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Pharmaceuticals in Belgrade's wastewater: Impact on surface waters and environmental risk assessment

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Abstract: In the Belgrade region, direct discharges of untreated wastewater into the Sava and Danube rivers have a detrimental impact on water quality. This study investigates this impact by examining the presence of pharmaceuticals in Belgrade's wastewater and corresponding surface water, alongside the associated environmental risk. A highly sensitive and selective LC–MS/MS method was developed, validated and applied to the analysis of water samples. Thirteen out of seventeen target pharmaceuticals were detected in wastewater, while nine compounds were found in surface water, with diclofenac reaching the highest concentration (1.1 μg L^{-1}). Metoprolol, carbamazepine and diclofenac were the most prevalent in both wastewater and surface water samples. Risk assessment indicated that diclofenac and azithromycin posed a high environmental risk (*RQ* > 1), while a mixture toxicity assessment suggested a cumulative hazard at all sampling sites. Additionally, the study evaluated pharmaceutical removal efficiencies in two wastewater treatment plants in Serbia, revealing variable efficiencies and even negative removal rates for some compounds, highlighting the inadequacy of conventional treatment plants in effectively eliminating these substances. The results emphasize the urgency of regulatory actions and the need for adequate treatment technologies to reduce pharmaceutical pollution in aquatic environments.

Keywords: drugs; emerging pollutants; urban wastewater; water quality; WWTP; ecological assessment.

INTRODUCTION

Pharmaceuticals are increasingly recognised as significant environmental contaminants due to their rising production and widespread use, posing potential risks

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to aquatic ecosystems. Municipal sewage discharge is a major source of these contaminants. Pharmaceuticals enter wastewater through multiple pathways: *i*) excretion of parent compounds and metabolites following human consumption; *ii*) improper disposal of unused drugs; *iii*) hospital and healthcare facility discharges; *iv*) pharmaceutical manufacturing effluents.[1–4] These originate either as pharmaceutical waste (unused drugs disposed of through drains) or human metabolites (excreted after therapeutic use). Human excretion is the predominant pathway, with 30–90 % of administered doses excreted as parent compounds or metabolites.[5] Following administration, many pharmaceuticals undergo hepatic metabolism and are conjugated to facilitate excretion. The concern is that certain metabolites may retain or exceed the toxicity and persistence of the parent compounds.[6] However, current environmental risk assessment studies are predominantly focused on parent compounds. Numerous studies have confirmed the widespread occurrence of pharmaceuticals in wastewater and surface waters at concentrations capable of disrupting aquatic organisms and potentially affecting human health.[7–11]

This issue is particularly concerning in the Republic of Serbia, as less than 21 % of the population is connected to wastewater treatment plants (WWTPs).[12] Belgrade, the capital, exemplifies this issue, as it completely lacks treatment facilities, resulting in the direct discharge of raw sewage into the Sava and Danube rivers. Furthermore, existing WWTPs are often insufficient for removing pharmaceuticals,[13] allowing these contaminants to enter surface water, groundwater, and even drinking water.[14–16] Ongoing research is necessary to better understand the fate of pharmaceutical pollutants, their impact on aquatic life, and the potential risks they pose to ecosystem stability.

This study aimed to: *i*) develop and validate a fast, simple, and sensitive liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the determination of selected pharmaceuticals in wastewater; *ii*) determine the concentrations of selected contaminants in Belgrade urban wastewater directly discharged to the Sava and the Danube rivers, as well as in the corresponding river water; *iii*) assess the environmental risk posed by contaminants detected in river water; *iv*) evaluate the removal efficiency of the analysed pharmaceuticals in two WWTPs. The pharmaceuticals selected for the study are among the most commonly used and/or frequently detected in the investigated area.

EXPERIMENTAL

Chemicals and reagents

High-purity (> 95 %) analytical standards of seventeen selected pharmaceuticals were obtained from domestic pharmaceutical companies (Hemofarm, STADA Group, Vršac, Serbia, and Zorka-Pharma, Šabac, Serbia). These included: antibiotics (trimethoprim, sulfamethoxazole, azithromycin, erythromycin); the drugs for the treatment of cardiovascular diseases (metoprolol, bisoprolol, enalapril, cilazapril, amlodipine, atorvastatin, clopidogrel, simvastatin); anxiolytic

drugs (bromazepam, carbamazepine, lorazepam, diazepam); and one non-steroidal anti-inflammatory drug (diclofenac).

The individual stock standard solutions of pharmaceuticals were prepared in methanol at a concentration of 100 mgL^{-1}. The working standard solutions were prepared by mixing the appropriate amounts of the individual stock standard solutions and diluting them with methanol. All solutions were stored at 4 °C. For the pH adjustment of the water samples, concentrated acetic acid and ammonia were used. All solvents used were HPLC-grade from J.T. Baker (Center Valley, USA) or Sigma–Aldrich, and all reagents were of analytical grade. Deionised water was obtained using the GenPure ultrapure water system (TKA, Niederelbert, Germany).

Sample preparation

In this study, we modified a previously developed sample preparation method[17] for determining pharmaceuticals in surface water and groundwater samples by replacing the methanol–dichloromethane elution solvent with a safer methanol–ethyl acetate mixture. The modified method, briefly described below, was validated for wastewater samples.

Waters (Milford, MA, USA) Oasis hydrophilic–lipophilic balance (HLB, 200 mg/6 mL) cartridges were utilised for the solid-phase extraction (SPE) of wastewater samples. Prior to the SPE procedure, wastewater samples were filtered through < 1μm glass fibre filters (Whatman GmbH, Dassel, Germany). SPE sorbent was preconditioned with 5mL of a methanol–ethyl acetate (1:1) mixture, followed by 5 mL of deionized water and 5mL of deionized water with its pH adjusted to 6.0. The wastewater sample (100 mL), adjusted to pH 6.0, was then passed through the cartridge, and the sorbent was dried by vacuum suction for 10 min. Afterwards, analytes were eluted using 15 mL of a methanol–ethyl acetate (1:1) mixture, and the extract was evaporated under a nitrogen stream and reconstituted in 1 mL with methanol. The extract was filtered through a 0.45 μm polyvinylidene difluoride (PVDF) filter from Roth (Karlsruhe, Germany) and analysed.

LC–MS/MS analysis

Water sample extracts were analysed using LC–MS/MS based on the method developed by Grujić *et al.*,[18] which was further expanded to include drugs for the treatment of cardiovascular diseases, allowing the determination of 17 pharmaceuticals. Briefly, a Surveyor LC system (Thermo Fisher Scientific, Waltham, MA, USA) was used for the separation of the analytes on the reversed-phase Zorbax Eclipse® XDB-C18 column (75 mm×4.6 mm i.d., 3.5 μm particle size; Agilent Technologies, Santa Clara, USA). A pre-column (12.5 mm×4.6 mm i.d., 5 μm particle size; Agilent Technologies, Santa Clara, CA, USA) was installed in front of the separation column. The mobile phase consisted of water (A), methanol (B) and 10 % acetic acid (C). The gradient changed as follows (Fig. S-I, Supplementary material to this paper): 0 min, A 65 %, B 33 %, C 2 %; 12 min, B 98 %, C 2 %; 18 min, B 98 %, C 2 %; 18.01 min, B 100 %. The initial conditions were then re-established and held for 10 min. The flow rate of the mobile phase was 0.5 mL min^{-1}. An aliquot of 10 μL of the final extract was injected into the LC system. The LC system was coupled with an LCQ Advantage quadrupole ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The electrospray ionization (ESI) technique was used, and all analytes were analyzed in the positive ionization mode. The optimal source parameters were as follows: source voltage (4.5 kV), sheath gas (25 au, *i.e.*, 25 arbitrary units), and capillary temperature (290 °C). Fragmentation reactions used for identification and quantification, as well as the confirmation of selected analytes, are shown in Table S-I (Supplementary material).

The standard addition method was used to analyse the collected wastewater samples, compensating for matrix effects and incomplete analyte extraction. Each water sample was divided into six aliquots: four aliquots were spiked with a working standard solution at concentrations of 50–1000 ng L^{-1}, resulting in final extract concentrations of 5–100 μg L^{-1}, and the remaining two were not spiked.

Method validation

The linearity of the analytical response was studied using the matrix-matched standards (MMS) prepared at five concentration levels (5–100 μg L^{-1}). MMS were prepared by adding working standard solutions of the pharmaceuticals to the blank extracts obtained after the SPE procedure. The extraction recoveries and the repeatability of the method, expressed as the relative standard deviation (R*SD*), were determined by analyzing three replicate wastewater samples spiked at two concentration levels (100 and 1000 ng L^{-1}). The limits of detection (*LOD*s) and quantification (*LOQ*s) were determined as the minimum detectable amount of analyte with a signal-to-noise (*S*/*N*) ratio of 3 and 10, respectively,[19] using spiked samples at a concentration of 50 ng L^{-1}.

Water sample collection

The details related to sample collection are given in Supplementary material.

Risk assessment

The risk quotient (*RQ*) approach was used to assess the environmental risk of pharmaceuticals detected in surface water. Risk quotients were calculated using the equation:

$$RQ = \frac{MEC}{PNEC} \tag{1}$$

where *MEC* is the measured environmental concentration and *PNEC* is the predicted no-effect concentration of the detected compounds.[21] *PNEC* values were obtained from the Norman database,[22] using the lowest available values for each compound (Table S-III, Supplementary material). The risk to aquatic life may increase when pharmaceuticals are present in mixtures, as each compound contributes to toxicity, even if individual concentrations are below their *PNEC* values. Therefore, to fully estimate the risks to aquatic life arising from exposure to pharmaceuticals, the combined effects of their mixtures were considered. Risk at each sampling site was evaluated using the concentration addition concept,[23,24] with RQ_{mix} calculated as:

$$RQ_{mix} = \sum_{i=1}^{n} \frac{MEC_i}{PNEC_i} \tag{2}$$

Based on *RQ* values, the environmental risk was classified as: high ($RQ \geq 1$), medium ($0.1 \leq RQ < 1$), and low ($RQ < 0.1$).[25]

RESULTS AND DISCUSSION

Method validation

The developed analytical method was validated for wastewater samples, with linearity, recovery, repeatability, detection limits and quantification limits assessed (Table S-IV, Supplementary material). Linearity was evaluated using MMS (5––100 μg L^{-1}), corresponding to initial sample concentrations of 50–1000 ng L^{-1}. Determination coefficients (R^2) ranged from 0.990 to 0.999 for all tested analytes,

158 indicating adequate linearity. The recoveries ranged from 70 to 120 % for most
159 analytes, except for azithromycin and erythromycin (55–62 %), likely due to pH
160 adjustment to 6.0. Our previous study showed that pH values of 3.0 and 7.5 were
161 optimal for azithromycin and erythromycin, respectively.[18] However, pH 6.0 pro-
162 vided the best overall recoveries, making it the most suitable choice for simul-
163 taneous analysis. The resulting *RSD* values were all below 20 %. Low limits of
164 detection (3.3–50.0 ng L^{-1}) and quantification (11.1–166.7 ng L^{-1}) were obtained,
165 demonstrating the method's sensitivity, which is in agreement with values reported
166 in the literature[19] and its suitability for determining trace levels of selected analytes
167 in wastewater samples.

168 *Wastewater and surface water sample analysis*

169 The validated method was used to analyse selected pharmaceuticals in urban
170 wastewater samples discharged into the Sava and Danube rivers in Belgrade, as
171 well as in the corresponding surface water. As shown in Table I, 13 out of 17 target
172 pharmaceuticals were detected in urban wastewater, with concentrations ranging
173 from 15 ng L^{-1} (cilazapril) to 1.2 μg L^{-1} (sulfamethoxazole). The most ubiquitous
174 drugs were metoprolol, bisoprolol, enalapril, carbamazepine and diclofenac, det-
175 ected at all sampling sites, confirming their widespread use and persistence in was-
176 tewater. Sampling site WW1 had the highest number of detected compounds (13
177 out of 17), likely because it is the primary discharge point for Belgrade's waste-
178 water.

179 TABLE I. Pharmaceuticals detected in Belgrade urban wastewater at seven sampling sites
180 WW1–WW7 (n = 2)

Pharmaceutical	Concentration ± *SD*, ng L^{-1}						
	WW1	WW2	WW3	WW4	WW5	WW6	WW7
Trimethoprim	122±12	53±5	–	–	482±72	–	–
Metoprolol	290±22	154±13	434±58	36±3	305±32	143±20	294±8
Sulfamethoxazole	122±19	122±9	–	–	1184±152	–	–
Azithromycin	318±48	102±0	–	–	–	–	–
Bisoprolol	63±11	46±4	118±22	83±7	49±8	35±4	76±9
Enalapril	155±12	128±26	240±51	284±44	174±17	164±20	208±34
Cilazapril	15±2	89±16	22±4	–	–	–	–
Erythromycin	133±22	266±34	347±22	–	–	–	–
Carbamazepine	274±44	313±30	449±14	263±8	127±25	190±49	253±41
Lorazepam	171±24	84±3	318±19	167±33	77±10	105±22	–
Diazepam	163±22		–	–	–	–	–
Atorvastatin	71±5	25±4	–	–	–	–	–
Diclofenac	442±73	253±11	58±5	471±70	782±156	479±105	310±71

181 Pharmaceuticals in untreated wastewater have been studied across the Wes-
182 tern Balkans, with Petrović *et al.*[9] detecting trimethoprim, sulfamethoxazole, carb-
183 amazepine, lorazepam and atorvastatin in Novi Sad at concentrations similar to

ours, with carbamazepine being among the most abundant in both studies. However, diclofenac and metoprolol concentrations were higher in their study, with diclofenac exceeding 1 μg L^{-1}. Terzić *et al.*[26] investigated 44 pharmaceuticals in wastewater from Bosnia and Herzegovina, Croatia and Serbia, reporting a wide concentration range for many compounds. For pharmaceuticals detected in both studies, the concentrations measured in our samples fell within the reported ranges.

In the corresponding surface water samples, 9 out of 17 target pharmaceuticals were detected (Table II), with concentrations ranging from 19 ng L^{-1} (bisoprolol) to 1.1 μg L^{-1} (diclofenac). The most prevalent drugs in the surface water samples were metoprolol, carbamazepine, and diclofenac, detected at all sites. At sampling site SW8, 9 out of 17 substances were detected, with notably higher concentrations, likely due to its location in a small bay with limited water circulation.

TABLE II. Pharmaceuticals detected in Belgrade surface water at eight sampling sites SW1–SW8 (n = 2)

Pharmaceutical	Concentration ± *SD*, ng L^{-1}							
	SW1	SW2	SW3	SW4	SW5	SW6	SW7	SW8
Trimethoprim	145±11	–	–	–	–	–	–	334±54
Metoprolol	95±3	107±13	53±8	308±50	82±14	78±11	132±12	1012±150
Sulfamethoxazole	–	–	–	–	–			127±7
Azithromycin	–	–						58±1
Bisoprolol				33±2			19±2	661±124
Enalapril	–	–	–		–		–	1099±8
Carbamazepine	20±2	20±2	23±1	38±7	24±4	32±5	56±8	552±29
Diazepam	–	–	–	–		–	–	176±18
Diclofenac	316±47	157±2	267±50	225±45	375±75	1087±123	251±50	950±176

In a review paper,[27] the reported concentration ranges of pharmaceuticals in the Lower Danube River basin were: carbamazepine (3.94–945 ng/L), diclofenac (0.8–255 ng/L), sulfamethoxazole (30–204 ng/L) and trimethoprim (0.8–223 ng/L). Our results align with these ranges, except for diclofenac, which showed significantly higher concentrations at two sampling sites. Similarly elevated diclofenac levels were reported in the Danube near Budapest, Hungary (up to 931 ng/L).[28]

In most cases, the same compounds were found in both surface water samples and wastewater discharged into them, indicating the impact of Belgrade's municipal wastewater on surface water quality. The concentrations of most detected compounds were significantly lower in river water. However, for some compounds, such as diclofenac, similar or even higher concentrations were observed in surface water compared with wastewater. The highest detected diclofenac concentration in river water exceeded that observed in wastewater, likely due to its high environmental persistence.[29] Metoprolol, diclofenac and carbamazepine were the most frequently detected analytes in both sample types.

Hazard characterisation of detected pharmaceuticals

Hazard identification was conducted for each detected pharmaceutical according to the REACH/CLP criteria, evaluating five key hazard classes: persistence, bioaccumulation, toxicity, mobility and endocrine disruption.[30] A comprehensive summary of the hazard characterisation for all detected pharmaceuticals is provided in Table S-V (Supplementary material). The hazard assessment revealed that several detected pharmaceuticals exhibited concerning profiles: carbamazepine and erythromycin showed high persistence and toxicity, while azithromycin showed moderate-to-high persistence combined with high toxicity. Multiple pharmaceuticals (metoprolol, sulfamethoxazole, carbamazepine, erythromycin) demonstrated high mobility, raising concerns about their potential for widespread environmental distribution. Additionally, various compounds, including metoprolol, sulfamethoxazole, azithromycin, erythromycin, carbamazepine, lorazepam, diazepam and diclofenac, showed potential endocrine-disrupting activity. These hazard characteristics justify the inclusion of the detected pharmaceuticals in the risk assessment.

Risk assessment

The environmental risk associated with analytes detected in surface water samples was assessed, with the obtained *RQ*s presented in Fig. 2A. Based on the calculated *RQ* values, bisoprolol posed a low risk ($RQ < 0.1$), while most of the other detected analytes posed a moderate risk ($0.1 \leq RQ \leq 1$). However, diclofenac posed a high risk, with *RQ* values exceeding 1 at all sampling sites, reaching a maximum of 27.17 at site SW6. Azithromycin also exceeded the high-risk threshold ($RQ > 1$) at one site ($RQ = 3.05$ at SW8). Both diclofenac and azithromycin have been proposed for inclusion in the EU Priority Substances List in the field of water policy,[31] due to their widespread presence in water bodies and harmful effects on aquatic organisms. Other studies,[32–34] have also reported high risks for these substances, highlighting the need for further attention and regulatory consideration.

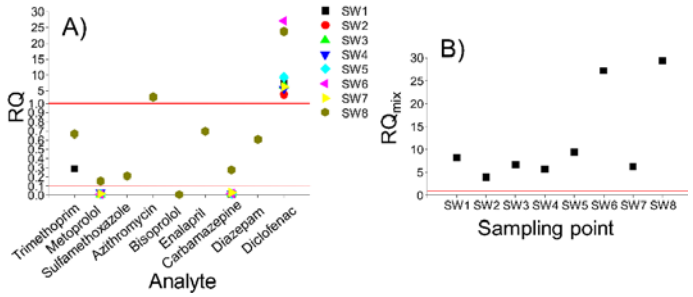

Fig. 2. A) Risk quotients (*RQ*s) for individual compounds at each surface water site; B) risk quotients for the compound mixture (RQ_{mix}) at each site.

The risk of the entire mixture of pharmaceuticals was also assessed at all surface water sampling sites. As shown in Fig. 2B, the RQ_{mix} significantly exceeded the threshold of 1 at all sites, with the highest value of 29.42 observed at site SW8. At this location, as well as at all other sites, diclofenac was the predominant contributor to the high RQ_{mix} value. However, even contaminants that individually do not pose a significant risk can contribute to an increased RQ_{mix} value and as part of a mixture, they may amplify environmental risk due to cumulative effects. These findings emphasise the need to assess the combined effects of contaminant mixtures, rather than focusing solely on individual compounds.

Pharmaceuticals detected in WWTPs and their removal rates

Pharmaceutical concentrations in wastewater samples from the selected WWTPs in Serbia (Table III) showed significant variations in both influent and effluent levels. Among the nine detected pharmaceuticals, the highest influent concentrations were recorded for carbamazepine (2.7 μg L^{-1}) at WWTP1 and diclofenac (5.9 μg L^{-1}) at WWTP2. Generally, influent concentrations exceeded those obtained for untreated wastewater from Belgrade (Table I), particularly for metoprolol, erythromycin and diclofenac. These variations may stem from differences in wastewater composition, such as differences in the proportion of domestic effluents.

TABLE III. Pharmaceuticals detected in influent and effluent samples of two WWTPs in Serbia (n = 2); *RE*: removal efficiency

Pharmaceutical	Concentration ± *SD*, ng L^{-1}		*RE* %	Concentration ± *SD*, ng L^{-1}		*RE* %
	Influent WWTP1	Effluent WWTP1		Influent WWTP2	Effluent WWTP2	
Trimethoprim	37±5	145±7	–292	142±13	109±22	23
Metoprolol	860±77	11630±15	–1252	644±112	3095±12	–381
Sulfamethoxazole	215±24	151±13	30	385±73	107±16	72
Bisoprolol	78±3	73±9	6	89±5	29±2	67
Enalapril	543±55	105±8	81	868±149	399±58	54
Erythromycin	1448±261	520±78	64	956±2	3833±25	–301
Carbamazepine	2675±589	389±62	85	1368±328	529±74	61
Lorazepam	148±2	242±5	–63	188±13	–	100
Diclofenac	1592±143	919±55	42	5927±948	324±35	95

Our results align with other studies reporting carbamazepine concentrations of up to 2150 ng L^{-1} in wastewater in Spain and 2499 ng L^{-1} in China,[35,36] as well as diclofenac levels reaching 4056 ng L^{-1} in Slovenia.[37] In contrast, Afonso-Olivares *et al.*[38] reported lower concentrations of carbamazepine, diclofenac and erythromycin compared with our study, while sulfamethoxazole and trimethoprim were found at higher levels. Differences in consumption patterns may account for regional variations in pharmaceutical concentrations.

All pharmaceuticals detected in influent samples were also present in effluent samples from both WWTPs, except for lorazepam (which was detected in only one effluent), indicating incomplete removal by conventional wastewater treatment. The highest effluent concentrations were observed for metoprolol (11.6 μg L^{-1}) at WWTP1 and erythromycin (3.8 μg L^{-1}) at WWTP2. The removal efficiencies (RE) of pharmaceuticals were evaluated based on influent and effluent concentrations. *RE* varied significantly, ranging from 6 % (bisoprolol) to 85 % (carbamazepine) at WWTP1 and 23 % (trimethoprim) to 100 % (lorazepam) at WWTP2. Gros *et al.*[14] suggested that pharmaceuticals may persist in WWTP effluents due to low adsorption onto activated sludge or slow microbial degradation within the plant's retention time. Consequently, conventional secondary treatment with activated sludge achieves only partial removal, with low *RE* commonly reported for certain pharmaceuticals.[15,39] Additionally, several pharmaceuticals (trimethoprim, lorazepam and metoprolol in WWTP1; erythromycin and metoprolol in WWTP2) exhibited higher concentrations in effluent than in influent samples, resulting in negative *RE*. This phenomenon, documented in other studies,[14,15,37] may result from pharmaceutical retention within the WWTP and subsequent episodic release. Another possible explanation is enzymatic deconjugation of conjugated metabolites, leading to the regeneration of parent compounds during treatment.[40] Despite their similar configurations, the two WWTPs exhibited significant differences in pharmaceutical removal efficiency, which may be explained by differences in process parameters, such as hydraulic retention time and sludge retention time.[41] WWTP2 generally exhibited higher efficiency, particularly in the removal of diclofenac and lorazepam (95 and 100 %, respectively).

Overall, these results suggest that the wastewater treatment processes in the two WWTPs were insufficient to fully eliminate the studied contaminants, highlighting the need for advanced treatment strategies to reduce pharmaceutical contamination in surface waters.

CONCLUSION

This study assessed the presence and impact of pharmaceuticals in Belgrade's wastewater on surface waters, revealing considerable pollution and associated environmental risks. A sensitive and reliable LC–MS/MS method was developed, validated and successfully utilised for pharmaceutical analysis. The most frequently detected pharmaceuticals in wastewater and surface water were metoprolol, carbamazepine and diclofenac, with diclofenac reaching concentrations of up to 1.1 μg L^{-1} in surface water. Risk assessment revealed that diclofenac and azithromycin posed a high environmental risk in surface waters, while the cumulative risk of pharmaceutical mixtures exceeded safety thresholds at all sampling sites. The results from the two conventional wastewater treatment plants (WWTPs) demonstrated variable removal efficiencies, with some pharmaceuticals exhibiting

negative removal rates, suggesting potential issues in the treatment processes. Overall, the study underscores the need for advanced wastewater treatment technologies and regulatory measures to mitigate pharmaceutical contamination and its ecological risks.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/13395, or from the corresponding author on request.

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SUPPLEMENTARY MATERIAL TO

Pharmaceuticals in Belgrade's wastewater: impact on surface waters and environmental risk assessment

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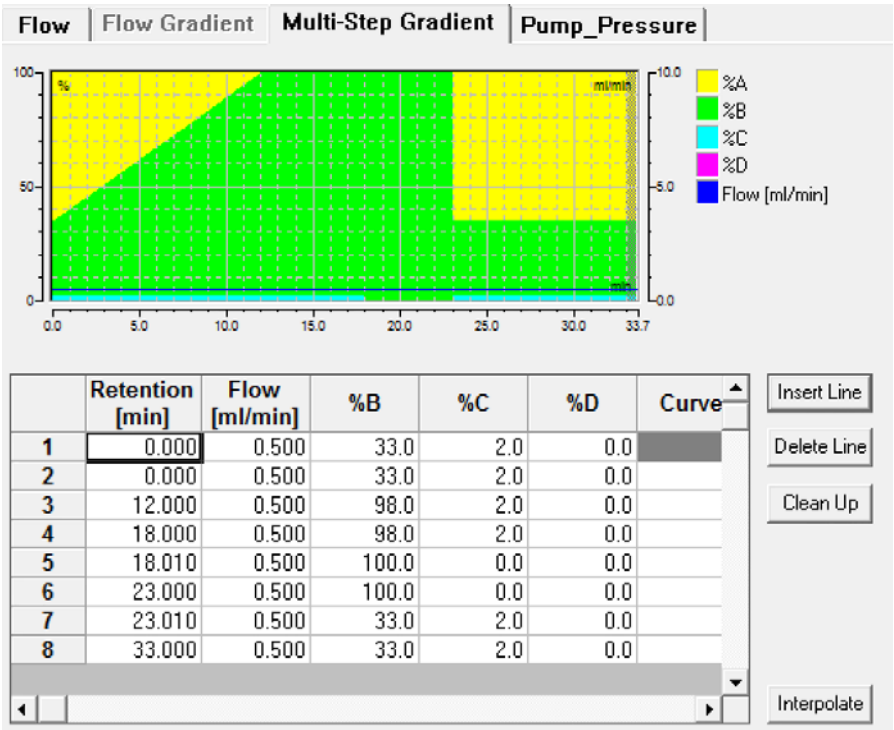

Fig. S-I. Mobile phase gradient profile.

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Water sample collection

Wastewater and surface water samples were collected in Belgrade, Serbia, near the confluence of the Sava and the Danube rivers (Table S-II). A map with marked sampling sites (WW1–WW7) is shown in Fig. S-1. Wastewater samples were taken at seven discharge points, which handle about 80 % of Belgrade's untreated wastewater. The Belgrade sewage system consists of 212 km of collectors, 1,439 km of pipe network, 32,750 drains and 53,394 sewage connections.[20] Sample WW1 was taken from the largest sewage canal, serving approximately 500,000 inhabitants. Samples were collected over 24 h using automatic samplers and combined into composite samples. Corresponding surface water samples were collected at eight sampling sites downstream of wastewater discharge. Three samples were collected from the Sava river (SW1–SW3, Fig. S-1), four samples from the Danube river (one before the confluence of the two rivers, SW4; three after the confluence, SW6–SW8), and one at the confluence of the Sava and Danube rivers (SW5). Surface water samples were collected by direct sampling from a boat in the middle of the river flow at a depth of about 50 cm. All water samples were collected in 1 L PET bottles and stored at 4 C until analysis (usually within 1–2 days after sampling). No precipitation occurred on the day of sampling.

Since Belgrade lacks a WWTP, influent and effluent samples were collected from two WWTPs located in small municipalities in Serbia (Sombor, WWTP1, and Velika Plana, WWTP2). The selected WWTPs provide primary and secondary treatment of wastewater using biologically active sludge. WWTP1 has a treatment capacity of 50,000 population equivalents (PE) or 9,300 m^3 day^{-1}, while WWTP2 has a capacity of 35,000 PE. Composite 24 h samples of influent and effluent wastewater were collected at each WWTP using automatic sampling devices. Water samples were stored in 1 L PET bottles and kept frozen without preservatives until preparation for analysis, which occurred a few days after sampling.

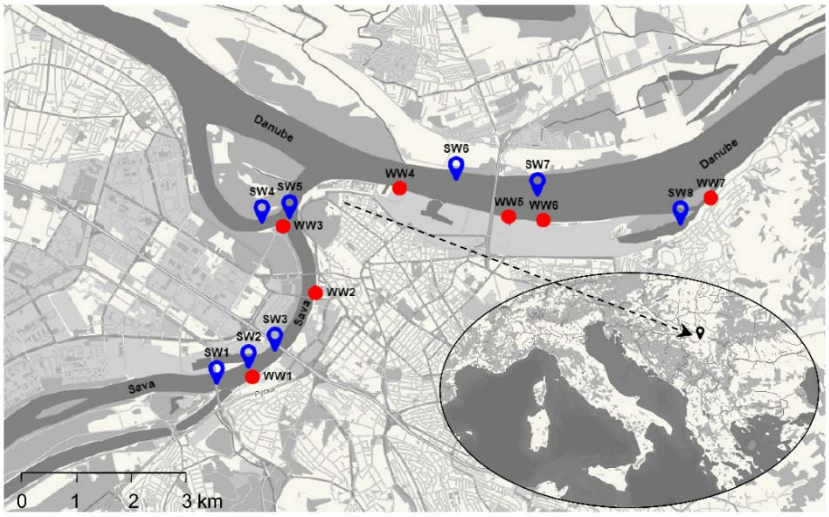

Fig. S-1. Sampling sites of wastewater (WW) and corresponding surface water (SW) in the Sava and the Danube rivers in Belgrade (Serbia).

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493 **Table S-I.** MS operating parameters for selected pharmaceuticals.

Pharmaceuticals	Precursor ion (*m/z*)	Quantification reaction	Collision energy (%)	Confirmation reaction	Collision energy (%)
Trimethoprim	291 $[M+H]^+$	291→230	44	291→123	44
Metoprolol	268 $[M+H]^+$	268→191	37	268→218	37
Sulfamethoxazole	254 $[M+H]^+$	254→188	34	254→156	34
Azithromycin	749 $[M+H]^+$	749→591	30	591→434	28
Bisoprolol	326 $[M+H]^+$	326→116	31	326→222	31
Enalapril	377 $[M+H]^+$	377→234	30	377→303	30
Cilazapril	418 $[M+H]^+$	418→211	25	211→183	32
Erythromycin	734 $[M+H]^+$	734→576	26	734→716	26
Bromazepam	316 $[M+H]^+$	316→288	36	288→261	35
Amlodipine	409 $[M+H]^+$	409→238	25	409→294	25
Carbamazepine	237 $[M+H]^+$	237→194	34	237→220	34
Lorazepam	321 $[M+H]^+$	321→303	32	303→275	26
Diazepam	285 $[M+H]^+$	285→257	40	257→228	39
Atorvastatin	559 $[M+H]^+$	559→466	25	559→440	25
Diclofenac	296 $[M+H]^+$	296→278	28	278→250	22
Clopidogrel	322 $[M+H]^+$	322→212	28	212→184	23
Simvastatin	419 $[M+H]^+$	419→285	21	419→199	21

494 **Table S-II.** The sampling site description.

Sample	Latitude	Longitude	Site description
Surface water (SW)			
SW1	44.7962	20.4259	The Sava; 4 km before the confluence
SW2	44.7989	20.4336	The Sava; 3.5 km before the confluence; downstream from the largest WW canal (WW1)
SW3	44.8019	20.4400	The Sava; 2.7 km before the confluence
SW4	44.8233	20.4368	The Danube; 500 m before the confluence
SW5	44.8241	20.4434	The confluence
SW6	44.8305	20.4828	The Danube; 3.6 km after the confluence
SW7	44.8279	20.5091	The Danube; 5.6 km after the confluence
SW8	44.8231	20.5359	The Danube; 8.5 km after the confluence; small bay
Wastewater (WW)			Number of inhabitants connected to WW canal
WW1	44.7976	20.4346	The largest WW canal; catchment area of 7,277 ha; 500,000
WW2	44.8118	20.4494	Catchment area of 113 ha; 23,000
WW3	44,8229	20.4419	The second large WW canal; catchment area of 2,620 ha; 225,000
WW4	44.8293	20.4694	Catchment area of 92 ha; 17,000
WW5	44.8245	20.4953	Catchment area of 1,112 ha; 165,000
WW6	44.8239	20.5035	Catchment area of 50 ha; 5,000
WW7	44.8277	20.5430	Catchment area of 116 ha; 7,000

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Table S-III. Lowest PNEC values of detected pharmaceuticals in freshwater obtained from NORMAN database.

Pharmaceuticals	Norman PNEC ID	CAS No.	Taxon. group	Scientific name	Applied AF	Justification	Derivation method	Lowest PNEC freshwater (μg L^{-1})	Ref
Trimethoprim	PNEC-ID-0348354	738-70-5	n.r. [a]	n.r.	0	n.r.	n.r.	0.5	1
Metoprolol	PNEC-ID-0348060	51384-51-1	PI	*Desmodesmus subspicatus*	50	n.r.	deterministic	8.6	2
Sulfamethoxazole	PNEC-ID-0348126	723-46-6	P	*Synechococcus leopoliensis*	10	n.r.	deterministic	0.6	2
Azithromycin	PNEC-ID-0347903	83905-01-5	PI	*Microcystis aeruginosa*	10	n.r.	deterministic	0.019	2
Bisoprolol	PNEC-ID-0348209	66722-44-9	PIV	n.r.	50	n.r.	deterministic	92	3
Enalapril	PNEC-ID-0031812	75847-73-3	fish	-	1000	[b]	deterministic	1.58	4
Carbamazepine	PNEC-ID-0347929	298-46-4	I	*Daphnia pulex*	50	n.r.	deterministic	2	2
Diazepam	PNEC-ID-0257661	439-14-5	F	*Danio rerio*	10	[c]	deterministic	0.29	5
Atorvastatin	PNEC-ID-0348226	134523-00-5	PIV	n.r.	10	n.r.	deterministic	8.5	6
Diclofenac	PNEC-ID-0348270	15307-86-5	n.r.	n.r.	0	n.r.	n.r.	0.04	7

[a] n.r. – not reported

[b] One predicted short-term L(E)C50 from each of three trophic levels (i.e., base set)

[c] Long-term results (e.g., EC10 or NOECs) from at least three species (normally fish, Daphnia and algae) representing three trophic levels

503 **Table S-IV.** Validation parameters of the analytical method: recoveries and relative standard
504 deviations (RSD), limits of detection (LOD) and quantification (LOQ), and linearity correlation
505 coefficient (R^2).

Pharmaceuticals	Recovery, % (RSD, %) Spiking level, ng L^{-1}		LOD, ng L^{-1}	LOQ, ng L^{-1}	R^2
	100	1000			
Trimethoprim	88 (8)	92 (5)	11.0	36.8	0.993
Metoprolol	87 (12)	87 (5)	3.3	11.1	0.993
Sulfamethoxazole	83 (5)	70 (8)	30.0	100.0	0.999
Azithromycin	58 (7)	55 (11)	5.2	17.4	0.999
Bisoprolol	71 (12)	84 (11)	5.2	17.2	0.997
Enalapril	117 (19)	110 (11)	30.0	100.0	0.993
Cilazapril	88 (2)	101 (7)	3.9	13.2	0.997
Erythromycin	56 (17)	62 (10)	4.1	13.7	0.997
Bromazepam	89 (11)	93 (7)	25.0	83.3	0.998
Amlodipine	81 (4)	96 (2)	23.4	77.9	0.998
Carbamazepine	107 (8)	101 (8)	3.6	11.8	0.999
Lorazepam	83 (13)	93 (6)	14.3	47.6	0.996
Diazepam	86 (10)	95 (4)	21.1	70.3	0.990
Atorvastatin	95 (2)	83 (5)	19.6	65.5	0.999
Diclofenac	83 (20)	92 (13)	23.1	76.9	0.993
Clopidogrel	87 (7)	94 (1)	9.3	30.9	0.999
Simvastatin	118 (19)	107 (7)	50.0	166.7	0.998

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Table S-V. Hazard identification of detected pharmaceuticals.

Pharmaceutical	Persistence	Bioaccumulation	Toxicity	Mobility	Endocrine Disruption (ED)	Ref.
Trimethoprim	Moderate	Low	Moderate	High	Not reported	8
Metoprolol	Moderate	Low	Low	Moderate–high	Possible functional ED	9-11
Sulfamethoxazole	Moderate	Low	High	Moderate–high	May exhibit endocrine toxicity	12-14
Azithromycin	Moderate–high	Low–moderate	High	Moderate–high	May have endocrine effects	15
Bisoprolol	Moderate	Low	Low	Moderate	Not reported	16,17
Enalapril	Moderate	Low	Low	Moderate	Not classified as ED	18,19
Cilazapril	Moderate	Low	Low	Moderate	Not reported	20
Erythromycin	High	Low	High	High	Potential disruptor	21-23
Carbamazepine	High	Low	High	High	Potential to function as an ED	24-26
Lorazepam	Moderate	Low	Moderate	Moderate	Functional ED uncertainty	27
Diazepam	Moderate–high	Low–moderate	Moderate	Moderate	Potential to interact with endocrine function	28,29
Atorvastatin	Low–moderate	Low	Moderate	Moderate	Not reported	30,31
Diclofenac	Moderate-high	Low	Moderate-high	Moderate-high	Possible ED	32,33

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