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Analytical and bioanalytical assessments of organic micropollutants in the Bosna River using a combination of passive sampling, bioassays and multi-residue analysis

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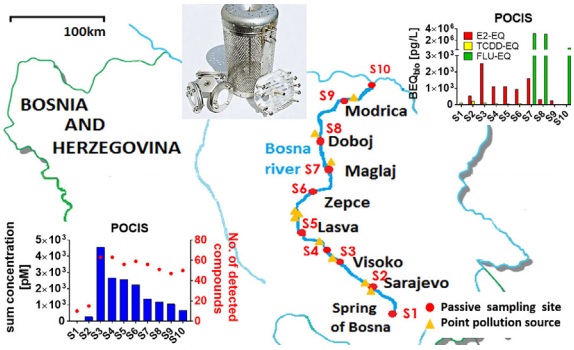
HIGHLIGHTS

- Impact of insufficient water treatment on river water quality was assessed.
- Passive sampling, multi-residue analysis and battery of bioassays were combined.
- Sarajevo was identified as a major pollution source in Bosna river.
- Diazinon occurred at most sites in concentrations posing risk to aquatic biota.
- Most bioactivities (except estrogenicity) were not explained by detected compounds.

GRAPHICAL ABSTRACT



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ABSTRACT

Complex mixtures of contaminants from multiple sources, including agriculture, industry or wastewater enter aquatic environments and might pose hazards or risks to humans or wildlife. Targeted analyses of a few priority substances provide limited information about water quality. In this study, a combined chemical and effect screening of water quality in the River Bosna, in Bosnia and Herzegovina was carried out, with focus on occurrence and effects of contaminants of emerging concern. Chemicals in water were sampled at 10 sites along the Bosna River

Abbreviations: AR, androgen receptor; AhR, arylhydrocarbon receptor; BiH, Bosnia and Herzegovina; BEQ, bioanalytical equivalent concentration; CECs, contaminants of emerging concern; CI, contamination index; CUPs, currently used pesticides; DCM, dichloromethane; DDT, 1,1,1-trichloro-2,2-bis(4-chlorphenyl)ethane; DHT, dihydrotestosterone; DHT-EQ, dihydrotestosterone equivalent; DS, downstream; E1, estrone; E2, 17β-estradiol; E2-EQ, 17β-estradiol equivalent; E3, estriol; EC_{50}, concentration at which the effect reaches 50% of the effect in positive control; EE2, 17α-ethinylestradiol; ER, estrogen receptor; Flu-EQ, flutamide equivalent; HCH, hexachlorocyclohexane; HI, hazard index; HQ, hazard quotient; LOD, limit of detection; LOQ, limit of quantification; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; POCIS, polar organic chemical integrative sampler; OCPs, organochlorine pesticides; PRC, performance reference compound; REP, relative effect potency; RB, river basin; SPE, solid phase extraction; SPMD, Semi permeable membrane device; TCDD, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; TCDD-EQ, TCDD equivalent; US, upstream; WWTP, waste water treatment plant.

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by use of passive sampling. The combination of semipermeable membrane devices (SPMDs) and polar organic chemical integrative samplers (POCIS) enabled sampling of a broad range of contaminants from hydrophobic (PAHs, PCBs, OCPs) to hydrophilic compounds (pesticides, pharmaceuticals and hormones), which were determined by use of GC–MS and LC-MS (MS). *In vitro*, cell-based bioassays were applied to assess (anti)androgenic, estrogenic and dioxin-like potencies of extracts of the samplers. Of a total of 168 targeted compounds, 107 were detected at least once. Cumulative pollutant concentrations decreased downstream from the city of Sarajevo, which was identified as the major source of organic pollutants in the area. Responses in all bioassays were observed for samples from all sites. In general, estrogenicity could be well explained by analysis of target estrogens, while the drivers of the other observed effects remained largely unknown. Profiling of hazard quotients identified two sites downstream of Sarajevo as hotspots of biological potency. Risk assessment of detected compounds revealed, that 7 compounds (diazinon, diclofenac, 17β-estradiol, estrone, benzo[*k*]fluoranthene, fluoranthene and benzo[*k*]fluoranthene) might pose risks to aquatic biota in the Bosna River. The study brings unique results of a complex water quality assessment in a region with an insufficient water treatment infrastructure.

1. Introduction

Sustainable management of water resources relies on regular monitoring of status and trends of qualities of surface waters, which allows identification of hazards and or risks posed by multiple anthropogenic stressors (Geissen et al., 2015). Chemical pollution of water resources is considered one of the main causes of impairment of aquatic ecosystems and losses of biodiversity (Vörösmarty et al., 2010). The ever-increasing multitude of chemicals entering aquatic environments constitutes a challenge for monitoring schemes, because most of these compounds typically occur at rather small (sub-ng L^{-1}) concentrations. However, some of these are sufficiently potent or have the potential to be accumulated to concentrations such that they can elicit biological effects. Moreover, some chemicals might undergo biotic or abiotic transformation forming very complex environmental mixtures where most individual components can only hardly be identified (Ginebreda et al., 2014). These compounds, known as contaminants of emerging concern (CECs), comprise many different chemical and usage pattern groups, *i.e.* personal care products, human and veterinary pharmaceuticals, surfactants and surfactant-derived compounds, X-ray contrast media, pesticides, disinfection by-products, algal toxins, flame retardants, plasticizers, UV-filters, industrial compounds and transformation products (Sima et al., 2014). CECs together with priority pollutants such as PAHs, legacy and currently used pesticides (CUPs) might cause adverse effects in aquatic biota and pose risks to human health.

Passive sampling presents a promising approach for surface water monitoring of CECs, because it provides a sensitive measurement of dissolved concentrations that is integrated over time (C_{free}). Due to its proportionality to the chemical activity and chemical potential, C_{free} is considered a key parameter in understanding chemical's exposure of aquatic organisms (Reichenberg and Mayer, 2006). Passive sampling enables integrative collection of contaminants over an extended period of time and captures residues from episodic events, which typically remain undetected when using grab sampling (Alvarez et al., 2004; Vrana et al., 2005). Passive samplers are available for sampling of a wide variety of compounds, *e.g.* semipermeable membrane device (SPMD) for hydrophobic substances such as PAHs or PCBs (Huckins et al., 1993) and polar organic chemical integrative sampler (POCIS) for hydrophilic substances such as polar pesticides and pharmaceuticals (Alvarez et al., 2004). Passive samplers are non-mechanical devices, that require minimal resources of personnel and equipment for sampling, and they constitute a well-defined sampling medium with a constant uptake capacity. Because of the integrative character of sampling, they accumulate a sufficient amount of sampled chemicals for detection of small concentrations in water and samples for multiple analyses including bioassays (Jones et al., 2015; Moschet et al., 2014; Vrana et al., 2014). Passive sampling has been successfully combined with *in vitro* bioassays in many earlier studies (Emelogu et al., 2013; Jalova et al., 2013; Jarosova et al., 2012).

In vitro bioassays, as sensitive, rapid and cost-effective screening tools, have been applied previously to detect micro-pollutants in water (Escher et al., 2014; Jalova et al., 2013; Neale et al., 2015). Unlike target chemical analysis, bioanalytical tools take into account possible mixture effects of all chemicals present in the sample (Altenburger et al., 2015). Bioassays can, therefore, help to provide a more holistic picture of possible hazards of complex environmental mixtures of priority pollutants and CECs to aquatic biota (Connon et al., 2012).

The Bosna River Basin (RB), with a total surface area of 10,809.83 km^2 and a population of almost one million is the most populated and developed region in Bosnia and Herzegovina (BiH). The Bosna River is about 275 km long and receives pollution from several points and diffuse sources. The major point sources comprise various industries, including among others, leather, pulp and paper, steel making, oil refining, thermal power, municipalities (Sarajevo, with no WWTP in operation at the time of sampling, Zenica and Doboj) and landfills (Sarajevo and Zenica). Diffuse pollution originates from agriculture and households, because only about 50% of population is connected to sewerage systems. Releases of untreated effluents from municipalities and industrial facilities often dominated by old and generally less effective technologies are considered a key environmental problem in the region (Smital et al., 2013).

The objective of the study, results of which are presented here, was to characterize, in some detail, water quality in the Bosna River affected by untreated wastewaters. This was achieved by use of a combination of passive sampling, a battery of *in vitro* bioassays and targeted chemical analyses for several compound classes. This approach evaluated chemical and ecotoxicological status at 10 sampling sites along the river. The specific goals of this study were to: 1) screen for potencies of response in bioassays as well as quantification of 168 targeted compounds in extracts of SPMD and POCIS samples; 2) estimate proportions of observed responses in bioassay that could be explained by targeted chemicals and 3) identify hotspots by use of contamination profiling and chemicals posing risk to aquatic biota by means of hazard assessment.

2. Materials and methods

2.1. Study area

Passive samplers (SPMD and POCIS) were deployed at 10 locations along the Bosna River, BiH, for 28–43 days from mid-October to mid-November 2012 (Fig. 1, Table S1 in Supplementary Materials SM2). Detailed information on individual sampling sites, exact dates of sampler's deployment, physicochemical parameters of river water during deployment and retrieval of the samplers, and estimation of sampled volumes are provided in Table S1 in SM2. The sampling sites were selected to span the whole waterway from the source, in the south, upstream of Sarajevo to the confluence with the Sava River near Modrica in the north. In order to evaluate absolute and relative sources of target compounds

Fig. 1. Sites (S1-S10) of deployment of passive samplers in autumn 2012 along the Bosna River, BiH.

and biological effects, sampling sites were situated upstream (US) and downstream (DS) of major known point sources of pollution entering the River Bosna (municipalities, industries and landfills; Fig. 1).

2.2. Sampling and sample processing

Two passive sampler cages were co-deployed at each sampling location. One contained samplers intended for bioassay screening and the other one samplers for chemical analyses. Cages, made of perforated stainless steel, were commercially available (www.exposmeter.com). At each sampling location, 3 POCIS samplers and 3 replicate SPMD samplers were placed into each protective cage. Cages with samplers were installed in the river water, usually from bridge pillars, approximately 1 m below the surface and fixed in place by use of weights, buoys and ropes. At the end of exposure, samplers were collected and checked for formation of biofilms or damage. While samplers were being deployed and collected, an additional field control of each sampler type was exposed to air only and processed identically to the deployed in-stream samplers. The field control was used to assess potential sampler contamination during transportation, storage and handling. Potential contamination arising from the manufacturing process, sampler components, laboratory storage, processing and analytical procedures, was assessed by analysis of fabrication control passive samplers (3 replicates for each sampler type). Analysis of fabrication controls also served to determine the initial concentration of PRCs in the SPMD samplers before exposure (Booij et al., 2007; Huckins et al., 2002).

2.2.1. SPMDs

SPMDs, purchased from Exposmeter AB, Tavelsjö, Sweden (www.exposmeter.com), consisted of an LDPE (Low-density polyethylene) membrane filled with triolein (1 mL, 95% purity), with nominal dimensions 2.54 × 91.4 cm, exposure surface area of 460 cm^2 and wall-thickness of 75–90 μm. Samplers designated for chemical analyses contained 2 μg $sampler^{-1}$ of individual performance reference compounds (PRCs; D_{10}-Acenaphthene, D_{12}-Benzo(e)pyrene, D_{12}-Chrysene, D_{10}-Fluorene, D_{10}-Phenanthrene). No PRCs were added to the samplers intended for toxicological analysis. The volume of the sampler was 4.95 mL (triolein + membrane). SPMDs were stored at −20 °C in gas-tight metal containers before use.

After exposure, collected SPMD samplers were processed according to Vrana et al. (2014). In brief, they were cleaned of mud and debris, placed in a cooled container and transported to the laboratory. Accumulated compounds were extracted by dialysis to hexane (two times for 24 h). The volume of dialysates was reduced and extracts were further cleaned up by gel permeation chromatography (GPC) with dichloromethane as a mobile phase. The SPMD extracts for toxicological analysis were solvent exchanged to 100 μL DMSO. SPMD extracts for chemical analyses were reduced in volume and further fractionated by use of silica gel or sulfuric acid modified silica gel for PAHs, PCBs and OCPs analyses. The SPMD samplers and their extracts were stored at −20 °C.

2.2.2. POCIS

POCIS samplers, consisting of membrane-sorbent-membrane layers compressed between two stainless-steel support rings, were purchased from Exposmeter AB, Sweden (www.exposmeter.com) under the commercial name EWH-Pharm - Exposmeter Water Hydrophilic Pharmaceuticals. The membrane was made of microporous polyethersulphone (PES) with 0.1 μm pore size. The samplers with the surface area of 45.8 cm^2 contained 200 mg Oasis HLB powder adsorbent. Following exposure, each POCIS sampler was dismantled and the sorbent was by means of Milli-Q water transferred into an empty 3 mL SPE cartridge fitted with a polypropylene frit. The sorbent phase was dried by applying gentle vacuum on a vacuum manifold. The mass of recovered sorbent was determined gravimetrically from the mass difference of the SPE cartridge with and without sorbent. For one POCIS, the analytes were eluted from the sorbent with 2 × 3 mL of elution mixture (MeOH:DCM, 1:1 v/v). The eluate was then evaporated under mild stream of nitrogen and reconstituted into 3 mL of MeOH. Extract of was divided into two aliquots intended for bioassays and chemical analyses of CUPs. A solvent of samples for toxicological analysis was exchanged for 0.5 mL of DMSO. Parallelly exposed individual POCIS were used for the chemical analyses as described in 2.3.3 and 2.3.4. The POCIS samplers and their extracts were stored until analyses at −20 °C.

2.3. Chemical analyses

Chemical analyses of 168 target compounds (134 in POCIS and 34 in SPMD extracts) in 4 compound classes were conducted by use of state-of-the-art GC–MS(-MS) and HPLC-MS(-MS).

2.3.1. Hydrophobic compounds analyzed in SPMD extracts

Hydrophobic compounds were determined according to a method described elsewhere (Vrana et al. 2014). In brief, identification and quantification of PAHs were conducted using 6890 N GC (Agilent Technologies, Palo Alto, CA, USA), which was equipped with a 30 m × 0.25 mm × 0.25 μm HP5-MS column (Agilent, USA) coupled to 5972 MS operated in electron impact ionization mode. PCBs and OCPs analysis was conducted on GC–MS/MS 6890 N GC (Agilent Technologies, Palo Alto, CA, USA) equipped with a 60 m × 0.25 mm × 0.25 μm DB5-MS column (Agilent J&W, USA) which was coupled to a Quattro Micro GC–MS/MS (Waters, Micromass, Manchester, UK) and operated in electron ionization mode. Details of sample processing and instrumental analysis are provided in SM1-Section 1.

2.3.2. Currently used pesticides analyzed in POCIS extracts

Agilent 1290 series (Agilent Technologies, Waldbronn, Germany) HPLC coupled to MS-MS AB Sciex Qtrap 5500 (AB Sciex, Concord, ON, Canada) with electrospray ionization (ESI) was used for analyses of CUPs. A Phenomenex SecureGuard C18 guard column (Phenomenex, Torrance, CA, USA) followed by a Phenomenex Synergy Fusion C-18 end capped column (100 mm × 2 mm i.d., 4 μm particles) was used

for separation of target compounds. Quantifications were based on isotopically labelled internal standards. Method details are given in SM1-Section 2 and in Brumovský et al. (2016).

2.3.3. Estrogens analyzed in POCIS extracts

POCIS for analyses of estrogens were processed as described in Skodova et al. (2016). Estrogens were analyzed by use of HPLC Agilent 1200 Series (Agilent Technologies, Waldbronn, Germany) coupled to MS-MS (Agilent 6410 Triple Quad; Agilent Technologies, Waldbronn, Germany) after derivatization with dansyl chloride (Lin et al. 2007). An ACE 3 C18 column (150 mm × 4.6 mm, 3 μm) coupled with a pre-column was used for chromatographic separation. Quantification was based on internal standards (E2-d4, E3-d2) and a 9-point calibration curve. Dansyl chloride derivatives exhibited a fragment ion *m/z* of 171, present in the MS-MS spectra of all investigated compounds. Detailed description of POCIS extraction, clean-up, derivatization and LC-MS-MS analysis of estrogens is given in SM1 – Section 2.

2.3.4. Pharmaceuticals analyzed in POCIS extracts

POCIS for analyses of pharmaceuticals were processed as described in Fedorova et al. (2014). Pharmaceuticals, illicit drugs and metabolites analyses in POCIS samples were carried out by use of an Accela 1250 LC pump and Accela 600 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) with an HTS XT-CTC autosamplers (CTC Analytics AG, Zwingen, Switzerland) coupled to a Q-Exactive mass spectrometer and a triple stage quadrupole MS/MS TSQ Quantum Ultra mass spectrometer (both from Thermo Fisher Scientific, San Jose, CA, USA). A HypersilGold aQ column (50 mm × 2.1 mm ID × 5 μm particles; Thermo Fisher Scientific, San Jose, CA, USA) was employed for separation of the target analytes. The POCIS extracts were supplemented with isotope-labelled internal standards prior to dilution with ultrapure water (1:1) and analyzed by use of conventional LC injection (10 μL of sample per injection). The validated method was described in full detail in previous papers (Fedorova et al., 2014, 2013; Grabic et al., 2012).

2.4. In vitro bioassays

Three cell-based reporter gene bioassays were used to examine ER-, (anti)AR- and AhR-mediated potencies and cytotoxicity of organic extracts of passive samplers. DMSO (0.5% v/v, Sigma Aldrich, Czech Rep.) was used as a solvent for extracts and reference compounds. All assays were conducted in 96-well microplates and included six dilutions of extracts in triplicate to characterize a dose-response curve for each sample. Samples were always tested in at least two independent experiments. A brief description of the bioassays is provided below, while more details on the bioassay methods and test conditions are provided in the SM1 – Section 3.

2.4.1. AR-mediated potency

Androgenicity and antiandrogenicity of extracts were assessed using MDA-kb2 cells (Wilson et al., 2002). These are human breast cancer cells transfected with a promoter containing androgen responsive elements driving expression of luciferase, as detailed in Jálová et al. (2013). In brief, the MDA-kb2 cells were exposed to calibration of reference compound, solvent control and sample extracts, in L-15 medium for 24 h at 37 °C. Standard reference calibration curves for androgenic response were produced with dihydrotestosterone (DHT; eight-point dilution series: 3.3 pM–100 nM). To assess antiandrogenicity, the cells were co-exposed to competing endogenous ligand DHT (100 pM) together with sample extracts, solvent control or calibration of standard antiandrogen flutamide (FLU; five-point dilution series: 110 nM–100 μM). The intensity of luciferase luminescence was measured with prepared luciferase reagent (Pavlíková et al., 2012).

2.4.2. ER-mediated potency

Estrogenicity of extracts was assessed by use of MVLN cells (Demirpence et al., 1993). These are human, breast carcinoma cells transfected with a promoter containing estrogen responsive elements driving expression of luciferase, as earlier described in Jálová et al. (2013). In brief, MVLN cells were exposed to sample extracts, calibration reference and solvent control in DMEM/F12 medium for 24 h at 37 °C. Standard calibration was conducted using 17β-estradiol (E2; six-point dilution series 1–500 pM) and the intensity of luciferase luminescence was assessed by use of Promega Steady Glo Kit (Promega, USA).

2.4.3. AhR-mediated potency

Dioxin-like activities, mediated through the aryl hydrocarbon receptor (AhR) were assessed by use of H4G1.1c2 cells (CAFLUX assay), rat hepatoma cells which contain a GFP reporter gene under control of dioxin-responsive elements (Nagy et al. 2002). In brief, the H4G1.1c2 cells were exposed to extracts, calibration reference and solvent control in DMEM medium for 24 h at 37 °C. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) served as standard reference compound and calibration curves were established by use of six-point dilution series (1–500 pM). The intensity of fluorescence as a measure of receptor activation was assessed after medium replacement with phosphate buffer.

2.4.4. Cytotoxicity

Combination of three dyes, according to Schirmer et al. (1998) with slight modifications, was used to assess cytotoxicity of the sample extracts. The intensity of fluorescence was measured after 30 min of incubation with CFDA-AM (5-carboxyfluorescein diacetate acetoxy- methyl ester, Invitrogen, Basel, Switzerland) reflecting cell membrane integrity and with AlamarBlue (AB, Invitrogen, Basel, Switzerland) showing cellular metabolic activity (530/590 nm and 485/520 nm, respectively). Afterwards, lysosomal membrane integrity was assessed by measurement of absorbance (540 nm) after 2 h incubation with neutral red (NR, Sigma-Aldrich, Buchs, Switzerland). Cell viability was also assessed by microscopic inspection.

2.4.5. Bioassay data analysis

Responses to sample extracts were expressed as percents of the maximum response observed in the calibration reference curves (% DHT_{max}/% $E2_{max}$/% $TCDD_{max}$). The response of the solvent control was subtracted from both sample and calibration responses. Nonlinear logarithmic regression of dose-response curves of calibration reference and samples was used for calculation of effect concentrations equivalent to 50% of maximal response (EC_{50}; Graph Pad Prism 6, GraphPad® Software, San Diego, California, USA). Bioanalytical equivalent concentrations (BEQ_{bio}) were calculated by relating the EC_{50} values of calibration reference (DHT-EQ, E2-EQ, TCDD-EQ) with the concentration of the tested sample inducing the same response (Escher and Leusch, 2012; Jalova et al., 2013). Percentage of the maximal luminescence inhibition in the calibration curves of reference antiandrogen flutamide co-exposed with competitive concentration of DHT (100 pM) were used to characterize the antiandrogenic effects expressed as FLU-EQ based on EC_{50} levels. LOQs for individual samples in each bioassay were calculated as 3-fold the standard deviation (SD) of the average response of solvent control on each assay plate according to Könemann et al. (2018). Results of cytotoxicity evaluation were expressed as a fraction of control (FOC) ranging from 0 to 1 and corresponding to a relative decrease of fluorescence/absorbance of samples related to solvent control.

Potency balance calculations using the ratio between BEQ_{chem} (based on chemical analysis) and BEQ_{bio} values (based on bioassays) were carried out to quantify the proportion of the response of bioassays that could be explained by detected chemicals. Total BEQ_{chem} was calculated as a sum of BEQ_{chem} values of individual compounds, for which relative effect potency (REP) value was available. REPs for target

compounds based on identical or similar biological models were collected in open scientific literature or ToxCast dashboard (US EPA, 2015).

2.4.6. Calculation of dissolved water concentrations from passive sampler data

2.4.6.1. SPMD. Concentrations of PAHs, PCBs and OCPs dissolved in water were calculated from amounts accumulated in SPMDs by exactly following the previously described method (Vrana et al. 2014). Briefly, the calculations were based on an assumption that the amounts of analytes absorbed by samplers follow a first-order approach to equilibrium. Aqueous concentrations of individual compounds were calculated from the mass absorbed by the SPMD, the *in situ* sampling rate of the compounds (R_S) and their sampler-water partition coefficients (K_{SW}). Nonlinear least squares method according to Booij and Smedes (2010) was used to estimate the R_s values on the basis of dissipation of PRCs from SPMDs during exposure. Fraction f of individual PRCs (D_{10}-acenaphthene, D_{10}-fluorene, D_{10}-phenanthrene and D_{10}-chrysene and D_{12}-benzo[*e*]pyrene), that had remained in the SPMD after exposure, was considered as a continuous function of their K_{SW}, with R_S as an adjustable parameter. A model for water-boundary layer controlled uptake, derived by Rusina et al. (2010) was used to estimate R_S of individual target compounds as a function of their molar mass.

For the purpose of comparison of toxic potencies of extracts from SPMDs from different sampling sites, the measured bioanalytical equivalent concentrations (BEQ_{bio}) in extracts [ng.SPMD^{-1}] were translated to water concentrations $C_{W\text{-}BEQ}$ [ng L^{-1}] at the individual sites as shown in Jálová et al. (2013). Linear uptake of the compounds that exhibit bioassay response in the extracts was assumed since their physicochemical properties are not known. SPMDs for toxicological analysis were not spiked with PRCs, and thus their sampling rate were for each sampling site calculated as R_S values of co-deployed SPMDs for chemical analysis for a compound with a medium molar mass (MW = 300 g mol^{-1}).

2.4.6.2. POCIS. Linear uptake from water during the entire sampling period was assumed to assess the concentrations of polar pesticides and pharmaceuticals dissolved in water from amounts accumulated in POCISs. Because the use of PRCs in POCIS is questionable and the variation of published R_S data is related not only to compound physicochemical properties but also to differences in exposure conditions such as temperature, water flow rates, salinity, pH, and fouling, it is currently not possible to select unbiased substance specific *Rs* values in POCIS in a specific deployment situation (Harman et al., 2012).

Harman et al. (2012) reviewed all published POCIS calibration data and the median values for all reported *Rs* values are 0.18 and 0.19 L d^{-1} (stagnant and turbulent exposures, respectively). Therefore, we applied a constant value of R_S of 0.2 L d^{-1} for all compounds as well as for bioassay responses.

2.5. Contamination profiling

Toxicity profiles based on a set of *in vitro* bioassays were translated into site-specific contamination profiles by use of an approach outlined by Hamers et al. (2010). The measured bioassay response of each extract was divided by the response measured in the extract from the upstream reference site S1, which was considered unaffected by anthropogenic pressures. The ratio between the response of downstream sites (S2-S10) and a reference site (S1), contamination index (CI), was regarded as a measure of contamination by each of the tested endocrine effects. If no potency was detected at the reference site S1, one-half of the LOQ was used to calculate the CI. Contamination index 1.0 was assigned for the effects less than the LOQ.

2.6. Hazard assessment

Assessments of hazards of detected target compounds were conducted by use of the lowest predicted no effect concentrations (PNEC) values derived by the NORMAN Network to identify compounds with hazard potential (Dulio et al., 2018; Working Group on Prioritisation of Emerging Substances, 2013). In order to protect aquatic biota, ecotoxicological threshold values, known as the lowest NORMAN PNECs, were determined on the basis of experimental data, existing environmental quality standards (EQSs), or *in silico* predictions. Hazard quotients (HQs) were calculated (Eq. 1), where: c_i is the calculated dissolved concentration of an individual compound in water at a particular sampling site, and PNEC is the lowest NORMAN PNEC value. Compounds with HQs < 1.0 are less than the threshold for a specified level of effect, while those with HQs exceeding 1 might pose risk to aquatic life. Overall hazard index (HI) was calculated by summation of all HQs of compounds detected at each sampling site and concentrations below LOQ were considered zero.

$$\mathrm{HQ} = \frac{c_i}{\mathrm{PNEC}} \quad (1)$$

3. Results and discussion

3.1. Chemical analyses

All passive samplers were successfully retrieved from sampling sites except for the POCIS sampler deployed at site S2, where two out of three POCIS discs were damaged and therefore not enough extract was available to carry out the analysis of pharmaceuticals and several CUPs. Samplers deployed at site S10 were retrieved after 43 instead of the planned 30 days due to flooding of the Sava River. Of the 168 target compounds, 103 compounds were detected in extracts of samplers from at least one sampling site. Specifically, 71 out of 134 compounds (52.9%) were found in POCIS and 32 out of 34 (94.1%) in SPMD extracts. 65 (38.7%) compounds never exceeded their LOQ. The concentrations for all individual compounds are listed in detail in SM2 in both the format ng POCIS^{-1} or ng SPMD^{-1} as well as recalculated based on the derived sampling rates to ng L^{-1} (SM2 – Tables S2, S3). Summary results of the chemical analyses showing the number of detected compounds and their sum concentrations at each site in pM and pmol sampler^{-1} are reported in Tables 1 and SM2-Table S4, respectively.

Concentrations of targeted compounds less than the LOQ were considered zero in the calculations of summary concentrations. Total sum molar concentrations of all compounds (in pM) detected at each site in relation to the observed bioactivities expressed as contamination profiles are presented in Fig. 2. There is a clear trend of decreasing cumulative concentration from S3 downstream to S10 in the POCIS samples, while no such pattern can be seen in case of hydrophobic compounds determined in the SPMD. Most analyzed compounds were undetectable at the reference site S1 (spring of Bosna) with only a few compounds detected in concentrations near their LOQs. The major source of pollution to the Bosna River was Sarajevo, the capital with a population of about 300,000 (Milinovic, 2013). Detailed results of chemical analyses are listed in SM2-Table S2 and S3.

3.1.1. Passive sampler performance characteristics

3.1.1.1. SPMD. Sampling rates for SPMDs, expressing the equivalent volume of water from which a compound is extracted per day, were calculated from *in situ* dissipation of PRCs. A graphical presentation of fitting the PRC data to the model described in 2.4.6., as well as results of the R_S calculation, are shown in Supplementary information, Section 1.3. Since R_S weakly depends on molar mass, the Table S1 shows site specific sampling rates for a model compound with a molar mass of 300 g mol^{-1},

Table 1
Sum of concentrations of target compounds detected in SPMD and POCIS extracts expressed in pM for different compound classes. Numbers of detected target compounds within each compound class at each sampling site are given in parentheses. Concentrations of target compounds less than the LOQ were considered zero in calculations of sums. "n.a." stands for "not analyzed".

Sampler	Compound class	Total nr. of target compounds	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
			Spring of Bosna	Sarajevo, DS	Visoko, US	Visoko, DS	Lasva confluence, US	Zepce, US	Maglaj, US	Doboj, US	Modrica, US	Modrica, DS
SPMD	PAHs[b]	15	7.2 (9)	2.3×10^{2} (14)	2.5×10^{2} (14)	1.9×10^{2} (14)	1.3×10^{2} (14)	2.8×10^{2} (14)	1.9×10^{2} (14)	3.3×10^{2} (14)	2.3×10^{2} (14)	2.2×10^{2} (14)
	PCBs	7	7.3×10^{-2} (7)	4.7×10^{-1} (7)	6.2×10^{-1} (7)	6.3×10^{-1} (7)	4.7×10^{-1} (7)	2.5×10^{-1} (7)	3.4×10^{-1} (7)	3.6×10^{-1} (7)	2.6×10^{-1} (7)	3.8×10^{-1} (7)
	OCPs	12	3.8×10^{-2} (5)	2.4×10^{-1} (11)	1.7×10^{-1} (9)	6.5×10^{-1} (11)	3.8×10^{-1} (11)	2.9×10^{-1} (10)	1.9×10^{-1} (10)	2.0×10^{-1} (10)	1.6×10^{-1} (10)	2.2×10^{-1} (10)
POCIS	CUPs	52	2.9 (7)	2.4×10^{2} (11[a])	1.8×10^{2} (13)	1.8×10^{2} (15)	2.1×10^{2} (13)	2.5×10^{2} (15)	2.6×10^{2} (15)	2.2×10^{2} (15)	1.3×10^{2} (13)	1.2×10^{2} (13)
	Estrogens	5	0 (0)	2.9×10^{1} (4)	1.6×10^{1} (4)	1.9×10^{1} (4)	0 (0)	7.0 (4)	4.1 (4)	3.4 (4)	2.1 (3)	1.6 (3)
	Antibiotics	11	0 (0)	n.a.	6.9×10^{2} (6)	5.3×10^{2} (7)	4.6×10^{2} (6)	2.9×10^{2} (6)	2.3×10^{2} (6)	1.8×10^{2} (6)	1.7×10^{2} (6)	1.2×10^{2} (6)
	Antidiabetics	2	0 (0)	n.a.	1.6 (2)	1.6 (2)	8.1×10^{-1} (2)	6.5×10^{-1} (2)	6.7×10^{-1} (2)	0 (0)	0 (0)	1.6×10^{-1} (1)
	Antihistamins	6	0 (0)	n.a.	1.9×10^{1} (2)	2.0×10^{1} (2)	7.7 (2)	8.1 (1)	8.3 (1)	6.4 (1)	5.4 (1)	2.3 (1)
	Cancer treatment	1	0 (0)	n.a.	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Cardiovascular	10	1.2×10^{-1} (1)	n.a.	3.9×10^{2} (10)	2.6×10^{2} (9)	2.3×10^{2} (9)	1.9×10^{2} (8)	1.4×10^{2} (8)	1.2×10^{2} (8)	9.7×10^{1} (8)	7.2×10^{1} (8)
	NSAIDS	1	0 (0)	n.a.	2.1×10^{2} (1)	2.8×10^{2} (1)	1.6×10^{2} (1)	1.2×10^{2} (1)	9.4×10^{1} (1)	6.7×10^{1} (1)	5.2×10^{1} (1)	3.4×10^{1} (1)
	Psychoactive	20	2.1×10^{-1} (1)	n.a.	3.0×10^{2} (12)	2.6×10^{2} (11)	2.4×10^{2} (11)	1.9×10^{2} (11)	2.3×10^{2} (10)	1.9×10^{2} (6)	1.8×10^{2} (7)	1.4×10^{2} (7)
	Statins	4	0 (0)	n.a.	1.5×10^{1} (4)	1.2×10^{1} (3)	4.1 (3)	3.3 (2)	1.9 (1)	2.4 (1)	1.1 (1)	9.4×10^{-1} (1)
	Illicit drugs	8	0 (0)	n.a.	9.9 (3)	8.6 (3)	1.0×10^{1} (3)	6.8 (3)	5.2 (3)	3.2 (3)	1.4 (2)	1.3 (3)
	Metabolites	5	0 (0)	n.a.	2.8×10^{1} (4)	2.5×10^{1} (4)	1.7×10^{1} (4)	1.1×10^{1} (4)	7.7 (3)	8.8 (4)	6.3 (3)	5.5 (4)
	Others	9	2.9 (1)	n.a.	2.7×10^{3} (2)	1.1×10^{3} (2)	1.2×10^{3} (2)	1.2×10^{3} (2)	4.0×10^{2} (2)	3.8×10^{2} (2)	4.1×10^{2} (2)	1.8×10^{2} (2)

[a] At site S2, only 40 target CUPs were analyzed.
[b] Concentration of naphthalene is not included in the reported sum of PAHs because of poor recoveries and its presence in blanks.

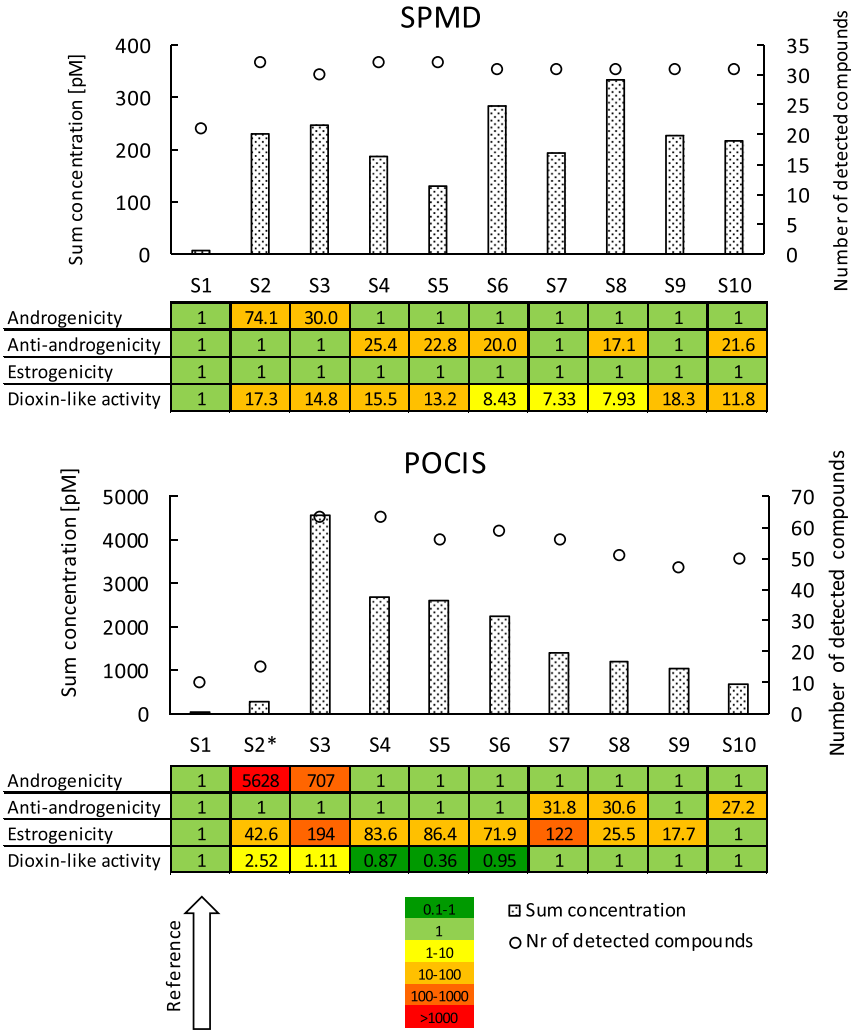

Fig. 2. Profiles of contamination at sampling sites S2-S10 combined with the total number and cumulative concentration (pM) of detected compounds in SPMD (top) and POCIS (bottom) extracts. Colors green to red indicate to what extent the bioassay responses exceed the response of the bioassay at reference site S1 (on a logarithmic scale). *77 pharmaceuticals and 16 target CUPs were not analyzed in the POCIS extract from site S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$R_{s,300}$. $R_{s,300}$ values ranged from 5.7 to 10.5 L d^{-1}. The volume of water extracted for a compound that remains in the integrative uptake phase during the entire sampling period ranged from 154 to 285 L.

Compound specific data treatment was not possible for interpretation of toxic potencies BEQ (ng/SPMD) of SPMD extracts, since the BEQ comprise many unknown substances. In order to translate toxic potencies to aqueous concentrations, we applied site specific *R*s derived from PRC data for a compound with $MW = 300$ (a compound with medium molecular size) as a compromise. This is justifiable since *R*s present only a very weak function of MW ($Rs = B \times MW^{-0.47}$) and thus, the uncertainty introduced by accepting an assumed MW of 300 for all compounds active in a bioassay is less than a factor 2, when assuming that active ingredients are within the range of MW from 200 to 700 (*i.e.* $700^{-0.47}/200^{-0.47} = 1.8$), which is a typical range for xenobiotics that are absorbed by SPMDs.

3.1.1.2. POCIS. Unlike the data from SPMDs, site specific sampling rates cannot be derived for POCIS. Therefore, we decided to apply a constant R_S value of 0.2 L d^{-1} for all compounds as well as for bioassay responses, acknowledging the resulting uncertainty of the reported data, which ultimately renders them semi-quantitative. Despite this introduced uncertainty, passive sampling with POCIS provides time-integrated concentrations of pollutants, in contrast to spot sampling. If the uncertainty of water concentrations estimated from passive sampling is lower than the variability of environmental concentrations, data obtained by passive sampling represent the contamination in the water body equally or better than the low frequency spot sampling that is currently applied in regulatory monitoring of surface waters (Miège et al., 2015).

3.1.2. Hydrophobic compounds

Most hydrophobic compounds were detected at all sampling sites including site S1. PAHs occurred at the greatest concentrations (sum of the 16 US EPA PAHs with the exception naphthalene 7.9–3.3 × 10^2 pM) compared to the other classes of hydrophobic compounds *i.e.* PCBs and OCPs (sum concentrations 3.8 × 10^{-2}-6.5 × 10^{-1} pM). Results for naphthalene are not reported because of its poor extraction recoveries and high levels found in blank samples. Within the class of PAHs, compounds with 3 and 4 condensed aromatic rings were detected at the greatest concentrations and occurred at all sampling sites. Concentrations of acenaphthene were 10-fold greater at site S6 and downstream compared with the sites upstream of S6. This indicates presence of a specific local pollution source for this compound, possibly the thermal power plant in Kakanj. Spatial concentration profiles of the remaining PAHs, PCBs and OCPs were less variable. Concentrations of detected compounds were comparable with those sampled by SPMDs during a survey in 2008 and 2009 when the river was screened for Stockholm Convention persistent organic pollutants (Harman et al.,

2013). The comparison for selected compounds is shown in Supplementary information, Section 1.4. Similar concentrations of dissolved hydrophobic contaminants collected by passive samplers in central European rivers were reported previously (Prokeš et al., 2012; Jálová et al., 2013 and Vrana et al., 2014). Hydrophobic compounds adsorbed on suspended particulate matter were not addressed by the present study, but an additional load of these compounds on solid phase can be expected to contribute to the overall burden.

3.1.3. CUPs

Concentrations of most CUPs were less than the LOQ and at sites S6, S7, and S8 with the greatest occurrence of CUPs, only 15 out of 52 compounds were detected. In total, 20 CUPs (38.5%) were detected at least once. Carbendazim, diuron and isoproturon occurred at all sampling sites including the reference site S1 and their concentrations ranged from 6.0×10^{-2}–5.6 ng L^{-1}. The greatest concentrations were detected in case of diazinon and prometryn (53 ng L^{-1} and 17 ng L^{-1} at sites S2 and S5, respectively) and these two compounds occurred at all sampling sites except for the reference site (S1). In the EU, both diazinon, a non-systemic organophosphate insecticide, and prometryn, a systemic triazine herbicide, have been banned for use in plant protection products since 2007 and 2002, respectively (European Commission, 2016). However, both these compounds along with other banned pesticides (*e.g.* atrazine, diuron, isoproturon) have been commonly detected in European surface waters despite the ban (Neale et al., 2015; Szekacs et al., 2015). Several studies have prioritized diazinon among the pesticides of major concern in surface waters (Guha et al., 2016; Kuzmanovic et al., 2014a). Greatest cumulative concentrations of CUPs were observed at sites S7, S6 and S2 (2.6×10^2 pM, 2.5×10^2 pM, and 2.4×10^2 pM). Comparable concentrations of CUPs determined in European surface waters and WWTP effluents were published previously (Jálová et al., 2013; Loos et al., 2013; Neale et al., 2015 and Tousova et al., 2017).

3.1.4. Estrogens

Natural estrogens E1, E2 and E3 were detected at all sites in a range of 2.0×10^{-2}–5.8 ng L^{-1} except for sites S1 and S5. Greatest concentrations were observed at sites S2-S4. Concentrations of E3 at sites S2-S4 were 10-fold greater than those measured in the Danube River (Neale et al., 2015) or Sava River (Tousova et al., 2017), where neither E1, E2 nor EE2 were detected. EE2, a synthetic estrogen contained in hormonal contraceptives, was less than the LOQ of 1.0×10^{-2}–3.0×10^{-2} ng L^{-1} at all sampling sites. This might be related to the 8- to 9-fold lesser prevalence of hormonal contraceptives in BiH compared to western European countries like Belgium or the UK due to cultural and economic reasons as well as limited availability of hormonal contraceptives (Boussen, 2012). Pollution of European rivers with E2 and EE2 is a ubiquitous phenomenon, however, analysis of these compounds still presents a challenge because LODs of most current monitoring techniques are still greater than the proposed environmental quality standard EQS values (4×10^{-1} and 3.5×10^{-2} ng L^{-1} for E2 and EE2, respectively) under the WFD (Tiedeken et al., 2017). This fact stresses the need of routine application of bioanalytical tools for monitoring of estrogenic potency in surface waters, because bioassays can integrate the effect of multiple compounds contained in complex environmental mixtures and are more sensitive than most chemical analytical methods (Jarošová et al., 2014; Kunz et al., 2017). LC-MS-MS is widely recognized as the most sensitive technique for the identification and quantification of estrogens in environmental samples and the present study confirms its compliance with the WFD requirements as LODs for E2 was 2.0×10^{-3} ng L^{-1} and for EE2 ranged from 1.0×10^{-2} to 3.0×10^{-2} ng L^{-1} (Tiedeken et al., 2017).

3.1.5. Pharmaceuticals

Analyses of 77 pharmaceuticals in 11 subclasses resulted in detection of 47 compounds (61%) at least once. The most frequently detected compounds, disopyramide, carbamazepine and caffeine, occurred at all sampling sites. The greatest cumulative concentration of pharmaceuticals was observed at site S3 (4.4×10^3 pM) and gradually decreased downstream to site S10. The same trend was observed for numbers of pharmaceuticals detected at individual sites. Concentrations of carbamazepine and caffeine, indicator compounds of municipal waste water pressure (Buerge et al., 2003; Clara et al., 2004), were greatest at site S3 (42 and 4.9×10^2 ng L^{-1}, respectively) and differences between concentrations at site S3 and other sites were especially pronounced in case of caffeine. Concentrations of carbamazepine and caffeine detected in the Danube River were an order of magnitude lesser than in the present study (Neale et al., 2015). Within the subclass of antibiotics, 11 compounds were targeted and 6 were detected at 8 sampling sites (S3–S10). Sulfamethoxazole, trimethoprim and clarithromycin reached the greatest concentrations ranging from a few to hundreds of ngL^{-1}. Similar concentrations of sulfamethoxazole and clarithromycin in Sava river were reported by Tousova et al. (2017). However, concentrations of trimethoprim were 10-fold less than in the present study. Cardiovascular drugs were frequently detected (8 out of 10 compounds were detected at 8 sites) with greatest concentrations observed for valsartan and atenolol (76 ng L^{-1}, and 21 ng L^{-1}, respectively), which have been reported to be ubiquitous and persistent in aquatic environments (Ebele et al., 2017). Comparable concentrations of atenolol were detected in the Po and Lambro Rivers in Northern Italy (Calamari et al., 2003). Diclofenac, a nonsteroidal anti-inflammatory drug, was detected at 8 sampling sites (S3–S10) at concentrations ranging from 10 to 82 ng L^{-1}. Similar concentrations of diclofenac in river water and WWTP effluents were previously reported (Marsik et al., 2017 and Loos et al., 2013). However, a review paper reported a wide range of concentrations reaching μg·L^{-1} (Tiedeken et al., 2017). Concentrations of psychoactive drugs ($n = 20$) were rather small (hundreds of pg L^{-1} or single ngL^{-1}) with the exception of carbamazepine. Seven illicit drugs were determined, whereas cocaine, and its metabolite benzoylecgonine, and methadone were detected at 8 sites and 3,4-methylenedioxymethamphetamine (MDMA) was detected at 7 sites. Similar levels of cocaine were reported in Czech and Welsh rivers by Fedorova et al. (2014) and Kasprzyk-Hordern et al. (2008), respectively.

3.2. In vitro bioassays

The responses of SPMD blank sample extracts did not significantly differ from the response of solvent controls in all *in vitro* bioassays used in the present study. Minor estrogenic and dioxin-like activities were observed for POCIS blank sample extracts. Effect equivalents of these responses were subtracted from respective effect equivalents detected in POCIS samples. None of the SPMD or POCIS extracts elicited cytotoxicity up to the greatest tested concentration in any of the used mammalian cell lines (0.5% v/v SPMD or POCIS extract mL^{-1}). An overview of *in-vitro* bioassay results in pg L^{-1} is given in Table 2 (results in pmol L^{-1} are shown in SM2-Table S5), and more details are reported in SM2-Table S6. REP values used for mass balance calculations were available for anti-androgenicity (12 compounds), estrogenicity (8 compounds) and dioxin-like potency (16 compounds). No REPs were available for androgenicity. Complete mass balance calculations, REPs with literature references and resulting contributions of each compound to the observed potencies in bioassays are shown in SM2-Table S7.

3.2.1. AR-mediated potency

Androgenic potencies greater than LOQ (DHT-EQ. 1×10^{-2}–3.5 pg L^{-1} and 0.59–55 pg L^{-1} for SPMD and POCIS, respectively) were detected at sites S2 and S3. Concentrations of DHT-EQs detected in extracts of SPMDs were 10 pg L^{-1} (S2) and 4.2 pg L^{-1} (S3). Androgenic potency detected in POCIS extracts was almost two orders of magnitude greater (DHT-EQ. 1.7×10^3 and 2.1×10^2 pg L^{-1} at sites S2 and S3, respectively). The DHT-EQ concentrations detected in POCIS extracts

Table 2
BEQ_{bio} and BEQ_{chem} values in pg L^{-1} for SPMD and POCIS extracts determined in *in vitro* bioassays and chemical analyses. Percentage of effect that can be explained by the detected chemicals is given in parentheses. The uncertainty of BEQ_{bio} is expressed with standard deviation (n = 2).

Bioassay	Sampler	Sampling site	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
			Spring of Bosna	Sarajevo, DS	Visoko, US	Visoko, DS	Lasva confluence, US	Zepce, US	Maglaj, US	Doboj, US	Modrica, US	Modrica, DS
AR: DHT-EQ	SPMD	BEQ_{bio}	$<2.8 \times 10^{-1}$	10 ± 2.8	4.2 ± 4.7	<3.5	<2.5	$<1 \times 10^{-1}$	$<1.5 \times 10^{-1}$	$<1.6 \times 10^{-1}$	$<2.8 \times 10^{-1}$	$<1 \times 10^{-2}$
	POCIS	BEQ_{bio}	$<5.9 \times 10^{-1}$	$1.7 \times 10^{3} \pm 1 \times 10^{3}$	$2.1 \times 10^{2} \pm 1.8 \times 10^{2}$	<55	<53	<1.1	$<9.5 \times 10^{-1}$	<38	<41	<2.7
Anti-AR: FLU-EQ	SPMD	BEQ_{bio}	$<4 \times 10^{3}$	$<4.2 \times 10^{3}$	$<3.6 \times 10^{3}$	$5 \times 10^{4} \pm 1.2 \times 10^{4}$	4.5×10^{4}	4.0×10^{4}	$<5.6 \times 10^{3}$	3.4×10^{4}	$<5.4 \times 10^{3}$	4.3×10^{4}
		BEQ_{chem}	3	20	20	20 (0.04%)	10 (0.03%)	20 (0.05%)	20	10 (0.03%)	10	20 (0.05%)
	POCIS	BEQ_{bio}	$<2.0 \times 10^{5}$	$<2.3 \times 10^{5}$	$<2.1 \times 10^{5}$	$<3.4 \times 10^{5}$	$<3.3 \times 10^{5}$	$<3.3 \times 10^{5}$	$3.2 \times 10^{6} \pm 9.9 \times 10^{5}$	$3.1 \times 10^{6} \pm 6.4 \times 10^{5}$	$<2.3 \times 10^{5}$	$2.8 \times 10^{6} \pm 7.2 \times 10^{5}$
		BEQ_{chem}	2×10^{2}	4×10^{3}	3×10^{3}	2×10^{3}	1×10^{3}	2×10^{3}	2×10^{3} (0.06%)	2×10^{3} (0.06%)	1×10^{3}	1×10^{3} (0.04%)
ER: E2-EQ	SPMD	BEQ_{bio}	$<3.7 \times 10^{-1}$	$<3.9 \times 10^{-1}$	$<7.8 \times 10^{-1}$	<1.0	$<2.7 \times 10^{-1}$	$<2.3 \times 10^{-1}$	$<1.6 \times 10^{-1}$	$<1 \times 10^{-1}2$	$<2.7 \times 10^{-1}$	$<1.7 \times 10^{-1}$
		BEQ_{chem}	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	POCIS	BEQ_{bio}	<30	5.4×10^{2}	$2.5 \times 10^{3} \pm 2.3 \times 10^{3}$	$1.1 \times 10^{3} \pm 1.2 \times 10^{2}$	$1.1 \times 10^{3} \pm 8.8 \times 10^{2}$	$9.2 \times 10^{2} \pm 8.7 \times 10^{2}$	1.6×10^{3}	3.3×10^{2}	2.3×10^{2}	<70
		BEQ_{chem}	10	1.7×10^{3} (305%)	9.88×10^{2} (40%)	8.7×10^{2} (82%)	10 (0.84%)	3.9×10^{2} (43%)	2.1×10^{2} (14%)	1.9×10^{2} (59%)	1.5×10^{2} (64%)	1.0×10^{2}
AhR: TCDD-EQ	SPMD	BEQ_{bio}	$<7.9 \times 10^{-1}$	6.9 ± 1.4	$5.9 \pm 6.8 \times 10^{-1}$	$6.2 \pm 5.5 \times 10^{-1}$	$5.2 \pm 8.8 \times 10^{-1}$	3.6 ± 1.7	$2.9 \pm 5 \times 10^{-2}$	$3.2 \pm 1.2 \times 10^{-1}$	7.3 ± 3.1	4.7 ± 2.2
		BEQ_{chem}	7×10^{-2}	5.6×10^{-1} (8.2%)	7.2×10^{-1} (12%)	5.6×10^{-1} (9.1%)	4.1×10^{-1} (7.8%)	6.1×10^{-1} (18%)	7.0×10^{-1} (24%)	4.5×10^{-1} (14%)	4.4×10^{-1} (6.1%)	5.5×10^{-1} (12%)
	POCIS	BEQ_{bio}	$<1.2 \times 10^{2}$	$2.2 \times 10^{2} \pm 1.8 \times 10^{2}$	96 ± 53	<76	31 ± 5.2	82 ± 13	<12	<12	<28	<17
		BEQ_{chem}	1×10^{-3}	3.0×10^{-2} (0.02%)	2.0×10^{-2} (0.02%)	1.0×10^{-2}	1.0×10^{-2} (0.04%)	1.0×10^{-2} (0.02%)	7.0×10^{-2}	6.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}

were consistent with the data reported in earlier studies addressing river water (König et al., 2017; Tousova et al., 2017) or WWTP effluents (Bain et al., 2014; Jalova et al., 2013).

Anti-androgenic potencies greater than the LOQ (FLU-EQ. 1.2×10^3 – 5.6×10^3 pg L^{-1}) were detected in extracts of SPMD at 5 sites (S4, S5, S6, S8, S10) and FLU-EQ ranged between 3.4×10^4–5.1×10^4 pg L^{-1}. Based on the potency balance, 0.03–0.05% of the response in the bioassay could be explained and benzo[*a*]pyrene was identified as the major contributor (REP was available for only 2 compounds). In the case of extracts of POCIS, 3 sites (S7, S8, S10) exhibited measurable potencies, with FLU-EQs of 3.2×10^6, 3.1×10^6 and 2.8×10^6 pg L^{-1}, respectively (LOQ 1.4×10^5–3.4×10^5 pg L^{-1}). Approximately from 0.04–0.06% of the observed anti-androgenicity could be explained and diazinon was the primary contributor (REP was available for 10 compounds). The anti-androgenic potency of river waters determined by use of passive sampling was reported in earlier studies (Jálová et al., 2013; Liscio et al., 2014). Anti-androgenic effects were observed more frequently in SPMDs, which is consistent with previous results (Creusot et al., 2013). Concentrations of anti-androgenicity measured in river water affected by untreated waste water, by use of large volume SPE and MDA-kb2 cells (König et al., 2017), were 10-fold less than the concentrations in extracts of POCIS observed during the present study. Similar to our results, only a minor portion of the observed anti-androgenicity (up to 3%) could be explained by targeted chemicals (König et al., 2017). When the yeast androgen screen assay (YAS), was used to measure anti-androgenicity of river water potency measured in extracts of SPMDs were almost 100-fold greater than those observed in extracts of POCIS (Liscio et al., 2014 and Chen and Chou 2016). More than 31 compounds were identified that could have contributed to the observed anti-androgenicity (Liscio et al., 2014). These chemicals accounted for >50% of potency observed in the bioassay. This list of compounds included several pharmaceuticals with confirmed anti-androgenic potency in the YAS (*i.e.* carbamazepine, citalopram, codeine, diclofenac, diltiazem, irtesartan, trimethoprim and venlafaxine), which were also detected in our study and could therefore possibly contribute to the anti-androgenic potency (REPs for MDA-kb2 not available). The main drivers of anti-androgenicity were not identified since the major portion of the effect could not be explained by target chemicals.

3.2.2. ER-mediated potency

No estrogenic potency was detected at concentrations greater than the LOQ (E2-EQ. 0.12–1 pg L^{-1}) in any of the SPMD extracts. POCIS extracts elicited estrogenic potency above the LOQ (E2-EQ. 30–1.1×10^2 pg L^{-1}) at 8 sampling sites (S2–S9) and the E2-EQ ranged from 2.3×10^2 to 2.5×10^3 pg L^{-1}. S3 was the site with greatest estrogenic potency. These results are consistent with those reported for surface waters during previous studies (Jalova et al., 2013; Jarosova et al., 2012; Jugan et al., 2009; Tousova et al., 2017). Potency balance calculations revealed that 0.84–305% of the estrogenicity could be explained by target compounds (REP was available for 8 compounds), dominated by natural estrogens (E1, E2 and E3). This is in line with earlier findings of Miège et al. (2009), who identified estrogens as the main drivers of estrogenicity in river water. Neale et al. (2015), who assessed estrogenicity in 22 river water samples, reported that 0.31 to 61% of the observed potency could be explained by target chemicals and that E1 along with a phytoestrogen genistein were the main drivers. At site S2 (Sarajevo DS), with BEQ_{chem} based on detected estrogens exceeding the BEQ_{bio} by >300%, antiestrogenic effects might be present and mask part of the estrogenic potency elicited by the detected estrogens. Antiestrogenic effects have been commonly detected in river waters and they are believed to result from the combined action of a multitude of chemicals present in complex environmental mixtures (Gehrmann et al., 2016; Oh et al., 2006).

3.2.3. AhR-mediated potency

AhR-mediated (dioxin-like) potency was detected in all SPMD extracts except for site S1. The TCDD-EQ ranged from 2.9 to 7.3 pg L^{-1} (LOQ 0.4–0.84 pg L^{-1}). These concentrations are consistent with previously reported potencies measured in SPMD extracts of WWTP effluents and river water (Jálová et al., 2013). Potency balance calculations identified benzo[*b*]fluoranthene, benzo[*k*]fluoranthene and chrysene as the main drivers of the observed potencies, with detected compounds explaining 6.1–24% of the potencies measured in the CAFLUX assay. REP values were available for 9 compounds. Similarly, 2–10% of the dioxin-like potency detected in SPMDs deployed in a drinking water reservoir in China could be explained by analysis of PAHs and PCBs (Wang et al., 2014). According to their results, the contribution of PCBs to the overall potency was negligible, which is consistent with the findings in the study, results of which are reported here. POCIS extracts showed AhR-mediated potency at 6 sites (S1, S2, S3, S4, S5 and S6) with TCDD-EQ. 31–2.2×10^2 pg L^{-1} (LOQ 12–1.2×10^2 pg L^{-1}). The greater frequency of AhR mediated potency detected in extracts of SPMD compared to POCIS samplers was observed previously in an earlier study (Creusot et al., 2013). Concentrations detected in POCIS extracts were as much as 10-fold greater than AhR-mediated potency observed in extracts of POCIS, collected in headwaters in the Czech Republic (Jarosova et al., 2012) or in large volume SPE samplers placed in the Danube River (König et al., 2017; Neale et al., 2015). Only 0.02–0.04% of the effect detected in POCIS could be explained and propiconazole contributed most to potency measured in the bioassay, REPs were available for 7 compounds. In the Danube River, 3–71% of detected AhR potency could be explained by three chemicals, daidzein, terbuthylazine and carbaryl (Neale et al., 2015). Concentrations of terbuthylazine and carbaryl in extracts of the POCIS were less than the LOQ or small. Daidzein, a natural isoflavone, was not analyzed in our study. Significant amounts of AhR-mediated potency in river waters has been reported to be of anthropogenic origin, particularly from treated and untreated wastewater (Long and Bonefeld-Jørgensen, 2012). Potential contributors could include also polar derivatives of PAHs, some pharmaceuticals, CUPs or other weakly or moderately polar natural compounds. A larger list of target compounds associated with REP values of particular compounds is needed for successful identification of the main AhR-mediated potency drivers.

3.3. Contamination profiling

A profile of integrated effects of mixtures at various locations in the Bosna, based on potencies observed in the three bioassays (Table 2) for extracts of the two types of sampler at each location were developed based on comparison to the reference site (S1), which was defined as the location least affected by direct and indirect inputs from human activities, including urbanization and industrialization. Contamination indices (CI), the ratio between the response of downstream sites (S2–S10) and a reference site (S1), in combination with the overall cumulative concentration and number of detected target compounds for each sampling site are shown in Fig. 2. None of the sampling sites downstream of S1 can be considered as uncontaminated as the reference site because all sites exceeded a CI of 1.0 for at least two endpoints. The CI profiles, as well as the cumulative concentration and the number of detected hydrophobic compounds, differed less between individual sites in extracts of SPMD than in extracts of POCIS. Extracts of POCIS from S1 elicited dioxin-like potency, which exceeded the response in the extract from site S5, which resulted in a CI of 0.36. Contamination indices indicate that the most contaminated sites were S2 and S3. The greatest cumulative concentrations and numbers of detected compounds were observed for the extract of the POCIS at S3. A complete analysis for S2 was not available. This result implies that the city of Sarajevo constituted the major source of contaminants relevant for the observed AhR-mediated potency. The trend of decreasing cumulative concentrations

in extracts of POCIS samplers downstream of S3 cannot be clearly seen in CIs and no patterns between CIs and cumulative concentrations in extracts of SPMD were observed, despite extensive, multi-residue analyses. Observed potencies could not be assigned to specific compounds, which is a common case for complex environmental matrices (Weller, 2012). Novel approaches proposed for future monitoring schemes, including a combination of non-target identification of chemicals (Peng et al., 2016), screening of effects (Sun et al., 2017, 2016), mixture toxicity modelling and effect-directed analysis (Altenburger et al., 2015) will facilitate identification of compounds responsible for adverse effects in aquatic ecosystems.

3.4. Hazard assessment

Hazard quotients could not be calculated for all compounds. NORMAN lowest PNEC values were available for 167 of 168 target compounds (99.4%). Concentrations of 7 compounds exceeded PNECs at least at 2 sampling sites (Table 3). Hazard quotient (HQ) of the insecticide, diazinon, exceeded 1.0 at all 9 sites, where it was detected and its HQs, which were as great as 4.4, were greatest of all compounds. HQs of diclofenac, a NSAID, and two estrogens, E1 and E2 2, exceeded HQ of 1.0 at 2 sites. In this study, the PNEC for EE2 was less than its LOQ so no HQ could be calculated. Therefore, EE2 might still pose a potential risk to aquatic biota even though it was never detected. In the class of PAHs, HQs of benzo[*b*]fluoranthene exceeded 1.0 at 6 sites and those of fluoranthene and benzo[*k*]fluoranthene exceeded 1.0 at 2 sites. Complete results of hazard assessment and a list of the lowest NORMAN PNEC values are shown in SM2- Table S8. In a previous study that applied a similar methodology of assessment of samples collected in 4 European river basins, diazinon, diclofenac and fluoranthene were also identified and prioritized as most hazardous (Tousova et al., 2017). Diazinon was ranked among the most hazardous compounds in several Iberian, North European and US rivers (Kuzmanovic et al., 2014; von der Ohe et al., 2011). Diclofenac was identified as a driver of hazard in Greek rivers (Thomaidi et al., 2015 and Kosma et al., 2014). Those authors also found several compounds from the class of antibiotics, including sulfamethoxazole, trimethoprim and clarithromycin to exceed HQs of 1.0 to aquatic biota. These antibiotics were also detected in the study, results of which are presented here. However, their concentrations did not exceed their, respective PNECs. Of PAHs, benzo[*k*]fluoranthene (Smital et al., 2013) and fluoranthene (Von der Ohe et al., 2011) have been prioritized previously the most hazardous. The overall hazard index (HI), resulting from the summation of all HQs at each sampling site, indicates that all sites downstream of the reference site S1 might cause adverse effects to aquatic biota as their HIs exceed 1.0.

3.5. Limitations of the research and its environmental implications

Beside advantages of passive sampling techniques compared to grab sampling techniques such as time integrative sampling of bioavailable contaminants and lower achievable detection limits, passive sampling also suffers from several limitations. As mentioned earlier, toxic potencies measured in passive samplers can be translated into equivalent toxic potencies in water only when making assumptions of fully integrative uptake of all compounds present in the sampled mixtures and when calculations are done with averaged sampling rates over a broad range of compound properties. These approximations are necessary since the identity of compounds causing the observed effects remains largely unknown. The application of models for improvement of measurement accuracy that relate sampling parameters to physicochemical compound properties is thus precluded. The approximation of sampling parameters is ultimately associated with an increased uncertainty of reported data. The uncertainty of SPMD-derived aqueous concentrations is generally lower than that of POCIS data, since for SPMD site specific sampling rates can be derived using PRC approach, and sampling rates of nonpolar compounds sampled by SPMDs only weakly depend on molecular structure (Lohmann et al., 2012). In our study, only a limited set of five deuterated PAHs as PRCs was applied for estimation of SPMD sampling rates. The accuracy of estimation can be improved by application of a broader range of PRC compounds, as has been shown by Booij and Smedes (2010). POCIS data has to be considered semi-quantitative since the uptake mechanism of polar compounds from water is not fully understood, PRC approach cannot be applied for *in situ* sampling rate correction, and also a larger variability of sampling rates on physicochemical compound properties and environmental factors (water flow, pH, temperature) is expected than for SPMDs (Miège et al., 2015). The application of a single constant POCIS sampling rate value of 0.2 $L\,d^{-1}$ for all compounds was thus chosen as a compromise in a situation when the effect of environmental variables and compound properties on sampling rate could not be fully controlled or quantified. The elevated measurement uncertainty can be accepted as long as it is lower than the variability of environmental concentrations, which may be dramatic in dynamic river systems such as the Bosna river investigated in our study. The obtained semi-quantitative data cannot be, in general, directly applied for checking compliance with environmental quality criteria, however, they are very suitable for screening of areas and pollutants of concern and identification of areas, where a focused monitoring can be performed at a later stage using conventional monitoring methods.

4. Conclusions

The study which assessed water quality of the Bosna River found concentrations of contaminants or observed potency of mixtures that

Table 3
Target compounds with hazard quotient (HQ) values exceeding 1 (in bold) and overall hazard index (HI) at individual sampling sites.

Compound name	PNEC [ng L^{-1}]	S1 Spring of Bosna	S2 Sarajevo, DS	S3 Visoko, US	S4 Visoko, DS	S5 Lasva confluence, US	S6 Zepce, US	S7 Maglaj, US	S8 Doboj, US	S9 Modrica, US	S10 Modrica, DS	Frequency of PNEC exceedance [%]
17β-estradiol	4.0×10^{-1}	<LOD	**2.0**	**1.1**	7.5×10^{-1}	<LOD	3.0×10^{-1}	1.8×10^{-1}	1.5×10^{-1}	1.3×10^{-1}	1.0×10^{-1}	20
Benzo[*b*] fluoranthene	1.7×10^{-1}	<LOD	**1.2**	**1.3**	**1.2**	8.5×10^{-1}	**1.2**	**1.6**	9.0×10^{-1}	9.0×10^{-1}	**1.7**	60
Benzo[*k*] fluoranthene	1.7×10^{-1}	<LOD	8.8×10^{-1}	**1.2**	8.6×10^{-1}	6.0×10^{-1}	9.7×10^{-1}	**1.1**	6.8×10^{-1}	7.1×10^{-1}	7.4×10^{-1}	20
Diazinon	$1.2 \times 10^{+1}$	<LOD	**4.4**	**2.2**	**2.2**	**2.5**	**3.7**	**3.6**	**2.7**	**1.6**	**1.6**	90
Diclofenac	$5.0 \times 10^{+1}$	<LOD	n.a.	**1.3**	**1.6**	9.3×10^{-1}	6.9×10^{-1}	5.6×10^{-1}	4.0×10^{-1}	3.1×10^{-1}	2.0×10^{-1}	22
Estrone	3.6×10^{0}	<LOD	**1.6**	7.0×10^{-1}	**1.1**	<LOD	2.3×10^{-1}	1.4×10^{-1}	1.3×10^{-1}	1.0×10^{-1}	8.1×10^{-2}	20
Fluoranthene	6.3×10^{0}	1.3×10^{-2}	9.1×10^{-1}	9.3×10^{-1}	6.7×10^{-1}	5.0×10^{-1}	9.7×10^{-1}	9.5×10^{-1}	**1.2**	8.5×10^{-1}	**1.4**	20
Total hazard index		3.9E-01	1.5E + 01	1.5E + 01	1.4E + 01	9.7E + 00	1.3E + 01	1.3E + 01	1.0E + 01	7.9E + 00	1.0E + 01	

did not differ significantly from those measured in other European rivers, even in those areas with more advanced wastewater treatment technologies and infrastructure. Chemical analyses revealed frequent occurrences of pesticides, which were banned for use in plant protection products in the EU, *e.g.* diazinon, carbendazim, isoproturon, diuron, prometryn, metolachlor. Diazinon occurred at most sites at concentrations which might cause adverse effects on aquatic biota. These compounds are of concern and should be included in regular monitoring and possible mitigation measures. With the exception of estrogenicity, potencies of endocrine effects observed *in vitro*, by use of bioassays, could not be explained by targeted compounds. Natural estrogens were found to be largely responsible for the observed estrogenic potency. These results emphasize the need to apply bioassays as a complementary tool in routine monitoring of water quality, because chemical analysis alone cannot indicate effects elicited by mixtures of compounds occurring at small concentrations in environmental mixtures. It should be further enhanced in future studies by application of progressive approaches combining effect-based screening, modelling of mixtures responses and effect-directed analysis together with non-target identification of chemicals to prioritize the most relevant toxicants and effect drivers.

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Appendix A. Supplementary data

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Supplementary Materials 1 to:

Analytical and bioanalytical assessments of organic micropollutants in the Bosna River using a combination of passive sampling, bioassays and multi-residue analysis

Authors: Zuzana Toušová, Branislav Vrana, Marie Smutná, Jiří Novák, Veronika Klučárová, Roman Grabic, Jaroslav Slobodník, John Paul Giesy, Klara Hilscherová

SECTION 1: SPMD – Sample processing, instrumental and data analyses

SPMD samplers were cleaned by a stream of tap water from debris and mud, followed by drying with paper cloth. Pre-cleaned samplers were immersed in diluted hydrochloric acid (1 mol.L^{-1}) to remove the carbonates that precipitated from hard water during field exposure. The sampler was then washed by water, dried with acetone and placed into 250 mL glass container with a ground joint glass stopper. 100 mL of n-hexane was added to each sampler container to fully immerse the sampler in solvent. PAH surrogates (100-500 ng/sampler of D_8-naphthalene, D_{10}-anthracene, D_{10}-pyrene, D_{12}-benza(a)anthracene, D_{12}-benzo(k)fluoranthene, D_{12}-benzo(a)pyrene and D_{12}-benzo(g,h,i)perylene) PCB surrogates (10 ng/sampler of PCB4, PCB29 and PCB185) and D_6-lindane (100 ng/sampler) in hexane were spiked into each container (samplers intended for chemical analyses). The extraction was performed using dialysis, based on diffusion and equilibrium partitioning of compounds through the LDPE membrane into solvent. After 24 h the dialysates were transferred into clean marked sample bottles and the dialysis was repeated with another 100 mL hexane for further 24 hours. Dialysates were combined and evaporated to 10 mL at rotary vacuum evaporator at 40 °C. The extract was then further reduced in volume under stream of nitrogen and subjected to clean-up by gel permeation chromatography (GPC). The sample was processed by GPC to remove the remaining interferences such as triolein and sulfur prior to analysis by GC-MS. The sample (500 μL) was injected into GPC apparatus equipped with a fraction collector (ECOM, Prague, Czech Republic) and fractionated using a high performance SEC column (Agilent PL Gel 5 μm 50 Å, 7.5 x 300 mm). The mobile phase was dichloromethane (1 mL min^{-1}). The collected fraction containing the compounds of interest extended from 6.6-10.3 mL. The volume was reduced to 100 μL by stream of nitrogen. Sample was then reconstituted to 1 mL by n-hexane. SPMDs for chemical analyses were subjected to further cleanup on silica gel.

1.1 PAHs

1.1.1 Clean up for chemical analysis of PAHs

Each column contained 2 g of deactivated silica gel, which was prepared by drying at 120 °C for 8 hours and deactivated by 6 % water. The column was conditioned by flushing 25 mL of dichloromethane-hexane mixture (50/50, v/v). After application of sample to the column, analytes were eluted by 50 mL of the same solvent mixture. The eluate was evaporated to 10 mL by rotary vacuum evaporator at 40 °C and further reduced in volume by a stream of nitrogen to volume 500 µL. Terphenyl internal standard was added to the samples prior to GC-MS analysis of PAHs.

1.1.2 Instrumental analysis of PAHs

The analysis of PAHs was performed using 6890N GC (Agilent, USA) equipped with a 30m × 0.25mm × 0.25µm HP5-MS column (Agilent, USA) coupled to 5972 MS operated in electron impact ionization mode. The analysis of PAHs in samples was preceded by calibration in the range 0-1000 ng mL^{-1}. The working conditions: pulsed split-less injection of 2 µL at 250 °C; helium flowrate 1.9 mL/min constant flow; column temperature program was from 70 °C (2 min isothermally), then increase with rate 25 °C/min to 150 °C, then at 3 °C/min to 200 °C, then at 8 °C/min to 250 °C, then isothermally 20 minutes. The time of analysis was 51.87 min. The MS detector was set to 320 °C and 70 eV for EI. The measurements were done in single ion monitoring mode (SIM) and for each compound 2-3 characteristic ions were used for detection and quantification. The determination of compound in a sample was performed from the peak area for highest characteristic ion in mass spectrum of compound by external calibration method and concentration were corrected using recovery of surrogates, that were added to the sampler containers before extraction. Details of instrumental analysis, including retention times and characteristic ions for qualitative and quantitative analysis of PAHs are given in Klučárová et al. (2013).

1.2 PCBs and OCPs

1.2.1 Clean-up for chemical analysis of PCBs and OCPs

SPMD extracts for PCB analysis were cleaned up using columns containing 5 g of sulfuric acid modified silica gel. The modified silica gel was prepared by mixing 50 g of activated silica gel with 33 mL of 98% H_2SO_4. Analyte elution was performed using 40 mL of dichloromethane/hexane (50/50, v/v) mixture. The eluate was reduced in volume to 1 mL by a nitrogen stream. PCB121 internal standard was added to the samples prior to GC-MS analysis of PCBs.

1.2.2 Instrumental analysis of PCBs and OCPs

PCB analysis was performed using GC-MS/MS 6890N GC (Agilent, USA) equipped with a 60m x 0.25mm x 0.25µm DB5-MS column (Agilent J&W, USA) coupled to Quattro Micro GC MS MS (Waters, Micromass, Manchester, UK) operated in EI+ ionization mode. At least 2 MRM transitions were recorded for each

compound analyzed. Injection was done in split-less mode at 280°C and 1 µL sample was loaded. Helium was used as carrier gas at the flow of 1.5 mL min^{-1}. The column temperature program was from 80°C (1 min hold), then 15°C min^{-1} to 180°C, and finally 5°C min^{-1} to 300°C (5 min hold). Raw data were processed using TargetLynx software (Waters, Micromass, Manchester, UK). Further details of analysis of PCBs and OCPs are given in Vrana et al. (2014).

1.3 Calculation of SPMD sampling rates

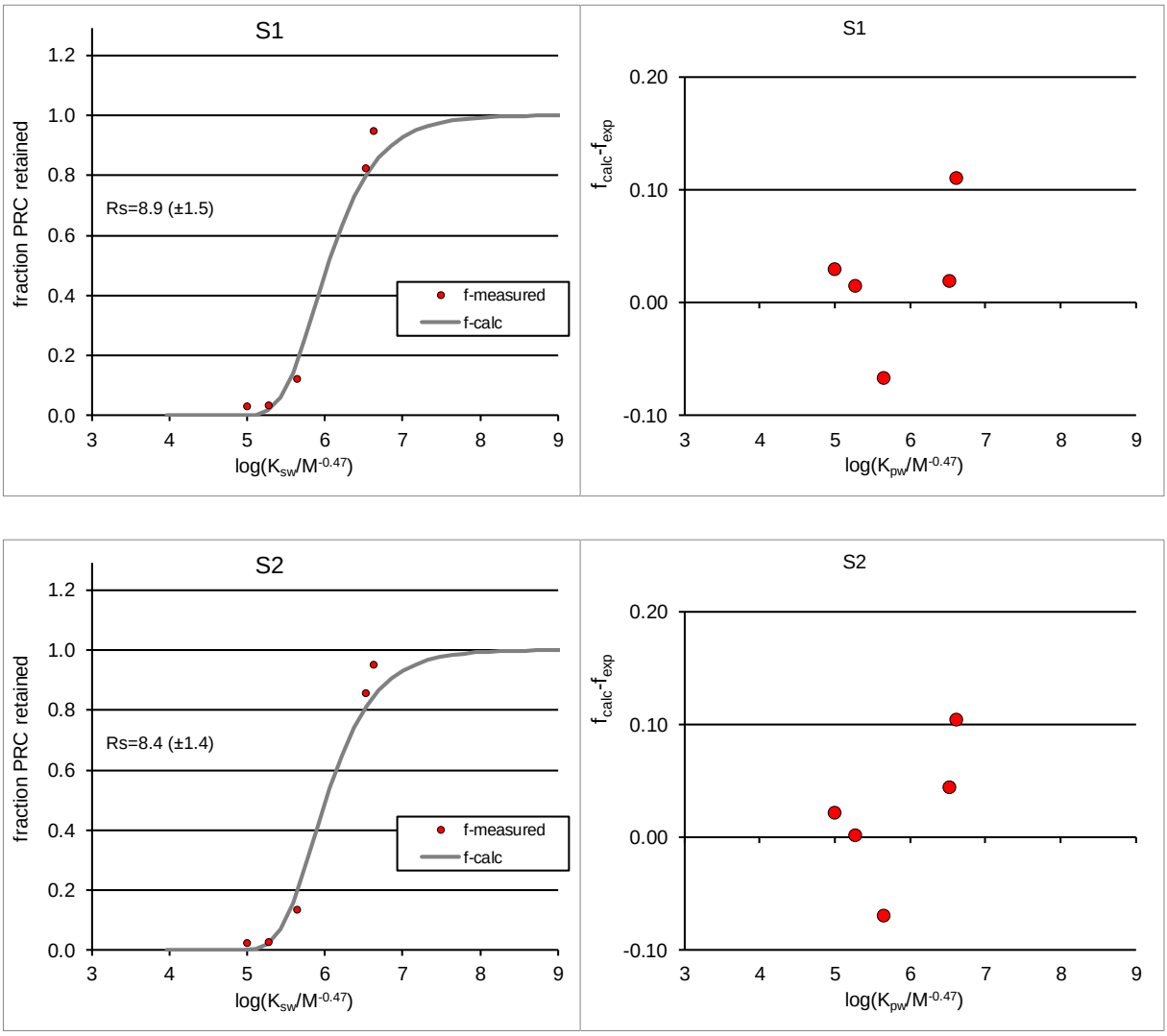


Figure S1. Sampling rate R_S estimation from PRC dissipation from SPMD during exposure in 10 deployments in the Bosna river, using nonlinear least squares method according to model described in 2.4.6. in the main text (left-hand graphs). The *f*(PRC) remaining in the SPMD after exposure were fitted by a continuous function of their K_{SW} and *MW* using a model described in detail in Vrana et al. (2014). The drawn lines represent the best nonlinear least- squares fit of the data. The number in the graph shows the $R_{s,300}$ for a model compound with a molecular mass of 300. The right hand-graph. K_{sw} values of PRCs applied in the calculations were 5.00, 5.28, 5.65, 6.54 and 6.63 for D_{10}-acenaphthene, D_{10}-

fluorene, D_{10}-phenanthrene and D_{10}-chrysene and D_{12}-benzo(e)pyrene, respectively. The K_{sw} values were calculated from logK_{ow} values using Equation 4 in Vrana et al. (2014).

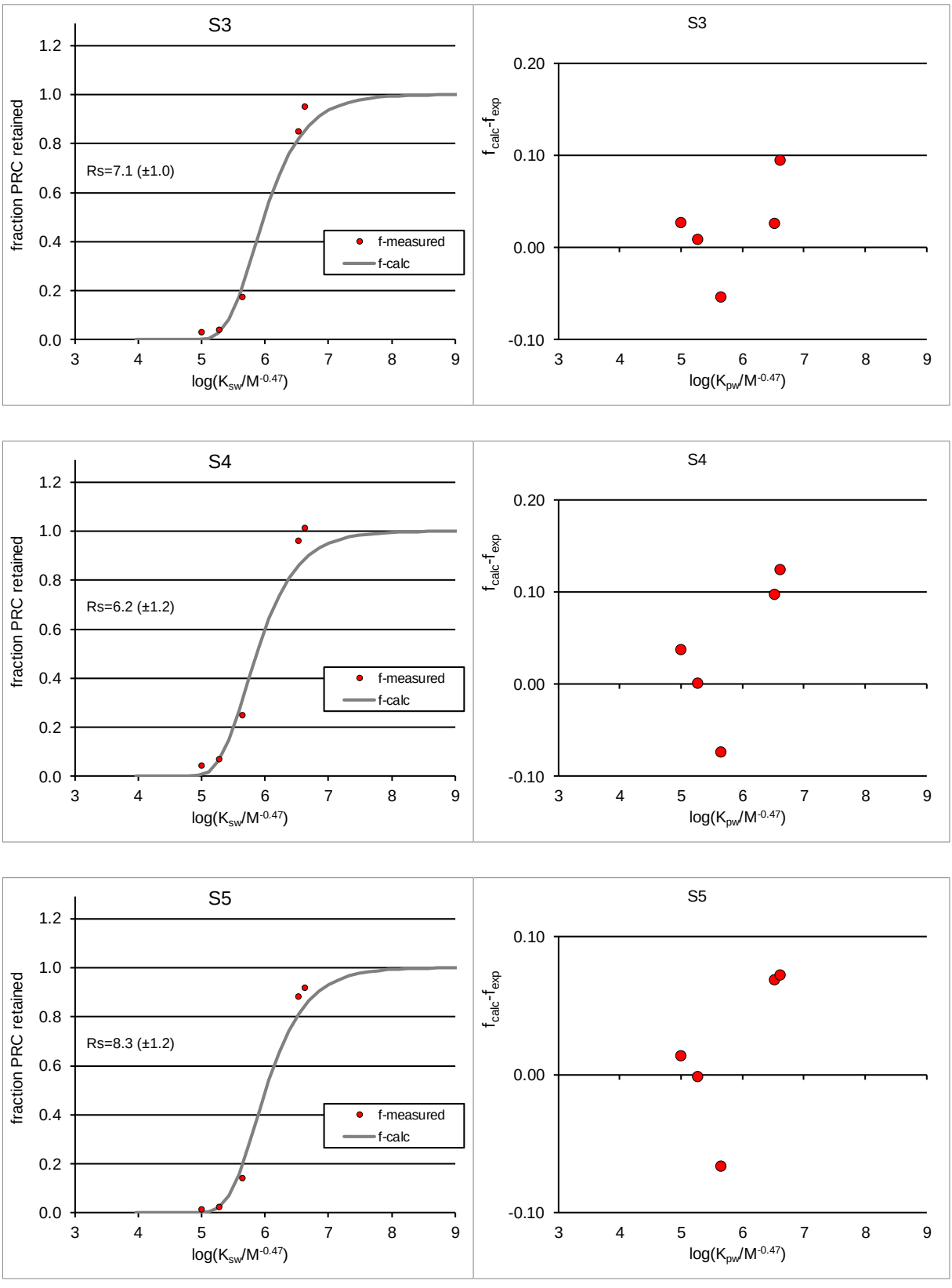


Figure S1 continued

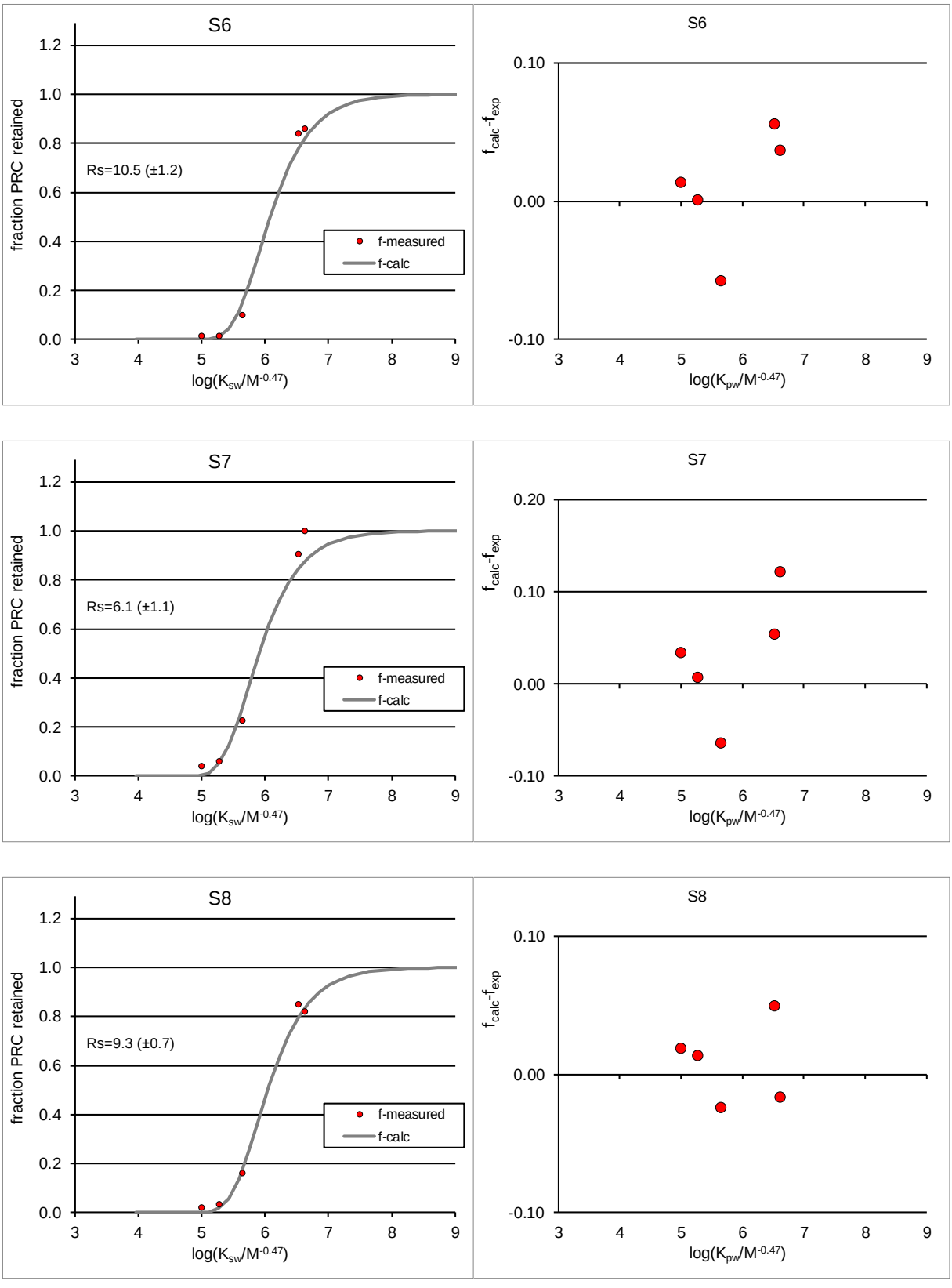


Figure S1 continued

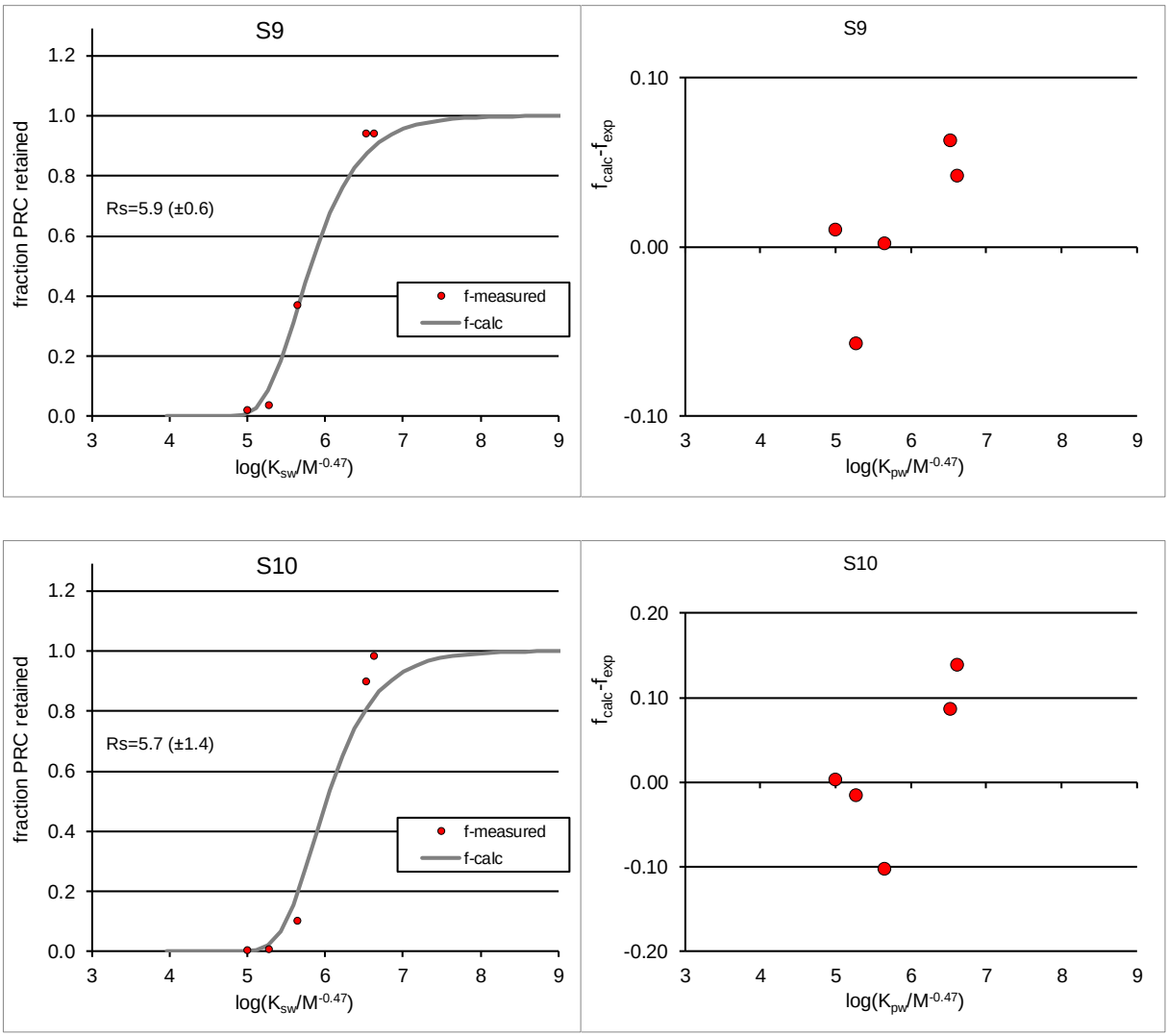


Figure S1 continued

Table S1 Results of sampling rate $R_{S,SPMD}$ estimation from PRC dissipation from SPMD during exposure in 10 deployments in the Bosna river, using nonlinear least squares method according to the procedure described in section 2.4.6 *Calculation of dissolved water concentrations from passive sampler data; SPMD* in the main text. The fraction of individual PRCs that remained in the SPMD after exposure was fitted by a continuous function of their K_{SW} and molar mass MW. Details of the procedure are described in Vrana et al. (2014).

Model output	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**
[1]*t* (d)	29	29	32	28	29	27	31	28	26	43
[2]estimated *FA*	129.2	122.4	103.6	90.5	121.6	154.0	89.6	135.6	86.5	83.2
Standard error of *FA*	21.8	20.4	14.5	18.2	18.2	17.9	15.8	10.6	9.0	20.0
Coef. Variation of *FA*	0.17	0.17	0.14	0.20	0.15	0.12	0.18	0.08	0.10	0.24
residual Std. error	0.067	0.067	0.058	0.089	0.060	0.045	0.076	0.031	0.048	0.097
Degrees of freedom	4	4	4	4	4	4	4	4	4	4
[3]$R_{s,300}$ (L d^{-1})	8.9	8.4	7.1	6.2	8.3	10.5	6.1	9.3	5.9	5.7
Std. error of $R_{s,SPMD}$	1.5	1.4	1.0	1.2	1.2	1.2	1.1	0.7	0.6	1.4
[4]V_w at MW=300 (L)	257	243	227	174	242	285	190	260	154	245

[1]Sampler exposure time

[2]fitting parameter *FA* from Equation 3 in: Vrana et al., 2014

[3]$R_{s,300}$ represents the sampling rate of a model compound with a molecular mass of 300.

[4]Estimated sampled volume of water for a compound with molecular mass of 300.

1.4 Comparison of SPMD-derived aqueous concentrations in our study 2013 with the survey data in 2008

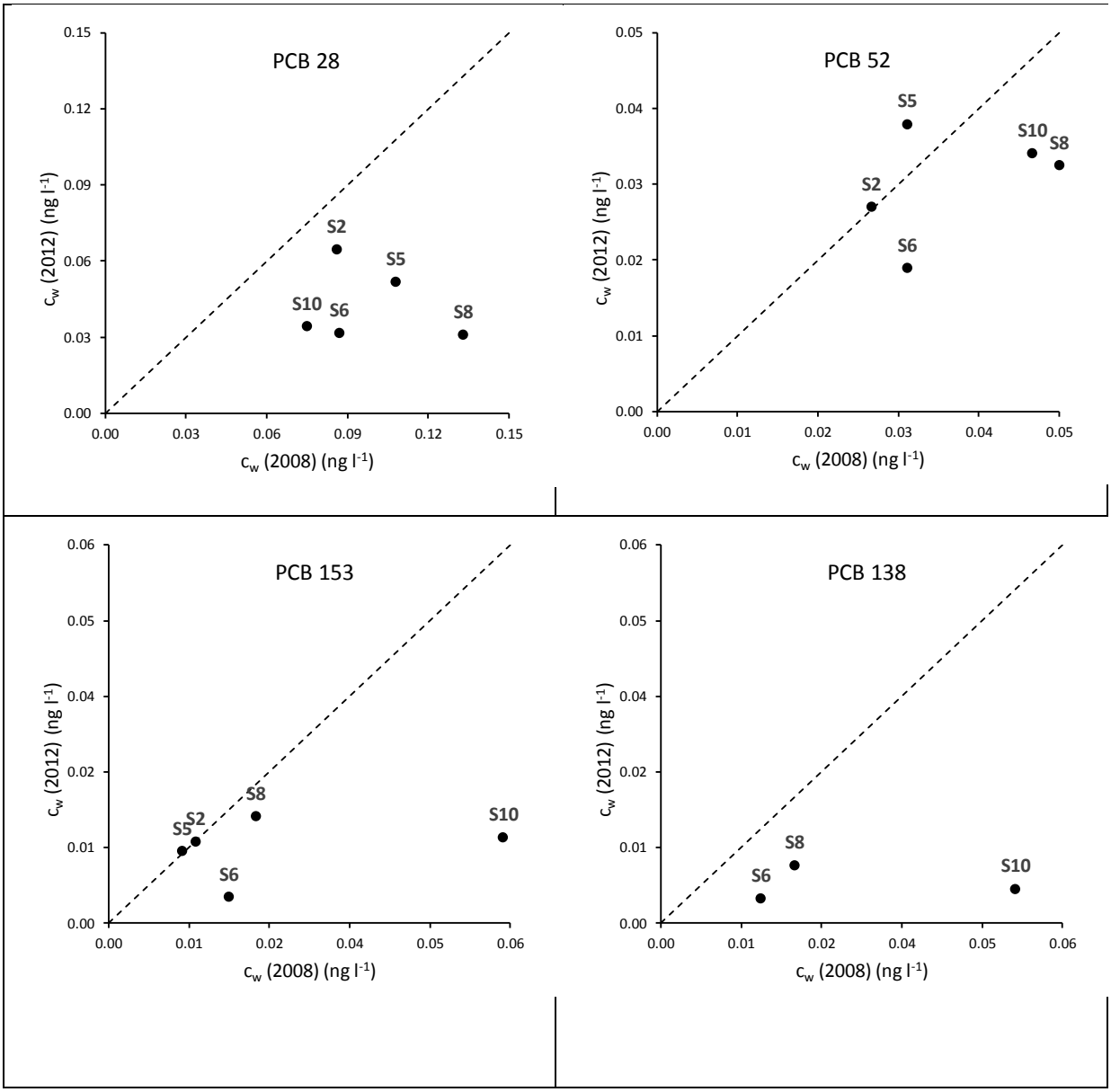
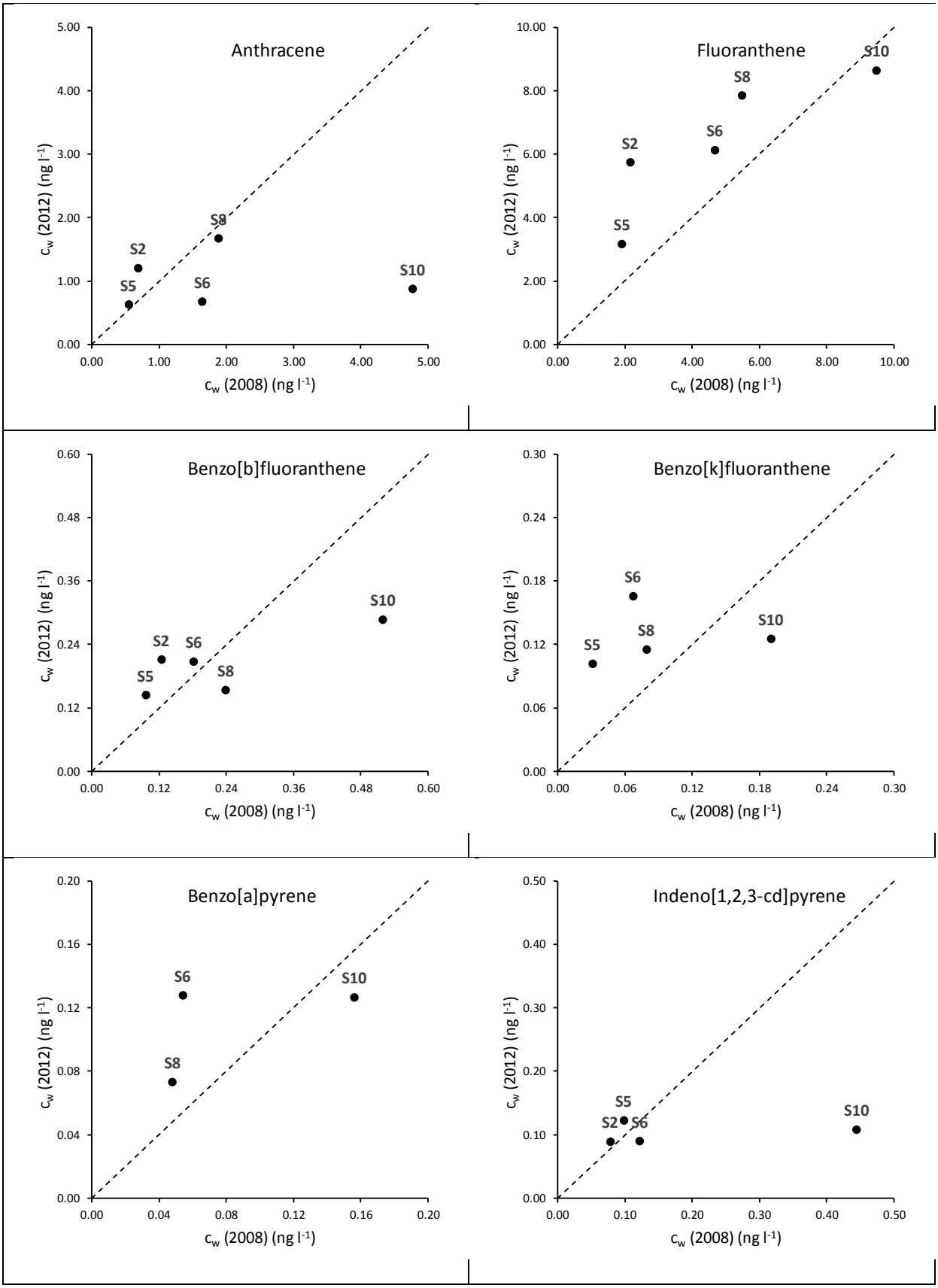


Figure S2. Comparison of SPMD-derived aqueous concentrations (C_w) of selected PCBs at 5 sites in Bosna river (y-axis) with data obtained using the same sampling technique in 2008 published by Harman et al. (2013) (x-axis). The dashed lines represent equality of the plotted variables. Comparison was possible only at 5 sites that were sampled during both sampling events.


Anthracene
Fluoranthene
Benzo[b]fluoranthene
Benzo[k]fluoranthene
Benzo[a]pyrene
Indeno[1,2,3-cd]pyrene
c_w (2012) (ng l^{-1})
c_w (2008) (ng l^{-1})
S2
S5
S6
S8
S10

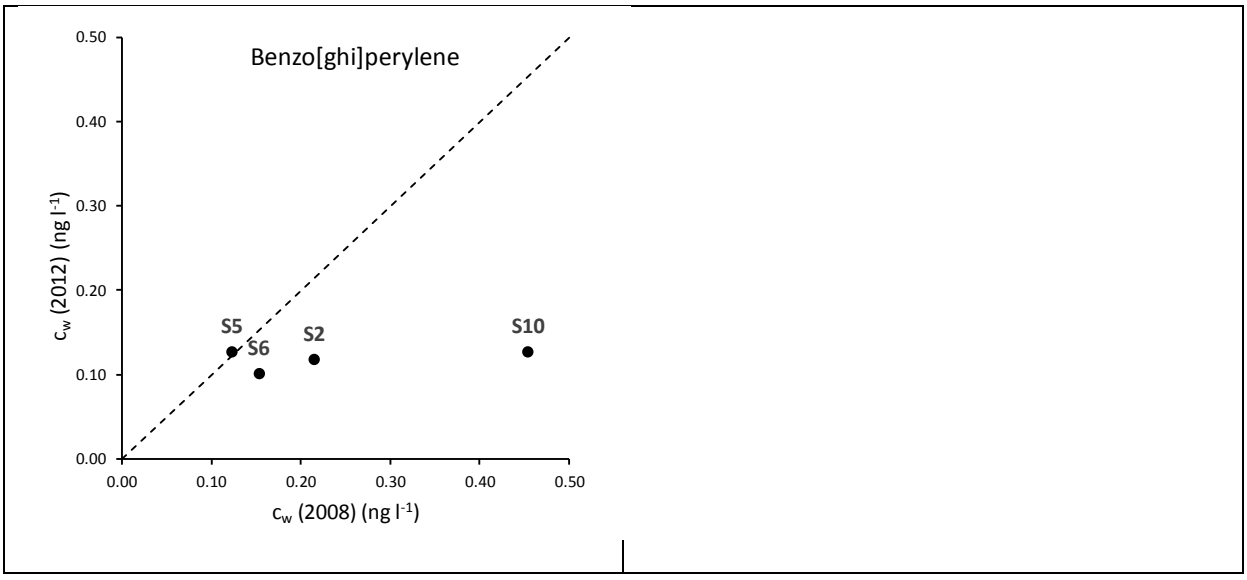


Figure S3. Comparison of SPMD-derived aqueous concentrations (C_w) of selected PAHs at 5 sites in Bosna river (y-axis) with data obtained using the same sampling technique in 2008 published by Harman et al. (2013) (x-axis).

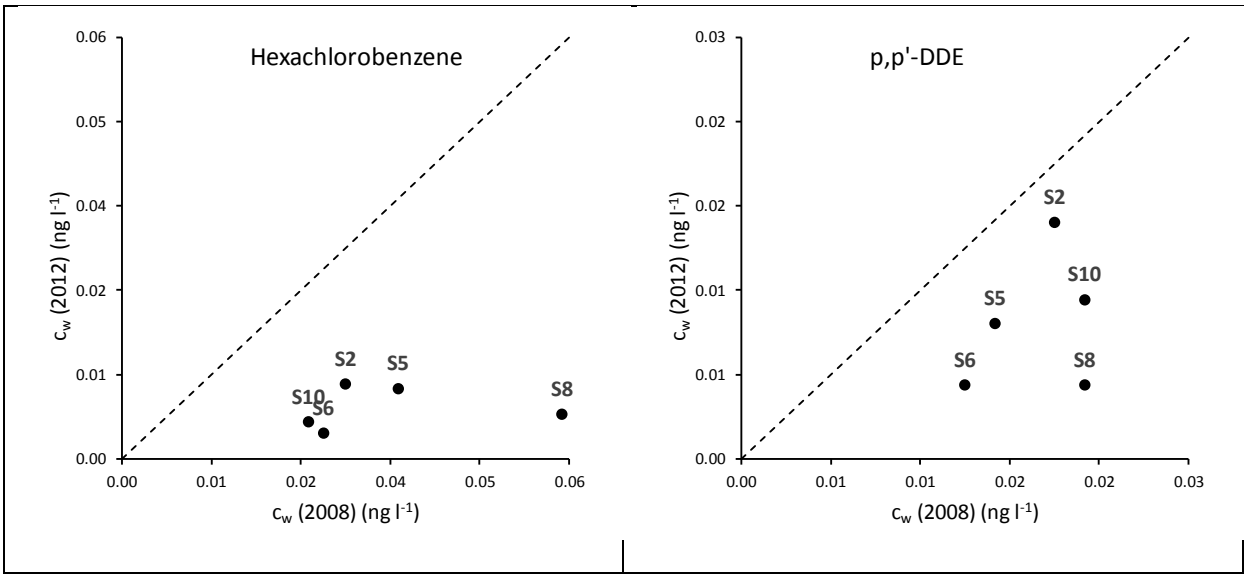


Figure S4. Comparison of SPMD-derived aqueous concentrations (C_w) of selected organochlorinated pesticides at 5 sites in Bosna river (y-axis) with data obtained using the same sampling technique in 2008 published by Harman et al. (2013) (x-axis).

SECTION 2: POCIS – Sample processing and instrumental analyses

2.1 CUPs

2.1.1 Instrumental analysis of CUPs

Analyses were performed with the HPLC apparatus Agilent 1290 series (Agilent Technologies, Waldbronn, Germany), which consisted of a vacuum degasser, a binary pump, a thermostated autosampler (10°C), and a thermostated column compartment kept at 30°C. The column was Phenomenex Synergi Fusion C-18 endcapped (4 µm) 100 x 2 mm i.d., equipped with Phenomenex SecureGuard C18 guard column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 5 mM ammonium acetate in water (A) and 5 mM ammonium acetate in methanol (B). The binary pump gradient was non-linear (increase from 20% B at 0 min to 80% B at 1 min, then increase to 90% B at 5 min, then 90% B for 8 min and 5 min column equilibration to initial conditions (20% B)); the flow rate was 0.25 mL/min. 10 µL of individual sample was injected for the analyses. The mass spectrometer was an AB Sciex Qtrap 5500 (AB Sciex, Concord, ON, Canada) with electrospray ionization (ESI). Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 5.5 kV; desolvation temperature, 400°C; Curtain gas 15 psi, Gas1 40 psi, Gas2 30 psi. In Scheduled MRM mode m/z transitions presented in Table S1 were monitored. Quantifications of analytes were based on isotopically labelled internal standards. Chromatographic retention times, mass spectrometer parameters and instrumental limits of quantification (LOQ) are given in Brumovský et al. (2016).

2.2 Estrogens

2.2.1 Materials and chemicals for analysis of estrogens

Acetonitrile (99.9 %), formic acid (98 %), 17α-ethinylestradiol (98 %), 17β-estradiol (98 %), 17α-estradiol (98 %), estriol (99.3 %), estrone (99.3 %), dansyl chloride (99 %), sodium bicarbonate, sodium hydroxide, methanol (99,9%) (purchased from Sigma-Aldrich, Germany), acetone (99.8 %), dichloromethane (99.8 %), n-hexane (95 %) (purchased from LAB-SCAN, Poland), deuterated standards - 17α-ethynylestradiol-2,4,16,16-d4 (98.8%, CDN-Isotopes, Canada), 17β-estradiol-2,4,16,16-d4 (99% CDN-Isotopes, Canada), 16α-hydroxy-17β- estradiol-2,4-d2 (98.4%, CDN-Isotopes, Canada), redistilled water (Osmonic 2, Czech republic);

SPE Vacuum Manifold (J. T. Bakter Inc.) and Florisil Strata cartridges FL-PR, 500 mg, 3 mL (Phenomenex) were used for the extraction experiments and cleaning process.

2.2.2 Extraction of POCIS samplers for analysis of estrogens

Oasis HLB sorbent was spiked with EE2-d4, which was used as a surrogate standard for assessment of the efficiency of the sample processing. For elimination of organic contamination, the sorbent was washed with 7.5 mL of solvent, methanol : water (40:60) and 7.5 mL redistilled water. The accumulated estrogens were recovered by elution with 50 mL acetone. The sorbent was dried and weighted. The extract was reduced to 1 mL by TurboVap and evaporated to dryness using a stream of nitrogen. Dried sample was dissolved in 1 mL of solvent, DCM : hexane (50:50).

2.2.3 Purification of extract from sorbents

Purification was performed through a Florisil cartridge (500 mg, 3 mL) as a follows: the extract of sorbents was percolated and elution was achieved with 5 mL acetone : hexane (75:25). Before elution, the cartridge was activated with DCM : hexane (50:50). Purified samples were evaporated to near-dryness under a stream of nitrogen and transferred into mini vials.

2.2.4 Derivatization

Ionization efficiency of estrogens can be improved by converting them into suitable derivatives, which allows quantification in lower concentration range. Precolumn derivatization technique according to Lin et al. (2007) was performed as follows (Figure S1). The solution of estrogens in acetone (20 µL) and $NaHCO_3$ (50 µL, 100 mmol·L^{-1}, pH 10.5, NaOH) was mixed (Vortex, 1 min). Dansyl chloride in acetone (50 µl, 1mg·ml^{-1}) was added to the solution. After the incubation (3 min, 60 °C) and evaporation to dryness under a stream of nitrogen, the sample was dissolved in 40 % methanol.

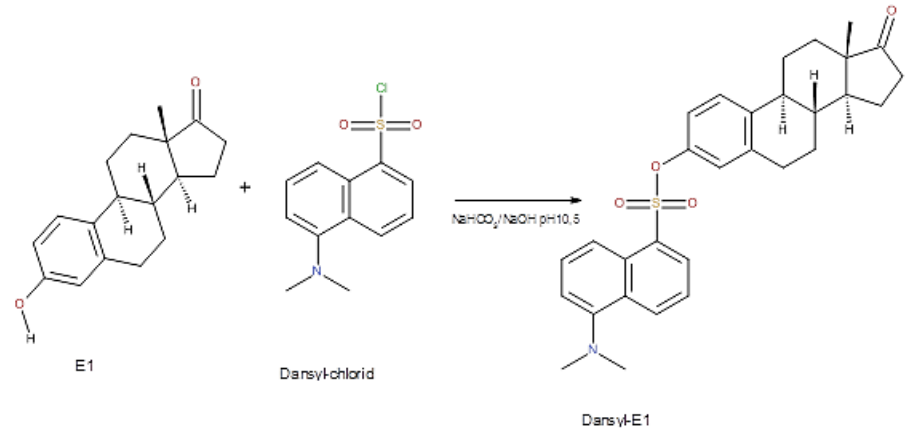


Figure S5: Derivatization of estrogens with dansyl-chloride

2.2.5 Chromatographic and MS-MS conditions

Analysis of estrogens was performed using LC (HPLC Agilent 1200 Series) with mass spectrometry (MS-MS Agilent 6410 Triple Quad) following the pre-column derivatization with dansyl-chloride. An ACE 3 C18 column (150 mm × 4.6 mm, 3 µm) was used for chromatographic separation. The column temperature was 25 °C. The injected sample volume was 10 µL. A solution of formic acid (7 mmol L^{-1}) in water and in ACN were used as a mobile phase for gradient elution: 0 min 60% ACN, 3.5 min 60% ACN, 17 min 92% ACN, 18 min 60% ACN, 25 min 60% ACN. Ionization was performed with an electrospray source in positive ionisation mode. Desolvation was achieved using nitrogen as a nebuliser and drying gas. The pressure of the nebulizer was 50 psi and the capillary voltage was 4500 V. Gas temperature was 320 °C and flow 8 L min^{-1}. Quantification was accomplished using internal standards (E2-d4, E3-d2) and an 9-point calibration curve. Dansyl-chloride derivatives exhibited a fragment ion m/z of 171, which can be found in the MS-MS spectra of all investigated compounds. MRM transitions, fragmentation and collision energy of individual estrogen derivatives are shown in Table S2.

Table S2: MRM transition, fragmentation and collision energy used for MS/MS analysis of estrogens.

Estrogen derivatives	Precursor ion (m/z)	Product ion (m/z)	Fragmentation [V]	Collision energy [V]
dansyl-E3	522	171 (156)	250	42
dansyl-EE2	530	171 (156)	250	40
dansyl-E2β	506	171 (156)	250	42
dansyl-E2α	506	171 (156)	250	42
dansyl-E1	504	171 (156)	250	38

dansyl-EE2-D	534	171 (156)	250	40
dansyl-E2-D	510	171 (156)	250	42
dansyl-E3-D	524	171 (156)	250	42

SECTION 3: *In vitro* bioassays

3.1 AhR-mediated activity

Dioxin-like activity mediated through AhR receptor was assessed using H4G1.1c2 cell line (CAFLUX assay), rat hepatoma cells containing a GFP reporter gene under control of dioxin-responsive elements, according to Nagy et al. (2002). H4G1.1c2 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum Mycoplex (PAA, Austria) at 37°C and 5% CO_2. H4G1.1c2 cells were seeded in black clear bottom 96-well microplate at density of 30,000 cells/well in DMEM and incubated for 24 h. Cells were then exposed to samples, calibration reference or solvent control in DMEM medium for another 24 h. Standard calibration was performed with 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, six-point dilution series 1–500 pM). At the end of exposure, the exposure medium was removed, cells were rinsed with PBS, and the intensity of fluorescence was measured (395 nm, 509 nm) on POLARstar OPTIMA microplate reader (BMG Labtech, Germany).

3.2 ER-mediated potency

Estrogen receptor mediated potency was evaluated by use of the MVLN bioassay, a human breast carcinoma cell line transfected with the luciferase gene under control of estrogen receptor activation (Demirpence et al., 1993; Hilscherova et al., 2002; Jálová et al., 2013). MVLN cells were routinely cultured in medium DMEM/F12 supplemented with 10% fetal bovine serum Mycoplex (PAA, Austria) at 37°C and 5% CO_2. MVLN cells were seeded in white clear bottom 96-well microplate at density of 20,000 cells/well in DMEM/F12 supplemented with 10% dialyzed fetal calf serum (PAA, Austria), which was additionally dextran/charcoal treated to further decrease background concentrations of hormones. After 24 h of incubation, cells were exposed to samples, calibration reference or solvent control in DMEM/F12 medium for another 24 h. Standard calibration was performed with17β-estradiol (E2; dilution series 1–500 pM). At the end of exposure, the exposure medium was removed, cells were rinsed with PBS, lysed and mixed with Promega Steady Glo Kit luciferase reagent (Promega, USA). Plates were shaken for 10 minutes and the intensity of luminescence was measured on BioTek Synergy™ Mx microplate reader (BioTek Instruments, USA).

3.3 AR-mediated potency

(Anti)androgenicity was assessed in a bioassay with MDA-kb2 cells, a human breast carcinoma cell line stably transfected with luciferase reporter gene under control of functional endogenous androgen receptor (AR) and glucocorticoid receptor (GR), according to Wilson et al. (2002). MDA-kb2

cells were routinely cultured in L-15 Leibovitz medium supplemented with 10% fetal bovine serum Mycoplex (PAA, Austria) at 37°C. MDA-kb2 were seeded in white clear bottom 96-well microplate at density of 25,000 cells/well in L-15 Leibovitz medium supplemented with 10% dextran/charcoal treated dialyzed fetal calf serum and after 24 h incubation exposed to samples, calibration reference or solvent control in L-15 Leibovitz medium for another 24h. Standard calibration was performed with dihydrotestosterone (DHT; eight-point dilution series 3.3 pM–100 nM, Sigma Aldrich, Czech Republic). At the end of exposure, the exposure medium was removed and cells were rinsed with PBS, lysed with Luciferase cell culture lysis 5X reagent (Promega, USA) and shaken for 10 minutes. Luciferase reagent, prepared according to Wilson et al. (2002), was added and the intensity of luminescence was measured on BioTek Synergy™ 4 microplate reader (BioTek Instruments, USA).

3.4 Cytotoxicity

For assessment of cell viability, a combination of three dyes was used: AlamarBlue (AB) as a measure for cellular metabolic activity, 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) as a measure for cell membrane integrity and Neutral Red (NR) as a measure for lysosomal membrane integrity, according to Schirmer et al. (1998) with slight modifications. The exposure medium was discarded after 24 h exposure of the cells to extracts, calibration reference and solvent control. Cells were gently rinsed with PBS and 100 µL dye solution 1, containing 5% v/v AB and 4µM CFDA-AM in fresh exposure medium, was added to each well. After incubation for 30 minutes, fluorescence was quantified using the POLARstar OPTIMA microplate reader (BMG Labtech, Germany) at respective excitation/emission wavelengths of 530/595nm for AB and 485/530nm for CFDA-AM. Dye solution 1 was discarded after the fluorescence measurement and 100 µL dye solution 2, containing 0.005% (w/v) NR in fresh exposure medium, was added to each well. Cells were incubated for 120 minutes, afterwards dye solution 2 was discarded, cells were gently rinsed with PBS and 100 µL lysis buffer, containing 1% (v/v) acetic acid in 50% (v/v) ethanol in water, was added to each well. Cells overlaid with the lysis buffer were shaken for 15 minutes and thereafter absorbance at 540 nm was measured using BioTek Synergy™ 4 microplate reader (BioTek Instruments, USA).

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Table S4: Sum concentrations of target compounds detected in SPMD and POCIS extracts expressed in pmo
classes. Concentrations of target compounds below the LOQ were considered zero in the calculations of sur

Sampler	Compound class	Unit	S1 **Spring of Bosna**	S2 **Sarajevo, DS**	S3 **Visoko, US**	S4 **Visoko, DS**	S5 **Lasva confluenc e, US**
SPMD	PAHs**	$ng.SPMD^{-1}$	1.84E+03	5.58E+04	5.62E+04	3.23E+04	3.09E+04
	PCBs	$ng.SPMD^{-1}$	1.88E+01	1.14E+02	1.40E+02	1.09E+02	1.14E+02
	OCPs	$ng.SPMD^{-1}$	9.9E+00	5.9E+01	3.8E+01	1.1E+02	9.3E+01
POCIS	CUPs	$ng.POCIS^{-1}$	1.67E+01	1.39E+03	1.16E+03	9.88E+02	1.23E+03
	Estrogens	$ng.POCIS^{-1}$	0.00E+00	1.69E+02	1.05E+02	1.05E+02	0.00E+00
	Antibiotics	$ng.POCIS^{-1}$	0.00E+00	n.a.	4.42E+03	2.99E+03	2.67E+03
	Antidiabetics	$ng.POCIS^{-1}$	0.00E+00	n.a.	9.95E+00	8.83E+00	4.71E+00
	Antihistamins	$ng.POCIS^{-1}$	0.00E+00	n.a.	1.24E+02	1.09E+02	4.47E+01
	Cancer treatment	$ng.POCIS^{-1}$	0.00E+00	n.a.	0.00E+00	0.00E+00	0.00E+00
	Cardiovascular	$ng.POCIS^{-1}$	7.36E-01	n.a.	2.52E+03	1.48E+03	1.35E+03
	NSAIDS	$ng.POCIS^{-1}$	0.00E+00	n.a.	1.35E+03	1.55E+03	9.12E+02
	Psychoactive	$ng.POCIS^{-1}$	1.14E+00	n.a.	1.92E+03	1.47E+03	1.36E+03
	Statins	$ng.POCIS^{-1}$	0.00E+00	n.a.	9.37E+01	6.80E+01	2.38E+01
	Illicit drugs	$ng.POCIS^{-1}$	0.00E+00	n.a.	6.35E+01	4.78E+01	5.99E+01
	Metabolites	$ng.POCIS^{-1}$	0.00E+00	n.a.	1.81E+02	1.42E+02	9.86E+01
	Others	$ng.POCIS^{-1}$	1.70E+01	n.a.	1.72E+04	5.98E+03	7.21E+03

*At site S2 only 40 out of 52 target CUPs were analyzed

** Concentration of naphthalene is not included in the reported sum of PAHs because of poor recoveries and its prese

l SPMD^{-1} or pmol POCIS^{-1} for different compound
ns. "n.a." stands for "not analyzed"

S6	S7	S8	S9	S10
Zepce, US	**Maglaj, US**	**Doboj, US**	**Modrica, US**	**Modrica, DS**
8.04E+04	3.64E+04	8.67E+04	3.51E+04	5.32E+04
7.21E+01	6.43E+01	9.46E+01	3.96E+01	9.41E+01
8.3E+01	3.5E+01	5.3E+01	2.4E+01	5.3E+01
1.36E+03	1.50E+03	1.21E+03	6.85E+02	1.07E+03
3.81E+01	2.38E+01	1.94E+01	1.13E+01	1.38E+01
1.58E+03	1.34E+03	9.92E+02	8.60E+02	1.01E+03
3.50E+00	3.86E+00	0.00E+00	0.00E+00	1.35E+00
4.39E+01	4.78E+01	3.59E+01	2.79E+01	1.99E+01
0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1.00E+03	8.02E+02	6.81E+02	5.01E+02	6.21E+02
6.28E+02	5.47E+02	3.78E+02	2.68E+02	2.94E+02
1.02E+03	1.34E+03	1.06E+03	9.14E+02	1.20E+03
1.78E+01	1.09E+01	1.34E+01	5.39E+00	8.02E+00
3.64E+01	3.01E+01	1.82E+01	7.07E+00	1.10E+01
5.80E+01	4.45E+01	4.94E+01	3.26E+01	4.73E+01
6.28E+03	2.30E+03	2.14E+03	2.15E+03	1.57E+03

ence in blanks