Occurrence and ecotoxicological risk assessment of emerging contaminants in urban wastewater treatment plant

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Abstract. This is the first study of a broad range of chemical classes of emerging contaminants conducted by analyzing influent and effluent samples from the wastewater treatment plant of the city Topola, in Serbia. The list of compounds is extensive and this paper provides a better understanding of the environmental burden from different classes of emerging contaminants. The samples were prepared using an optimized solid-phase extraction method and analyzed by liquid chromatography-tandem mass spectrometry. Removal patterns of selected compounds are discussed based on their physico-chemical properties and detected concentrations. Significant removal efficiencies, exceeding 70%, were found for the majority of investigated pharmaceuticals, pesticides, steroids, and sweeteners. Ecotoxicological risk assessment was performed by using two complementary methods: (1) an individual substance approach, based on the calculation of risk quotients (RQs) for each substance as the ratio of Predicted Environmental Concentration (PEC) and Predicted No Effect Concentration (PNEC), and (2) mixture risk assessment ("the cocktail effect") based on the summation of individual RQs. The classical approach (ERA method with individual substances) identified amlodipine as the riskiest substance in WWTP effluent. The mixture ERA approach revealed new risks, which were not recognized by the classical ERA method, indicating that individually "safe" emerging compounds can contribute to a significant risk of the whole effluents.

Keywords: Emerging contaminants, Wastewater treatment plant, Liquid chromatography-tandem mass spectrometry, Ecotoxicological risk assessment.

1 Introduction

The list of chemical compounds that can be frequently found in the literature as "emerging substances" is constantly growing. The presence of these compounds in the aquatic environment and wastewater has been well documented [1-6] and is known to pose an adverse long-term risk to human health and aquatic ecosystems

even at low concentrations [7-10]. Urban wastewater treatment plants (WWTPs) have been designed to remove high levels of conventional pollutants such as oil and grease, coliform fecal bacteria, nitrogen, phosphorus, and organic matter. However, many studies have shown that the removal of emerging contaminants remains very poor, and therefore the effluents from wastewater treatment plants still represent one of the main sources of emerging substances discharges into the aquatic environment [11-13]. As a consequence, these pollutants are continuously released in trace amounts (typically ranging from ng/L to μg/L) into receiving watercourses. It should be also noted that these substances are usually not detected individually, but rather as a complex mixture, so their "cocktail effects" should be taken into account when assessing the risks to humans and the environment.

In the WWTPs, emerging substances undergo various processes, such as adsorption onto suspended matter, biodegradation, or chemical degradation. The removal efficiency of these substances varies significantly depending on their physico-chemical properties, operational parameters of the plant, and the type of treatment process applied. The main aim of this work is to determine the occurrence and fate of a broad range of chemical classes of emerging compounds in WWTP of the city Topola, in Serbia. The list of compounds analyzed in this paper is extensive and comprises 23 different pharmaceuticals, 16 pesticides, 20 steroids (combination of steroid hormones and sterols), and 8 sweeteners (seven artificial and one natural). Finally, the present study is aimed to develop and apply a new ecotoxicological risk assessment (ERA) method for emerging substances released from WWTP effluents into freshwater watercourses. So far, the majority of published papers [14-16] were based on the analysis of substance measured in environmental waters and did not consider the risk posed by the release of the pollutants by WWTP discharges. The ERA method applied in this work will assess the risk of each pollutant alone based on the comparison of Predicted Environmental Concentrations (PEC) and Predicted No Effect Concentration (PNEC) values, following European guidelines [17], but also it will take into account the "cocktail effect" due to the mixture of emerging substance in WWTP effluents in the territory, using a recently developed procedure [18-20].

2 Materials and methods

2.1 Chemicals and reagents

Analytical standards of selected pharmaceuticals were supplied from Hemofarm (STADA Group, Vršac, Serbia), whereas pesticide standards were obtained from Riedel-de Haën (Seelze, Germany). Steroid analytical standards were purchased from Steraloids Inc. (Newport, US) and analytical standards of artificial sweeteners were obtained from Sigma-Aldrich (Buchs, Switzerland), except sucralose which was purchased from TCI Europe (Zwijndrecht, Belgium). All standards of the investigated compounds were of high purity grade (> 95%). The list of 67 initially selected analytes, their chemical classes and physico-chemical properties are presented in Table 1.

Table 1. Analytes selected for investigation: chemical class, molecular weight (M_w) and water solubility (WS).

Analyte	Chemical class	M_w, g/mol	WS[a], mg/L
Pharmaceuticals			
Trimethoprim	Used in combination with sulfonamide antibiotics	290	400
4-Acetylaminoantipyrine (4-AAA)	The final metabolite of analgesic/antipyretic metamizole	245	-
4-Formylaminoantipyrine (4-FAA)	The final metabolite of analgesic/antipyretic metamizole	231	-
Sulfamethoxazole	Sulfonamide antibiotic	253	610
Azithromycin	Macrolide antibiotic	748	7.1
Erythromycin	Macrolide antibiotic	733	1.4
Midecamycin	Macrolide antibiotic	814	Soluble in acidic water
Clarithromycin	Macrolide antibiotic	748	1.693[b]
Roxithromycin	Macrolide antibiotic	837	181
Doxycycline	Tetracycline antibiotic	444	630[b]
Metoprolol	Antihypertensive, β-blocker	267	4,780
Bisoprolol	Antihypertensive, β-blocker	325	2,240
Enalapril	Antihypertensive, ACE inhibitor	376	16,400
Cilazapril	Antihypertensive, ACE inhibitor	417	27.5[b]
Amlodipine	Antihypertensive, calcium channel blocker	408	75.3
Atorvastatin	Antihyperlipemic, statin	558	$1.1 \cdot 10^{-3}$
Simvastatin	Antihyperlipemic, statin	418	0.03
Clopidogrel	Anticoagulant	321	50.8
Bromazepam	Sedative, benzodiazepin	315	175
Lorazepam	Sedative, benzodiazepin	320	80.0
Diazepam	Sedative, benzodiazepin	284	50.0
Carbamazepine	Antiepileptic	236	17.7[b]
Diclofenac	Analgesic/antipyretic	296	2.4
Pesticides			
Acephate	Organophosphate insecticide	183	$8.2 \cdot 10^5$
Monocrotophos	Organophosphate insecticide	233	$1.0 \cdot 10^6$
Dimethoate	Organophosphate insecticide	229	23,300
Malathion	Organophosphate insecticide	330	143
Carbendazim	Benzimidazole fungicide	191	29.0
Imidacloprid	Neonicotinoid insecticide	255	610
Acetamiprid	Neonicotinoid insecticide	222	4,200
Monuron	Phenylurea herbicide	198	230
Diuron	Phenylurea herbicide	232	42.0
Linuron	Phenylurea herbicide	249	75.0
Carbaryl	Carbamate insecticide	201	110
Carbofuran	Carbamate insecticide	221	320
Simazine	Triazine herbicide	201	6.2
Atrazine	Triazine herbicide	215	34.7

Analyte	Chemical class	M_w, g/mol	WS[a], mg/L
Propazine	Triazine herbicide	229	8.6
Tebufenozide	Diacylhydrazine insecticide	352	0.83
Steroids			
Estriol	Steroid hormone	288	500
Estrone (E1)	Steroid hormone	270	30.0
Equilin	Steroid hormone	268	1.4
Norethindrone	Steroid hormone	298	7.0
17α-Ethinylestradiol (EE2)	Steroid hormone	296	11.3
17β-Estradiol (E2)	Steroid hormone	272	3.6
17α-Estradiol	Steroid hormone	272	3.9
Levonorgestrel	Steroid hormone	312	2.1
Mestranol	Steroid hormone	310	0.30
Epicoprostanol	Human/animal sterol	388	$3.4 \cdot 10^{-4}$ [b]
Epicholestanol	Human/animal sterol	388	$3.5 \cdot 10^{-5}$
Coprostanol	Human/animal sterol	388	$2.0 \cdot 10^{-2}$
Cholestanol	Human/animal sterol	388	$8.8 \cdot 10^{-5}$
Cholesterol	Human/animal sterol	386	$9.5 \cdot 10^{-2}$
Cholestanone	Human/animal sterol	386	$2.9 \cdot 10^{-4}$ [b]
Desmosterol	Plant sterol	384	$2.0 \cdot 10^{-4}$
Stigmasterol	Plant sterol	412	$1.1 \cdot 10^{-5}$
Campesterol	Plant sterol	400	$2.8 \cdot 10^{-5}$
β-Sitosterol	Plant sterol	414	$1.3 \cdot 10^{-5}$
Sitostanol	Plant sterol	416	$9.8 \cdot 10^{-6}$
Artificial sweeteners			
Acesulfame	Sulfamate ester	163	$9.1 \cdot 10^{5}$
Saccharin	Benzisothiazole	183	789.2
Cyclamate	Salt of cyclamic acid	179	$1.0 \cdot 10^{6}$
Sucralose	Disaccharide	397	$2.3 \cdot 10^{4}$
Aspartame	Dipeptide	294	564.7
Neohesperidin dihydro-chalcone; NHDC	Dihydrochalcone	612	$2.0 \cdot 10^{3}$
Neotame	Dipeptide	378	14.4
Stevioside	Diterpene glycoside	805	$4.5 \cdot 10^{3}$

[a]Source: https://www.srcinc.com

[b]Source: US EPA. [2012]. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.11. US EPA, Washington, DC, US.

The stock standard solutions were prepared in methanol at a concentration of 100 µg/mL. The working standard solutions in the concentration range 10–1,000 ng/mL were prepared by mixing the appropriate amounts of the stock standard solutions and dilution with methanol. All solutions were preserved at –4 °C. All solvents used were HPLC grade from J.T. Baker (Center Valley, US) or Sigma-Aldrich (St. Louis, US). Ammonium acetate and concentrated acetic acid were of the analytical grade. Deionized water was obtained by passing the distilled water through a GenPure ultrapure water system (TKA, Niederelbert, Germany).

2.2 Studied area

The samples were collected from a small wastewater treatment plant of town Topola, in the Republic of Serbia. The incoming load of the facility in population equivalent is 8000, and the annual mean incoming flow rate of the WWTP influent is 1089 m^3/day. The annual mean flow rate of the WWTP effluent is 978 m^3/day. Only households and catering facilities are connected to the city sewerage network, and therefore mechanical and biological treatments are applied in the plant. The secondary treatment process is based on an activated sludge system. Wastewater effluents are discharged to the river Kamenica. The average flow of the recipient *i.e.* receiving watercourse Kamenica at the time of the sample collection was 30 m^3/s.

2.3 Sample preparation procedure

Wastewater samples were prepared for the analysis using solid-phase extraction (SPE). The used SPE protocol has been previously developed for the isolation and preconcentration of selected pharmaceuticals and pesticides from the water matrix [21]. The optimized method showed high recoveries for all investigated analytes (ranging from 63.9% to 141.7%). Briefly, the volume of 100 mL of the wastewater was adjusted at pH=6. The OASIS HLB cartridges, used for extraction and preconcentration of the target analytes, were preconditioned with 5 mL of methanol/dichloromethane mixture (1:1, *v/v*) followed by 5 mL of deionized water. Wastewater samples were loaded onto cartridges, and afterwards the cartridges were dried under vacuum for 10 min. The elution of analytes was performed with 15 mL of methanol/dichloromethane mixture (1:1, *v/v*). Extracts were evaporated and reconstituted to 1 mL with methanol. The final extracts were filtered through 0.45 μm polyvinylidene difluoride (PVDF) filters, acquired from Roth (Karlsruhe, Germany), into the autosampler vials and analyzed.

2.4 Calibration

The standard addition method was used for calibration. This calibration approach is often used when it is necessary to take into account the matrix effect (*i.e.* ion suppression or enhancement) and the incomplete analyte extraction. Each water sample was split into six aliquots. Four aliquots were used for the preparation of the calibration solutions by spiking the wastewater samples with working standard solution at the concentrations of 1–250 μg/L (for pharmaceuticals, pesticides, steroid hormones, and sweeteners) and 10–5000 μg/L (for sterols).

2.5 LC-MS^2 analysis of emerging contaminants

After extraction, samples were analyzed by liquid chromatography-tandem mass spectrometry using DionexUltiMate® 3000 LC system (Thermo Fisher Scientific, Waltham, US) coupled to linear ion trap LTQ XL (Thermo Fisher Scientific). For efficient separation of all analytes, three chromatographic columns were used. Separa-

tion of pharmaceuticals, pesticides, and steroid hormones was performed using reverse-phase Zorbax Eclipse® XDB-C18 column (75 mm×4.6 mm, 3.5 μm, Agilent Technologies, Santa Clara, US), whereas LiChrospher RP-18 EC column (250 mm×4.6 mm, 5 μm, Cronus, SMI-LabHut Ltd., UK) was employed for the chromatographic separation of sterols. Separation of artificial sweeteners was achieved on Luna® C8 column (3.0 mm × 150 mm, 3 μm) from Phenomenex, Torrance, US. In front of the separation columns, a precolumn was installed (12.5 mm×4.6 mm, 5 μm, Agilent Technologies).

For chromatographic separation of pharmaceuticals and pesticides the mobile phase consisted of water (A), methanol (B), and 10% acetic acid (C). When analyzing pharmaceuticals, mobile phase gradient (with the flow rate of 0.6 mL/min) changed as follows: 0 min, A 65%, B 33%, C2%; 12 min, B 98%, C2%; 18 min, B 100%. In the case of pesticides, mobile phase gradient changed in the following manner: 0 min, A 66%, B 33%, C 1%; 7.5 min, A 41%, B 58%, C 1%; 25 min, B 100%. The flow rate of the mobile phase was 0.5 mL/min. The mobile phase for chromatographic separation of steroids consisted of water (A) and methanol (B). Steroid hormones were separated using the following mobile phase gradient (flow rate 0.8 mL/min): 0 min, A 45%, B 55%; 13 min, B 100%. For sterols mobile phase was changed as follows: 0 min, B 100%; 12 min, A 10%, B 90%; 15 min, B 100%. The flow rate of the mobile phase was held at 1.5 mL/min. The mobile phase for the separation of sweeteners consisted of water (A), methanol (B), and 0.1 mol/L aqueous solution of ammonium acetate (D). The mobile phase gradient was changed in the following manner: 0 min, A 84%, B 15%, D 1%; 8 min, A 84%, B 15%, D 1%; 13 min, A 34%, B 65%, D 1%; 15 min, B 100%; 20 min, B 100%. The flow rate was 0.33 mL/min. In all chromatographic methods the initial conditions were re-established and held for 10 min. An aliquot of 10 μL of the final extract was injected into the LC system.

Two ionization interfaces of mass spectrometer were used for obtaining stabile ions of the selected analytes. All pharmaceuticals and pesticides were successfully ionized by the electrospray ionization (ESI) technique in the positive mode. The same ionization technique in the negative mode was used for the selected sweeteners. The optimal ESI source working parameters for monitoring all ions were source voltage of 4.5 kV and capillary temperature of 290 °C. Atmospheric pressure chemical ionization (APCI) in the positive mode was applied in the steroid analysis. The optimized APCI parameters were capillary temperature of 200 °C and vaporizer temperature of 400 °C. Fragmentation reactions of the precursor ion to the most intense fragment ion were used for identification and quantification of each analyte. Additional transitions were used for the confirmation of positive results. Detailed information on five separate analytical methods, including mass spectrometric parameters for the data acquisition, as well as fragmentation reactions for quantification and conformation of all selected analytes can be seen in Tables 2-6.

Table 2. MS operating parameters for selected pharmaceuticals: analytes' fragmentation reactions for quantification and confirmation purposes and optimal collision energies (CE).

Pharmaceuticals	Precursor ion (*m/z*)	Quantification reaction	CE (%)	Conformation reaction	CE (%)
Trimethoprim	291[M+H]$^+$	291→230	44	291→123	44
4-AAA	246[M+H]$^+$	246→228	28	246→204	28
4-FAA	232[M+H]$^+$	232→204	30	232→214	30
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
Doxycycline	445[M+H]$^+$	445→428	25	445→460	25
Enalapril	377[M+H]$^+$	377→234	30	377→303	30
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
Midecamycin	814[M+H]$^+$	814→614	25	814→596	25
Carbamazepine	237[M+H]$^+$	237→194	34	237→219	34
Clarithromycin	748[M+H]$^+$	748→590	24	748→558	24
Roxithromycin	837[M+H]$^+$	837→679	23	837→558	23
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
Cilazapril	418[M+H]$^+$	418→211	25	211→183	32

Table 3. MS operating parameters for selected pesticides: analytes' fragmentation reactions for quantification and confirmation purposes and optimal collision energies (CE).

Pesticides	Precursor ion (*m/z*)	Quantification reaction	CE (%)	Conformation reaction	CE (%)
Acephate	184[M +H]$^+$	184→143	40	184→113	40
Monocrotophos	224[M+H]$^+$	224→193	38	224→167	38
Carbendazim	192[M+H]$^+$	192→160	34	160→132	35
Imidacloprid	256[M+H]$^+$	256→210	25	256→175	25
Acetamiprid	223[M+H]$^+$	223→126	36	223→187	36
Dimethoate	230[M+H]$^+$	230→199	26	199→171	22
Monuron	199[M+H]$^+$	199→72	30	–	–
Carbaryl	202[M+H]$^+$	202→145	25	145→117	31
Simazine	202[M+H]$^+$	202→124	36	202→132	36
Carbofuran	222[M+H]$^+$	222→165	32	165→123	27
Atrazine	216[M+H]$^+$	216→174	38	174→146	35
Diuron	233[M+H]$^+$	233→72	34	–	–
Propazine	230[M+H]$^+$	230→188	35	230→146	35
Linuron	249[M+H]$^+$	249→182	35	249→160	35
Malathion	331[M+H]$^+$	331→285	24	285→127	20
Tebufenozide	375[M+Na]$^+$	375→225	34	375→319	34

Table 4. MS operating parameters for selected steroids: analytes' fragmentation reactions for quantification and confirmation purposes and optimal collision energies (CE).

Steroids	Precursor ion (*m/z*)	Quantification reaction	CE (%)	Conformation reaction	CE (%)
Steroid hormones					
Estriol	271[M–H_2O+H]$^+$	271→253	20	271→197	20
Estrone	271[M+H]$^+$	271→253	20	271→197	20
Equilin	269[M+H]$^+$	269→251	23	269→211	23
Norethindrone	299[M+H]$^+$	299→281	23	299→263	23
17α-Ethinylestradiol	279[M–H_2O+H]$^+$	279→133	25	279→205	25
17β-Estradiol	255[M–H_2O+H]$^+$	255→159	22	255→133	22
17α-Estradiol	255[M–H_2O+H]$^+$	255→159	22	255→133	22
Levonorgesterel	313[M+H]$^+$	313→295	22	313→277	22
Mestranol	293[M–H_2O+H]$^+$	293→147	26	293→173	26
Sterols					
Epicoprostanol	371[M–H_2O+H]$^+$	371→149	24	371→261	24
Epicholestanol	371[M–H_2O+H]$^+$	371→149	24	371→261	24
Coprostanol	371[M–H_2O+H]$^+$	371→149	24	371→261	24
Cholestanol	371[M–H_2O+H]$^+$	371→149	24	371→261	24
Cholesterol	369[M–H_2O+H]$^+$	369→243	24	369→287	24
Cholestanone	387[M+H]$^+$	387→369	19	387→243	19
Desmosterol	367[M–H_2O+H]$^+$	367→257	26	367→161	26
Stigmasterol	395[M–H_2O+H]$^+$	395→297	24	395→311	24
Campesterol	383[M–H_2O+H]$^+$	383→243	25	383→257	25
β-Sitosterol	397[M–H_2O+H]$^+$	397→243	25	397→257	25
Sitostanol	399[M–H_2O+H]$^+$	399→149	24	399→163	24

Table 5. MS operating parameters for selected sweeteners: analytes' fragmentation reactions for quantification and conformation purposes and optimal collision energies (CE).

Sweeteners	Precursor ion (*m/z*)	Quantification reaction	CE (%)	Confirmation reaction	CE (%)
Acesulfame	162[M–H]$^-$	162→82	27	162→102	27
Saccharin	182[M–H]$^-$	182→106	37	182→62	37
Cyclamate	178[M–H]$^-$	178→80	36	178→96	36
Sucralose	433[M+Cl]$^-$	433→397	14	433→395	14
Aspartame	293[M–H]$^-$	293→261	29	293→200	29
NHDC	611[M–H]$^-$	611→491	20	611→387	20
Neotame	377[M–H]$^-$	377→345	18	377→200	18
Stevioside	641[M–$C_6H_{11}O_5$–H]$^-$	641→479	22	641→317	22

2.6 Removal efficiency of selected emerging contaminants

To determine the removal efficiency of selected emerging contaminants during the wastewater treatment process, the following equation Eq. (1) was applied:

$$\text{RE}\ (\%) = \frac{C_i - C_e}{C_i} \times 100 \quad (1)$$

With: C_i - the quantified concentration of the pollutant in the WWTP influent, in µg/L; C_e - the quantified concentration of the pollutant in the WWTP effluent, in µg/L.

2.7 Ecotoxicological risk assessment for the receiving watercourse

For the collected WWTP effluent sample, an ecotoxicological risk assessment was performed by linking it to each individual pollutant, but also with the mixture of pollutants ("the cocktail effect") that is being released into the receiving watercourse. ERA methods are usually carried out by comparison of the Predicted Environmental Concentration of a substance in watercourses and Predicted No Effect Concentration at which no pharmacological effect is expected to occur for a specific organism. A PNEC value is generally derived using ecotoxicity testing data.

Predicted Environmental Concentration (PEC) calculation

The PEC value of each detected emerging contaminant in the receiving watercourse was calculated by taking into account the WWTP and watercourse flow rates and the concentration of each analyte quantified by LC-MS^2 effluent analysis using the following Eq. (2):

$$PEC = \frac{\text{WWTP flow rate}}{\text{Watercourse flow rate}} \times C_e \quad (2)$$

With PEC - Predicted Environmental Concentration of the pollutant in µg/L; WWTP flow rate - the flow rate of the WWTP in m^3/s; Watercourse flow rate - the flow rate of the WWTP's receiving watercourse in m^3/s and C_e - the detected concentration of the pollutant in the WWTP effluent.

Ecotoxicological risk assessment of the individual micropollutant

Ecotoxicological risk assessment is usually performed by the calculation of the risk quotient (RQ) (EC, 2003) using Eq (3):

$$RQ = \frac{PEC}{PNEC} \quad (3)$$

With RQ - Risk Quotient of the pollutant detected WWTP effluent; PEC - Predicted Environmental Concentration of the pollutant in µg/L and PNEC - Predicted No Effect Concentration of the pollutant in µg/L. PNECs are usually calculated based on critical concentrations, *e.g.* EC50 (median effective concentration), LC50 (median lethal concentration), and NOEC (no-observed-effect concentration) [22].

An RQ value below 1 is associated with insignificant ecotoxicological risk, and an RQ value above 1 to a potential ecotoxicological risk for watercourses. The environmental risk ranking categories are as follows: RQ < 0.01 is insignificant, <0.1 low risk, $0.1 \leq RQ \geq 1$ medium risk and RQ > 1 high risk [16].

Ecotoxicological risk assessment of the micropollutant mixture

The risk quotient of the micropollutant mixture was calculated according to the procedure suggested by Gosset et al. (2020) [19] following Eq (4):

$$RQ_{mix} = \sum_{1}^{n} \frac{PEC}{PNEC} = \sum_{1}^{n} RQ \quad (4)$$

With RQ_{mix} - Risk quotient of the mixture of pollutants; RQ - Risk quotient of the individual pollutant detected in WWTP. Similar to comparing RQ of individual substances to 1, the same is done for RQ_{mix}.

3 Results and discussion

All four classes of investigated organic contaminants were detected in the influent and effluent samples of the investigated WWTP in various concentration ranges summarized in Fig. 1-3. The widespread occurrence of sterols (Fig. 1) was detected in WWTP influent, with the maximum concentration of cholesterol (4128 µg/L), followed by coprostanol (3705 µg/L). Since cholesterol is the most abundant sterol in the human organism, it is expected to have high concentrations in sewage-contaminated samples. A high concentration of coprostanol can be explained by the fact that coprostanol is a sterol produced in the digestive tract of humans and higher vertebrates by hydrogenation of cholesterol and it comprises 40-60% of the total sterols excreted in human feces [23]. However, none of the monitored sterols were detected in effluent samples.

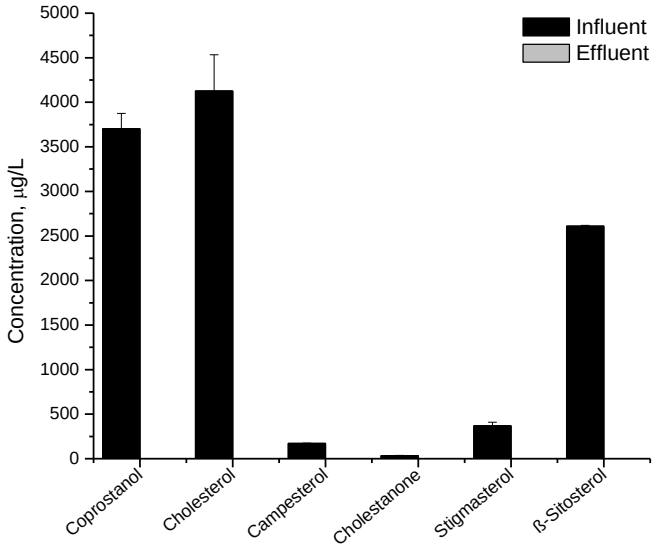

Fig 1. Representation of the mean influent and effluent concentrations ± standard deviation of detected sterols.

Notable concentrations in the WWTP influent were also recorded in the case of metamizole metabolites, 4-FAA and 4-AAA (up to 107 µg/L), bisoprolol (83 µg/L), and clopidogrel (61 µg/L) (Fig. 2).

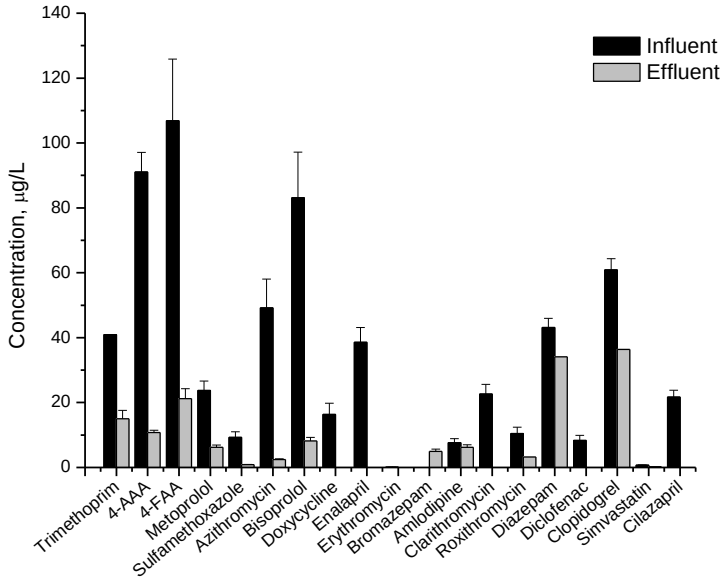

Fig 2. Representation of the mean influent and effluent concentrations ± standard deviation of detected pharmaceuticals.

Pesticides acephate and carbendazim were found at the concentrations of 254 µg/L and 83 µg/L, respectively, whereas out of all monitored sweeteners, saccharin was detected at the highest concentration of 39 µg/L. It can be also concluded that for the majority of compounds the concentrations in the effluent sample are significantly lower, indicating partial removal of these compounds in WWTP.

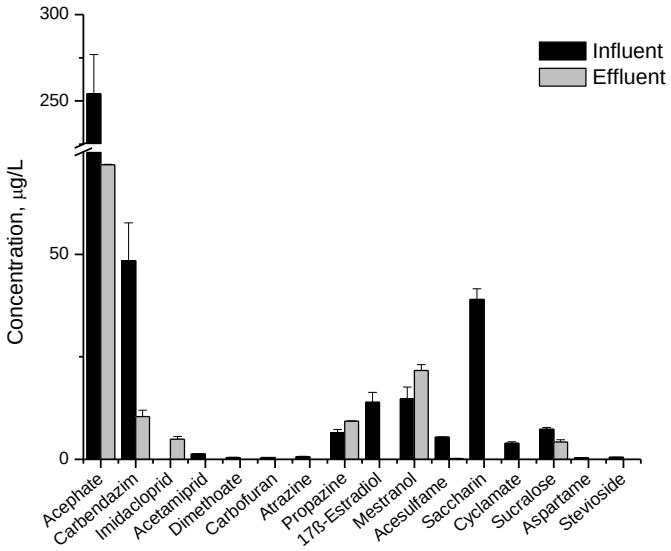

Fig. 3. Representation of the mean influent and effluent concentrations ± standard deviation of detected pesticides, steroid hormones and sweeteners.

3.1 Removal efficiency of the detected micropollutants

Overall, removal efficiency (RE) of the target compounds was over 70% for 30 detected analytes (Fig. 4). Differences in RE of pharmaceuticals (ranging from 18% to 100%) can be attributed to different physical and chemical properties of the compounds, to distinct mechanisms of degradation, sorption/sedimentation processes and uptake by active sludge [24]. Complete removal of sterols in WWTP (100%) can be associated with their physico-chemical properties, *i.e.* low water solubility and polarity, indicating that their primary mechanism of removal is adsorption onto active sludge particles. High RE was also observed in the case of pesticides and sweeteners, with the exception of sucralose (RE = 42%). For sucralose, sorption cannot be considered as its dominant removal mechanism due to the high value of water solubility and low molecular weight (Table 1).

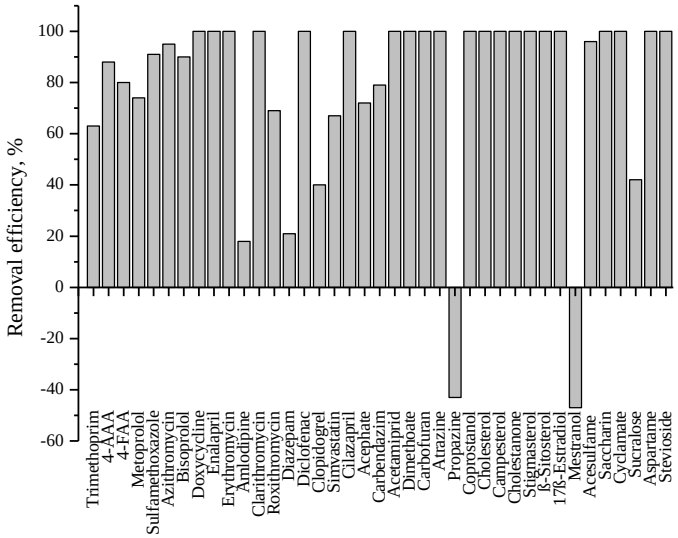

Fig. 4. Removal efficiencies of each investigated analyte detected in WWTP.

However, some organic compounds detected in WWTP showed a negative removal rate (Fig. 4). For example, propazine and mestranol were enriched in the process of wastewater treatment. By analyzing the effects of various processes on the removal of target micropollutants, it can be concluded that the biological treatment stage could not only decompose some organic compounds but also increase the concentration of some precursor compounds [25].

3.2 Ecotoxicological risk assessment of the detected emerging contaminants

The ecotoxicity of each of the studied pollutants detected in the present paper is presented by the PNEC values. The wide variability of PNEC values can be noted from Table 2. The three most toxic compounds recorded were pharmaceuticals amlodipine, azithromycin, and trimethoprim, with PNEC values between 0.28 and 9.4 ng/L. Their very high toxicity is directly linked to the significant toxicity of these molecules for aquatic organisms, such as fish, green algae, and daphnid. Other ecotoxic micropollutants were the pesticide propazine (PNEC = 40 ng/L), the pharmaceuticals (*e.g.* roxithromycin PNEC = 10 ng/L; metoprolol PNEC=100 ng/L; and sulfamethoxazole PNEC = 590 ng/L). The least ecotoxic pollutants detected were artificial sweeteners acesulfame and sucralose, with PNEC values of 2200 and 930 µg/L, respectively.

The risk quotients (RQ) calculated for each analyte detected in WWTP effluent and risk quotient of the mixture of pollutants (RQ_{mix}) are reported in Table 6. RQ values were obtained by comparing the PEC values calculated by Eq. (2) and PNEC values from the literature survey.

Table 6. Median pollutant concentrations in effluent (C_e), Predicted Environmental Concentrations (PEC) of each pollutant, Predicted No Effect Concentrations (PNEC) and the associated Risk Quotients (RQ).

Analyte	C_e, µg/L	PEC, µg/L	PNEC, µg/L	PNEC reference	RQ
Trimethoprim	15.01	$5.66 \cdot 10^{-3}$	0.0058	[19]	$9.76 \cdot 10^{-1}$
4-AAA	10.78	$4.07 \cdot 10^{-3}$	-*		-
4-FAA	21.19	$8.00 \cdot 10^{-3}$	-		-
Metoprolol	6.17	$2.33 \cdot 10^{-3}$	0.1	[19]	$2.33 \cdot 10^{-2}$
Sulfamethoxazole	0.84	$3.18 \cdot 10^{-4}$	0.59	[19]	$5.39 \cdot 10^{-4}$
Azithromycin	2.41	$9.10 \cdot 10^{-4}$	0.0094	[26]	$9.68 \cdot 10^{-2}$
Bisoprolol	8.18	$3.09 \cdot 10^{-3}$	72	[27]	$4.29 \cdot 10^{-5}$
Bromazepam	4.93	$1.86 \cdot 10^{-3}$	17.4	[28]	$1.07 \cdot 10^{-4}$
Amlodipine	6.19	$2.34 \cdot 10^{-3}$	0.00028	[29]	8.35
Roxithromycin	3.19	$1.20 \cdot 10^{-3}$	0.01	[19]	$1.20 \cdot 10^{-1}$
Diazepam	34.08	$1.29 \cdot 10^{-2}$	0.1	[26]	$1.29 \cdot 10^{-1}$
Clopidogrel	36.38	$1.37 \cdot 10^{-2}$	1.6	[30]	$8.58 \cdot 10^{-3}$
Simvastatin	0.24	$8.98 \cdot 10^{-5}$	22.8	[31]	$3.94 \cdot 10^{-6}$
Acephate	71.83	$2.71 \cdot 10^{-2}$	110	[32]	$2.46 \cdot 10^{-4}$
Carbendazim	10.41	$3.93 \cdot 10^{-3}$	1.5	[33]	$2.62 \cdot 10^{-3}$
Imidacloprid	4.90	$1.85 \cdot 10^{-3}$	121	[34]	$1.53 \cdot 10^{-5}$
Propazine	9.30	$3.51 \cdot 10^{-3}$	0.04	[35]	$8.77 \cdot 10^{-2}$
Mestranol	21.64	$8.17 \cdot 10^{-3}$	130	[36]	$6.28 \cdot 10^{-5}$
Acesulfame	0.21	$7.92 \cdot 10^{-5}$	2200	[37]	$3.60 \cdot 10^{-8}$
Sucralose	4.20	$1.58 \cdot 10^{-3}$	930	[38]	$1.70 \cdot 10^{-6}$
RQ_{mix}					9.79

*The PNEC values were not available in the literature.

Among the all detected compounds in the WWTP effluent, amlodipine was identified as the riskiest pollutant with an RQ value of 8.35. In the case of this compound, even with the low value of the predicted/measured concentration in the receiving streams, the high toxicity resulted in a low PNEC value, which led to a high RQ value.

The medium risk was recorded for pharmaceuticals trimethoprim, roxithromycin, and diazepam, indicating that, despite the fact that these compounds are partially removed in the treatment process, there is still a significant risk to aquatic organisms. RQ values lower than 0.1 were obtained for pesticide propazine and pharmaceuticals metoprolol and azithromycin, showing that these compounds are of low environmental risk. For the remaining emerging pollutants detected in WWTP effluent, the calcu-

lated RQ values were below 0.01, demonstrating insignificant risk to the aquatic environment.

In this paper ecotoxicological risk associated with the whole mixture of previously identified pollutants is also considered, since previous studies [18,19] have shown that the classic single substance ERA approach is not sufficient to reliably assess the risk associated with a complex mixture of pollutants that are independently affecting aquatic biota. Calculated RQ_{mix} (9.79, Table 6) has significantly exceeded the threshold value of 1. The obtained result demonstrates the significance of the mixture approach, revealing that individually "safe" emerging compounds can contribute to a significant risk of the whole effluents. Several recent studies [19,20,39] have drawn similar conclusions about "the cocktail effect" of mixtures of micropollutants in receiving waters.

4 Conclusion

The results obtained from this study showed the presence of widespread contamination by emerging contaminants, including both influent and effluent samples from the WWTP in the vicinity of city Topola, in Serbia. Most of the substances investigated were found at similar concentrations, with the exception of sterols, which were detected at significantly high levels (>10-100 fold). The removal efficiency of the analyzed WWTP varied, depending on the compound, in the range of 18-100%.

The ecotoxicological risk assessment of the individual micropollutant recognized pharmaceutical amlodipine as the compound of environmental concern, while for the majority of the selected emerging compounds the risk was determined as insignificant when considered individually. However, in order to assess environmental risk properly, the "the cocktail effect" of the entire mixture should be taken into account.

The overall results showed that an ecotoxicological risk cannot be excluded in the investigated area. Even though only one substance individually exceeded the ERA threshold, the combination of the detected compounds in the WWTP effluent poses an environmental risk. There is still a need for obtaining data on the acute and chronic ecotoxicity of different trophic levels to achieve a more robust and reliable ERA.

Furthermore, this work highlights the need of inclusion certain emerging pollutants (*e.g.* pharmaceuticals) in regular monitoring programs at national and international level. Additionally, the scale of the study should be increased and expanded to WWTP with higher treatment capacity to confirm the current result obtained for this territory at a national scale. Each territory has specific pollution characteristics, which is why further research is required.

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