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Levels of regulated POPs in fish samples from the Sava River Basin. Comparison to legislated quality standard values

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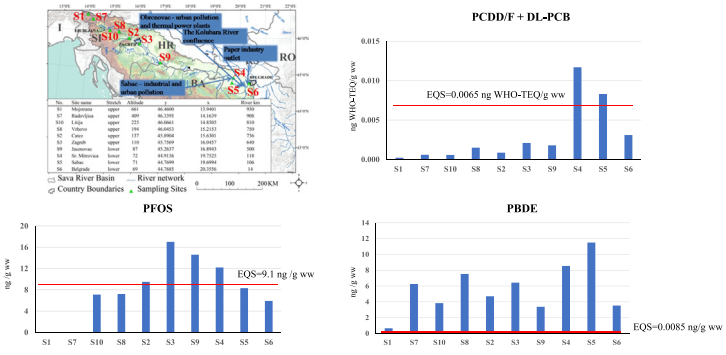
HIGHLIGHTS

- Fish samples from the lower stretch of the river showed PCDD/F+DL-PCB levels above the EQS.
- PCDD/Fs+DL-PCBs and NDL-PCBs exceeded the maximum levels for fish (as food product) in 20% of the samples.
- PBDE concentrations exceed the EQS up to more than a thousand times.
- Data suggest that anthropogenic impact is observed in the Sava River Basin.

GRAPHICAL ABSTRACT



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ABSTRACT

Fish samples of different species (i.e. rainbow trout (*Onchorhynchus mykiss*), barbel (*Barbus barbus*) and European chub (*Squalius cephalus*)) were collected from the Sava River Basin for a preliminary investigation of the levels of PCDD/Fs, PCBs, PBDEs and PFAS as a whole. Concentrations of PCDD/Fs, in terms of pg WHO-TEQ/g ww, were below the maximum limit established at the Commission Regulation (EU) No 1259/2011. On the contrary, when DL-PCBs were also included, levels increase up to 11.7 pg WHO-$TEQ_{PCDD/Fs+DL\text{-}PCBs}$/g ww in a particular case, with two samples out of a total of ten exceeding the maximum set at this EU Regulation and the EQS established at the European Directive regarding priority substances in the field of water policy (0.0065 ng WHO-$TEQ_{PCDD/Fs+DL\text{-}PCBs}$/g ww). A similar trend was also observed for NDL-PCBs, whit the same two samples, from the lower stretch of the river basin, exceeding the maximum limit allowed at the EU Regulation (125 ng/g ww). For PBDEs, levels found in all the samples exceeded the EQS (0.0085 ng/g ww) up to more than a thousand times and 40% of the samples presented PFOS values above the EQS. Data from this study were compared to values reported at the literature for fish from other geographical areas.

1. Introduction

Nowadays, there is a common position among the scientific community and the different competent authorities about the adverse effects of

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Persistent Organic Pollutants (POPs) on the environment and the exposed organisms, including human beings. The high toxicity, low degree of degradability and high persistence of these compounds have forced to adopt global measures for their control. In this sense, in 2001 the Stockholm Convention (SC) listed a number of chlorinated POPs as target compounds to be forbidden, eliminated or reduced by mean of the Best Available Techniques (BATs) (UNEP, 2001). Polychlorinated dibenzo *p* dioxins and polychlorinated dibenzofurans (PCDD/Fs), together with polychlorinated biphenyls (PCBs) were already included in the first list of substances. Later on, in 2009, the list of target chemicals of the SC was enlarged with 9 additional POPs, such as some polybromodiphenyl ethers (PBDEs) as well as perfluorinated compounds (PFASs) (i.e. perfluorooctane sulfonic acid (PFOS), its salts and its precursor perfluorooctane sulfonyl fluoride), among others (UNEP, 2009).

The particular concern about the unwanted effects of pollutants, including POPs, in the aquatic environment, is also reflected at the present European Directive regarding priority substances in the field of water policy (Directive 2013/39/EU). Environmental Quality Standards (EQS) have been set in the framework of this Directive for several substances, for some compounds not only in water but also in biota (e.g. fish). The Water Framework Directive (WFD) established EQS values for biota below which no harmful effects are expected to wildlife or humans. Monitoring conducted on biota is particularly important in the case of hydrophobic substances that tend to mostly accumulate in sediments and/or the fat tissues of living organisms. PCDD/Fs, PCBs and PBDEs are examples of lipophilic POPs that are hardly found in aqueous matrices and for which biota standards have been proposed. For PBDEs, biota EQS is referred to the sum of the concentrations of congeners BDE-28, BDE-47, BDE-99, BDE-100, BDE-153 and BDE-154 and has been set at 0.0085 ng/g wet weight (ww), while the EQS in inland surface waters for the same sum of congeners is 0.14 μg/L (maximum allowable concentration). These EQS should be taken into account in river basin management plans covering the period 2015 to 2021, and should be met by the end of 2021. On the other hand, PCDD/Fs and PCBs together with PFOS are among the new chemicals that were included in 2013 in the list of priority substances in the field of water policy. In this case, the EQS should be taken into account for monitoring programmes by the end of 2018, and should be met by the end of 2027. For PCDD/Fs and PCBs, EQS has only been established for biota and it is expressed in toxic equivalents according to the World Health Organisation 2005 Toxic Equivalence Factors (WHO-TEQ) for the sum of PCDD/Fs and "dioxin-like" PCBs (DL-PCBs) (0.0065 ng WHO-TEQ/g ww). The EQSs set for PFOS are 36 μg/L, as maximum allowable concentration in inland surface waters, and 9.1 ng/g ww in biota.

Apart from the environmental implications, the knowledge about POP levels in biota is also important from the point of view of human health. It is well known that at least 90% of the human exposure to PCDD/Fs and PCBs is estimated to come from food consumption, with fish and other related products contributing in an important way to this intake (Kiviranta et al., 2004; Bocio et al., 2007). In this sense, the WFD has derived EQS values in biota to ensure that humans are protected against adverse effects of consuming contaminated fish products. Therefore, the approach to derive these EQS is based on setting the same values already included in previous European Regulations as maximum levels for fish as food product. When these maximum levels are not available, human toxicological indicators (e.g. tolerable daily intake, reference dose) are considered to calculate the final EQS (EC. Guidance Document No. 27, 2011a).

In 1997 the International Agency for Research on Cancer (IARC) classified 2,3,7,8 tetra chlorodibenzo *p* dioxin carcinogenic to humans (Group 1) and more recently PCBs, as the whole family of compounds, have also been included in this group (IARC, 2016). Later on, in 2001, the European Union set maximum levels for PCDD/Fs at a wide range of food categories (e.g. fish) for the first time. These values have been revised along the last two decades and the latest update of the European Regulation also includes maximum levels for PCDD/Fs and DL-PCBs together and for the sum of the six most representative non-dioxin-like PCBs (NDL-PCBs) (Commission Regulation (EU) No 1259/2011). On the contrary, there are no limits established for PBDEs in food and the IARC has not classified the carcinogenicity of any PBDE congener. Despite there is an agreement that ingestion is one of the major routes of exposure to these compounds, particularly trough the consumption of fatty fish (Daso et al., 2010), the Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA) has not been able to set a tolerable daily intake (TDI) with the information available (EFSA, 2011). Therefore, based on the Opinion of this Expert Panel, the European Commission issued a Recommendation in 2014 with the aim to obtain more data about the concentrations of PBDEs in food in order to perform a further assessment (Commission Recommendation of 3 March, 2014). In regards to PFASs, fish consumption (Pérez et al., 2014) together with drinking water (Llorca et al., 2012a; Schwanz et al., 2016) have been identified as central sources of PFASs contamination in humans. In this sense, even though there is not a specific regulatory framework setting maximum allowable levels of PFASs in food products, a TDI of 150 ng/kg body weight per day for PFOS was established in 2008 as a result of the risk assessment on PFASs performed by the EFSA's CONTAM Panel (EFSA, 2008). However, nowadays these values are susceptible to be changed in order to be more restrictive, for instance the Environmental Protection Agency (US EPA) indicated in 2016 a reference dose of 20 ng/kg body weight per day (US EPA, 2016).

In this study, the levels of dioxin-like substances (PCDD/Fs and DL-PCBs), NDL-PCBs, PBDEs and PFASs were determined in fish samples collected along the Sava River Basin (SRB) during a sampling campaign performed in 2015. The SRB is one of the most significant sub basins of the Danube River Basin and, to the best of our knowledge, this is the first study reporting levels of this whole set of POPs in that geographical area. The main goal was to quantify the concentrations of all families of compounds and to discuss these preliminary findings from a regulatory point of view considering fish both, as biota as described in terms of the water policy Directive and as a food product that can be consumed by specific human sub-populations which obtain it from freshwater fishing. In addition, the POP levels have been compared to those reported in the literature for fish from other river basins and inland surface waters in order to assess the degree of contamination of the SRB.

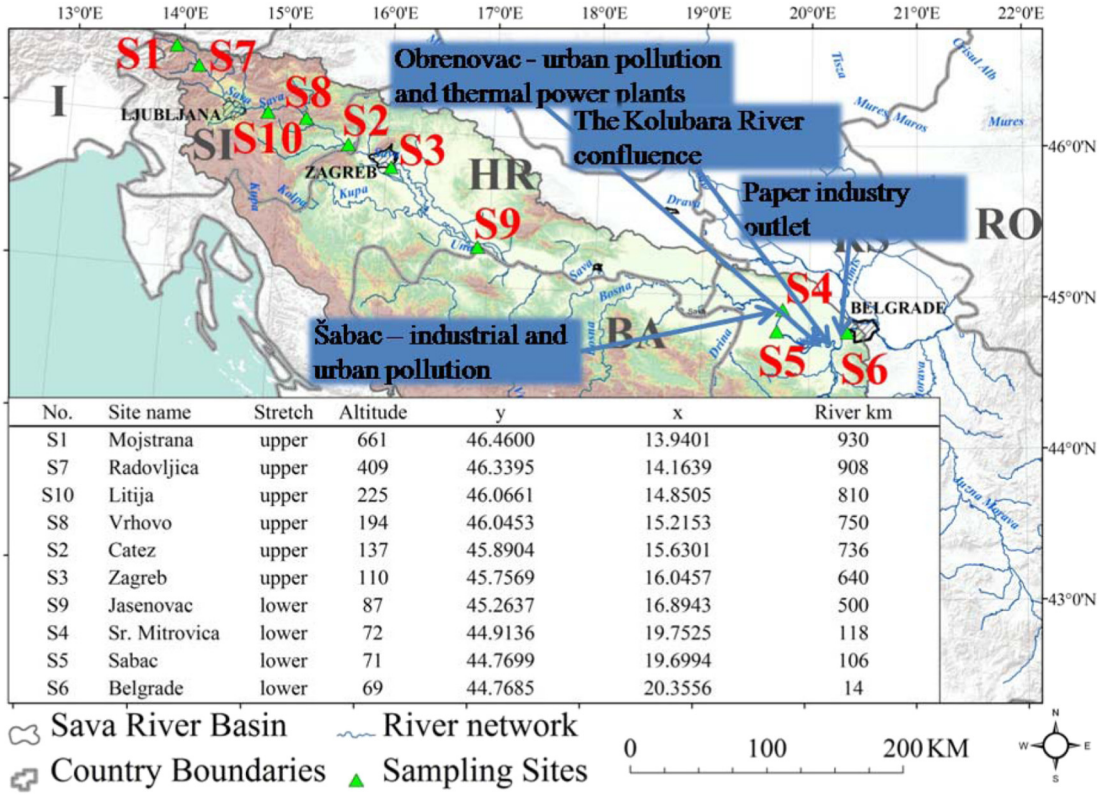
2. Materials and methods

2.1. Study area and sample collection

The SRB covers a wide geographic area (Fig. 1) with a total of 97,713 km^2 and including population of about 8.5 million inhabitants. It is a macro region, an area that includes the territories of six countries – Slovenia (SI), Croatia (HR), Bosnia and Herzegovina (BA), Serbia (RS), Montenegro (ME), with a minor part of the basin also extending to Albania (AL).

The SRB is one of the most significant sub basins of the Danube River Basin, with a share of 12%. The landscape within the SRB is diverse, the elevation varying between approx. 69 m above sea level (m a.s.l.) at the mouth of the Sava River in Belgrade (Serbia) and 2864 m a.s.l. (Triglav, Slovenian Alps). Mean elevation of the basin is approximately 545 m a.s.l.

In terms of land cover/land use, most of the basin is covered by forest and semi-natural areas (54.7%) and agricultural surfaces (42.4%), while the share of artificial surfaces is 2.2%. The basin is affected by water scarcity, due either to climatic or societal reasons, and also by significant environmental pressures. The upper part is largely influenced by hydromorphological pressures, and central stretches by agricultural activities and biological processes related to eutrophication, while the lower part is influenced mostly by stressors related to high pollution



No.	Site name	Stretch	Altitude	y	x	River km
S1	Mojstrana	upper	661	46.4600	13.9401	930
S7	Radovljica	upper	409	46.3395	14.1639	908
S10	Litija	upper	225	46.0661	14.8505	810
S8	Vrhovo	upper	194	46.0453	15.2153	750
S2	Catez	upper	137	45.8904	15.6301	736
S3	Zagreb	upper	110	45.7569	16.0457	640
S9	Jasenovac	lower	87	45.2637	16.8943	500
S4	Sr. Mitrovica	lower	72	44.9136	19.7525	118
S5	Sabac	lower	71	44.7699	19.6994	106
S6	Belgrade	lower	69	44.7685	20.3556	14

Fig. 1. Geographic area of the Sava River Basin (SRB) and location of sampling sites.

from industrial processing, along with untreated municipal waste water discharge (Giulivo et al., 2017).

Due to the heterogeneity of the studied river stretches (>930 km of the river covered by the investigation), it was not possible to collect the same specie for analysis in all the sampling sites. Thus, the aim was to collect species with a similar feeding behavior and, if possible, taxonomically close. In this sense, fish from three different species, i.e. rainbow trout (*Onchorhynchus mykiss*), barbel (*Barbus barbus*) and European chub (*Squalius cephalus*), were collected between 1st and 9th September 2015 at 10 different locations along the river basin (Fig. 1). A pooled sample of between 1 and 9 individual fish, depending on the fish species and size of the animals, was obtained from each site. Data on the processed individuals in each sampling location (i.e. species, number of individual fish per pool, mean length, mean weight and fat content of the pooled samples) are presented in Table 1.

2.2. PCDD/F and PCB analysis

Whole fish samples were lyophilized, ground and homogenized as a general procedure before the analysis of all POPs. Then, for PCDD/F and DL-PCB analysis, 10 g of lyophilized sample were extracted in a Soxhlet for ~24 h with toluene:cyclohexane (1:1, v/v) after being spiked with known amounts of mixtures of ^{13}C-PCDD/Fs and ^{13}C-DL-PCBs (Supporting information Section S1). Next, the extracts were rotary evaporated and kept in an oven overnight (105 °C) in order to eliminate the solvent prior to gravimetrical fat determination. Residues were dissolved in 5 mL of *n* hexane and organic components, fat and other interfering substances were removed using a silica gel column modified with sulphuric acid (44%, w/w). Further sample purification and fractionation were carried out using multilayer silica, basic alumina and carbon columns. The method allowed obtaining two fractions: fraction 1, containing the DL-PCB congeners, and fraction 2 containing the PCDD/F congeners. Both fractions were rotary concentrated and transferred into 2 mL vials. Then, the remaining solvent was reduced to dryness by a gentle stream of nitrogen. Final extracts were obtained by adding nonane containing known amounts of recovery standards (Supporting information Section S1).

For NDL-PCB analysis, about 1–3 g of lyophilized sample were spiked with known amounts of ^{13}C-NDL-PCBs (Supporting information Section S1) and then extracted in a Soxhlet ~24 h using *n* hexane:dichloromethane (1:1, v/v). After that, the extracts were rotary concentrated and transferred to *n* hexane. Organic components, fat and other interfering substances were removed in the same way that for PCDD/F and DL-PCB analysis. Then, fractionation of the extracts was carried out with a Florisil column. The extracts obtained were concentrated to ca. 1 mL using a rotary evaporator. Each extract was then transferred into

Table 1
Data on the processed individuals in each sampling location.

Sampling site	S1	S7	S10	S8	S2	S3	S9	S4	S5	S6
Species	*Onchorhynchus mykiss*	*Onchorhynchus mykiss*	*Squalius cephalus*	*Squalius cephalus*	*Barbus barbus*	*Barbus barbus*	*Squalius cephalus*	*Barbus barbus*	*Barbus barbus*	*Squalius cephalus*
Number of fish per pool	2	2	2	4	8	9	2	1	1	1
Mean length (cm)	31	24	16	16	12	15	23	33	42	29
Mean weight (g)	320	154	58	52	28	55	145	530	568	555
Fat content (%)	2.2	9.8	2.2	1.2	8.9	7.3	2.6	6.7	9.8	9.6

a 2 mL vial and concentrated under a gentle stream of nitrogen to dryness. To evaluate the recovery of the analytes, final extracts were obtained by adding a known amount of $^{13}C_{12}$-PCB 118 in nonane.

PCDD/Fs, DL-PCBs and NDL-PCBs extracts were analyzed by GC-HRMS on a Agilent Technologies 7890A gas chromatograph (Agilent, Palo Alto, CA, USA) coupled to a Micromass Premier (Waters, Manchester, UK) high resolution mass spectrometer (EBE geometry) controlled by a Masslynx data system. All extracts were injected, as well as standards, in nonane, with a CTC combipal autosampler (CTC Analytics AG, Zwingen, Switzerland) under data control system. Chromatographic separation for PCDD/Fs and DL-PCBs was achieved with a DB5-ms (Agilent, Folsom, CA, USA) fused-silica capillary column (60 m × 0.25 mm I.D., 0.25 μm film thickness), while NDL-PCBs chromatographic separations were performed using a DB-XLB (Agilent, Folsom, CA, USA) fused-silica capillary column (60 m × 0.25 mm I.D. × 0.25 μm film thickness). Helium at 1 mL/min was used as carrier gas.

Electron ionization (EI) mode was used with an electron energy of 32 eV, trap current of 500 μA and an acceleration voltage of 8000 V, operating in the selected ion monitoring (SIM) mode at a resolving power of 10,000 (10% valley definition). The ion source and transfer line were set at 250 and 280 °C, respectively. Quantification was carried out by the isotopic dilution method. Further details about the GC-HRMS analysis are reported in Section S2 of the Supporting information.

2.3. PBDE analysis

For PBDEs, 1 g of lyophilized sample was spiked with ^{13}C-PBDEs (Supporting information Section S1) and samples were kept in the fridge overnight to equilibrate. Extraction was carried out by pressurized liquid extraction (PLE). Samples were loaded into an 11 mL extraction cell. Dead volume was filled with hydromatrix. The extraction cell was filled with *n* hexane:dichloromethane (1:1, v/v) until the pressure reached 1500 psi (1 psi = 6894.76 Pa) and heated to 100 °C. After an oven heat-up time of 5 min under these conditions, two static extractions of 10 min at constant pressure and temperature were developed. After this static period, fresh solvent was introduced to flush the lines and cell, and the extract was collected in the vial. The flush volume amounted to 100% of the extraction cell. The extraction was cycled twice. Extracts were concentrated to dryness, kept in the oven at 95 °C for 2 h and lipid content was determined gravimetrically. Then the extracts were treated with sulphuric acid in order to remove lipids. After acid treatment, the organic phase was cleaned through solid phase extraction (SPE) using Al-N cartridges (5 g) conditioned with *n* hexane and eluted with *n* hexane:dichloromethane (1:2, v/v). Extracts were concentrated to incipient dryness and reconstituted in 40 μL of toluene for the instrumental analysis (Barón et al., 2014).

Instrumental analysis was carried out with GC–MS-MS using an Agilent Technologies 7890A gas chromatograph coupled to a 7000A GC–MS Triple Quadrupole. Chromatographic separation was carried out with a DB-5 ms column (15 m × 0.25 mm I.D., 0.1 μm film thickness). Electron ionization (EI) was selected as ionization mode (Barón et al., 2014). However, due to low sensitivity to decabrominated analytes using GC-EI-MS-MS, BDE-209 was determined with GC-negative chemical ionization (NCI)-MS (Eljarrat et al., 2004).

2.4. PFAS analysis

For the analysis of PFASs in fish, the procedure described by Llorca et al. (2012b) was employed. Extracts and the posterior analysis were performed in triplicates. Briefly, 0.5 g of lyophilized sample was spiked with 20 μL of a mixture of internal standards (100 ng/mL) and left at equilibrium for 20 min (Supporting information). The extraction procedure was based on alkaline digestion. The weighted sample was mixed with a solution of 10 mL of methanol with 10 mM sodium hydroxide. After homogenisation, the mixture was digested for 2 h in an orbital shaker. After this time, the mixture was centrifuged at 4000 rpm and 17 °C for 20 min. Then, 4 mL of the supernatant was dried with a gentle stream of N_2, reconstituted in 100 μL of a mixture water:methanol (9:1, v/v) and directly injected for purification in an on-line clean-up system (Thermo Fisher EQuan™) based on turbulent flow chromatography (TFC). For the purification, two columns were used, Cyclone (50 mm × 0.5 mm, 60 μm particle size, 60 Å pore size) and C18 XL (50 mm × 0.5 mm, 60 μm particle size, 60 Å pore size), connected in tandem. After purification, the extracts were directly pumped to the analytical column.

LC separation was achieved using a Thermo Scientific Aria TLX-1 system (Thermo Fisher Scientific, Franklin, MA, USA) equipped with a Hypersil GOLDTM PFP LC analytical column (50 × 3) (Thermo Scientific). Mobile phases were (A) aqueous ammonium acetate 20 mM and (B) methanol ammonium acetate 20 mM, used under gradient conditions. The total run time for each injection was 9 min with a flow rate of 0.4 mL/min. The injection volume was set at 20 μL.

The LC system was coupled to a Thermo Scientific Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA), equipped with a Heated IonSpray source. All analyses were performed operating in the negative electrospray ionization (ESI (−)) mode. The acquisition was performed in selected reaction monitoring mode (SRM) to obtain enough identification points (two transitions for each compound) according to current legislations (Commission Decision, 2002/657/EC). Selected *m/z* for each compound can be seen elsewhere (Llorca et al., 2012b).

2.5. QA/QC measures

For PCDD/F and DL-PCB the results were expressed as individual concentrations as well as in WHO-TEQ (World Health Organization Toxic Equivalent) using the Toxic Equivalency Factors (TEFs) revised in 2005 (Van den Berg et al., 2006). TEQ values were calculated in upperbound assuming the method limit of detection (MLOD) for those congeners below this limit. The MLODs were calculated for each congener in each particular sample. Recoveries were in the range of 60 to 120% as indicated in the corresponding EU Regulation (Commission Regulation (EU) No 644/2017).

For PBDEs, recovery ranges were between 59 and 77% with a reproducibility varying from 1.3 to 11.4%. Method limits of detection (MLODs) and method limits of quantification (MLOQs) varied between 4 and 335 pg/g ww and from 13 to 1113 pg/g ww, respectively.

In the case of PFAS, MLOQs were experimentally evaluated by spiking matrix blank, previously analyzed, with a mixture of selected compounds at a low concentration. MLODs and MLOQs varied between 0.09 and 2.69 ng/g ww and from 0.31 to 8.97 ng/g ww, respectively (Pignotti et al., 2017).

Strict quality assurance/quality control procedures (QA/QC) were followed for all the POP considered in this study, including the injection of calibration standards and analysis of procedural blanks (covering extraction, cleanup and instrumental determination) in parallel with the samples. The blanks either did not present the selected compounds or they were present at a very low levels compared to concentrations found in the samples (e.g. NDL-PCBs), therefore no subtraction of blanks was performed to obtain the final results on the samples.

Analysis of reference materials was also carried out as well as regular participation in interlaboratory exercises with optimum results. In addition, the methods for PCDD/F and PCB analysis are accredited under the ISO/IEC 17025:2005.

3. Results and discussion

3.1. PCDD/Fs and PCBs

Concentrations of individual PCDD/F and PCB congeners for the different fish samples, as well as their corresponding total WHO-TEQs (2005), are shown in Table 2. For PCDD/Fs, the isomer distribution of

Table 2
Concentrations of individual PCDD/F and PCB congeners (pg/g ww), as well as WHO-TEQ values (upperbound) (pg WHO-TEQ_{2005}/g ww) and total NDL-PCB values (ng/g ww) in fish samples.

Sampling site	S1	S7	S10	S8	S2	S3	S9	S4	S5	S6
2,3,7,8 - TCDD	<0.002	0.10	0.01	0.03	0.03	0.05	0.06	0.14	0.20	0.07
1,2,3,7,8 - PeCDD	0.01	0.04	0.01	0.02	0.04	0.06	0.06	0.16	0.19	0.09
1,2,3,4,7,8 - HxCDD	<0.003	0.01	<0.004	<0.01	0.02	0.02	0.02	0.03	0.03	0.03
1,2,3,6,7,8 - HxCDD	0.01	0.02	<0.004	0.01	0.04	0.04	0.03	0.08	0.07	0.05
1,2,3,7,8,9 - HxCDD	0.005	0.01	<0.004	<0.01	0.03	0.02	0.02	0.03	0.03	0.03
1,2,3,4,6,7,8 - HpCDD	0.01	0.04	0.01	0.04	0.12	0.12	0.05	0.07	0.07	0.07
OCDD	0.11	0.32	0.06	0.15	0.35	0.40	0.14	0.10	0.13	0.13
2,3,7,8 - TCDF	0.07	0.40	0.20	0.27	0.72	1.0	1.1	4.0	5.3	1.7
1,2,3,7,8 - PeCDF	0.01	0.04	0.01	0.01	0.05	0.08	0.06	0.26	0.33	0.16
2,3,4,7,8 - PeCDF	0.02	0.11	0.02	0.04	0.10	0.22	0.18	1.0	1.1	0.41
1,2,3,4,7,8 - HxCDF	<0.004	0.01	0.003	<0.01	0.01	0.03	0.01	0.04	0.05	0.05
1,2,3,6,7,8 - HxCDF	<0.004	0.01	0.003	0.01	0.02	0.02	0.02	0.05	0.06	0.04
2,3,4,6,7,8 - HxCDF	<0.004	0.01	0.003	<0.01	0.02	0.03	0.01	0.03	0.04	0.05
1,2,3,7,8,9 - HxCDF	<0.004	<0.004	0.01	<0.01	0.003	0.004	<0.01	0.004	<0.01	0.005
1,2,3,4,6,7,8 - HpCDF	0.01	0.01	0.01	0.03	0.02	0.04	0.04	0.02	0.02	0.03
1,2,3,4,7,8,9 - HpCDF	<0.004	<0.01	<0.005	<0.01	<0.003	<0.004	<0.01	<0.004	<0.003	<0.005
OCDF	0.01	0.01	<0.003	0.01	0.02	0.01	<0.01	0.004	0.01	0.01
WHO-$TEQ_{PCDD/F}$	0.03	0.22	0.04	0.09	0.19	0.30	0.31	1.0	1.3	0.48
PCB-81	<0.4	1.8	2.0	6.5	3.0	5.2	6.0	33	19	41
PCB-77	3.2	41	57	149	78	140	165	519	564	602
PCB-123	4.2	9.5	26	88	26	58	68	510	329	94
PCB-118	197	735	1968	5100	1601	3860	3980	27,197	16,501	5128
PCB-114	5.0	16	35	136	47	98	96	612	400	132
PCB-105	67	213	662	1727	590	1493	1384	7779	5481	1831
PCB-126	1.8	3.2	4.2	11	5.6	15	12	91	60	23
PCB-167	21	43	106	415	114	339	273	2962	1535	510
PCB-156	34	80	184	742	225	627	436	2654	1667	674
PCB-157	7.5	16	41	130	44	112	94	549	342	134
PCB-169	0.3	0.5	0.4	1.3	0.6	1.5	<1.0	7.1	4.1	1.9
PCB-189	3.3	6.6	11	57	16	62	36	113	78	55
WHO-TEQ_{DL-PCB}	0.19	0.37	0.53	1.4	0.66	1.8	1.5	10.6	7.0	2.7
WHO-$TEQ_{PCDD/F+DL-PCB}$	0.22	0.60	0.57	1.5	0.86	2.1	1.8	11.7	8.3	3.1
PCB-28	72	906	456	2295	1644	2226	1228	1980	5060	4418
PCB-52	110	617	503	1358	1215	2211	2230	4887	9690	8630
PCB-101	260	773	1229	3279	1812	5089	4979	24,290	24,716	6456
PCB-153	834	1190	1711	6248	2373	8996	6823	82,482	52,043	14,464
PCB-138	512	822	1326	4744	1770	7099	4990	41,093	31,336	9619
PCB-180	259	474	411	2474	849	3798	2145	13,108	9240	4024
∑NDL-PCB (ng/g ww)	2.0	4.8	5.6	20	9.7	29	22	168	132	48

toxic congeners was characterized by the presence of some of the lowest chlorinated compounds, particularly 2,3,7,8 TCDF and 2,3,4,7,8 PeCDF, contributing 50% and 10% (mean values), respectively, to the concentration of the sum of PCDD/F congeners. On the contrary, congeners with a higher degree of chlorination were found in minor proportions, with the exception of OCDD that contributed in a 16% (mean value) (Fig. 2a). The highest PCDD/F concentrations, expressed as total WHO-TEQ, were found in samples S4 and S5, with values equal to and slightly higher than 1.0 pg WHO-$TEQ_{PCDD/F}$/g ww, respectively. A similar trend was observed for PCB levels, being S4 and S5 the samples that showed the highest concentrations for DL-PCBs, expressed as total WHO-TEQ concentrations (10.6 and 7.0 pg WHO-TEQ_{DL-PCB}/g ww, respectively), as well as for NDL-PCB (as the sum of the 6 congeners analyzed), in this case values up to 168 ng/g ww (S4) and 132 ng/g ww (S5) were found. The PCB congener distribution was in good agreement with that usually observed in biota, being PCB-118 the most abundant among DL-PCBs followed by PCB-105 and two hexachlorinated isomers (PCB-167 and PCB-156) (Fig. 2b). On the other hand, NDL-PCBs showed, as expected, higher concentrations than DL-PCBs, PCB-153 and PCB-138 were the predominant congeners of this group (Fig. 2c).

WHO-TEQ results for PCDD/Fs in all the samples analyzed were below to the limit value of 3.5 pg WHO-$TEQ_{PCDD/F}$/g ww established by the EU Regulation for freshwater fish species considered as food product (Commission Regulation (EU) No 1259/2011). On the contrary, the calculated WHO-TEQ levels for the sum of PCDD/Fs and DL-PCBs in S4 and S5 (11.7 and 8.3 pg WHO-$TEQ_{PCDD/F+DL-PCB}$/g ww, respectively) were clearly higher than the corresponding limits established at the same EU Regulation (6.5 pg WHO-$TEQ_{PCDD/F+DL-PCB}$/g ww), which is also the EQS established at the European Directive regarding priority substances in the field of water policy (Directive 2013/39/EU). In addition, these two samples exceed the limit value of 125 ng/g ww indicated at the EU Regulation for the sum of NDL-PCBs in freshwater fish species.

Even though it was not possible to select pristine sites for sampling purposes in the Sava River since there is always some anthropogenic influence (e.g. agriculture, urban waste waters and/or industry), fish samples from the lower stretch of the river basin (S9, S4, S5 and S6) are more contaminated in terms of PCDD/F and PCB concentrations than those from the upper part (S1, S7, S10, S8, S2 and S3). In particular, S4 and S5 sampling sites are located in the area of Sremska Mitrovica and Sabac, respectively, where there is important urban and industrial pollution influence (i.e. power plant stations and paper industry). Sample from S6 (a sampling site located at the area of Belgrade) should show similar values than those from S4 and S5, but despite high levels were found compared to the samples of the upper part of the SRB, they were notably lower than those reported from S4 and S5. In this sense, the fish species and size of the individuals should also be considered to justify both, the high levels of S4 and S5 samples and the differences with S6 sample. S4 and S5 fish were barbel and present the highest mean weight and length among all the samples, while S6 has similar size characteristics than these two barbel samples, but instead it was a pooled sample of chub fish.

PCDD/F and PCB results from the present study are consistent with data recently published by Fliedner et al. about levels in freshwater fish species (i.e. *Squalius cephalus*, *Abramis brama* and *Perca fluviatilis*) also sampled in September 2015 from the middle section of the German

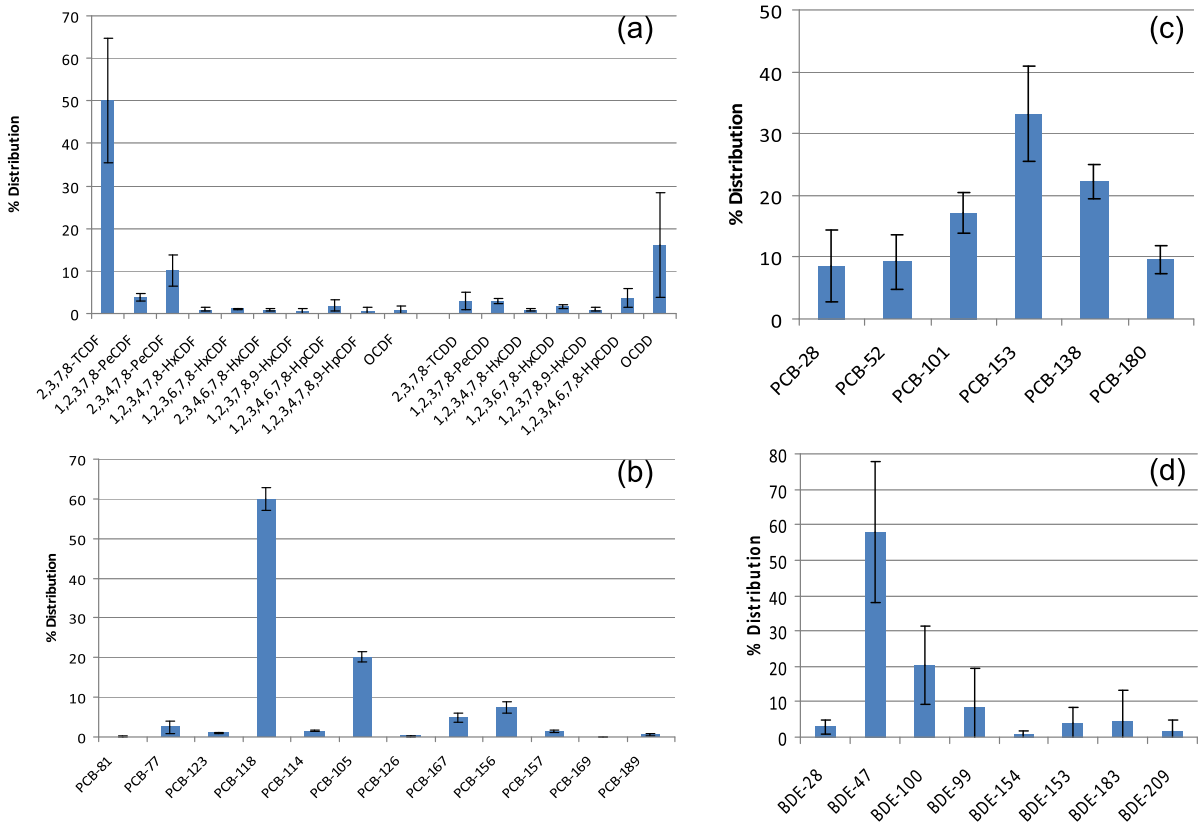


Fig. 2. Congener distribution of PCDD/Fs (a), DL-PCBs (b), NDL-PCBs (c) and PBDEs (d). Mean values and standard deviation (n = 10).

Danube. These authors found values in the range of 1.74 to 11.4 pg WHO-TEQ/g ww for the sum of PCDD/Fs and DL-PCBs, therefore with some samples above the EQS value, while levels of NDL-PCBs were high (21.7–119 ng/g ww) but, in that case, for all the samples still below the limit value of 125 ng/g ww set at the EU Regulation (Fliedner et al., 2018). In addition, higher concentrations of PCDD/Fs (0.43–4.12 pg WHO-TEQ/g ww) but similar concentrations of DL-PCBs (1.23–15.6 pg WHO-TEQ/g ww) were reported in bream samples caught in other European rivers (i.e. Elbe, Rhine) in the area of Germany (Fliedner et al., 2016). Lower levels were found in freshwater whitefish from the Turia river (Spain) (Bordajandi et al., 2003) and the Roya river (Italy) (Squadrone et al., 2015); and from the Qiantangjiang river in China (Han et al., 2007), the Chenab river in Pakistan (Eqani et al., 2015) and the Nile river in the Cairo region (El-Kady et al., 2007). On the contrary, levels of PCDD/Fs and PCBs were higher in freshwater fish sampled in the geographical area of the Great Lakes and the Hudson river in USA when compared to the results from the SRB and other European rivers (Wan et al., 2010; Skinner, 2011; Pagano et al., 2018).

3.2. PBDEs

PBDEs were detected in all fish samples as can be seen in Table 3. The sum of PBDEs ranged from 0.65 to 11.5 ng/g ww (from 11.9 to 461 ng/g lipid weight (lw)). As observed for PCDD/Fs, DL-PCBs and NDL-PCBs, the highest levels were found in samples S4 and S5. Eight different PBDE congeners were detected, tri-BDE-28, tetra-BDE-47, penta-BDE-99, penta-BDE-100, hexa-BDE-153, hexa-BDE-154, hepta-BDE-183 and deca-BDE-209. BDE-47 was the most abundant compound, contributing between 12 and 81% (mean value of 58%) of total PBDE burden (Fig. 2d). The contribution of BDE-99 and BDE-100 was also significant, with contributions of up to 32% and 49% respectively. This PBDE pattern is the same as that presented in previous studies on biota (Van Leeuwen and de Boer, 2008; Ben Ameur et al., 2011; Van Ael et al., 2013; Santín et al., 2013).

PBDE values from this work were also compared to those presented in other studies. A recent article (Eljarrat and Barceló, 2017) reviewed data published in the last five years in different countries around the world. PBDE levels from the SRB were within the concentration ranges

Table 3
Concentrations of individual PBDE congeners as well as total PBDE values, expressed in ng/g ww, in fish samples.

Sampling site	S1	S7	S10	S8	S2	S3	S9	S4	S5	S6
BDE-28	0,04	0,08	0,12	0,24	0,09	0,08	0,15	0,04	0,34	0,13
BDE-47	0,08	3,25	1,84	4,61	3,57	5,24	2,08	5,68	8,16	1,71
BDE-100	0,32	0,67	0,81	1,52	0,52	0,85	0,64	1,80	2,52	0,53
BDE-99	0,21	1,71	0,12	0,07	0,23	0,17	0,13	0,18	0,19	0,17
BDE-154	<MLOQ	0,20	<MLOD	0,11	<MLOD	<MLOD	0,05	0,17	<MLOD	<MLOD
BDE-153	<MLOD	0,27	<MLOD	0,93	0,28	<MLOD	0,26	0,65	<MLOD	<MLOD
BDE-183	<MLOD	<MLOD	0,93	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	0,63
BDE-209	<MLOQ	0,09	0,03	0,07	<MLOQ	0,06	0,06	<MLOQ	0,29	0,36
ΣPBDEs	0,65	6,26	3,83	7,53	4,70	6,43	3,37	8,54	11,5	3,53

Table 4
Concentrations of PFASs, expressed in ng/g ww, in fish samples.

Sampling site	S1	S7	S10	S8	S2	S3	S9	S4	S5	S6
PFBA	<MLOD	<MLOQ	<MLOQ	<MLOD	7.1	5.8	<MLOD	<MLOQ	<MLOD	<MLOQ
PFPeA	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
PFHxA	<MLOQ	8.5	11.6	2.6	33.2	<MLOQ	0.5	<MLOQ	4.2	10.0
PFHpA	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
PFOA	2.6	<MLOQ	2.5	3.7	<MLOQ	<MLOQ	3.1	3.9	8.0	4.2
PFNA	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
PFDA	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
PFUnA	<MLOD	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ
PFDoA	<MLOD	<MLOD	<MLOQ	6.4	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ
PFTrA	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
PFHxDA	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
PFODA	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
PFBS	<MLOQ	<MLOQ	<MLOQ	1.8	<MLOQ	3.5	<MLOQ	<MLOQ	<MLOQ	<MLOQ
PFHxS	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
PFOS	<MLOQ	<MLOQ	7.1	7.2	9.5	17.0	14.6	12.2	8.3	5.9
PFDS	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
FOSA	<MLOD	<MLOD	<MLOQ	3.7	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ

in European fish (between <LOD and 353 ng/g ww). Higher contamination was found in Asia and North America studies, with concentration levels ranging between 0.03 and 1726 and 0.075–4806 ng/g ww, respectively.

As biota EQS refers only to the sum of the concentrations of congener numbers 28, 47, 99, 100, 153 and 154, the sum of PBDEs was recalculated taking into account only these PBDE congeners. However, values remained practically the same since only BDE-183 and BDE-209 were removed and they were not the most predominant in the samples. Then, the sum of PBDEs now ranged from 0.65 to 11.2 ng/g ww, being always higher than the EQS value set at 0.0085 ng/g ww.

Once observed that PBDE levels exceeded the EQS value, it is important to know the extent to which this excess occurs. PBDE data from the present study exceeded up to more than a thousand times the EQS. However, this is not only the case in the SRB, as the same situation also occurs in other European countries and in other areas of the world (Eljarrat and Barceló, 2017). In this sense, it should be noted that EQS value for PBDEs developed for biota under the WFD is criticized by some authors (Jürgens et al., 2013). The EQS was calculated to protect human consumers based on observed effects of only one congener (BDE-99) on rats and including very large safety factors (EC. PBDE EQS dossier, 2011b). Indeed, in developing the proposed PBDE EQS the authors of the dossier on PBDEs thought that 44.4 ng/g ww for the sum of 6 BDEs is sufficient to protect wildlife predators. Hence the current EQS for PBDEs seems to be controversial and it should be revised as soon as new toxicological data will be available. The alternative EQS value of 44.4 ng/g ww would improve the situation, since none of the fish samples analyzed would exceed the value established as EQS. In this sense, it will be interesting to assess the results obtained in the present work taking into account future revised EQS included in the WFD (SCHEER, 2017), that will derive from new human toxicological data, not just for PBDEs but for all the other families of compounds.

3.3. PFASs

The most frequently detected compound was PFOS, present in 80% of the samples of the SRB, followed by PFOA and PFHxA, both in the 70% of the samples (See Table 4). It should be highlighted that PFOS and PFOA are the more persistent ones and PFHxA is the final degradation compound of the new generation of PFASs, used as substitution compounds of PFOS and PFOA. Also, in general, PFOS was found at the highest concentrations, in levels in the range from 5.9 to 17.0 ng/g ww, with four samples exceeding the EQS for this compound, being S4 one of them. But the peak concentration was found for PFHxA, with 33.2 ng/g in a single sample of barbel. These results agree with previous works where PFOS was the more frequently encountered PFAS and also was predominant in fish. For example, in the Czech Republic rivers Hloušková et al. reported total concentrations of PFASs in the range between 0.15 and 877 ng/g ww, and extremely high levels of PFOS 842 ng/g ww (Hloušková et al., 2013). The same group of authors reported as well the concentrations of PFASs in fish from the upper Labe River with maximum levels of PFOS reaching 61.3 ng/g ww (Svihlikova et al., 2015). In another study carried out on European eel, Giari et al., 2015 showed the presence of PFOS (up to 6.28 ng/g ww) and PFOA (up to 92.77 ng/g ww) at similar concentrations to the ones detected in this work. In addition, in Spain PFOS was also found to be the most ubiquitous PFAS in fish samples collected at the mouth of the Ebro River, with $\sum$PFASs ranging from 63.8 to 938 ng/g ww (Pignotti et al., 2017), in agreement with a previous work were PFOS was at levels between 0.01 and 1280 ng/g ww also in fish samples from the Ebro River (Lorenzo et al., 2016). It should be noted that in these cases in Spain and the Czech Republic the concentrations in fish were higher than in the present work due to the medium flows, hydrological conditions and the influence of industrial and urban wastes in those cases. In other studies, the concentrations were much similar to the present study and also PFOS was predominant. For example, in different studies carried out in China (Loi et al., 2011; Xu et al., 2014), United States (Stahl et al., 2014) and Tierra del Fuego (Argentina) (Llorca et al., 2012b). Notwithstanding, in all the cases the most ubiquitous compound was the banned PFOS.

4. Conclusions

In summary, despite the limited number of samples analyzed, data suggest that anthropogenic impact is observed in the SRB in terms of the presence of PCDD/Fs, PCBs, PBDEs and PFASs. From a total of ten samples, in one of them (S4) all the EQS for the evaluated POPs were exceeded, while in another one (S5) the EQS for the sum of PCDD/Fs and DL-PCBs was also exceeded. Four samples presented PFOS values above the EQS and for PBDEs all samples were several orders of magnitude above the EQS. Based on these concentrations in fish, there is a need to establish a surveillance monitoring programme on POPs in the SRB and to evaluate the potential risk for human health due to the consumption of these fish by the population in this area. From the point of view of toxicity and human health protection it would also be interesting to study the overall toxicity of a sample when different families of POPs exceed the EQS, cumulative of synergic effects could take place that should be considered.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.07.371.

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