



Untreated wastewater impact and environmental risk assessment of artificial sweeteners in river water and sediments of the Danube River Basin in Serbia

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Abstract

Artificial sweeteners are receiving increasing attention as newly recognized emerging contaminants that mainly reach the aquatic environment through the discharge of municipal wastewater containing large amount of these compounds. In this study, the impact of raw untreated wastewater discharges on the levels and the water/sediment distribution of artificial sweeteners in the Danube River and its largest tributaries in Serbia was evaluated, and a comprehensive assessment of environmental risks for freshwater and benthic organisms was performed. Acesulfame and sucralose were detected in all river water samples (100%), while saccharin (59%) and cyclamate (12%) were less frequently found, indicating long-term continuous sewage-derived pollution. Aspartame (100%) and neotame (60%) were the only artificial sweeteners recorded in the sediment samples due to their preference to sorb to particulate matter in the water/sediment system. In terms of ecotoxicological risk, a low risk for aquatic organisms was determined at the detected levels of saccharin in river water, while a high to medium risk was found for benthic biota at the concentrations of neotame and aspartame detected in sediments. The largest contribution to the pollution of the Danube River Basin with artificial sweeteners, and consequently the highest environmental risk, was determined in the two largest cities, the capital Belgrade and Novi Sad, which raises the issue of transboundary pollution.

Keywords Artificial sweeteners · Untreated wastewater · River water · River sediments · Environmental risk assessment · The Danube

Introduction

Artificial sweeteners are synthetic sugar substitutes widely utilized in human nutrition and animal feed due to their high sweetening intensity and low caloric value (Kroger et al. 2006; Luo et al. 2019a). They are also common ingredients in pharmaceuticals and oral hygiene products. Among the most popular artificial sweeteners are acesulfame, saccharin,

cyclamate, aspartame, sucralose, neohesperidin dihydrochalcone (NHDC) and neotame, which are used in large quantities in major economic markets of the world (Luo et al. 2019b). Despite the safety assessment that consumption of artificial sweeteners below acceptable daily intake may not pose a health risk to consumers (Carocho et al. 2017), scientists are divided in terms of their harmful side effects. Some clinical and epidemiological studies indicate that consumption of these substances is associated with metabolic derangements and an increased risk of excessive weight gain, type 2 diabetes, metabolic syndrome, and cardiovascular disease (Swithers 2013; Pepino 2015). For this reason, natural sweeteners like stevia are attracting consumer attention. Steviol glycosides are extracted from the leaves of *Stevia rebaudiana* and stevioside is the most abundant glycoside (Carocho et al. 2017; Ciriminna et al. 2019).

The decade-long use of artificial sweeteners in food products and medicines has resulted in their ubiquitous environmental occurrence. Numerous studies have reported the presence of sweeteners in different environmental compartments, including

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surface (Loos et al. 2009; Scheurer et al. 2009; Berset and Ochsenbein 2012; Perkola and Sainio 2014; Moldovan et al. 2018), costal (Baena-Nogueras et al. 2018; Guo et al. 2021), and groundwater (Buerge et al. 2009; Gan et al. 2013), as well as sediments (Bernot et al. 2016; Fu et al. 2020) and soil (Gan et al. 2014; Li et al. 2021). The major sources of artificial sweeteners in the environment are effluents from wastewater treatment plants (WWTPs) and untreated municipal wastewater (Gan et al. 2013; Tran et al. 2014). Most of the ingested acesulfame, saccharin, cyclamate, and sucralose are excreted unchanged (Renwick 1986; Roberts et al. 2000), while aspartame, NHDC, neotame, and stevioside are metabolized to a greater extent (O'Brien Nabors 2001; Carocho et al. 2017). Diffuse sources of sweeteners are animal farming and agriculture due to their application in animal feed (Li et al. 2021).

Of all commonly used artificial sweeteners, acesulfame and sucralose show the highest resistance in wastewater treatment and persistence in the aquatic ecosystem (Buerge et al. 2009; Fu et al. 2020). Other sweeteners display variable degrees of removal in wastewater treatment processes and biodegradation in the environment (Gan et al. 2013; Subedi and Kannan 2014; Luo et al. 2019a). For example, the removal efficiency of saccharin and cyclamate in WWTP processes is higher than 90% (Scheurer et al. 2009; Baena-Nogueras et al. 2018), while aspartame elimination is reported to be around 70% (Subedi and Kannan 2014). Due to high specificity for sewage wastewater and much higher concentrations than other wastewater indicators, artificial sweeteners are considered as possibly the best tracers of municipal wastewater in the environment in recent years (Fu et al. 2020; Spoelstra et al. 2020). Acesulfame and sucralose are the most frequently used persistent markers that indicate older sewage pollution of the aquatic environment, whereas saccharin and cyclamate are used as indicators of recent (fresh) wastewater contamination (Tran et al. 2014; Zirlwagen et al. 2016).

The widespread presence and persistency of artificial sweeteners in the natural environment and their potential harmful impact on human health have induced interest in ecotoxicological research and studies of their impact on aquatic life. Due to the limited data on environmental risks resulting from continuous exposure to low levels of these compounds, artificial sweeteners are a newly recognized class of emerging pollutants (Luo et al. 2019a; Praveena et al. 2019). They are currently not included in routine monitoring programs at the European level and may be candidates for future legislative regulation, depending on research on their (eco)toxicity, potential health effects, and data on monitoring their occurrence in different environmental compartments. A study on the ecotoxicity of acesulfame showed that irradiation products produced in the UV disinfection process of water treatment were more toxic to goldfish (*Carassius auratus*) than the parent compound (Ren et al. 2016). It was

also determined that upon prolonged exposure to intense solar irradiation, the photodegradation products are > 500 times more toxic than acesulfame itself to marine bacteria *V. fischeri* (Sang et al. 2014). The presence of sucralose in the aquatic environment can affect the physiology and locomotion behavior of *Daphnia magna* and this deviation from normal behavior may ultimately have ecological consequences (Wiklund et al. 2012). In addition, sucralose concentrations similar to those detected in the environment cause significant increases in oxidative stress biomarkers in common carp (*Cyprinus carpio*) (Saucedo-Vence et al. 2017). Oxidative stress is considered one of the main action mechanisms of toxicants and its biomarkers indicate the level of general damage to biomolecules. Since both persistent sweeteners can chelate heavy metals, increased environmental levels of acesulfame and sucralose can increase the mobility and toxicity of heavy metals accumulated in sediments. The release of heavy metals from sediments has been found to inhibit the growth of green algae (*Scenedesmus obliquus*) (Liu et al. 2020). As for biodegradable artificial sweeteners, ecotoxicity studies have shown that some of them can also cause negative effects on aquatic organisms, especially after prolonged exposure to environmental concentrations. For instance, aspartame affects the growth of duckweed (*Lemna minor*) and impairs the overall condition of the plant (Kobetičová et al. 2018).

The Danube River and its tributaries are an important resource for drinking water production and agricultural irrigation for about 83 million people living in the basin of this river. It is a unique river ecosystem with the highest freshwater biodiversity in Europe. Present aquatic species are very sensitive to increased contamination from municipal wastewater discharges, agriculture, and industry, and are particularly susceptible to bioaccumulation (Stagl and Hattermann 2016). There is a limited number of European studies on surface water pollution with artificial sweeteners and only a few have addressed the Danube River and its tributaries (Loos et al. 2009; Scheurer et al. 2009; Moldovan et al. 2018). About 92% of the Republic of Serbia (RS) lies within the Danube River Basin, accounting for 10% of the total basin. The key source of water pollution in the RS is wastewater discharged into the recipient without any treatment since only 8.1% of wastewater generated by agriculture, forestry and fishing, households, and industry is treated (Statistical Office of the RS 2022). Unlike the neighboring European Union (EU) countries, there is not a single WWTP that has been constructed along the entire course of the Danube in the RS, so the environmental risk from exposure to artificial sweeteners in the aquatic environment is expected to be high, especially in highly populated areas with high wastewater burden, raising the issue of transboundary pollution. Considering the importance of the Danube River, the aim of this study was to evaluate the potential threat to the

Danube River ecosystem from the unregulated discharge of untreated municipal wastewater along the entire course of the Danube in the RS and to assess the environmental risk for freshwater and benthic biota in the investigated area. To the best of our knowledge, a comprehensive assessment of the aquatic environment pollution with artificial sweeteners that includes both water and sediment matrix has not yet been reported in Europe. In addition, an integrated environmental risk assessment for both aquatic and benthic organisms was performed for the first time.

Materials and methods

Chemicals and reagents

High purity ($\geq 98\%$) standard substances of seven sweeteners (acesulfame, saccharin, cyclamate, aspartame, NHDC, neotame, and stevioside) were obtained from Sigma-Aldrich (Buchs, Switzerland), while sucralose was purchased from TCI Europe (Zwijndrecht, Belgium). Selected artificial sweeteners are the most commonly found on the market in the RS and are approved for use as food additives (Regulation No. 53/2018). The chemical structures and physicochemical properties of the chosen sweeteners, such as octanol–water partitioning coefficient (K_{ow}), organic carbon–water partitioning coefficient (K_{oc}), and water solubility, are shown in

Table S1 (Supplementary Material). An individual standard solution of each sweetener was prepared in methanol at the concentration of $100 \mu\text{g mL}^{-1}$. Working standard solutions were prepared by mixing individual standard solutions and diluting 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 prepared using a GenPure ultrapure water system (TKA, Niederelbert, Germany).

Sampling site description and sample collection

The Danube River Basin, covering area of $801,463 \text{ km}^2$ and 10% of continental Europe, is the most international river basin in the world as it extends into the territories of 19 countries. The Danube River is the longest river in the EU (2857 km) and its catchment area forms one of the continent's largest and most important water systems, which millions of people rely on for drinking water and agricultural practice (Mănoiu and Crăciun 2021). The Danube River valley dominates the north of the RS. The Danube flows through the country at a length of 588 km and is divided into the upper and lower sections (Fig. 1). The upper section covers the stretch from the Hungarian border to the capital Belgrade. The lower section covers the stretch from Belgrade to the Bulgarian border, with the Iron Gate I and II hydroelectric power plants located in the border area with

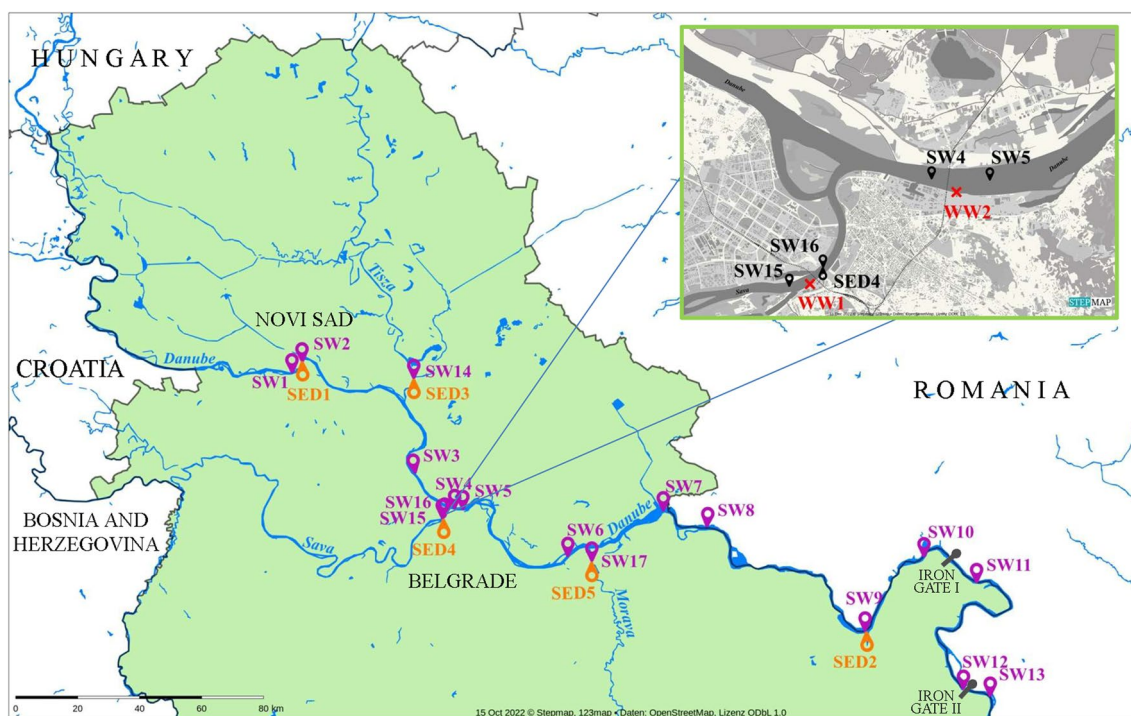


Fig. 1 Map of the Danube River and its main tributaries in the Republic of Serbia with the surface water (SW) and sediment (SED) sampling sites, as well as two wastewater canals (WW) in the capital Belgrade

Romania. Three major Danube tributaries on the territory of the RS are the Tisza, the Sava, and the Morava. The Tisza River is the longest tributary of the Danube (966 km), covering an area of 157,186 km², with only 7% of its catchment area in the RS. The Sava is the Danube's largest tributary in terms of discharge and the second largest according to the catchment area (97,713 km²). It flows into the Danube in Belgrade and its lower reach in the RS comprises 16% of the basin. The Morava River is 185 km long and its basin (6126 km²) is entirely placed on the RS territory. It flows through the most fertile area of central Serbia. Agricultural land covers about 57% of the RS, and 30% is forested (ICPDR 2023).

In order to investigate the impact of untreated wastewater discharge on the aquatic environment and biota of the Danube in the RS, river water and sediment samples were collected in April 2021 at 17 sampling sites in the Danube River Basin (Fig. 1, Table S2). Thirteen samples of river water (SW1–SW13) were taken to cover the entire course of the Danube in the RS, without any treatment facility, and in this way, around 410 km of the river was investigated. Two Danube sediments representing the upper (SED1) and lower (SED2) stretch of the river were collected. Sampling locations included the two largest Serbian cities—the capital Belgrade (over 1,600,000 inhabitants) and Novi Sad (over 350,000 inhabitants) which are heavily affected by municipal wastewater discharge due to high population density. Samples of river water and sediment were also collected from the Danube's tributaries—the Tisza (SW14, SED3), the Sava (SW15 and SW16, SED4), and the Morava (SW17, SED5), to assess their contribution to artificial sweeteners load entering the Danube. River water samples were collected by direct sampling, from a boat, in the middle of the river course at a depth of about 50 cm, while river sediments were taken from the river bottom using a Van Veen grab sampler. In addition, raw municipal wastewater was sampled from the two largest sewage canals in Belgrade (WW1 on the Sava and WW2 on the Danube) at the point of discharge into receiving rivers (Fig. 1, Table S2). To evaluate their impact on the level of artificial sweeteners in the river water, samples of surface water were collected before and after each of the two wastewater discharges. All samples were collected in PVC containers and stored in a freezer, without preservation agent.

Sample preparation

River and wastewater samples were thawed and filtered through 1–3- μ m glass fiber filter (Whatman GmbH, Dassel, Germany). The previously developed and optimized solid-phase extraction (SPE) method (Gvozdić et al. 2020) was used to isolate and preconcentrate artificial sweeteners. In the SPE procedure, a water sample (50 mL) with pH adjusted to 3.0 was loaded onto Oasis HLB cartridge

(200 mg/6 mL; Waters, Milford, USA), and the analytes were eluted with 10 mL of methanol. The extract was then evaporated under nitrogen stream to a volume of 0.5 mL, filtered through 0.45- μ m PVDF filter (Roth, Karlsruhe, Germany) into a glass autosampler vial and analyzed.

Sediment samples were thawed and air-dried in a thin layer at room temperature for several days in the dark. They were then homogenized and sieved through 500- μ m pore size sieve to separate gravel, debris, and other impurities. Sediments were prepared for analysis using a previously optimized ultrasonic extraction (USE) method (Gvozdić et al. 2020). An amount of 2 g of river sediment was extracted using 5 mL of methanol in an ultrasonic bath for 15 min. The sample was then centrifuged for 10 min at 4000 rpm, and the supernatant was decanted. The procedure was repeated two more times. The final extract (15 mL) was evaporated to a volume of 0.5 mL, filtered through 0.45- μ m PVDF filter and analyzed.

Liquid chromatography–tandem mass spectrometry analysis

The analysis of the prepared samples was carried out using liquid chromatography with tandem mass spectrometry (LC–MS/MS), as the method of choice for the detection of sweeteners traces in complex environmental matrices. LC and MS operating parameters for the determination of eight selected artificial sweeteners were developed and optimized in a previous study (Gvozdić et al. 2020). LC analysis was performed using a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation of analytes was carried out on a Luna® C8 reverse-phase column, 150 mm \times 3.0 mm i.d. and 3- μ m particle size (Phenomenex, Torrance, USA). The mobile phase consisted of deionized water, methanol, and 0.1 mol L⁻¹ aqueous solution of ammonium acetate. MS/MS analysis was performed using LTQ XL (Thermo Fisher Scientific) linear ion trap mass spectrometer, coupled with a HPLC system. Electrospray ionization (ESI) in negative mode was used as the ionization technique. Selected reaction monitoring (SRM) was used as the acquisition mode. LC and MS operating parameters for selected sweeteners, including LC mobile-phase gradient, MS parameters for data acquisition, and fragmentation reactions for quantification and confirmation purposes, are shown in Table S3 in Supplementary Material.

Quality assurance and quality control

Extraction blanks were analyzed in each sample batch in the same manner as environmental samples to account for possible contamination during sample preparation and laboratory processing. For surface and wastewater samples, the blank was 50 mL of deionized water extracted using the SPE procedure.

For the sediment samples, an extraction blank was prepared using the established USE method without sediment. Selected artificial sweeteners were not detected in the blanks. Recoveries of analytes for river water samples were in the range of 69–109%, while for wastewater samples they ranged from 75 to 100% (Table S4, Supplementary Material). Lower recoveries were obtained for the sediment samples (up to 64%), with exceptionally low values achieved for aspartame, NHDC, and neotame (17–35%), which is consistent with the difficult extraction of artificial sweeteners from the sediment matrix. The relative standard deviations were lower than 20%.

The limits of detection (LODs) and quantification (LOQs) were calculated using the signal-to-noise (S/N) ratios. The LOD and LOQ of each investigated sweetener were estimated as the minimum detectable concentration with S/N ratio of 3 and 10, respectively, using water and sediment samples spiked at low concentrations (Table S4). Low detection and quantification limits were obtained for selected artificial sweeteners in surface water (1.2–14.7 ng L⁻¹ and 4.0–49.0 ng L⁻¹, respectively), while slightly higher values were achieved for wastewater samples (4.4–18.7 ng L⁻¹ and 14.7–62.3 ng L⁻¹, respectively). For river sediments, LOD and LOQ values were in the range of 0.3–1.5 ng g⁻¹ and 1.0–5.0 ng g⁻¹, respectively.

The standard addition method was applied as a calibration technique for the quantification of detected artificial sweeteners in water and sediment samples. After filtration, each river water and wastewater sample were divided into six 50-mL portions. Calibration solutions were prepared by spiking with working standard solutions at 10–200 ng L⁻¹ (for river water samples) and 100–30,000 ng L⁻¹ (for wastewater samples). For sediment samples, the calibration solutions were prepared by spiking four 2-g portions of each sediment sample at 5, 50, 250, and 500 ng g⁻¹. All fortified samples were extracted using the SPE or USE procedure.

Environmental risk assessment

Environmental risk assessment was performed by calculating risk quotients (RQs) for artificial sweeteners detected in each river water and sediment sample. RQs were calculated by dividing the measured environmental concentration (MEC) of each sweetener with the corresponding predicted no-effect concentration (PNEC) for freshwater or sediment (Alves et al. 2021; Kondor et al. 2022). In principle, the PNEC is calculated by dividing the lowest short-term EC50 (median effective concentration), short-term LC50 (median lethal concentration), or long-term NOEC (no observed effect concentration) by the appropriate assessment factor (AF). The assessment factors reflect the degree of uncertainty in extrapolation from laboratory toxicity test data for a limited number of species to the natural environment (EC 2003; Sharma et al. 2019). Depending on available ecotoxicological data on different organisms,

AF of 1000 is applied when at least one short-term EC50 or LC50 is available from each of the three trophic levels of the base set (fish, Daphnia, and algae), and AF of 100 is applied when one long-term NOEC value (either fish or Daphnia) is available. The assessment factors applied for long-term tests are smaller as the uncertainty of the extrapolation from laboratory data to the natural environment is reduced (EC 2003).

The PNEC values used in the study were obtained from the Norman Ecotoxicology Database (NORMAN 2023) as the lowest PNECs, preferably based on experimental ecotoxicity data, but also predicted by QSAR (quantitative structure–activity relationship) analysis for aquatic organisms. The lowest PNECs for all artificial sweeteners (except cyclamate) presented in the Norman Ecotoxicology Database were derived using the LC50 values determined by Aalizadeh et al. (2017) for the water flea *Daphnia magna* by applying reliable prediction models. Since most of the lowest PNECs were derived for freshwater (PNEC_{fw}), the lowest PNECs for sediments (PNEC_{sed}) were calculated using the following equation (Eq. (1), NORMAN 2023):

$$PNEC_{sed} = PNEC_{fw} \times 2.6 \times (0.615 + 0.019 \times K_{oc}) \quad (1)$$

Environmental risk to aquatic or benthic organisms is classified into four categories: “negligible/no risk” (RQ < 0.01), “low risk” (0.01 < RQ < 0.1), “moderate/medium risk” (0.1 < RQ < 1), and “high risk” (RQ ≥ 1) (Sharma et al. 2019; Kondor et al. 2022). In order to evaluate “cocktail effect” of the mixture of artificial sweeteners detected in each water and sediment sample, the RQ_{mix (water/sediment)} was calculated, based on the concentration-addition method for risk assessment of mixtures, as the sum of the RQ values obtained from the individual sweetener concentrations (Eq. (2), Kondor et al. 2022).

$$RQ_{mix (water/sediment)} = \sum_{i=1}^n RQ_i \quad (2)$$

In addition, to determine the overall ecotoxicological risk at sampling sites where both water and sediment samples were collected, RQ_{mix sum} was calculated considering the risk assessment results for both types of samples (Eq. (3), Kondor et al. 2022).

$$RQ_{mix sum} = \sum_{i=1}^n RQ_{mix water} + \sum_{i=1}^n RQ_{mix sediment} \quad (3)$$

Results and discussion

Artificial sweeteners detected in river water

Artificial sweeteners were found at all investigated sampling sites of the Danube River (SW1–SW13), as well

as in its tributaries the Tisza, the Sava, and the Morava (SW14–SW17). The results of artificial sweeteners detected in the Danube River Basin are summarized in Table 1. Acesulfame and sucralose were the most frequently detected artificial sweeteners in surface water samples, in the concentration ranges of 41–83 ng L⁻¹ and 42–206 ng L⁻¹, respectively. The widespread presence of acesulfame and sucralose in the aquatic environment can be explained by the fact that they are metabolically inert (Renwick 1986; Roberts et al. 2000) and are consequently ubiquitous in municipal wastewater that is ultimately discharged into the receiving waters. Furthermore, the two sweeteners are highly soluble in water (Table S1) and quite stable in the environment, and thus are regarded as indicators of wastewater-derived pollution (Lim et al. 2017; Ribbers et al. 2019; Fu et al. 2020).

The levels of acesulfame were relative uniform along the entire course of the Danube in the RS (Fig. 2) and similar to the levels in the tributaries. The generally balanced

concentrations of acesulfame in the Danube River Basin in the RS can be explained by the high dilution capacity of the Danube and evidently less pronounced influence of locations with a high wastewater burden. Namely, the concentrations of this sweetener were the same before and after (75 ng L⁻¹ and 76 ng L⁻¹, samples SW4 and SW5; and 51 ng L⁻¹, both samples SW15 and SW16) of the two major municipal canals that discharge wastewater to the Danube and the Sava in Belgrade (WW1 and WW2, Fig. 1). Regarding sucralose, the highest concentration was detected in the Danube in Belgrade (206 ng L⁻¹, sample SW5), after a major wastewater discharge. Untreated wastewater discharges in highly populated areas, such as Belgrade and Novi Sad, apparently have high impact on sucralose levels in river water. The recorded concentrations of this artificial sweetener in the Danube and the Sava rivers, downstream from the sewage discharges in Belgrade (206 ng L⁻¹, sample SW5; 111 ng L⁻¹, sample SW16) were much higher than those detected upstream (89 ng L⁻¹, sample SW4; 68 ng L⁻¹, sample SW15).

Table 1 Detected concentrations of selected artificial sweeteners in surface water (SW), wastewater (WW) and sediment (SED) samples

		Concentration ± SD (ng L ⁻¹)					
		Acesulfame	Saccharin	Cyclamate	Sucralose	Aspartame	Neotame
		Surface water					
Danube	SW1	64 ± 11	55 ± 11	– ^a	119 ± 15	–	–
	SW2	57 ± 5	90 ± 9	–	138 ± 35	–	–
	SW3	41 ± 3	77 ± 8	–	153 ± 11	–	–
	SW4	75 ± 13	92 ± 19	129 ± 19	89 ± 9	–	–
	SW5	76 ± 17	396 ± 37	118 ± 15	206 ± 18	–	–
	SW6	46 ± 6	–	–	76 ± 8	–	–
	SW7	62 ± 11	–	–	71 ± 3	–	–
	SW8	50 ± 10	–	–	95 ± 9	–	–
	SW9	63 ± 6	93 ± 11	–	94 ± 14	–	–
	SW10	41 ± 12	58 ± 7	–	68 ± 6	–	–
	SW11	80 ± 8	–	–	70 ± 15	–	–
	SW12	50 ± 7	109 ± 16	–	69 ± 14	–	–
	SW13	82 ± 13	–	–	78 ± 9	–	–
Tisza	SW14	50 ± 3	–	–	97 ± 4	–	–
Sava	SW15	51 ± 5	–	–	68 ± 7	–	–
	SW16	51 ± 4	104 ± 19	–	111 ± 23	–	–
Morava	SW17	83 ± 5	124 ± 20	–	42 ± 9	–	–
		Wastewater					
Sava	WW1	5383 ± 164	35,683 ± 840	16,576 ± 581	4039 ± 499	580 ± 20	170 ± 2
Danube	WW2	7488 ± 57	28,225 ± 544	23,179 ± 930	4756 ± 503	293 ± 31	53 ± 8
		Concentration ± SD (ng g ⁻¹)					
		Sediments					
Danube	SED1	–	–	–	–	117 ± 20	48 ± 9
	SED2	–	–	–	–	139 ± 17	9 ± 3
Tisza	SED3	–	–	–	–	48 ± 18	–
Sava	SED4	–	–	–	–	292 ± 33	21 ± 5
Morava	SED5	–	–	–	–	134 ± 14	–

^a(–) not detected

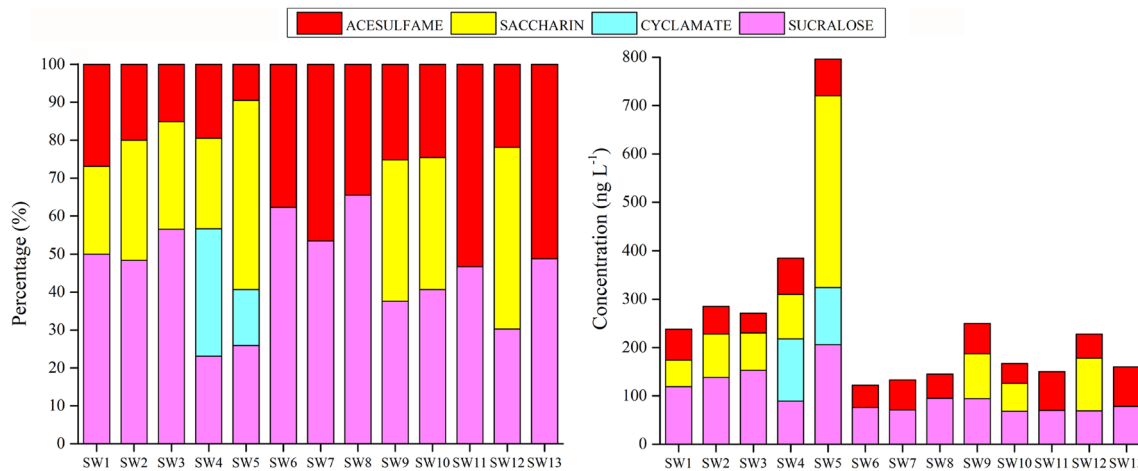


Fig. 2 Percentage (left) and concentration (right) of four artificial sweeteners detected in surface water along the entire course of the Danube River in the Republic of Serbia

Compared to the results from other European countries, the levels of acesulfame and sucralose in the Danube River Basin in the RS were generally significantly lower. High concentrations of these compounds were reported in five rivers in Spain (120–1620 ng L⁻¹ and 40–3600 ng L⁻¹, respectively, Arbeláez et al. 2015) and in twelve rivers in Finland (28–9600 ng L⁻¹ and up to 1000 ng L⁻¹, Perkola and Sainio 2014). However, a number of studies have shown very high levels of acesulfame, with sucralose detected at levels similar to those found in our study. For instance, in the Prut River on the Romanian-Moldavian border, as the second longest tributary of the Danube, concentrations of 120–750 ng L⁻¹ and 15–22 ng L⁻¹ were determined for acesulfame and sucralose, respectively (Moldovan et al. 2018). In a similar study of the Rhine River and its tributaries in Germany, high levels for acesulfame (379–3044 ng L⁻¹) were recorded, while sucralose was detected in a significantly lower range (18–175 ng L⁻¹, Ruff et al. 2015). Previous study of four rivers in Germany showed similar concentration trend, with acesulfame being the dominant sweetener (270–2700 ng L⁻¹, Scheurer et al. 2009), found at 730 ng L⁻¹ in the Danube River, whereas sucralose was detected in much lower concentrations (10–110 ng L⁻¹, and 20 ng L⁻¹ in the Danube). These results are consistent with monitoring data from 120 river water samples measured in 27 European countries (Loos et al. 2009) which showed sucralose concentrations ranging from 16 to 924 ng L⁻¹, with lower levels detected in Germany and Eastern Europe (Hungary, Bulgaria, Greece, etc.), suggesting a lower use of sucralose as an artificial sweetener in food products in these countries. Since acesulfame and sucralose are metabolically and environmentally stable and show limited removal in WWTPs (Buerge et al. 2009; Subedi and Kannan 2014), the concentrations detected in other European countries reflect the high effluent burden

in highly populated areas and different sweetener consumption patterns.

Saccharin was detected in ~60% of surface water samples in the concentrations range 55–396 ng L⁻¹, with the maximum value measured in the Danube River in Belgrade (sample SW5, Fig. 2), downstream from the major sewage canal (WW2, Fig. 1). Cyclamate was found in only two samples, from the Danube in Belgrade, at the concentrations of 118 and 129 ng L⁻¹ (samples SW5 and SW4). According to the highest levels detected in raw wastewater (samples WW1 and WW2), these two artificial sweeteners are most often used, and their concentrations in the Danube River Basin in the RS are consistent with the population density in the catchment and the high anthropogenic burden by untreated municipal wastewater. The much lower detection frequency of these two sweeteners in river water, compared to acesulfame and sucralose, is related to their limited stability and fast biodegradation in the environment (Luo et al. 2019a). The results of some studies also suggest a higher degradation rate of cyclamate in comparison to saccharin (Bergheim et al. 2015; Luo et al. 2019a). The absence of cyclamate in most of the investigated river samples could be therefore explained by the rapid environmental degradation, as well as by the lower amount of consumed cyclamate that is discharged into the receiving water (samples WW1 and WW2, Table 1). In addition, saccharin and cyclamate are considered as a very useful tool to identify recent wastewater contamination (Tran et al. 2014; Zirlwagen et al. 2016). Both persistent and biodegradable artificial sweeteners can be used to distinguish between past and recent sewage pollution of surface waters (Tran et al. 2014). According to the levels of saccharin and cyclamate, the most significant continuous input of fresh municipal wastewater into the river ecosystem of the Danube in the RS takes place in Belgrade

(samples SW4 and SW5). Less pronounced sewage contamination of the Danube River occurs in less populated areas along the river's course, as indicated by the absence of both biodegradable sweeteners. However, very high concentration of saccharin was recorded in a sparsely populated agricultural area by the Morava River (124 ng L^{-1} , sample SW17). The elevated levels could be explained by the application of livestock manure, as saccharine and NHDC are the only two artificial sweeteners permitted in animal feed in the RS (Regulation on Animal Feed Quality 2010). Saccharin is also a degradation product of some sulfonylurea herbicides (Berger and Wolfe 1996), which is another possible entry route into the aquatic environment.

Fresh untreated wastewater pollution in Belgrade was also confirmed using the cyclamate/acesulfame ratio as suggested by Zirlwagen et al. (2016). It is cited study; it was determined that this ratio is a powerful indicator for distinguishing between treated and untreated wastewater, and a more reliable parameter for wastewater input than absolute concentration due to the advantage that it is not affected by dilution. Our results showed that the ratio values for raw untreated wastewater were indeed the same (3.1 for both WW1 and WW2 samples) regardless of the different absolute concentrations of the two artificial sweeteners. For the only two river water samples in which cyclamate was detected (samples SW4 and SW5), the values of the cyclamate/acesulfame ratio (1.7 and 1.5, respectively) were about two times lower than in the raw wastewater. Given that the ratio value in the cited study was considerably lower for treated wastewater (about three orders of magnitude lower than untreated), our results confirm the continuous input of fresh untreated sewage water into the Danube in the capital city of Belgrade.

Concentrations of saccharin and cyclamate in the Danube River Basin in the RS were generally similar to those reported in rivers of other European countries. Two artificial sweeteners were detected in twelve rivers in Finland (up to 490 ng L^{-1} and up to 210 ng L^{-1} , respectively, Perkola and Sainio 2014) and in the catchment area of the Rhine River in Germany ($14\text{--}241 \text{ ng L}^{-1}$ and $14\text{--}106 \text{ ng L}^{-1}$, Ruff et al. 2015). In a previous study of four rivers in Germany (the Rhine, the Neckar, the Danube, and the Main), similar levels of detected sweeteners were observed ($10\text{--}350 \text{ ng L}^{-1}$ for saccharin and $30\text{--}320 \text{ ng L}^{-1}$ for cyclamate, Scheurer et al. 2009), with a concentration of 40 ng L^{-1} found in the Danube River for both artificial sweeteners. Saccharin and cyclamate concentrations similar to those found in the Danube in Germany were detected in the Prut River, the Danube tributary ($36\text{--}46 \text{ ng L}^{-1}$ and $15\text{--}27 \text{ ng L}^{-1}$, respectively, Moldovan et al. 2018). In addition, a study of five rivers in Spain showed that saccharin was not found in any of the investigated river water samples, while cyclamate was recorded at a level of up to 80 ng L^{-1} (Arbeláez et al. 2015).

Variable detection levels of these compounds in surface water are associated with dietary preferences and amounts of sweeteners in consumer products, as well as with different elimination rates in WWTPs.

The other four investigated sweeteners (aspartame, neotame, NHDC, and stevioside) were not found in any of the river water samples, which is in accordance with literature data showing their very rare detection (e.g., Gan et al. 2013). However, aspartame and neotame were detected in municipal wastewater, although at very low concentrations (up to 580 ng L^{-1} and 170 ng L^{-1} , respectively), similar to studies showing their significantly lower levels in influents compared to other artificial sweeteners (Gan et al. 2013; Subedi and Kannan 2014).

Artificial sweeteners detected in river sediments

Aspartame and neotame were the only detected artificial sweeteners in the investigated sediment samples of the Danube River and its major tributaries (the Tisza, the Sava, and the Morava) in the RS. Conversely, these two artificial sweeteners were not detected in any of the river water samples. The results can be explained by their rapid metabolism in the human body (O'Brien Nabors 2001) and high level of degradation in the environment (Berset and Ochsenein 2012; Gan et al. 2013). In addition, they show high removal efficiency in WWTPs (Gan et al. 2013). Given their high $\log K_{ow}$ and $\log K_{oc}$ values, as well as their low water solubility (Table S1), aspartame and neotame have high sorption affinity for organic-rich sediment particles and partition into sediment in a water/sediment system.

Aspartame was found in all sediment samples in the concentration range of $48\text{--}292 \text{ ng g}^{-1}$ (Table 1). The highest level of aspartame was recorded in the Sava River (sample SED4), in Belgrade, as highly populated capital city. According to the detected levels of the two sweeteners in untreated wastewater (samples WW1 and WW2), aspartame is consumed more than neotame through food products and beverages. This could be the reason why neotame is less frequently detected and, at lower levels, in sediments than aspartame.

There are only a few studies that have investigated artificial sweeteners in surface water sediments. In a national survey of trace organic compounds in sediments of rivers, streams, and creeks in the USA, sucralose was found in 12.5% of analyzed sediment samples with a maximum concentration of 16 ng g^{-1} (Bernot et al. 2016). An investigation of Canadian urban streams with combined storm water and sewer overflow showed that aspartame, one of ten wastewater micropollutants selected as potential sanitary tracers of sewage contamination, was not detected in any of the sediment samples analyzed (Hajj-Mohamad et al. 2014). In a study on traces of seven artificial sweeteners

in 16 lakes in Wuhan (China), acesulfame, saccharin, and cyclamate were detected in almost all sediment samples, with saccharin being the dominant sweetener with a maximum concentration of 4.2 ng g⁻¹ (Fu et al. 2020). Sucralose was found in 12% of the analyzed lake sediments, while aspartame, neotame, and NHDC were not detected. The reported levels of acesulfame, saccharin, cyclamate, and sucralose in densely populated areas of China are close to the LODs determined for our method, which could explain the lack of their detection in the sediments of the Danube River Basin in the RS.

Table 2 Environmental risk assessment for artificial sweeteners detected in sediment (SED) samples of the Danube River and its tributaries with *PNEC_{sed} values from the NORMAN Ecotoxicology Database (NORMAN, 2022)

Sample	Aspartame RQ *385 µg kg ⁻¹	Neotame RQ *20.9 µg kg ⁻¹	RQ _{mix sediment}
SED1	0.30 (medium risk)	2.30 (high risk)	2.60 (high risk)
SED2	0.36 (medium risk)	0.43 (medium risk)	0.79 (medium risk)
SED3	0.12 (medium risk)	–	0.12 (medium risk)
SED4	0.76 (medium risk)	1.01 (high risk)	1.77 (high risk)
SED5	0.35 (medium risk)	–	0.35 (medium risk)

Environmental risk assessment

The results of the environmental risk assessment associated with the detected artificial sweeteners are presented in Table S5 (for river water samples) and Table 2 (for sediment samples). It was found that the concentrations of acesulfame, cyclamate, and sucralose recorded in the surface waters of the Danube and its tributaries do not represent a risk (RQs < 0.01) to aquatic biota in the Danube River Basin in the RS. In fact, only saccharin levels detected in the Danube after major municipal wastewater discharge in Belgrade (sample SW5, Fig. 3) may pose a certain risk to aquatic organisms. According to the PNEC_{fw} value for saccharin (35.9 µg L⁻¹, Table S5, Supplementary Material), a low risk (0.01 < RQ < 0.1) was determined. The sum of individual RQs of different sweeteners (RQ_{mix water}, Table S5) additionally indicates an increased risk of the mixture due to the “cocktail effect” for sample SW4 (0.014, low risk), taken from the Danube upstream of the major sewage canal in Belgrade.

The levels of artificial sweeteners detected in all five river sediments indicate significant ecotoxicity of aspartame and neotame at the levels recorded, posing a medium to high risk to benthic organisms (Table 2, Fig. 3). The concentrations of aspartame found in five sediments from the Danube River Basin point to a medium risk for sediment-dwelling organisms (0.1 < RQ < 1), according to the PNEC_{sediment} value (385 µg kg⁻¹). However, based on the calculated ecotoxicity of neotame (PNEC_{sediment} = 20.9 µg kg⁻¹), a high risk (RQ ≥ 1) was found for benthic organisms at concentrations

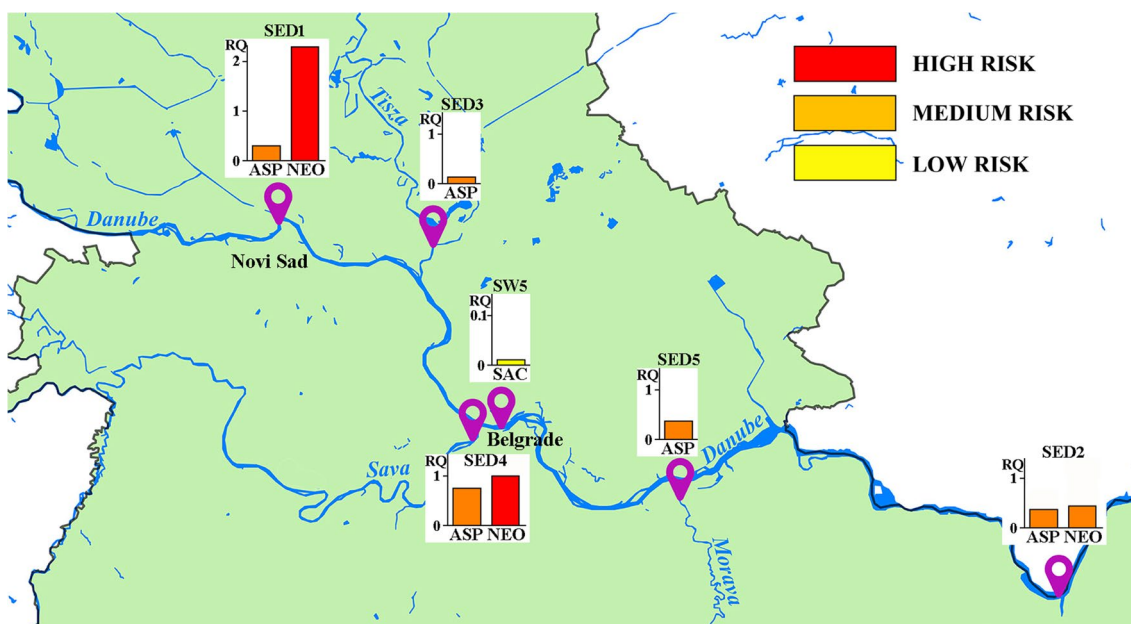


Fig. 3 Locations in the Danube River Basin with elevated concentrations of artificial sweeteners (saccharin, SAC; aspartame, ASP; neotame, NEO) in surface water (SW) or sediments (SED) that pose a risk to aquatic or benthic organisms

recorded in sediments of the Danube (sample SED1) and the Sava (SED4). For sample SED2, another sediment for the Danube River, medium risk was determined. According to the mixture risk approach, calculated $RQ_{\text{mix sediment}}$ (Table 2) confirmed medium or high risk for sweeteners detected in sediment samples, indicating a potentially detrimental effect on sediment biota. In addition, the overall ecotoxicological risk at the sampling sites where both surface water and sediments were collected ($RQ_{\text{mix sum}}$, Table S6) was not significantly increased due to the negligible contribution of risk to aquatic organisms compared to the dominant impact of risk to the benthic organisms.

It is evident that the sediments of the Danube River Basin are of great concern with regard to the detected artificial sweeteners, as a high- or medium-level risk to aquatic organisms was determined for each of the analyzed sediments. Given that sediments are considered a major sink of organic pollutants, they can also act as a secondary source of pollution in the case of resuspension. The results of the study revealed the highest ecotoxicological risk in the Danube River Basin in the RS in the two largest cities—the capital Belgrade and Novi Sad, both heavily impacted by untreated municipal wastewater and high population density. Medium risk was determined in smaller cities on the Danube and its tributaries—the Morava and the Tisza. The obtained results indicate the improvement of treatment and management of sewage wastewater and the need to include artificial sweeteners in routine monitoring programs.

Conclusions

Levels of four artificial sweeteners found in surface water of the Danube River Basin in Serbia reflect the high anthropogenic burden by untreated municipal wastewater. Persistent sweeteners acesulfame and sucralose, which were detected in all river water samples, indicate long-term wastewater-derived pollution. Although most often used according to high levels in sewage water, saccharin and cyclamate were found less frequently due to rapid environmental degradation. The most significant continuous input of untreated wastewater into the river ecosystem of the Danube takes place in the two largest Serbian cities, Belgrade and Novi Sad, leading to an increase in the levels of sucralose, saccharin, and cyclamate in the river water. The levels of acesulfame were generally uniform along the entire course of the Danube and similar to the levels in the tributaries. Compared to results reported for rivers in other European countries, acesulfame and sucralose concentrations were generally significantly lower, while saccharin and cyclamate levels were generally similar to those in European rivers, due to Serbia's smaller population and different sweetener consumption patterns. In river sediments, only aspartame and neotame were

detected as a result of their high sorption affinity for organic-rich sediment particles and preferential partitioning into sediment in the water sediment system. Aspartame was found in all sediment samples and the highest level was recorded in the capital Belgrade. Based on the low levels found in untreated wastewater, neotame is an artificial sweetener that is less commonly consumed and therefore less frequently detected in river water.

Depending on the recorded levels, persistence, and bioaccumulation potential, the detected artificial sweeteners have different impacts on aquatic organisms and the ecosystem as a whole. The ecotoxicological risk assessment of sweeteners found in the river water of the Danube and its tributaries in Serbia singled out only saccharin as a compound with a low environmental risk. However, sweeteners detected in river sediments pose a significantly greater environmental risk. A medium risk to benthic organisms was found at the recorded levels of aspartame, and neotame was determined to be an artificial sweetener of high environmental concern due to its high risk to sediment biota. The results presented in the study clearly show that untreated wastewater discharge in the two largest, densely populated cities is the major source of artificial sweetener contamination in the Danube River Basin in Serbia. Considering future climate change and population growth, as more intense rainfall and flooding cause overwhelming the drainage systems and sewer overflows, and higher temperatures lead to lower river flow regimes, continuous discharge of untreated wastewater could cause a detrimental decline in river water quality. As this may also become a transboundary issue by endangering the water quality of EU countries downstream, the much-needed sewage pollution control and stricter environmental regulation on food additives are necessary.

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Declarations

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