, with coprostanol being expectedly the dominant one (35.6 %–54.6 % for the dissolved phase and 41.8 %–63.6 % for the suspended phase), since it constitutes about 60 % of the total sterols in the human feces (Leeming et al., 1996). It was found in the range of 3.4–12 μg L^{-1} in the dissolved phase and 110–229 μg L^{-1} in the suspended phase of the wastewater samples. As there are no reported sterol concentrations in raw sewage wastewater in the available studies, the detected levels were compared with those recorded in WWTP influents. However, the previously determined average amount of suspended solids in water samples (Section 2.3) suggests that raw sewage from wastewater canals generally contains a lower amount of suspended material (average 98 ± 42 mg) than WWTP influents (average 146 ± 62 mg). Therefore, the sterol concentrations in the suspended phase of the raw sewage samples detected in this study are likely to be lower than their levels in the WWTP influents from other studies. In fact, the highest coprostanol concentrations detected in the dissolved phase of samples from the two major sewage canals (12 and 11 μg L^{-1}, samples WW1 and WW5), with mean annual flow of 48,723,120 and 19,552,320 m^3/annually, were also found to be significantly lower than those detected in WWTP influents of other studies with lower mean annual inflows. Detected coprostanol concentrations are lower than those detected in influents of two WWTPs in Hungary (37–46 μg L^{-1} in dissolved phase, 22–488 μg L^{-1} in SPM, Andrási et al., 2013), WWTP in France with capacity of 1800 PE and an inflow of 160 m^3 day^{-1} (99 μg L^{-1} in dissolved phase, Jeanneau et al., 2011), WWTP in Germany with an influent flow of 23,000 m^3 day^{-1} (84 μg L^{-1} in dissolved phase, Beck and Radke, 2006) and six WWTPs in Canada with an influent flow in the range from 900 to 30,300 m^3 day^{-1} (394–914 μg L^{-1} in dissolved phase, Furtula et al., 2012b). Lower coprostanol concentrations in wastewater canals compared to WWTP influents could be explained by the fact that canals are constantly affected by the river water level and are often flooded.

Cholesterol was the second highly abundant human/animal sterol in sewage wastewater, recorded at the levels of 1.9–5.9 μg L^{-1} in the dissolved phase. Cholesterol-rich food (primarily meat and cooking oil) is a major source of large amounts of this sterol in household wastewater (Leeming et al., 2015). The observed cholesterol levels are much lower than those reported in the influents of previously mentioned WWTPs in Hungary (7.6–43 μg L^{-1}, Andrási et al., 2013), France (122 μg L^{-1}, Jeanneau et al., 2011), Germany (91 μg L^{-1}, Beck and Radke, 2006) and Canada (459–1061 μg L^{-1}, Furtula et al., 2012b). Cholesterol concentrations in the suspended phase of wastewater samples (40–97 μg L^{-1}) are also lower than those found in WWTP influents in Hungary (95–442 μg L^{-1}, Andrási et al., 2013). As previously for coprostanol, lower cholesterol concentrations could be explained by the substantial impact of the high river water level, as well as the lower amount of suspended material.

As for the five determined phytosterols, β-sitosterol was again the most abundant, found in the range of concentrations of 0.97–3.6 μg L^{-1} in the dissolved phase and 11–70 μg L^{-1} in the SPM of wastewater samples. Compared to WWTP influents, these levels are lower than those found in Hungary (7.0–42 μg L^{-1} in dissolved phase, 76–130 μg L^{-1} in suspended phase, Andrási et al., 2013), France (28 μg L^{-1} in dissolved phase, Jeanneau et al., 2011), Germany (16 μg L^{-1} in dissolved phase, Beck and Radke, 2006) and Canada (154–416 μg L^{-1} in dissolved phase, Furtula et al., 2012b).

In both water phases of wastewater samples, the distribution of sterols is dominated by coprostanol (7.5 ± 3.6 μg L^{-1} in the dissolved phase and 154 ± 43 μg L^{-1} in the SPM, average concentration), followed by cholesterol (4.1 ± 1.4 μg L^{-1} and 69 ± 21 μg L^{-1}) and β-sitosterol (2.5 ± 0.9 μg L^{-1} and 37 ± 22 μg L^{-1}). Other sterols are present in significantly lower amounts, such as cholestanol (0.56 ± 0.47 μg L^{-1} and 12 ± 4 μg L^{-1}), stigmasterol (0.56 ± 0.41 μg L^{-1} and 11 ± 8 μg L^{-1}), epicoprostanol (0.24 ± 0.13 μg L^{-1} and 3.7 ± 1.8 μg L^{-1}), campesterol (0.18 ± 0.07 μg L^{-1} and 1.8 ± 0.7 μg L^{-1}) and epicholestanol (0.13 ± 0.08 μg L^{-1} and 0.69 ± 0.29 μg L^{-1}). Desmosterol was not found in the dissolved phase of raw wastewater, but was detected at a low concentration of 0.81 ± 0.65 μg L^{-1} in SPM. The only noticeable difference in the sterol abundance pattern is in the detected levels of sitostanol, which is not detected in the dissolved phase, while in the suspended phase it was found at an average concentration of 4.1 ± 1.4 μg L^{-1}. Compared to river water, a more pronounced partition to SPM was found for human/animal sterols (92.1 ± 6.8 %) and phytosterols (95.0 ± 5.7 %) in wastewater, most likely due to the higher amount of suspended material.

In addition, a direct connection was established between the raw wastewater discharges and the high levels of two key human/animal sterols, coprostanol and cholesterol, in receiving river water. The two major sewage canals (WW1 and WW5, Table S2) are distinguished by high levels of coprostanol in the dissolved phase (12 and 11 μg L^{-1}, respectively, Table 2). The corresponding receiving surface water (samples SW3 and SW8) contains 384 and 232 ng L^{-1} of coprostanol in the dissolved phase (Table 1), the highest concentrations among all river water samples affected by raw sewage discharge. The same connection was observed between the highest cholesterol levels detected in the dissolved phase of the WW1 sample (5.9 μg L^{-1}) and in the corresponding receiving water (sample SW3, 853 ng L^{-1}). Regarding the suspended phase, the concentrations of coprostanol and cholesterol in sample WW3 (229 and 97 μg L^{-1}, respectively) were the highest among the investigated wastewater samples, and the corresponding SW5 sample stands out with the highest concentrations of these sterols (7553 and 6758 ng L^{-1}, respectively).

3.3. Sterol prevalence in river water and sterol ratios

The levels of detected sterols can also be presented in bar graphs to visually single out the most abundant sterols in each sample and in this way determine the sewage contamination level of surface water samples (Fig. 2). According to the bar graphs, it is evident that there is a difference

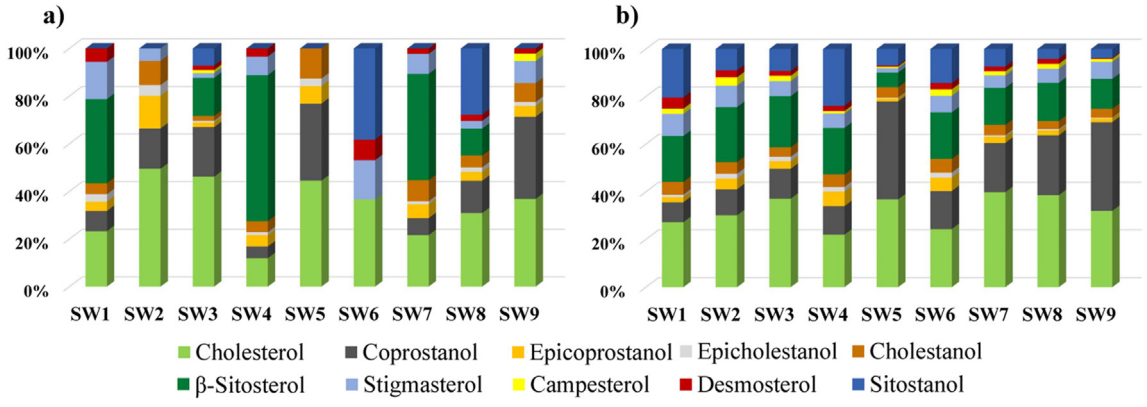

Fig. 2. Prevalence of sterols in dissolved phase (a) and suspended phase (b) of surface water (SW) samples.

between sterol distribution in the dissolved phase and the SPM of river water samples. Human/animal sterols are prevalent (above 60 % of total sterols) in the dissolved phase of samples SW2, SW3, SW5 and SW9 (~90 %, ~65 %, 100 % and ~ 80 %, respectively) and in the suspended phase of samples SW5, SW7, SW8 and SW9 (~80 %, ~65 %, ~65 % and ~70 %, respectively). Taking into account the composition of both phases of the water sample, the Danube samples SW5 and SW9 were singled out as the most contaminated. Sample SW9 was collected from a small bay, away from the main course of the Danube, with limited water flow (Fig. 1). It also showed the highest concentrations of all human/animal sterols among the investigated river water samples. Sample SW5 was taken just before the confluence of the Danube and the Sava River, downstream from the raw wastewater discharge (sample WW3).

On the other hand, high levels and prevalence of phytosterols were noted for the dissolved phase of samples SW1, SW4 and SW6 (~60 %, ~75 %, ~70 %, respectively), and SPM of sample SW1 (~60 %). The composition of sample SW1, taken from the Danube 18 km before the confluence, indicates a dominant biogenic input from higher plants in the rural area (Fig. 1). In addition, it was evident from the bar graphs that none of the four human/animal sterols were detected in the dissolved phase of sample SW6, and their prevalence in its SPM was among the lowest. The dominant phytosterol in the SW6 sample was sitostanol (Table 1), one of the major sterols in herbivore feces (Leeming et al., 1996), indicating a dominant non-human biogenic sterol input. Apparently, the confluence of the two rivers, from where the sample SW6 was collected, is the least contaminated, probably due to turbulent mixing and extensive dilution.

Based on the previous results (Fig. 2, Table 3), severe sewage contamination of both rivers was identified, and a more pronounced impact of raw wastewater discharges on the composition of the suspended phase was noted, compared to the dissolved phase of water sample. In fact, it was found that all detected sterols prefer to partition to SPM in the dissolved/suspended phase distribution, indicating the necessity to take both water phases into account when performing steroid-based environmental pollution research. In order to obtain a reliable and realistic picture of steroid contamination and steroid fate in the aquatic environment, it is crucial to determine the distribution of sterols between the dissolved and suspended water phases.

The abundance pattern of sterols detected in river water was different from that in raw sewage, showing a much higher influence of phytosterols. The SW9 sample was excluded from the calculation of average concentrations due to the extremely high levels of all detected sterols. In the dissolved phase, the dominant sterol was cholesterol (366 ± 226 ng L^{-1}), followed by β-sitosterol (285 ± 313 ng L^{-1}) and coprostanol (146 ± 121 ng L^{-1}), indicating a more pronounced non-human biogenic input. In the SPM of surface water samples, cholesterol was also present in the highest concentration (2629 ± 2055 ng L^{-1}), while the order of abundance was changed for coprostanol (1819 ± 2435 ng L^{-1}) and β-sitosterol (1150 ± 527 ng L^{-1}). Apparently, the raw wastewater discharges along both rivers have greater impact on the composition of SPM. Regarding other sterols, the abundance pattern in both water phases was the same: sitostanol (110 ± 178 ng L^{-1} in the dissolved phase and 838 ± 685 ng L^{-1} in SPM), stigmasterol (76 ± 50 ng L^{-1} and 427 ± 280 ng L^{-1}), cholestanol (63 ± 40 ng L^{-1} and 353 ± 236 ng L^{-1}), epicoprostanol (53 ± 31 ng L^{-1} and 198 ± 46 ng L^{-1}), desmosterol (33 ± 22 ng L^{-1} and 169 ± 155 ng L^{-1}), campesterol (25 ng L^{-1} in the dissolved phase of sample SW3 and 130 ± 68 ng L^{-1} in SPM) and epicholestanol (20 ± 10 ng L^{-1} and 73 ± 21 ng L^{-1}).

A more reliable way to determine the sewage contamination level of natural waters is to use sterol ratios. The calculated ratios of detected sterols and the identification of human fecal contamination in surface water samples are shown in Table 3. According to the reference values of ratio No. 1 (coprostanol/(coprostanol + cholestanol)), samples SW3, SW5, SW8 and SW9 show positive sewage contamination of both dissolved phase and suspended material, pointing to a high impact of raw wastewater discharges along both rivers. For other samples, the ratio values indicate that detected sterols are of mixed origin, both natural and anthropogenic, with a greater impact of human-derived pollution on the composition of SPM leading to a positive ratio value. Using the same reference values as for ratio No. 1, ratio No. 2 (coprostanol + epicoprostanol/(coprostanol + epicoprostanol + cholestanol)) compensates for the influence of the age of fecal contamination and additionally points to the sample SW2 as sewage-contaminated, besides samples SW3, SW5, SW8 and SW9.

Ratio No. 3 (coprostanol/cholesterol) recognized human-derived pollution only in the suspended material of samples SW5 and SW9. However, the dissolved phases of the two samples showed the highest ratio value (0.73 and 0.94, respectively) compared to the other samples (0.34–0.44, Table 3). Some authors have suggested that a much lower value of coprostanol/cholesterol ratio (>0.2) should indicate fecal pollution (Grimalt et al., 1990; Kong et al., 2015). According to the reference value of 0.06 for ratio No. 4 (coprostanol/(cholesterol + cholestanol)), all investigated samples show certain human fecal pollution. However, samples SW5 and SW9 stand out with higher ratio values for both the dissolved phase (0.57 and 0.77, respectively) and SPM (1.00 and 1.04) compared to other surface water samples (0.23–0.43 for the dissolved phase and 0.26–0.60 for SPM), indicating heavily contaminated sampling sites.

For the coprostanol/epicoprostanol ratio (ratio No. 5), a value higher than 1.5 was determined for both the dissolved phase and the SPM of samples SW1, SW3, SW5, SW8 and SW9, confirming the human waste input in both investigated rivers. Since there is no WWTPs in the investigated area, the values of ratio No. 6 (epicoprostanol/coprostanol) lower than 0.2 point to untreated sewage discharge and the presence of raw human-derived fecal matter. It was determined that only sample SW9 was positive for human fecal pollution, since both dissolved and suspended phases of the water sample showed ratio values below 0.2. Nevertheless, samples SW3,

Table 3
Calculated sterol ratios for dissolved phase* and *suspended phase*** of surface water (SW) samples used to determine human fecal contamination.

Ratio no.	SW1	SW2	SW3	SW4	SW5	SW6	SW7	SW8	SW9
1.	(~) 0.65*	(~) 0.62	(+) 0.91	(~) 0.52	(+) 0.72	nc	(~) 0.45	(+) 0.73	(+) 0.81
	*(~) 0.61***	*(~) 0.69*	*(+) 0.76*	*(+) 0.70*	*(+) 0.90*	*(+) 0.74*	*(+) 0.82*	*(+) 0.88*	*(+) 0.91*
2.	(+) 0.73	(+) 0.75	(+) 0.92	(~) 0.68	(+) 0.76	nc	(~) 0.59	(+) 0.78	(+) 0.83
	(~) 0.66	*(+) 0.76*	*(+) 0.80*	*(+) 0.78*	*(+) 0.91*	*(+) 0.79*	*(+) 0.84*	*(+) 0.89*	*(+) 0.91*
3.	(–) 0.36	(–) 0.34	(–) 0.45	(–) 0.41	(–) 0.73	nc	(–) 0.33	(–) 0.44	(–) 0.94
	(–) 0.31	*(–) 0.36*	*(–) 0.34*	*(–) 0.54*	*(+) 1.12*	*(–) 0.66*	*(–) 0.52*	*(–) 0.65*	*(+) 1.16*
4.	(+) 0.30	(+) 0.28	(+) 0.43	(+) 0.30	(+) 0.57	nc	(+) 0.23	(+) 0.38	(+) 0.77
	(+) 0.26	*(+) 0.31*	*(+) 0.31*	*(+) 0.44*	*(+) 1.00*	*(+) 0.53*	*(+) 0.47*	*(+) 0.60*	*(+) 1.04*
5.	(+) 2.15	(–) 1.23	(+) 10.97	(–) 1.03	(+) 4.38	nc	(–) 1.22	(+) 3.57	(+) 7.59
	(+) 3.91	*(+) 2.47*	*(+) 3.96*	*(+) 1.96*	*(+) 33.72*	*(+) 2.82*	*(+) 8.09*	*(+) 12.12*	*(+) 22.72*
6.	(~) 0.46	(–) 0.81	(+) 0.09	(–) 0.97	(~) 0.23	nc	(–) 0.82	(~) 0.28	(+) 0.13
	(~) 0.26	*(~) 0.41*	*(~) 0.25*	*(~) 0.51*	*(+) 0.03*	*(~) 0.35*	*(+) 0.12*	*(+) 0.08*	*(+) 0.04*

(+) certain human fecal contamination; (~) uncertain human fecal contamination; (–) no human fecal contamination;
nc - not calculated.
The ratio values for the suspened phase of the water samples (indicated by two asterisks **) are in italics.

SW5 and SW8 displayed a positive ratio value for one phase of the water sample (dissolved or suspended), while the other phase had a value very close to 0.2 (up to 0.28).

Comparing the values of the sterol ratios calculated for the dissolved and suspended phase (Table 3), it can also be observed that the ratio values (ratios No. 1–No. 5) obtained for the dissolved phase are generally lower than those for SPM. Due to the fractionation between the two phases of water samples in which sterols predominantly partition to SPM, and consequently higher levels of sterols in the suspended phase, higher values of e.g., ratio No. 1 (0.61–0.91) and ratio No. 3 (0.31–1.16) were obtained compared to those in the dissolved phase (0.45–0.91 and 0.33–0.94, respectively). Owing to the higher values of some ratios obtained for SPM, more samples were recognized as positive for human fecal pollution. In the case of ratio No. 6, with values lower than 0.2 indicating positive human-derived pollution, lower values were obtained for SPM (0.03–0.51) compared to the dissolved phase (0.09–0.97). Clearly, when tracing steroid-based environmental pollution and the fate of steroid compounds in the aquatic environment, it is necessary to consider and analyze both water phases.

The obtained results of sterol ratios and the conclusions drawn can be visualized using 3D scatter plots of the three most reliable sterol ratios No. 1, No. 3 and No. 6, for the dissolved and suspended phase of surface water samples, respectively (Fig. 3). Namely, in our previous work, these three ratios were found to be the most reliable for the assessment of the sewage pollution of sediments in the Danube River Basin (Matić Bujagić et al., 2016). Ratios No. 1 and No. 2 are very similar because they consider the same key biomarkers. Inclusion of epicoprostanol in ratio No. 2 to compensate for the impact of the age of fecal pollution did not significantly improve the assessment of fecal contamination of the Danube and the Sava rivers (Table 3). Therefore, ratio No. 1 was selected for 3D scatter plots as one of the most reliable and the most frequently used sterol ratios. Ratio No. 5 (coprostanol/epicoprostanol) is reciprocal to ratio No. 6, although the reference values of these two ratios are not reciprocal. Calculated values of ratio No. 5 indicate sewage-sourced pollution of almost all investigated river water samples, while ratio No. 6 was able to distinguish the level of fecal contamination (Table 3). Apparently, ratio No. 6 is more selective, which is why it was chosen as more reliable than ratio No. 5 to trace the source of fecal pollution. Compared to ratio No. 3 (coprostanol/cholesterol), ratio No. 4 (coprostanol/(cholesterol + cholestanol)) proved to be less selective as it showed certain sewage pollution of all river water samples. The reference value of 0.06 appears to be too low to reliably identify human-sourced fecal contamination. These three most reliable sterol ratios were applied to evaluate sewage pollution of the Danube and the Sava rivers using 3D scatter plots.

It is evident from the 3D plots that there is a difference between the samples that stood out as the most polluted in the dissolved vs. suspended phase of the water samples. In both instances, samples SW5, SW8 and SW9 were singled out as heavily contaminated. However, the SW3 sample additionally stood out in the dissolved phase, while the sample SW7 was singled out in SPM. Apparently, there is a very high impact of the largest municipal wastewater discharge (sample WW1, Fig. 1) on the composition of the dissolved phase of the SW3 sample. In fact, the highest level of coprostanol in the dissolved phase of all tested samples was recorded in the SW3 sample of the Sava River (384 mg L^{-1}, Table 1), with the SW9 sample excluded due to specific conditions at the sampling site. It can also be noticed that the concentrations of coprostanol in the dissolved phase are generally similar in the samples of both rivers (65–384 ng L^{-1}, the Sava samples SW2, SW3, SW4, vs. 71–232 ng L^{-1}, the Danube samples SW1, SW5, SW7, SW8).

Regarding the suspended phase, all samples from the Danube affected by municipal wastewater discharges in Belgrade showed high fecal pollution (Fig. 3b, samples SW5, SW7–SW9). Comparing the levels of coprostanol in the SPM of both rivers, it is evident that the Danube (951–7553 ng L^{-1}) is much more contaminated than the Sava River (306–531 ng L^{-1}). This can be explained by the different hydrodynamic conditions of the two rivers. As the average annual flow of the Danube is about 3.5 times higher than its tributary the Sava River (Simić et al., 2017), this high-energy flow can promote the mobilization of particles settled on the riverbed and resuspension of sediment material with accumulated contaminants. On the other hand, the lower-flow of the Sava River leads to the deposition of suspended material introduced by wastewater discharge, potentially increasing the sediment storage of pollutants in this reach.

3.4. Sterols detected in WWTPs and their removal rate

Sterol levels detected in the dissolved and suspended phase of influent and effluent samples from two WWTPs in the RS, as well as their removal rates during treatment, are presented in Table 4. The sterol profiles of the samples from the two WWTPs were similar to those in sewage wastewater samples (Table 2), with the most commons sterols being coprostanol, cholesterol and β-sitosterol. The levels of these sterols in WWTP influents are also comparable to their concentrations in the raw sewage (cholesterol up to 5.9 μg L^{-1}, coprostanol up to 12 μg L^{-1}, β-sitosterol up to 3.6 μg L^{-1}, Table 2).

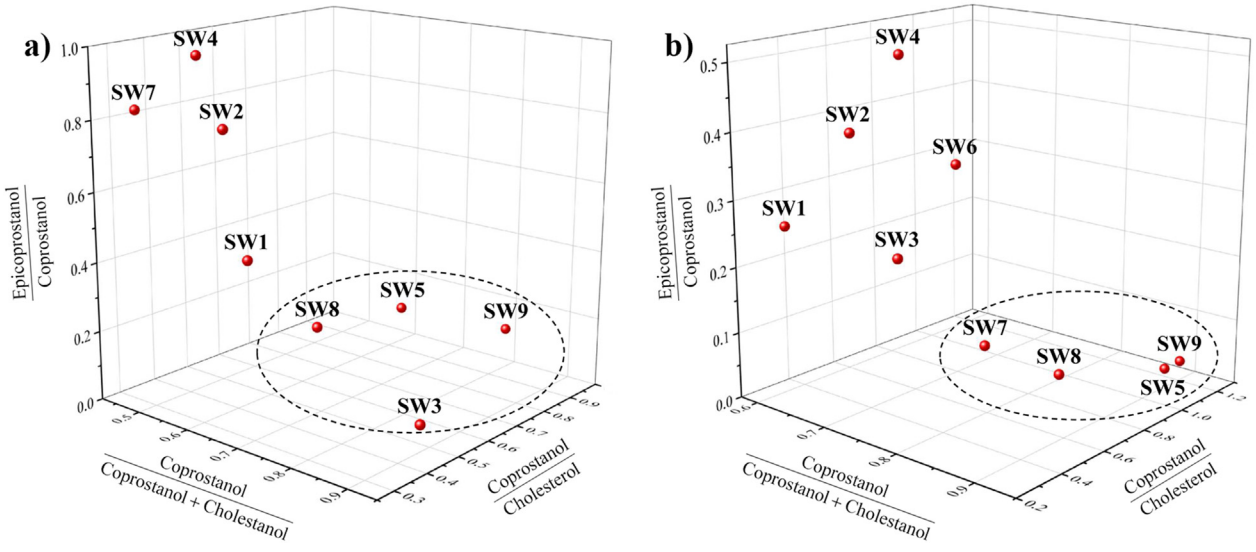

Fig. 3. 3D scatter plots of the three most reliable sterol ratios for dissolved phase (a) and suspended phase (b) of surface water (SW) samples with the most polluted samples marked.

Table 4
Detected sterol concentrations and removal efficiency (RE) in dissolved phase* and suspended phase** of wastewater treatment plant (WWTP) samples.

	Concentration ± SD, μg L^{-1}				Removal efficiency, %	
	WWTP1		WWTP2		WWTP1	WWTP2
	Influent	Effluent	Influent	Effluent		
Cholesterol	6.7 ± 1.4*	3.5 ± 1.1	7.5 ± 1.2	0.07 ± 0.01	48	99
	(111 ± 16)**	(18 ± 3)	(117 ± 8)	(2.4 ± 0.7)	(84)	(98)
Coprostanol	11 ± 3	5.3 ± 1.8	9.2 ± 1.1	0.11 ± 0.01	52	99
	(1210 ± 196)	(56 ± 7)	(91 ± 5)	(2.1 ± 0.4)	(95)	(98)
Epicoprostanol	0.45 ± 0.11	0.15 ± 0.04	1.1 ± 0.06	0.17 ± 0.01	67	85
	(6.8 ± 1.5)	(1.8 ± 0.3)	(3.3 ± 0.1)	(0.12 ± 0.02)	(74)	(96)
Epicholestanol	0.11 ± 0.02	0.07 ± 0.02	0.26 ± 0.05	0.04 ± 0.01	36	85
	(1.1 ± 0.1)	(0.19 ± 0.02)	(1.5 ± 0.1)	(0.09 ± 0.01)	(83)	(94)
Cholestanol	0.81 ± 0.19	0.58 ± 0.15	0.58 ± 0.12	0.09 ± 0.01	28	84
	(20 ± 1)	(4.3 ± 0.4)	(11 ± 1)	(0.90 ± 0.20)	(79)	(92)
β-Sitosterol	1.9 ± 0.6	1.1 ± 0.3	2.5 ± 0.1	–	42	100
	(103 ± 22)	(15 ± 3)	(50 ± 6)	(2.3 ± 0.3)	(85)	(95)
Stigmasterol	13 ± 3	0.38 ± 0.10	–	–	97	–
	(25 ± 3)	(3.4 ± 0.9)	(7.3 ± 0.1)	(0.35 ± 0.06)	(86)	(95)
Campesterol	0.25 ± 0.02	0.07 ± 0.01	–	–	72	–
	(3.2 ± 0.6)	(0.73 ± 0.08)	(2.9 ± 0.1)	(0.06 ± 0.01)	(77)	(98)
Desmosterol	0.13 ± 0.04	0.09 ± 0.02	–	–	31	–
	(2.8 ± 0.2)	(0.56 ± 0.04)	(4.1 ± 0.2)	(0.37 ± 0.08)	(80)	(91)
Sitostanol	0.71 ± 0.18	–	3.1 ± 0.7	–	100	100
	(8.4 ± 1.9)	(1.6 ± 0.2)	(85 ± 3)	(0.57 ± 0.05)	(81)	(99)

The removal efficiency (RE) of sterols in two WWTPs was evaluated and compared. It was estimated by comparing influent and effluent concentrations (Chang et al., 2011). Although the concentrations of the compounds in the influents of the two treatment plants were similar, WWTP2 was shown to be more efficient with much lower sterol levels in the outgoing effluents. Although both studied WWTPs have the same treatment technology, with mechanical treatment followed by biological treatment based on activated sludge, they show very different performances. Apparently, WWTP1 was not fully operational at the time of sampling as the results showed that it operated with lower efficiency than projected. The full capacity of 2000 PE was reached in 2021, one year after sampling was performed. Coprostanol removal rate was much higher in WWTP2 (99 % for the dissolved and 98 % for suspended phase) than in WWTP1 (52 % for the dissolved phase and 95 % for SPM). The obtained RE values for WWTP2, with a capacity of 8000 PE and an inflow of 1089 m^3 day^{-1}, are comparable to other studies that show a very high efficiency of coprostanol elimination. In two WWTPs in Hungary, the obtained REs were 88–98 % for the dissolved phase and up to 98 % for SPM (Andrási et al., 2013). WWTP in France, with a capacity of 1800 PE and an influent flow of 160 m^3 day^{-1}, showed 99 % RE for the dissolved phase (Jeanneau et al., 2011), while in six WWTPs in Canada, with inflows in the range of 900–30,300 m^3 day^{-1}, RE of 86–100 % was achieved for the dissolved phase (Furtula et al., 2012b).

Similar to coprostanol, the elimination of cholesterol during wastewater treatment process is more effective in WWTP2 (99 % for the dissolved phase and 98 % for SPM), compared to WWTP1 (48 % for the dissolved and 84 % for the suspended phase). The high RE values achieved in WWTP2 are similar to those obtained in WWTPs in Hungary (90–98 % for the dissolved phase and 89–99 % for SPM, Andrási et al., 2013), France (99 % for the dissolved phase, Jeanneau et al., 2011) and Canada (86–99 % for the dissolved phase, Furtula et al., 2012b).

Other detected human/animal sterols (epicoprostanol, epicholestanol and cholestanol) exhibited a similar elimination trend as coprostanol and cholesterol, with low RE obtained for the dissolved phase of wastewater treated in WWTP1 (as low as 28 % for cholestanol) and efficient removal obtained for SPM in WWTP1 (above 70 %), as well as for both dissolved and suspended phases of wastewater samples in WWTP2 (above 80 % and 90 %, respectively). The high REs of epicoprostanol, epicholestanol and cholestanol attained for the dissolved phase in WWTP2 are comparable to the results obtained in France (91 %, 100 %, up to 98 %, respectively, Jeanneau et al., 2011) and Canada (69–99 % for epicoprostanol, 82–100 % for epicholestanol, Furtula et al., 2012b).

Regarding phytosterols, β-sitosterol and desmosterol followed a removal trend similar to human/animal sterols. Very high REs for β-sitosterol were reached in WWTP2 (100 % for the dissolved phase and 95 % for SPM) and suspended phase of wastewater treated in WWTP1 (85 %), while low RE was obtained for the dissolved phase in WWTP1 (43 %). In similar studies, efficient removal of β-sitosterol was achieved in two WWTPs in Hungary (89 % and 92 % for the dissolved phase, and up to 99 % for SPM, Andrási et al., 2013), WWTP in France (99 % for the dissolved phase, Jeanneau et al., 2011), and six WWTPs in Canada (80–99 % for the dissolved phase, Furtula et al., 2012b). For desmosterol, high REs were achieved for SPM in both WWTPs (80 % and 91 %), while low removal rate was obtained for the dissolved phase of treated wastewater in WWTP1 (31 %).

As for other found phytosterols, stigmasterol, campesterol and sitostanol showed high RE in both studied WWTPs, for both the dissolved phase (97 %, 72 %, 100 %, respectively, in WWTP1 and 100 % for sitostanol in WWTP2) and SPM (86 %, 77 %, 81 %, respectively, in WWTP1 and 99 % for sitostanol in WWTP2). This is in agreement with comparable studies performed in France (up to 96 %, up to 100 %, 94 %, respectively, Jeanneau et al., 2011) and Canada (up to 96 %, 88–99 %, 87–100 %, respectively, Furtula et al., 2012b).

Generally, the efficiency of sterol removal in WWTP2 was high, with over 80 % RE achieved for all investigated sterols (84–100 % in dissolved phase and 91–99 % in SPM). On the other hand, WWTP1 showed a lower RE for the dissolved phase, with half of the detected compounds removed with <50 % RE, while the RE was higher for the suspended phase (74–95 %). The obtained results confirmed that WWTP1 was not fully operational at the time of sampling.

Finally, sterol ratios were calculated for raw wastewater samples (Table S7, Supplementary Material), as well as for influents and effluents of both WWTPs (Table S8), to determine possible values typical of untreated wastewater and ratios that can identify improvement in wastewater quality during WWTP treatment. As expected, all wastewater samples from sewage canals show positive fecal contamination. Of the six ratios tested, the values of three ratios No. 1, No. 2 (0.87–0.95 for both) and No. 6 (0.02–0.08) show narrow ranges that can be applied as a characteristic of sewage wastewater. It was also determined that only in the case of (coprostanol/(coprostanol + cholestanol)) ratio, for both water phases, values decreased from those suggesting certain human fecal contamination in the influent to those pointing to uncertain fecal contamination in the effluent. During sewage treatment in WWTP2, ratio No. 1 values decreased from 0.94 for the dissolved phase and 0.89 for SPM to 0.55 and 0.70, respectively.

4. Conclusions

Five human/animal sterols were detected in all investigated water samples, with cholesterol being the dominant compound in river water, while coprostanol was the most prominent in raw sewage and wastewater influents. Of the five investigated phytosterols, β-sitosterol was the most abundant. Human/animal sterol cholestanone and nine monitored steroid hormones were not detected, probably due to the limited performance of the applied methods for detecting analytes present in concentrations close to LODs, especially in the complex wastewater matrix. The sterol abundance pattern in river water was different from that in raw sewage, indicating a more pronounced non-human biogenic input and higher influence of phytosterols, as well as greater impact of wastewater discharges on the composition of SPM. Compared to similar studies, sterol levels detected in river water were much higher, while their concentrations in wastewater, both raw sewage and influents, were much lower, indicating severe contamination in the confluence area of the Danube and the Sava rivers in the RS capital. It was determined that the Danube was more contaminated by the municipal wastewater discharges than the Sava, as the sterol concentrations in the SPM of the Danube water samples were significantly higher, while their levels in the dissolved phases of the two rivers were similar. This can be explained by the different hydrodynamic conditions of the two rivers leading to different fate of particle-bound steroids in the aquatic environment. The confluence of the two rivers was found to be the least contaminated, probably due to turbulent mixing and extensive dilution.

The use of sterol ratios indicated positive sewage contamination of both dissolved phase and suspended material for most samples, pointing to the high impact of raw wastewater discharges along both rivers. However, for some samples, a greater impact on the composition of SPM was noted. This was confirmed using 3D scatter plots of the three most reliable sterol ratios that highlighted the difference between the most polluted samples according to the composition of the dissolved phase vs. suspended phase of water samples. Evidently, the sterol distribution between the dissolved and suspended water phases is vital for obtaining a realistic picture of steroid contamination and their fate in the aquatic environment. It was shown that all detected sterols prefer to partition to SPM in the dissolved/suspended phase distribution. The greater part of human/animal sterols and phytosterols present in river water samples (83.0 ± 11.9 % and 87.1 ± 15.2 %) and wastewater samples (92.1 ± 6.8 % and 95.0 ± 5.7 %) was bound to particulate matter compared to the dissolved phase, pointing to the need to consider and analyze both water phases in the tracing of steroid-based environmental pollution. It was also determined that the values of the sterol ratios (ratios No. 1–No. 5) obtained for the suspended water phase are generally higher than those for the dissolved phase. As for the ratio No. 6, with values lower than 0.2 indicating positive human-derived pollution, lower values were obtained for SPM compared to the dissolved phase. The evaluation of the extent of animal fecal contribution was limited due to the lack of two animal-specific markers (24-ethylcoprostanol and 24-ethylepicoprostanol) that should be included in future studies.

Finally, the efficiency of sterol removal in the fully operational WWTP was high and comparable to similar studies, with removal rates of over 80 % achieved for all investigated sterols, and coprostanol and cholesterol showing >98 % removal efficiency for both the dissolved and suspended water phases. It was determined that only (coprostanol/(coprostanol + cholestanol) ratio was sensitive enough to be affected by an improvement in the quality of treated wastewater, for both dissolved and suspended water phases, with values decreasing from 0.94 for the dissolved phase and 0.89 for SPM (certain human fecal contamination) to 0.55 and 0.70, respectively (uncertain fecal contamination).

CRediT authorship contribution statement

Zorica Jauković: Investigation, Writing - original draft, Visualization; **Svetlana Grujić**: Conceptualization, Writing - review & editing; **Ivana Matić Bujagić**: Methodology, Validation; **Anđelka Petković**: Resources; **Mila Laušević**: Funding acquisition; Supervision.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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