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# Bioanalytical and chemical characterization of organic micropollutant mixtures in long-term exposed passive samplers from the Joint Danube Survey 4: Setting a baseline for water quality monitoring

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ABSTRACT

Monitoring methodologies reflecting the long-term quality and contamination of surface waters are needed to obtain a representative picture of pollution and identify risk drivers. This study sets a baseline for characterizing chemical pollution in the Danube River using an innovative approach, combining continuous three-months use of passive sampling technology with comprehensive chemical (747 chemicals) and bioanalytical (seven *in vitro* bioassays) assessment during the Joint Danube Survey (JDS4). This is one of the world's largest investigative surface-water monitoring efforts in the longest river in the European Union, which water after riverbank filtration is broadly used for drinking water production. Two types of passive samplers, silicone rubber (SR) sheets for hydrophobic compounds and AttractSPE$^{TM}$ HLB disks for hydrophilic compounds, were deployed at nine sites for approximately 100 days. The Danube River pollution was dominated by industrial compounds in SR samplers and by industrial compounds together with pharmaceuticals and personal care products in HLB samplers. Comparison of the Estimated Environmental Concentrations with Predicted No-Effect Concentrations revealed that at the studied sites, at least one (SR) and 4–7 (HLB) compound(s) exceeded the risk quotient of 1. We also detected AhR-mediated activity, oxidative stress response, peroxisome proliferator-activated receptor gamma-mediated activity, estrogenic, androgenic, and anti-androgenic activities using *in vitro* bioassays. A significant portion of the AhR-mediated and estrogenic activities could be explained by detected analytes at several sites, while for the other bioassays and other sites, much of the activity remained unexplained. The effect-based trigger values for estrogenic and anti-androgenic activities were exceeded at some sites. The identified drivers of mixture *in vitro* effects deserve further attention in ecotoxicological and environmental pollution research. This novel approach using long-term passive sampling provides a representative benchmark of pollution and effect potentials of chemical mixtures for future water quality monitoring of the Danube River and other large water bodies.

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## 1. Introduction

There are several extensive water quality monitoring programs focused on the assessment of the contamination of large rivers in different regions worldwide (Blocksom et al., 2010; Chakraborty et al., 2016; Dahshan et al., 2016; Peng et al., 2018; Voloshenko-Rossin et al., 2015). The European Union's (EU) Water Framework Directive (WFD) aims at achieving a good water quality status across all water bodies in the EU. It recommends three types of monitoring (surveillance, operational, and investigative), and focuses on monitoring of priority substances and their risk to the aquatic environment (Allan et al., 2006). Surface waters are frequently polluted with complex mixtures of chemicals and there is a strong need for investigative monitoring addressing these mixtures and associated risks to aquatic organisms and human health. Aquatic biota is often exposed to mixtures of compounds from anthropogenic activities and natural sources. Many compounds are biologically active and may pose a hazard to aquatic wildlife or human health. The number of organic contaminants targeted by chemical analysis has been increasing over the last decades. However, current analytical methods still cannot comprehensively trace all components of complex environmental mixtures and they are not able to account for their combined effects (Escher et al., 2021). In a complementary approach, the occurrence of bioactive organic micropollutants may be efficiently assessed by cell-based *in vitro* bioanalytical tools, which detect the combined effects of all compounds with a common specific mechanism of action (Escher et al., 2018). Thus, the presence of all chemicals acting through the same mode of action in an environmental sample can be detected by bioassays, where their joint specific toxic potential is determined. The effects detected at the cellular level may then initiate adverse effects on higher levels of biological complexity. Many of them have been linked with adverse effects in organisms or populations through adverse outcome pathways (Ankley et al., 2010; Escher and Neale, 2021).

In environmental water quality monitoring, it is important to track the trends of pollution over longer time periods and to have sensitive analytical tools for their assessment. Various approaches are used for monitoring chemicals in water bodies, including a diverse range of sampling techniques, such as the widely applied grab and composite sampling, and more advanced techniques such as the *in situ* large-volume solid-phase extraction (Schulze et al., 2017) and passive sampling (Vrana et al., 2005).

In contrast to the conventional grab/spot sampling of water, passive sampling provides, due to its continuous sampling over several weeks, a more representative approach for the characterization of pollutant mixture composition and potential effects in sampled water bodies over a longer time frame. Passive sampling provides enough material for chemical analyses and bioanalytical assessment due to a high sampler uptake capacity. Practical advantages of passive sampling include low cost, operation that does not require any active source of energy (spontaneous compound accumulation), ease of sample storage due to small volumes of the sorptive phases and improved chemical stability when bound on solid phases. As other techniques, passive sampling also has its weaknesses. The main one is the uncertainty of estimating aqueous concentration from compound uptake, and some distortion of sampled compound mixture composition, associated with their partitioning from water to the sampler receiving phase. Passive sampling techniques have been successfully used previously for the analytical and bioanalytical assessment of mixture effects in aquatic environments (Creusot et al., 2014; De Baat et al., 2019; Hamers et al., 2018; Liscio et al., 2014; Nguyen et al., 2021; Sonavane et al., 2018; Tan et al., 2007; van der Oost et al., 2017a, b), including the Danube River (Novák et al., 2018) or rivers in its watershed (Toušová et al., 2019). Nevertheless, up to date, analytical and bioanalytical assessment has not been carried out in a major river like the Danube over several months covering an entire season. This data can provide representative information about the long-term pollution situation by averaging the variable concentrations of many contaminants.

The Danube is the largest river in the EU that flows 2826 km southeastwards from Germany to the Black Sea and collects water from a river basin that includes 19 countries, more than any other river in the world. The catchment area of the river basin was estimated to cover 801 093 $km^2$ (Sommerwerk et al., 2009), representing approximately 8% of the area of Europe (Natchkov, 1997). The river passes through numerous municipalities, including four capital cities (Vienna, Bratislava, Budapest, and Belgrade) and runs through nine countries (Germany, Austria, Slovakia, Hungary, Croatia, Serbia, Romania, Moldova, and Ukraine). The water from the Danube is used for various purposes, including as a source of drinking water, but it also receives treated and untreated wastewater and is affected by a wide spectrum of pollution sources from its catchment. Notably, about 83 million people live in the Danube River Basin and >20 million people depend directly on the Danube for their drinking water. The largest cities along the Danube in Slovakia, Hungary, and Serbia, like capitals Bratislava, Budapest and Belgrade, rely on over 50% of riverbank filtrated water for their drinking water supply (Kondor et al., 2020).

Given that the Danube drains a substantial area in many countries, joint research efforts addressing its contamination with organic micropollutants are essential for good water quality management reaching beyond the borders of individual states. The Joint Danube Survey (JDS), organized regularly (every six years) in the Danube, is one of the world's most extensive scientific multi-disciplinary riverine monitoring surveys (ICPDR, 2022). It is carried out at well-defined sampling sites along the whole Danube River. Its objective is to produce representative, reliable and comparable information on selected aspects of water quality for the length of the Danube River, including its major tributaries. Since Danube is used as a source of drinking water but also receives wastewater, the characterization of the presence of pollutants, potential mixture effects, and conducting risk prioritization of pollutants in samples collected over a long time period is of eminent importance for human and environmental health in this region.

Effect-based water quality monitoring using several *in vitro* assays was implemented next to chemical analyses to screen mixture effects for the first time in the previous Joint Danube Survey (JDS3) in 2013 (König et al., 2017; Neale et al., 2015; Novák et al., 2018). The results pointed to various relevant biological activities, namely activation of aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor isoform gamma (PPARγ), estrogen receptor isoform alpha (ERα), androgen receptor (AR), glucocorticoid receptor (GR), induction of adaptive stress response to oxidative stress (AREc32), and antagonism to AR (Alygizakis et al., 2019; König et al., 2017; Neale et al., 2015; Novák et al., 2018; Serra et al., 2020; Toušová et al., 2019). Nevertheless, the studies from the previous JDS campaigns analyzed only short term-collected samples that included grab samples (Loos et al., 2010), mobile passive sampling (Novák et al., 2018) and active large-volume solid-phase extraction (LVSPE) samples (König et al., 2017; Neale et al., 2017b). The mobile passive sampling within JDS3 was performed using a dynamic passive sampling system (Vrana et al., 2018) attached to a ship cruising through eight Danube stretches (from Germany to Romania). The sampling of each stretch took about five days, and in addition two stationary dynamic passive samplers were installed at one site for 4 and 5 days, respectively (Novák et al., 2018).

The main goal of this study was to provide information on representative pollution levels in the Danube in 2019, which can serve as a baseline for water quality monitoring in future Joint Danube Surveys. Investigation of temporal and spatial pollution trends over several years requires the use of representative sampling methods that can characterize contaminant patterns and levels in river water over longer time periods. In addition, some standardization of passive sampling and sample analysis methodology is required, similarly as has been demonstrated e.g. for passive samplers that are currently being applied in global monitoring of aquatic contaminants (Sobotka et al., 2022). In our study we propose potentially applicable methods based on

**Table 1**
Passive sampling sites in the Danube River. Samplers were deployed from May until September 2019.

| Site | Site name | Country | Latitude | Longitude | Distance from river mouth (km) | JDS4 site | River bank | Exposure (days) | Maximum water volume sampled by SR samplers (L)[a] | Water volume sampled by HLB samplers (L)[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **Jochenstein, water dam** | Germany | 48° 31.240′N | 13° 42.122′E | 2 205 | JDS 6 | Left | 101 | 2,257 | 73 |
| 2 | **Čunovo, dam of the water reservoir** | Slovakia | 48° 1.807′N | 17° 13.485′E | 1 855 | JDS 15 | Right | 103 | 11,541 | 73 |
| 3 | **DS Budapest, M0 bridge** | Hungary | 47° 23.230′N | 18° 59.460′E | 1 632 | JDS 24 | Middle | 105 | 5,971 | 73 |
| 4 | **Batina, bridge** | Croatia | 45° 50.632′N | 18° 51.315′E | 1 434 | JDS 29 | Right | 104 | 2,017 | 73 |
| 5 | **Pančevo, bridge** | Serbia | 44° 49.877′N | 20° 29.671′E | 1 154 | JDS 37 | Left | 104 | 2,886 | 73 |
| 6 | **Kladovo, jetty** | Serbia | 44° 36.784′N | 22° 36.820′E | 926 | JDS 41p[c] | Right | 104 | 4,917 | 73 |
| 7 | **Vidin-Calafat, bridge** | Bulgaria/Romania | 44° 0.293′N | 22° 56.840′E | 796 | JDS 43p[c] | Middle | 104 | 3,128 | 73 |
| 8 | **Ruse, harbor** | Romania | 43° 51.555′N | 25° 57.508′E | 490 | JDS 46p[c] | Right | 104 | 4,690 | 73 |
| 9 | **Galati, water company** | Romania | 45° 22.650′N | 28° 1.417′E | 152 | JDS 50p[c] | Left | 102 | 5,059 | 73 |

**Abbreviations and description:** SR – silicone rubber sheet samplers, HLB – AttractSPE HLB disks, JDS – Joint Danube Survey.

[a] The maximum volume of water extracted by the SR sampler with a surface area of 1000 $cm^2$ calculated for a model compound with a molecular mass of 300 (g/mol).

[b] The rounded estimated average volume of water extracted by HLB samplers calculated from the median of estimated sampled volume per compound ($V_{w,x}$ as described in Material and Methods 1.4, SM 2, A3). Only compounds detected in the passive samples from the Joint Danube Survey (JDS4) were used for the calculation of the median sampled volume. The exact value of 72.7 L was used to calculate relative extraction factors for evaluation of results from bioassays.

[c] The index 'p' means that the passive sampling site was in vicinity but not exactly at the position of the official JDS4 site.

representative passive sampling and demonstrate their use. Therefore, the main tasks of the present study were to passively sample a broad range of organic contaminants to determine their levels in water over a long period (a whole summer season) within the Joint Danube Survey 4 (JDS4), and to reveal the composition of the pollutant mixtures by extensive chemical analyses and their effects with *in vitro* bioassays. We further aimed to prioritize the chemical drivers of the detected effects by combining results from chemical analysis and bioassays. Moreover, we aimed to characterize the potential risks posed by organic pollutants that were quantified in passive sampler extracts, based on their Estimated Environmental Concentrations (EECs) and Predicted No-Effect Concentrations (PNECs), and to identify the respective mixture risk drivers. This combined approach enables prioritization of compounds and effects that require more attention in eco-toxicological and environmental pollution assessments or by regulatory bodies concerned with the Danube River and similar large water bodies.

## 2. Material and methods

### 2.1. Sampling strategy

The samples were collected at nine sites (Table 1 and Fig. 1) within the JDS4 during the passive sampling campaign lasting from the end of May till the beginning of September 2019 as described in detail in Liška et al. (2021). The sites were selected from 51 sites monitored within JDS4 to match locations where fish were collected for chemical analysis of pollutants in their tissues and where a more detailed survey was performed (Liška et al., 2021).

Two types of passive samplers were used: silicone rubber sheets (SR), which retain predominantly non-polar compounds, and adsorption solid-phase extraction samplers based on styrene–divinylbenzene, with hydrophilic moieties sorbent disks (HLB disks) retaining mostly polar compounds. Samplers were deployed in surface water using stainless steel frame holders at a depth of approximately 1 m below the water surface. Samplers were hung by ropes from bridges, buoys or jetties to keep the sampler holders floating. Both samplers accumulated organic compounds from the dissolved phase during exposure to Danube water for 101–105 days. Following exposure, samplers were cleaned from debris and fouling using local Danube water, inserted to glass storage jars, and brought cooled to 4 °C to the laboratory, where they were stored at −18 °C until processing.

#### 2.1.1. Passive sampling of polar compounds

The HLB disk sampler consisted of ten solid-phase extraction AttractSPE®Disks HLB (Affinisep, France) with 47 mm diameter. Each HLB disk was compressed between a stainless-steel ring and a supporting steel disk holder and fixed in position with stainless-steel bolts and nuts (Fig. S1). The exposed surface area of a single disk was 11.3 $cm^2$ and the total surface area of one sampler exposed to water was 113 $cm^2$ (one sided disk exposure). Before exposure, samplers were pre-conditioned and kept immersed in MilliQ water until exposure. Two sets of samplers were deployed in parallel at each site, one set was used for chemical analysis, the other set for analysis using *in vitro* bioassays. Both sampler sets were identical and not spiked with any performance reference compounds (PRCs). For processing, samplers were freeze dried and extracted in three steps with acetone and methanol. Extract volume was reduced by vacuum rotary evaporation, filtered and the extract in methanol was further reduced under nitrogen stream to 2 mL. Aliquots were taken for different types of analysis. For details on sample processing, extraction, and materials used see Supplementary material (SM 1, Text S1).

#### 2.1.2. Passive sampling of hydrophobic compounds

Passive samplers were made from silicone elastomer Altesil (Altec, UK) and applied as sheets of 9.5 × 5.5 cm of 500 μm thickness (Fig. S1). In each of these sheets two holes were punched which allow the sheets to


**Fig. 1.** Map of sampling sites with depicted capitals and major rivers in the Danube River watershed.

be fixed to the frame using cable ties. Before exposure, they were Soxhlet extracted in ethyl acetate. Two sets of SR passive samplers were deployed in parallel, where one set of SR samplers was spiked prior to exposure with 14 PRCs ($D_{10}$-biphenyl and 13 polychlorinated biphenyl (PCB) congeners that do not occur in technical mixtures) according to the procedure described in Smedes and Booij (2012), while the other set was not spiked. The samples from the spiked samplers were used for chemical analysis, and the non-spiked ones were subject to measurement using *in vitro* bioassays to avoid interference of PRCs in extracts with the bioassay response. Each SR sampler consisted of 10 silicone sheets, with a total surface area of 1000 $cm^2$. For processing, samplers were spiked with internal standards (only for chemical analysis) and Soxhlet extracted for 8 h with acetonitrile. The extracts of samplers were concentrated by Kuderna-Danish (KD) apparatus and the extract volume was adjusted to 2 mL acetonitrile. For chemical analysis, the extract was further azeotropically solvent exchanged in KD apparatus to hexane and aliquoted for compound group specific sample cleanup and instrumental analysis. For details on used sample processing, extraction, and materials see Supplementary material (SM 1, Text S1).

## 2.2. Chemical analyses

### 2.2.1. HLB disks

The concentrations of analytes in HLB disks were determined by target screening using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) and target analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS), the details of which are given in SM 1 (Text S2) and the methods used to determine the concentrations of each analyte are listed in SM 2 (A1).

The target analysis of HLB disks included: 101 pharmaceuticals and their metabolites, 105 pesticides and metabolites and 3 benzotriazoles. The instrumental analysis is described in Fedorova et al. (2014), Vrana et al. (2021b) and Fialová et al. (2023) and in SM 1 (Text S2.1.). The LC-HRMS target screening included 385 additional analytes from various compound classes. The instrumental method is described in Beckers et al. (2020). For calibration, a series of standards ranging from 1 to 1000 ng/mL were prepared in methanol.

In addition, 77 compounds showing endocrine activity (steroids and phenolic xenoestrogens) were quantified after an additional clean-up of the extracts using an aminopropyl column according to Labadie and Budzinski (2005). While ketosteroids and phenols were analyzed by LC-MS/MS in positive or negative ion mode, respectively, phenolic estrogens (estradiol, estrone, estriol, ethinylestradiol) were analyzed by LC-HRMS after derivatisation with dansyl chloride based on Backe (2015). Details are given in Backe (2015), König et al. (2017), and Hashmi et al. (2018) and in SM 1 (Text S2.1.).

### 2.2.2. SR samplers

The target analysis of SR included: 24 polycyclic aromatic hydrocarbons (PAHs), 7 indicator congener PCBs, 11 organochlorine pesticides (OCPs), including hexachlorobenzene, hexachlorocyclohexane isomers, DDT and its metabolites, 7 brominated diphenyl ether (BDE) congeners, hexabromocyclododecanes (HBCD) and 17 chlorinated cyclodiene pesticides. Selected compounds comprised pollutants regulated by the United Nations Stockholm Convention on POPs and PAHs regulated as priority substances in the aquatic environment in Europe and other countries. This set of compounds covers a broad range of properties including polarity and molar mass. For details of the methods used for target chemical analysis of SR extracts, see Sobotka et al. (2022).

### 2.3. Assessment of biological activities in extracts from passive samplers

A battery of *in vitro* reporter gene bioassays was used to assess biological activities, covering metabolism adaptation regulated by the aryl hydrocarbon receptor (AhR) and the gamma isoform of peroxisome proliferator-activated receptor (PPARγ), reactive modes of action based on adaptive stress response to oxidative stress (ARE), and specific modes of action like agonistic activation of estrogen (ER), androgen (AR), and glucocorticoid (GR) receptor as well as antagonistic effect on AR. The materials used for *in vitro* bioassays are reported in the SM (Text S3.1.).

To detect AhR activation, the AhR-CAFLUX assay based on H4G1.1c2 cell line was used (Nagy et al., 2002) and the details are in SM 1 (Text S3.2.). For the activation of PPARγ, ER and GR, GeneBLAzer (Thermo Fisher Scientific, Schwerte, Germany) assay technology was used, which is based on human embryonic kidney cells (HEK293) containing a stably transfected β-lactamase gene (Text S3.3.). The oxidative stress response was quantified with reporter gene AREc32 model based on the breast cancer cell line MCF7 and developed by Wang et al. (2006) as described in SM 1 (Text S3.4.).

The agonistic and antagonistic activities on AR were measured using MDA-kb2 cell line, which is based on MDA-MB-453 cell line, stably transformed with luciferase reporter gene (Wilson et al., 2002). For the details on the methods performed see SM1 (Text S3.5.).

All bioassays were run in agonistic mode, and MDA-kb2 also in antagonistic mode as described in Neale et al. (2017a). Cell viability in AhR-CAFLUX and MDA-kb2 models was measured with neutral red assay (Freyberger and Schmuck 2005). To assess cytotoxicity quantitatively in PPARγ GeneBLAzer, Erα GeneBLAzer, GR GeneBLAzer, and AREc32 the cell confluency was non-invasively measured using phase-contrast cell-imaging approach with software IncuCyte S3 in a cell imaging device IncuCyte S3 Live Analysis System (Essen BioScience, Germany) (Escher et al., 2019). Details on the bioassays are given in Table 2, details on the evaluation of data from cytotoxicity testing are in SM 1 (Text S4.1.), and bioassays can be found in SM 1 (Text S4.2.).

### 2.4. Data analysis

#### 2.4.1. Evaluation of bioassay data

The *in vitro* biological activities were reported as bioanalytical equivalent concentrations (BEQ) of a reference compound that would cause the same effect as the tested sample. The activities measured by *in vitro* biotests ($BEQ_{bio}$) were compared to activities predicted based on the measured concentration and relative potency of the detected compounds ($BEQ_{chem}$). The method used for derivation of bioanalytical limits of quantifiable effects is given in SM 1 (Text S4.2.3.).

### 2.5. Risk prioritization

To evaluate the potential risks posed to the aquatic environment by individual chemicals, two environmental risk estimates, i.e. Extent of Exceedance (*EoE*) and Frequency of Exceedance (*FoE*) were used before, as described in von der Ohe et al. (2011) and Dulio and von der Ohe (2013). For their calculation, the risk quotient of each compound at a given site was first calculated as the ratio of Estimated Environmental Concentrations (*EEC* derived as described in detail in chapter 3.1) and PNEC (*RQ*; Eq. (1)). The PNECs were used as thresholds to determine potential risk of a chemical at a given site and were downloaded from the NORMAN Ecotoxicology Database (NORMAN 2022). These were preferably based on experimental eco-toxicity data. Quantitative Structure-Activity Relationships (QSARs) predictions were used in case when experimental data were not available (von der Ohe et al., 2011).

$$RQ_i = \frac{EEC_i}{PNEC_i} \quad (1)$$

where $RQ_i$ is the local risk quotient of a compound *i* at a given site, $EEC_i$ is the *EEC* of compound *i* at that site (expressed per liter of river water), and $PNEC_i$ is the respective *PNEC* of compound *i*. On the basis of individual *RQ*s and the total number of sites, the $FoE_i$ was calculated (Eq. (2)) as:

$$FoE_i = \frac{\text{number of sites exceeding the RQ of 1}}{\text{total number of sites}} \quad (2)$$

The *FoE* score has a value between 0 and 1 and will be directly used for prioritization.

For the calculation of the $EoE_i$, the 95th percentile of all EECs for compound *i* across all sites was considered as (Eq. (3)):

$$EoE_i = \frac{EEC95_i}{PNEC_i} \quad (3)$$

The $EoE_i$ values were converted to a scale between 0 and 1 as described in von der Ohe et al. (2011). Substances with a 95th percentile of *EEC* that exceeded the lowest *PNEC* by a factor below 10 were assigned a score of 0.1. If the exceedance was >10 but below 100, the score was set to 0.2. The exceedance of the risk quotient of 1 indicates a potential risk posed by that compound to the aquatic environment, but *RQ*s smaller than 1 are usually disregarded. However, if the *RQ* of a compound is close to 1, it may additively contribute to the overall risk at a site. Thus, we have additionally derived a mixture risk contribution ($MRC_i$) score, indicating the number of sites at which compound *i* has *RQ* above 0.5 but below 1.0 (Eq. (4)). To reflect the lower risk of these compounds (i.e., with *RQ* between 0.5 and 1) as compared to single substance exceedances (RQ > 1), the resulting score is divided by a factor of 2 to calculate the final $MRC_i$ score (Eq. (4)).

$$MRC_i = \left(\frac{\text{number of sites with } RQ_i \text{ between 0.5 and 1.0}}{\text{total number of sites}}\right) \Big/ 2 \quad (4)$$

Finally, we have used a risk metric called Final score (Eq. (5)) that was used for the prioritization of compounds. The final score is the sum of *FoE*, *EoE* and *MRC* (Eq. (5)), in analogy to the NORMAN prioritisation framework (Dulio and von der Ohe, 2013).

$$\text{Final score} = EoE_i + FoE_i + MRC_i \quad (5)$$

The final score informs about both the individual risk and mixture contribution of a compound, i.e., RQs that are both above 1 and also between 0.5 and 1. The compounds with the highest Final score are expected to pose the highest overall risk to the aquatic environment.

## 3. Results and discussion

### 3.1. Evaluation of passive sampler data

In general, the uptake of an individual compound from water by a passive sampler can be described by a first-order uptake model to equilibrium according to Booij et al. (2007) and Vrana et al. (2018) as given in Eq. (6):

$$N_x = C_w K_{x,w} m_x DEQ_x \quad (6)$$

where $N_x$ is the compound́s amount taken up by the sampler × (SR sheets or HLB disks), $C_w$ is the freely dissolved aqueous concentration to which the sampler was exposed, $K_{x,w}$ is the compound-specific sorbent/water partition coefficient, $m_x$ is the sampler mass, and the term $DEQ_x$ denotes the degree of equilibrium attained by a compound during exposure. $DEQ_x$ takes values ranging from 0 to 1, it increases with sampler exposure time and indicates different sampling stages: linear or integrative stage when $DEQ_x < 0.2$, transitional stage *DEQ* 0.2–0.95, and equilibrium stage $DEQ_x > 0.95$. The product $K_{x,w} \times m_x \times DEQ_x$ has a unit of volume and can be interpreted as a compound-specific sampled volume of water, $V_{w,x}$. Eq. (6) states that $N_x$ is proportional to $C_w$, which provides an option to convert the passive sampling data to concentrations in the sampled water (Estimated Environmental Concentrations, *EEC*), provided that sampler- and compound-specific $K_{x,w}$ and sampler-,

compound-, and exposure-specific $DEQ_x$ are known. It is important to stress that this theory has been derived for individual compounds and its application to complex compound mixtures is not straightforward.

**Silicone rubber sheets.** Performance characteristics of SR sheets are well established. $K_{SR,w}$ values of many hydrophobic organic compounds are available from literature (Smedes 2018; Smedes et al., 2009) and $DEQ_{SR}$ values at each sampling site are assessed from dissipation of 14 performance reference compounds (PRCs) applying the mass transfer model by Rusina et al. (2010) and the approach by Smedes et al. (2009) as shown in Sobotka et al. (2022). This allowed the derivation of $C_w$ (*EEC*) for 75 compounds that were quantified in SR sampler extracts (SM 2, A2).

Unlike for HLB disks, the desired equilibrium partitioning of all sampled compounds between SR and water is not feasible even after a 3-month exposure. The SR samplers usually require a very long time to achieve complete equilibrium for very hydrophobic compounds. Vrana et al. (2019) have shown that samplers with a surface area of 400–800 $cm^2$ consisting of thin (100–500 μm) SR sheets exposed at sampling rates of 10–40 L/d for a period of up to four months reach partition equilibrium with water for compounds with log $K_{ow} \leq 5.5$. Very hydrophobic compounds, for which SR have very high uptake capacity ($K_{SR,w} \times m_{SR}$) are present in water at extremely low concentrations (often at pg/L level), and thus their diffusive flux from water to SR is slow. Moreover, the uptake kinetics to SR is site specific because it is affected mainly by local hydrodynamic conditions. This was confirmed also during passive sampling with SR in JDS4. Sampling kinetics and attainment of equilibrium in SR can be investigated using dissipation of PRCs dosed to SR intended for chemical analysis prior to their exposure in water. Knowing that the compound uptake and release in SR are isotropic processes (Booij et al., 2007), $DEQ_{SR} = 1$ can be confirmed for all compounds with log$K_{ow}$ less than that of a PRC which completely dissipated from SR. Inspection of the hydrophobicity profiles of PRC fractions remaining in the sampler log $K_{ow}$-$f$(PRC) shows that at JDS4 sites after a 3-month exposure partition equilibrium was attained in SR samplers for compounds with log $K_{ow}$<4.5–5 (Fig. S2). Based on the PRC release and isotropic exchange kinetics (Fig. S2) we quantified site-specific SR sampling rates (i.e. equivalent compound specific volume of water extracted per unit of time; $R_s$). Moreover, a compound is considered to be sampled integratively, when it is equally or more hydrophobic than a PRC that is released during exposure from SR to <50%. Based on that we also can conclude that hydrophobic compounds with log $K_{ow} > 5.5$ are sampled integratively (i.e. $DEQ_{SR} < 0.5$). This fact implies that, in general, a conversion of SR data to corresponding aqueous concentrations ($C_w$) or an extrapolation to equilibrium value $N^{\infty}_{SR,}$ at $DEQ_{SR} = 1$ is needed to identify spatial and temporal contaminant trends in aqueous phase. Without such conversion the comparison is obscured by different SR sampling rates/sampled volumes ($V_{w,SR}$) of compounds with log $K_{ow} > 5$ at different sites.

The maximum sampled water volume (Eq. (7))

$$V^{max}_{w,SR} = R^{M300}_{s} t \quad (7)$$

for a model compound with log $K_{ow} > 5$ and a molecular mass $M$ of 300, that was sampled time-integratively during 101–105 days exposure in Danube water, ranged from 2,017 to 11,541 L at sites 4 and 2, respectively (Table 1, Table S1).

For individual compounds, $C_w$ (EEC; and contamination trend) can be directly derived since measured $K_{SR,w}$ values are either available or can be estimated using linear solvation energy relationships (Ulrich et al., 2017), and $DEQ_{SR}$ can be calculated from site-specific PRC release (Fig. S2).

However, the identification of spatial or temporal trends of bioassay responses in SR extracts is complicated because identity and related properties (log $K_{ow}$) of compounds exhibiting effects in passive sampler extracts are unknown. For all compounds in the range of hydrophobicity log $K_{ow} > 5$, sampled volume $V^{bio}_{w,SR}$ can be approximated by the maximum volume of sampled water, $V^{bio}_{w,SR} = V^{max}_{w,SR}$. This approximation is realistic since for water boundary layer-controlled uptake to SR the sampling rate is only a weak function of molecular mass (Rusina et al., 2010). The disadvantage of this approach is that its application to compounds with log $K_{ow} \leq 5$ results in an underestimation of their real aqueous concentrations. This implies that the estimated $BEQ_{bio}$ in water, calculated from bioassay response in SR extracts, represents a realistic estimate of the joined effects (expressed as aqueous concentration) of active substances with log $K_{ow} > 5$, but an underestimation for more polar (but non-charged) compounds. This shortcoming is partially compensated by the usage of two samplers in parallel, which are complementary in coverage of the compound polarity range. We believe that effects of polar compounds with log $K_{ow} < 5$ are better assessed with HLB samplers.

**HLB disks.** In contrast to SR, the HLB sampler is designed to sample polar compounds. The uptake capacity of the HLB sampler for these compounds can be expressed as ($K_{HLB,w} \times m_{HLB}$). $K_{HLB,w}$ are generally lower than $K_{SR,W}$, and HLB samplers are expected to equilibrate faster than SR. In comparison with SR, performance characteristics of HLB disks are much less characterized for exposure durations of several months. Our recent study performed in a municipal wastewater effluent (Vrana et al., in preparation) demonstrated that for most compounds with log $K_{ow} < 5$ equilibrium has been reached between samplers and water within 3 months. Since HLB disk exposure in JDS4 lasted over 100 days, we assumed that for polar organic compounds under investigation HLB-water equilibrium was attained, i.e., $DEQ_{HLB} = 1$.

To further justify the assumption of $DEQ_{HLB} = 1$, we benchmarked the uptake by HLB disks using the published information. Several studies published laboratory-derived $K_{HLB,w}$ for HLB sorbent (Bäuerlein et al., 2012; Gao et al., 2022; Jeong et al., 2017, 2018, 2020), although specific values for AttractSPE™ HLB disks are not available. The manufacturer states that the HLB disks contain 90% (w/w) of HLB sorbent material (www.affinisep.com), and thus the values published for powder sorbent are roughly applicable also for HLB disks. The laboratory-derived $K_{HLB,w}$ values present the slope of linear sorption isotherms of compounds at environmentally relevant aqueous concentrations (in units of μg per litre or lower). These $K_{HLB,w}$ enable to estimate the maximum theoretical volume of water that can be sampled $V^{max}_{w,HLB} = K_{HLB,w} \times m_s$, since any non-linearity of isotherms towards sorbent saturation would result in lower equivalent sampled volumes at equilibrium. The published log $K_{HLB,w}$ (L/kg) comprise 123 values for various aquatic contaminants including pharmaceuticals, pesticides, personal care products and bisphenols, which range from 2.8 to 5.5 (median 4.4; Fig. S3), resulting in estimated $V^{max}_{w,HLB}$ from 1.5 to 856 L (median 66 L). In the early stage of exposure, sampling with HLB disks is integrative and the integratively sampled volume can be estimated by an equation analogical to Eq. (7), i.e. $V^{int}_{w,HLB} = R_{s,HLB} t$. The $R_{s,HLB}$ of a naked sorbent disk (without any membrane) has been shown to be surface-proportional to $R_{s,SR}$ (Vrana et al., 2018) and adopting this proportionality results in estimated $V^{int}_{w,HLB}$ between 228 and 1304 L at sites 4 and 2, respectively. When the $V^{int}_{w,HLB}$ volume exceeds the $V^{max}_{w,HLB}$, sorption equilibrium can be confirmed. The equilibrium condition is fulfilled for compounds with $K_{HLB,w} \leq V^{int}_{w,HLB}/m_s$, i.e. for compounds with log $K_{HLB,w} \leq 5$ at all sites. >90% of compounds with published $K_{HLB,w}$ values fulfill this criterion of equilibration during HLB disk exposure.

Further, we assumed that the capacity of sorption sites at HLB disk was not exhausted during exposure. The Langmuir equation below assumes that the sorbent has a limited number of sorption sites with similar affinity. When the concentration increases, the sorbent gets saturated when a maximum concentration on the sorbent is reached ($C_{max}$). This is the term equivalent to the total sorption capacity of the sampler. In the Langmuir model, at low $C_w$ ($C_s \ll C_{max}$), $K_{HLB,w}$ is constant and a linear sorption isotherm applies. Bäuerlein et al. (2012) measured sorption isotherms of selected polar organic compounds in HLB/water

**Table 2**
Description of the *in vitro* reporter gene bioassays used to assess biological activities in passive samples from the Joint Danube Survey 4 (JDS4).

| Endpoint (mode of action) | Bioassay/cell line | Bioanalytical laboratory | Reference compound | $EC_{10}$ or $EC_{IR1.5}$ or $IC_{20}$ of reference compound ± SEM (M)[a] | BEQ | Bioassay method reference | Type of cytotoxicity testing | EBT | Reference for EBT |
|---|---|---|---|---|---|---|---|---|---|
| *Xenobiotic metabolism* | | | | | | | | | |
| transactivation of aryl hydrocarbon receptor | CAFLUX /H4G1.1c2 | RECETOX | 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) | $2.5 \times 10^{-12} \pm 4.0 \times 10^{-14}$ | TCDD-EQ | Nagy et al., 2002 | neutral red assay | 106 pg/L TCDD EQs | Read-across from the EBT from Escher and Neale 2021 for the AhR CALUX (H4L7.5c2 (Brennan et al.) and translated into TCDD [b] |
| transactivation of peroxisome proliferator-activated receptor, isoform gamma | PPARγ GeneBLAzer/ HEK293H | UFZ | Rosiglitazone | $4.2 \times 10^{-10} \pm 3.9 \times 10^{-11}$ | Rosiglitazone-EQ | Invitrogen 2007a | cell confluence | 1.2 µg/L Rosiglitazone EQs | Escher and Neale, 2021 |
| *Reactive mode of action* | | | | | | | | | |
| adaptive response to oxidative stress | AREc32/ MCF-7 | UFZ | Benzo(a)pyrene (B(a)P) | $1.65 \times 10^{-7}$[c] | B(a)P-EQ | Escher et al., 2021 | cell confluence | 34 µg/L B(a)P EQ[d] | Escher and Neale, 2021 |
| *Specific mode of action* | | | | | | | | | |
| transactivation of estrogen receptor, isoform alpha | ERα GeneBLAzer/ HEK293T | UFZ | 17ß-estradiol (E2) | $1.7 \times 10^{-11} \pm 4.0 \times 10^{-13}$ | EEQ | Invitrogen 2007b | cell confluence | 0.34 ng/L EEQ | Escher et al., 2018 |
| transactivation of androgen receptor | AR agonism /MDA-kb2 | RECETOX | Dihydrotestosterone (DHT) | $2.3 \times 10^{-11} \pm 1.7 \times 10^{-12}$ | DHT-EQ | Wilson et al., 2002 | neutral red assay | NA | NA |
| androgen receptor antagonism | AR antagonism /MDA-kb2 | RECETOX | Flutamide (FLU) | $2.2 \times 10^{-7} \pm 2.2 \times 10^{-8}$ | FLU-EQ | Wilson et al., 2002 | neutral red assay | 3.5 µg/L FLU EQs | Escher et al., 2018 |
| transactivation of glucocorticoid receptor | GR GeneBLAzer/ HEK293T | UFZ | Dexamethasone (DEX) | $6.5 \times 10^{-10} \pm 2.7 \times 10^{-11}$ | DEX-EQ | Invitrogen 2007c | cell confluence | NA | NA |

**Abbreviations and description:** [a] - $EC_{IR1.5}$ was calculated for the reference compound benzo(a)pyrene, $IC_{20}$ was derived for flutamide, $EC_{20}$ was derived for TCDD, otherwise we expressed the effective concentrations as $EC_{10}$, [b] - calculated from EBT given in Escher and Neale (2021) by conversion of 250 ng/L B(a)P-EQ to 106 pg/L TCDD-EQ ($EC_{10}$ TCDD = 0.41 ng/L and $EC_{10}$ of B(a)P = 961 ng/L) based on their relative potencies, [c] - $EC_{IR1.5}$ of B(a)P taken from Lee et al. (2021), [d] – calculated from EBT-dichlorvos-EQ of 1.4 $mg_{dichlorvos}$/L from Escher and Neale (2021) by conversion to an EBT-B(a)P-EQ of 34 $\mu g_{B(a)P}$/L ($EC_{IR1.5}$ dichlorvos = 1.7 mg/L and $EC_{IR1.5}$B(a)P = 41.6 µg/L). BEQ - Bioanalytical equivalence concentration, NA – not available. RECETOX – Faculty of Science at Masaryk University (Brno, the Czech Republic), SEM – standard error of the mean, UFZ – Helmholtz Centre for Environmental Research (Leipzig, Germany).

system, including $C_{max}$. For example, for carbamazepine $C_{max}$ of 261 mmol/kg was determined, which corresponds to 62 g/kg sorbent. Applying $C_{max}$ to our HLB disk sampler with a sorbent mass of 2.64 g results in a total sorption capacity of 160 mg carbamazepine. In the Danube, the HLB disk sampler collected a maximum of 3.2 μg carbamazepine, which is <0.002% of $C_{max}$. Carbamazepine is among the targeted contaminants with the highest concentration. We assume that even in the presence of a mixture of several thousands of compounds with a similar concentration and affinity to the sorbent like carbamazepine in the Danube water the total saturation of sorption sites would not be reached. For trace contaminants, we thus assume linear isotherms and constant $K_{HLB,w}$.

When assuming constant $K_{HLB,w}$ values of individual compounds at all sites and equal $m_{HLB}$, Eq. (6) reveals that ratios of accumulated amounts $N_{HLB}$ (when $DEQ_{HLB} = 1$) at different sampling sites equal to the ratios of concentrations in water $C_w$ (EEC) at those sites (Eq. (8)):

$$\frac{N_{HLB,siteA}}{N_{HLB,siteB}} = \frac{C_{w,siteA}}{C_{w,siteB}} \quad (8)$$

This implies that, in an ideal situation, results for individual compounds obtained by equilibrium passive sampling at different sites or at different times (e.g. in different seasons, if we neglect the effect of water temperature, pH or salinity, fouling, dissolved organic carbon or competitive sorption on $K_{HLB,w}$) are directly comparable and enable the identification of spatial and temporal contaminant trends (Brack et al., 2016). This applies also for trends of effects caused by contaminant mixtures in passive sampler extracts, which may help to identify pollution hotspots or sites with elevated risks from chemical contamination. Equilibrium passive sampling thus provides a very good baseline for contamination assessment and for data comparison in future monitoring within regular Danube surveys, if sampling is performed with the same sampler design (i.e., identical sorbent material).

In reality, the $K_{HLB,w}$ values can be affected by environmental variables such as water temperature, salinity, pH, fouling, dissolved organic carbon content in water and also by competitive sorption of chemicals in water to the available sites at the sorbent. In the first approximation, we neglected these effects, and we assumed that their impact on $K_{HLB,w}$ at different sampling sites would be similar. This approximation is justifiable for this particular study because basic physicochemical parameters of the surface water during the sampler deployment period did not vary dramatically along the Danube River (Table S2; ICPDR, 2023). Nevertheless, one must acknowledge that contaminant mixture composition in passive samplers equilibrated with the water phase is affected by the affinity of compounds present in water to the HLB sorbent (i.e. different $K_{HLB,w}$ values), which results in inequal concentration of compounds with different affinities from water to sampler, following Eq. (6). As a result, the extract from HLB (at $DEQ_{HLB} = 1$ for all sampled compounds) is more enriched by compounds with high $K_{HLB,w}$ values compared to mixture composition in the sampled water. When comparing data to *PNECs* or other limit values derived as concentration in the aqueous phase, there is a need to convert $N_{HLB}$ to corresponding $C_w$ (EEC) using Eq. (6), which requires the knowledge of a compound-specific $K_{HLB,w}$ value.

In the above-mentioned study Vrana et al. (in preparation) we derived $K_{HLB,w}$ values from *in situ* HLB disks-water equilibration for 34 pharmaceuticals and 15 currently used pesticides and 1 industrial compound detected in our study (SM 2, A3), covering a range of contaminants typically present in municipal effluents. For 11 compounds, including 5 pesticides and 6 pharmaceuticals and personal care products, $K_{HLB,W}$ values derived in the proof of concept study can be compared to published laboratory-derived values (Table S3). The comparison shows a good agreement of laboratory- and field-derived values, with an average difference of ±0.4 log units. That corresponds to a potential difference within a factor of 3 of calculated aqueous concentrations, depending on the selection of applied $K_{HLB,W}$ values.

Substitution of these $K_{HLB,w}$ values to Eq. (6) at $DEQ_{HLB} = 1$ allowed to estimate $C_w$ for 359 compounds that were quantified in HLB sampler extracts from JDS4 (SM 2, A1 and A3). For 50 compounds where field-derived $K_{HLB,w}$ values were available (Vrana et al., in preparation), those values were applied for $C_w$ estimation. For the remaining over 300 compounds quantified in HLB extracts, experimental $K_{HLB,w}$ were not available and the required values were estimated from an established (Vrana et al., in preparation) empirical relationship between $K_{HLB,w}$ and the octanol–water distribution coefficient at pH 7.4 (log $D_{7.4}$) (ACDlabs 2022) shown in Eq. (9).

$$logK_{HLB,w} = 4.00 \pm 0.19 logD_{7.4} \quad (9)$$

The standard error of $K_{HLB,w}$ estimate using Eq. (9) is 0.5 log units, which results in the error of $C_w$ estimate from Eq. (6) within a factor of 3, when applying $K_{HLB,W}$ from the empirical model, and $DEQ_{HLB} = 1$.

The expression of a bioassay-based effect found in HLB extracts in an equivalent aqueous concentration of a reference compound $BEQ_{bio}$ (a concentration equivalent to $C_w$ in Eq. (6)) requires the knowledge of a mixture-matched $K^{bio}_{HLB,w}$ value, representing the mixture of compounds exhibiting the observed effects. That would allow estimating the mixture-matched volume of water, from which the HLB sampler extracted active compounds, i.e.

$$V^{bio}_{w,HLB} = K^{bio}_{HLB,w} \times m_{HLB} \quad (10)$$

We attempted to derive a rough estimate of $V^{bio}_{w,HLB}$ (L) that would provide information on the order of magnitude of water volume sampled by the HLB sampler relevant for the mixtures causing the effects (Eq. (10)). The estimate is based on the assumptions that a) the active compounds in the HLB extracts from the Danube have $K_{HLB,w}$ similar to those that were found and quantified in the same sampler by chemical analysis; b) $V^{bio}_{w,HLB}$ can be approximated by the median of $V_{w,HLB} = K_{HLB,w} \times m_{HLB}$ values of those compounds, which were quantifiable in all HLB sampler extracts (by chemical analysis), and for which $K_{HLB,w}$ values (either measured or estimated) were available from a proof-of-concept study Vrana et al. (in preparation). Since such an approach provides a rough estimate, we did not attempt to consider site-specific differences in sample composition or compound effect potencies when estimating $V^{bio}_{w,HLB}$. Instead, a uniform, non-specific, sampled water volume median $V^{bio}_{w,HLB}$ of 73 L (i.e. the apparent sampling rate 730 mL/day) was assigned to all Danube HLB samplers (SM 2, A3) and further used for estimation of relative extraction factors (*REF*) in bioassays. The median $V^{bio}_{w,HLB}$ of 73 L, derived using the above outlined approach, is in a good agreement to the median $V^{max}_{w,HLB}$ of 66 L, derived from the published laboratory-derived $K_{HLB,w}$.

For comparison, typical sampling rates $R_s$, i.e. sampled water volume per unit of time in polar organic chemical integrative samplers (POCIS) and Speedisk is 100 and 50 mL/day, respectively (Vrana et al., 2021b, Nguyen et al., 2021). Although POCIS and Speedisk samplers operate in integrative regime and our HLB sampler equilibrated with water due to long exposure, a comparison of sampling rates (actual or apparent) in the three passive sampler is helpful to consider whether the magnitude of estimated $V^{bio}_{w,HLB}$ is realistic. In integrative regime, the sampling rate is proportional to the sampler surface area ($R_S = k_o \times A$), where $k_o$ is the mass transfer coefficient (MTC); its value can be directly compared between samplers. The surface area $A$ of POCIS, Speedisk and HLB samplers is 46, 20 and 113 $cm^2$, respectively. The calculated apparent MTC in POCIS, Speedisk and HLB results in very similar values of 0.002, 0.003 and 0.006 L/d/$cm^2$, respectively. This demonstrates that a similar magnitude of $V^{bio}_{w,HLB}$ would be estimated empirically even if its calculation was based on the assumption of integrative uptake to HLB and the use of sampling rates published for similar adsorption based passive samplers.

Unfortunately, a simple direct comparison of HLB sampler data (in ng/sampler or ng/g) format from JDS4 using the above outlined approach cannot be made with historical data obtained using SDB-RPS

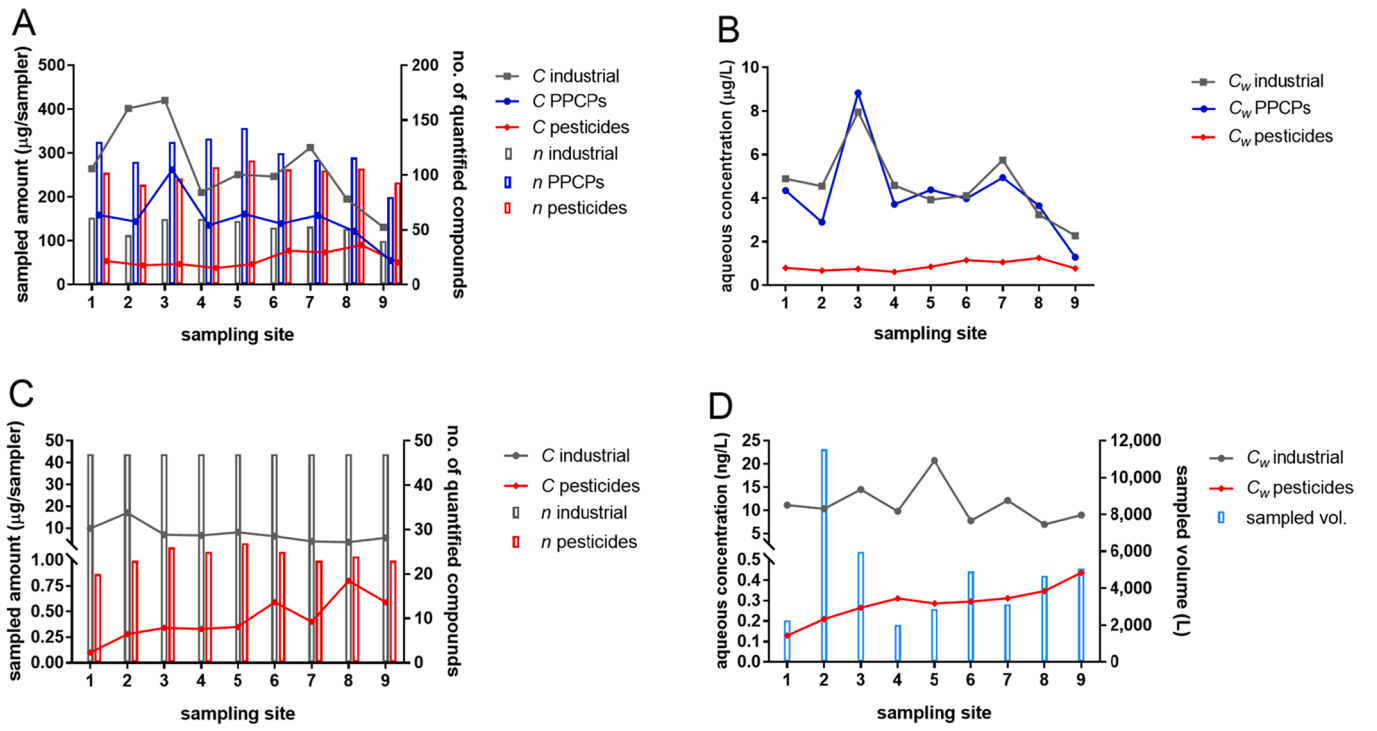


**Fig. 2.** (A) Spatial pattern of occurrence (total cumulative concentration) of groups of contaminants per sampler and number of detected chemicals per group in HLB samplers and (B) the sampled volume of water and estimated aqueous concentrations in HLB samplers recalculated per liter of river water. (C) Spatial pattern of occurrence (total cumulative concentration) of groups of contaminants in SR samplers and (D) the sampled volume of water and spatial pattern of occurrence (total cumulative concentration) of groups of contaminants in SR samplers recalculated per liter of river water. The group PPCPs includes in addition also some compounds used as food ingredients. *C* industrial = sum of sampled amount of industrial compounds per sampler, *C* PPCPs = sum of sampled amount of pharmaceuticals and personal care products per sampler, *C* pesticides = sum of sampled amount of pesticides per sampler, $C_w$ industrial = sum of concentrations of industrial compounds per liter of river water, $C_w$ PPCPs = sum of concentrations of pharmaceuticals and personal care products per liter of river water, $C_w$ pesticides = sum of concentrations of pesticides per liter of river water, *n* industrial = number of quantified industrial compounds, *n* PPCPs = number of quantified pharmaceuticals and personal care products, *n* pesticides = number of quantified pesticides. The categorization of chemicals into the groups and other information about the chemicals are given in Table 3 and Supplementary material 2 (A1 and A2). Some estrogenic compounds and ketosteroids are not included in the graphs as they were determined by target analysis only at selected localities.

Empore disks from the previous JDS3 survey (Novák et al., 2018). This is because in JDS3 sampling was done for only a short period of time (4–5 days). Despite the application of a dynamic passive sampler that maximized the sampling rates in JDS3, investigated compounds most likely did not attain distribution equilibrium between Empore disks and water (i.e., $DEQ < 1$). Even if equilibrium had been attained at all sites in JDS3, a direct sampler comparison would be difficult because two different sorbent materials with different $K_{x,w}$ were applied. The choice of the experimental design of passive sampling using sorbent disks in JDS3 and JDS4 has practical reasons. HLB disks, which seem to sorb a broader range of polar organic compounds than SDB-RPS disks, were not yet available in 2013. Moreover, equilibrium passive sampling was not feasible in JDS3, since sampling was done from a vessel moving downstream on Danube, and only a limited time (2–5 days) was available for sampling at each Danube stretch. Nevertheless, a comparison of sorbent disk-derived aqueous concentrations $C_w$ (*EEC*) in Danube between JDS3 and JDS4 is possible, when accepting the uncertainty that both samplings were not performed for the same period and at identical sites.

Similarly, $BEQ_{bio}$, calculated from bioassay response in HLB extracts, represents an estimate of the summed effects (expressed as aqueous concentration) of active substances in the water phase.

### 3.2. Chemical analyses

#### 3.2.1. Contaminant profiles along the water course of the Danube River

During exposure, passive samplers continuously sample compounds from the water phase, providing a representative image of pollution in the aqueous phase over the sampling period. Thus, our 3-month sampling within JDS4 provided a representative picture of the Danube water quality during the summer of 2019. We analyzed 671 and 76 compounds in HLB and SR samplers, respectively (SM 2, A1 and A2). The results document the presence of very complex pollutant mixtures across all study sites. The detected analytes included broad spectra of pesticides, pharmaceuticals, steroids, illicit drugs and their metabolites and transformation products, as well as industrial compounds, PAHs, PCBs, and other environmentally relevant chemicals. The spatial distribution trends of cumulative sampled amounts and Estimated Environmental Concentrations (*EECs*) and numbers of quantified compounds are shown in Fig. 2. Details and the categorization of analyzed compounds into groups according to their main usage can be found in SM 2 (A1 and A2). Regarding the estimated aqueous concentrations, the cumulative *EECs* of compounds, frequency of detection per group and the total number of analytes per group are shown in Table 3, while corresponding information per sampler is given in Table S4. The spatial distribution (in ng/sampler and the corresponding estimated ng/L aqueous concentrations) and the highest concentrations of the most abundant compounds in each group according to their main usage are given and discussed in SM 1 (Texts S5 and S6, Figs. S4–S35) and SM 2 (A4, A5, A6, and A7), respectively. The range of analytes has been significantly extended in this study compared to previous JDS campaigns. Loos et al. (2010) analyzed 34 compounds in grab water samples from JDS2, Novák et al. (2018) analyzed 204 compounds in Empore disk passive samplers and 81 compounds in SR passive samplers (JDS3), while Neale et al. (2015) analyzed 274 compounds in LVSPE extracts (JDS3).

**Table 3**
Sum of estimated aqueous concentrations (ng/L and pg/L) of groups of chemicals detected in passive samplers. The measured sampled amount/sampler is given in Table S4.

| Class of chemicals | Subclass of chemicals | Number of analyzed compounds | Number of detected compounds | ng/L (number of detected compounds with $K_{HLB,w}$) [a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | site 1 | site 2 | site 3 | site 4 | site 5 | site 6 | site 7 | site 8 | site 9 |
| *HLB samplers* | | | | | | | | | | | | |
| PPCPs and food related compounds | Antibiotics | 29 | 16 | 48 (11) | 14 (10) | 21 (12) | 31 (11) | 19 (15) | 20 (11) | 21 (12) | 14 (12) | 12 (9) |
| | Antidiabetics | 6 | 2 | ND | 0.01 (1) | 0.01 (1) | ND | 22 (2) | 36 (2) | 0.01 (1) | 0.02 (1) | ND |
| | Antifungals | 8 | 7 | 33 (5) | 114 (5) | 71 (4) | 43 (3) | 57 (5) | 50 (5) | 58 (4) | 54 (5) | 28 (2) |
| | Antihistamines | 13 | 7 | 4.3 (6) | 3.5 (6) | 5.2 (5) | 2.9 (5) | 1.5 (6) | 2.0 (6) | 1.6 (4) | 1.1 (6) | 1.1 (3) |
| | Antimicrobial preservatives | 5 | 4 | ND | 29 (1) | ND | 0.45 (1) | 3.3 (3) | ND | 9.4 (1) | 9.7 (1) | 18 (1) |
| | Antineoplastics | 10 | 4 | 23 (3) | 21 (3) | 38 (3) | 12 (3) | 14 (2) | 19 (2) | 25 (3) | 16 (3) | 16 (2) |
| | Antiparasitics | 6 | 3 | 6.7 (2) | 7.9 (2) | 21 (2) | 5.7 (2) | 7.7 (2) | 11 (2) | 9.4 (2) | 8.8 (2) | 5.2 (2) |
| | Antiseptics | 11 | 7 | 0.07 (1) | ND | 0.15 (1) | 0.16 (1) | 0.63 (1) | ND | 1.1 (1) | ND | ND |
| | Antivirals | 2 | 2 | 0.19 (1) | ND | 0.15 (1) | ND | ND | 2.7 (1) | 2.9 (1) | 2.4 (1) | 1.5 (1) |
| | Cardiovascular | 31 | 21 | 481 (15) | 147 (17) | 236 (18) | 170 (18) | 182 (16) | 23 (14) | 25 (13) | 14 (13) | 15 (9) |
| | Contrast agents | 2 | 1 | 15 (1) | 6.3 (1) | 13 (1) | 6.5 (1) | 2.0 (1) | 3.4 (1) | 2.0 (1) | 0.91 (1) | 0.24 (1) |
| | Food ingredients | 11 | 7 | 144 (6) | 172 (6) | 261 (6) | 159 (6) | 260 (7) | 178 (7) | 152 (6) | 99 (7) | 21 (4) |
| | Hormones | 17 | 7 | 1.4 (6) | NA[b] | 1.8 (6) | 3.4 (6) | 6.6 (7) | NA[b] | NA[b] | NA[b] | NA[b] |
| | Illicit drugs | 15 | 7 | 4.6 (6) | 3.7 (6) | 19 (6) | 2.0 (6) | 153 (7) | 27 (5) | 54 (5) | 19 (5) | 15 (3) |
| | Immunosuppressants | 4 | 1 | 5.2 (1) | ND | 8.5 (1) | 4.2 (1) | 6.3 (1) | 6.2 (1) | 11 (1) | 10 (1) | 6.0 (1) |
| | Lipid-lowering | 6 | 4 | 13 (3) | 43 (3) | 34 (3) | 18 (4) | 32 (3) | 33 (2) | 48 (4) | 33 (3) | 35 (3) |
| | Psychoactive | 73 | 53 | 2,773 (49) | 1,542 (43) | 3,055 (47) | 2,034 (50) | 1,520 (45) | 1,579 (47) | 1,847 (43) | 1,341 (41) | 732 (32) |
| | Sweeteners | 4 | 2 | 710 (2) | 788 (2) | 2,580 (2) | 1,104 (2) | 1,046 (2) | 1,096 (2) | 1,240 (1) | 834 (2) | 354 (1) |
| | Synthetic hormones | 39 | 4 | 0.08 (1) | NA | 0.04 (1) | 0.11 (2) | 0.23 (3) | NA | NA | NA | NA |
| | Urologicals | 3 | 2 | 11 (1) | ND | ND | ND | 16 (1) | 19 (1) | ND | 11 (2) | ND |
| | UV filters | 5 | 5 | 28 (3) | ND | 46 (1) | 83 (2) | 164 (5) | 120 (2) | 20 (1) | 106 (1) | ND |
| | Other pharmaceuticals | 13 | 5 | 51 (2) | 2.9 (2) | 2,416 (3) | 44 (3) | 876 (3) | 758 (3) | 1,419 (4) | 1,082 (3) | 32 (1) |
| Pesticides | Avicides | 3 | 2 | 4.4 (2) | ND | 3.7 (1) | 2.3 (1) | 2.0 (1) | ND | ND | ND | ND |
| | Fungicides | 51 | 38 | 84 (31) | 84 (24) | 74 (25) | 52 (29) | 75 (31) | 113 (27) | 68 (26) | 118 (30) | 45 (23) |
| | Herbicides | 120 | 73 | 422 (53) | 360 (53) | 366 (55) | 355 (60) | 376 (61) | 704 (60) | 697 (60) | 730 (57) | 384 (53) |
| | Insecticides | 43 | 19 | 66 (14) | 24 (12) | 134 (14) | 105 (15) | 152 (18) | 172 (16) | 180 (16) | 295 (17) | 153 (15) |
| | Repellents | 2 | 2 | 227 (2) | 210 (2) | 171 (2) | 101 (2) | 243 (2) | 166 (2) | 116 (2) | 115 (2) | 193 (2) |
| *HLB samplers* | | | | | | | | | | | | |
| Industrial | Dyes | 4 | 3 | 12 (2) | 12 (2) | 12 (2) | 9.6 (3) | 6.6 (2) | 6.2 (2) | 5.1 (2) | 4.3 (2) | 0.89 (1) |
| | General industrial | 82 | 42 | 3,586 (34) | 2,930 (25) | 5,381 (30) | 2,886 (34) | 2,523 (31) | 2,843 (29) | 4,416 (31) | 2,179 (30) | 1,637 (26) |
| | PFCs | 8 | 3 | 76 (3) | 51 (2) | 87 (3) | 48 (3) | 45 (2) | 56 (2) | 58 (2) | 46 (2) | 45 (1) |
| | Plastic additives | 16 | 10 | 710 (9) | 1,100 (8) | 1,123 (9) | 559 (8) | 907 (9) | 854 (10) | 904 (9) | 613 (9) | 464 (8) |
| | Rubber additives | 8 | 5 | 415 (4) | 288 (3) | 1,208 (5) | 721 (5) | 157 (4) | 262 (4) | 260 (4) | 208 (4) | 105 (2) |
| | Surfactants | 15 | 13 | NC[c] | NC | NC | NC | NC | NC | NC | NC | NC |

| Class of chemicals | Subclass of chemicals | Number of analyzed compounds | Number of detected compounds | pg/L (number of detected compounds) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | site 1 | site 2 | site 3 | site 4 | site 5 | site 6 | site 7 | site 8 | site 9 |
| *Silicone rubber (SR) samplers* | | | | | | | | | | | | |
| PAHs | PAHs | 29 | 28 | 11,074 (27) | 10,313 (28) | 14,434 (28) | 9,720 (27) | 20,677 (28) | 7,683 (27) | 12,060 (27) | 6,956 (27) | 8,746 (28) |
| Industrial | PCBs | 8 | 8 | 102 (8) | 63 (8) | 83 (8) | 126 (8) | 72 (8) | 129 (8) | 101 (8) | 69 (8) | 268 (8) |
| | BDEs | 10 | 10 | 8.7 (10) | 6.3 (10) | 7.4 (10) | 11 (10) | 7.2 (10) | 6.2 (10) | 6.6 (10) | 5.8 (10) | 2.7 (10) |
| Pesticides | Fungicides | 1 | 1 | 19 (1) | 9.0 (1) | 8.7 (1) | 12 (1) | 7.4 (1) | 7.6 (1) | 8.9 (1) | 10 (1) | 9.0 (1) |
| | Insecticides | 28 | 28 | 110 (22) | 201 (22) | 257 (25) | 299 (24) | 279 (25) | 288 (24) | 302 (22) | 337 (23) | 428 (21) |

**Abbreviations and description:** the number of analytes shows the total number of compounds in the respective class analyzed in the samplers, analytes are put into classes and subclasses according to their main use in the columns "class of chemicals" and "subclass of chemicals". BDEs – brominated diphenyl ethers, HLB – AttractSPE$^{TM}$ HLB disks, NA – not analyzed, ND – not detected, NC – not converted to aqueous concentrations, PAHs – polycyclic aromatic hydrocarbons, PFCs – perfluoroalkyl compounds, PCBs – polychlorinated biphenyls, PPCPs – pharmaceuticals and personal care products, and SR – silicone rubber samplers. The concentrations of individual compounds and their limits of quantification are given in SM 2 (A1 and A2). No compound from the subclass of plant growth regulators was detected (chlormequat, mepiquat, chlorpropham and daminozide) and thus this subclass is not included in this table. [a] – for these compounds EEC could be derived using $K_{HLB,w}$, [b] – only L-thyroxine was analyzed from the group of hormones in these samples but not found. [c] – sampled amounts of surfactants were not translated to aqueous concentrations because experimental $K_{HLB,w}$ values were not available for these compounds and the model equation (9) that is applied for estimation of $K_{HLB,w}$ from compound properties does not consider surfactant distribution in a sorbent-water system.

### 3.2.2. Interpretation of contaminant profiles in HLB disks

The results revealed very complex mixtures of contaminants across all sampling sites consisting of broad spectra of industrial chemicals, pesticides and pharmaceuticals. The most frequently detected chemicals in HLB samplers were herbicides (73 compounds were found at least once), psychoactive pharmaceuticals (53 compounds), general industrial chemicals (42 compounds), fungicides (38 compounds), and cardiovascular pharmaceuticals (21 compounds) (SM 2, A1). The numbers of quantified compounds compared to their cumulative sampled amounts and EECs are depicted in Fig. 2A and B. 381 of the 671 analyzed compounds were detected at least once in HLB samplers (SM 2, A1). The total number of compounds quantified at any one specific site ranged from 212 to 313 and from 67 to 74 in HLB and SR samplers, respectively (Fig. 2). For comparison, Novák et al. (2018) detected up to 103 and 70 compounds per river stretch (sampled during a ship cruise) of the Danube River in Empore disks and SR samplers within JDS3, respectively. Interestingly, the total sampled amounts of industrial compounds (including surfactants, Table S4) were higher at all sampling sites than cumulative sampled amounts of PPCPs and pesticides despite a lower number of analyzed industrial compounds compared to PPCPs and pesticides in HLB samplers (Fig. 2A). The cumulative estimated aqueous concentrations of industrial compounds (sum of EECs without surfactants, Table 3) and PPCPs were comparable but still higher than those of pesticides in HLB samplers (Fig. 2B). The pattern and levels of the cumulative sampled amount compared to cumulative aqueous concentrations of industrial compounds was affected by the fact that the sampled amount of surfactants, that were highly abundant at a few sites (Table S4), could not be translated into aqueous concentrations as their experimental $K_{HLB,w}$ values were not available and the model equation (9) that is applied for $K_{HLB,w}$ estimation does not consider surfactant distribution in a sorbent-water system (Fig. 2A and B). These sites with greater surfactant levels are connected to capital cities, particularly site 2 is downstream of Vienna and Bratislava, site 3 downstream of Budapest and site 5 downstream of Belgrade.

The five compounds with the highest concentrations in HLB samplers were hexa(methoxymethyl)melamine, lauramidopropylbetaine, triisobutylphosphate, sucralose and tetraglyme, ranging from 31 to 111 μg/sampler (corresponding to estimated water concentrations of 0.4–1.9 μg/L). In HLB samplers, the cumulative sampled amounts, estimated cumulative aqueous concentrations, and spatial distribution of individual chemicals at sites within the groups based on their use are described and shown in more detail in SM 1 (Text S5.1, Figs. S4–S27). Regarding the spatial distribution of compounds in HLB disks, site 1 that receives pollution from German part of Danube where is the highest population density in the Danube region (Hardi, 2012), was burdened with the highest load of antibiotics and cardiovascular pharmaceuticals compared to other sites (Figs. S8 and S10). The concentrations of cardiovascular pharmaceuticals were greater in upstream sites and strongly decreased in the lower Danube from site 6 (Figs. S9 and S10). The cumulative concentrations of herbicides were markedly higher at sites 6 – 8 than at other sites (Fig. S25), which indicates their origin from agriculture, that is an important economic activity in this area (Gajić et al., 2015).

### 3.2.3. Interpretation of contaminant profiles in SR samplers

A range of PAHs, PCBs, BDEs, and pesticides was analyzed in SR samplers. Industrial compounds clearly dominated over the pesticides in SR samplers (Fig. 2C). In general, the derived aquatic concentrations of pollutants in SR samplers were lower by about three orders of magnitude than in HLB samplers (Fig. 2B and D). Most of the compounds were detected in the group of PAHs (29 compounds found at least once), followed by insecticides (19 compounds), and BDEs (10 compounds) (Table 3 and SM 2, A2). The spatial distribution, cumulative sampled amounts, and estimated cumulative aqueous concentrations per group for compounds detected in SR samplers are given in SM 1 (Figs. S28–S35). The sum of PAHs that are priority substances under WFD was the highest at sites 3 and 7 (10 ng/L; SM 2, A2).

The highest concentrations of PAHs in the water column and sediment pore water downstream of Budapest have been observed also in a passive sampling campaign performed in 2013 in JDS3 (Belháčová-Minaříková et al., 2020; Vrana et al., 2018). The highest load of the sum of seven indicator PCB congeners was found at site 9 (0.26 ng/L; SM 2, A2), where the cumulative concentrations of all PCBs were also the highest, particularly due to elevated levels of PCB 28, and PCB 52 (Fig. S31). Interestingly, the sums of indicator PBDEs concentrations were almost 2 orders of magnitude lower than those of sum indicator PCBs. The highest sum of the WFD-regulated BDE congeners (10.3 pg/L) was found at site 4, followed by site 1 (SM 2, A2). Total concentrations of industrial compounds (PAHs, PCBs, and BDEs) were higher than those of organochlorine pesticides at all sampling sites since also more industrial compounds than pesticides were quantified in SR samplers (Fig. 2C, D). The highest concentrations of industrial compounds in water derived from SR samplers were observed at site 5 (Fig. 2D). The cumulative concentrations of organochlorine pesticides (mostly banned in the EU) in water sampled by SR samplers have shown a clear increasing trend down the river stream in SR samplers (Fig. 2D, Fig. S35, SM 2, A6, A7).

## 3.3. Risk prioritization based on chemical targeted analyses

Predicted and experimentally based *PNEC* values were available for 353 out of 359 and for 52 out of 75 compounds detected in the HLB and SR samplers for which aqueous concentrations could be estimated, respectively (SM 2, A10 - A12). For all prioritized compounds, there was at least one site with *RQ*s above 0.5, while twelve and two compounds exceeded the *RQ* of 1 at least at one site in the HLB and SR samplers, respectively (Table 4). The number of compounds with *RQ* ratios exceeding the value of 1 was the highest at site 8 (seven compounds), followed by sites 1, 2, and 5 (six compounds) in the HLB samplers. The overall cumulative risk at each site (i.e., the sum of the individual $RQ_i$) was the highest at the sites 1 and 3, reaching sum of *RQ*s of 127 and 116 in HLB samplers, based on the top 18 compounds, respectively (Table 4, SM 2, A10). Recently, Finckh et al. (2022) analyzed 54 WWTP effluents in various European countries and assessed the risk of organic chemicals by using three metrics: *RQ*s, Hazard Units based on species sensitivity distribution ratios and Toxic Units. They found that *RQ*, which has been used also in the present study, was the most sensitive metric. Noteworthy, there were four compounds in the present study that exceeded the *RQ* of 1 at all sites and their final scores, that included mixture risks (Eq. (5)), were the following: heptachlor epoxide (1.2) in SR samplers, as well as perfluorooctanesulfonic acid (PFOS; 1.2), hexa(methoxymethyl) melamine (1.2), and fipronil (1.1) in HLB samplers (Table 4). Nevertheless, the rather low effect threshold for heptachlor epoxide has been discussed as potentially too conservative (van der Oost et al., 2017b). It is alarming that some of the prioritized compounds exceeded the RQ by more than a factor of ten at all sites (i.e., PFOS and hexa(methoxymethyl)melamine) or at least at some sites (heptachlor epoxide and 17α-ethinylestradiol) (Table 4).

The *RQ* of 1 is usually considered as a threshold above which adverse effects cannot be excluded. However, compounds with *RQ*s slightly below 1 can additively contribute to the overall risk posed by all compounds present in a sample, commonly referred to as mixture risks. Therefore, we have also considered compounds with *RQ*s between 0.5 and 1.0 ($MRC_i$, Eq. (4)). Note that six compounds (terbuthylazine-2-hydroxy, metolachlor, flufenacet, imidacloprid, triclocarban, and diflubenzuron) in HLB samplers and one compound (alpha-endosulfan) in SR samplers were only prioritized based on their mixture contribution, while the other twelve (in HLB) and two (in SR) compounds had at least a single exceedance of *RQ* of 1 (Table 4). Hence, the overall relevance of compounds being prioritized solely based on their contribution to mixture risks is smaller.

Interestingly, some of the compounds prioritized based on the Final score in our study, i.e., PFOS, fipronil, and venlafaxine also posed a

**Table 4**
Compounds exceeding the Risk Quotient of 0.5 at least at one site. The compounds are ordered in descending order according to the Final score.

| Compound | *PNEC* (ng/L) | *PNEC* type | HLB samplers | | | | | | | | | *EoE* score | *FoE* | *MRC* | Final score[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *RQ* (*EEC*/*PNEC* ratio) | | | | | | | | | | | | |
| | | | site 1 | site 2 | site 3 | site 4 | site 5 | site 6 | site 7 | site 8 | site 9 | | | | |
| perfluorooctanesulfonic acid (PFOS) | 0.65 | AA-EQS | **68** | **51** | **73** | **41** | **60** | **71** | **78** | **58** | **69** | 0.20 | 1.0 | 0 | 1.2 |
| hexa(methoxymethyl) melamine | 57 | P-PNEC pred | **24** | **17** | **34** | **17** | **17** | **23** | **21** | **16** | **10** | 0.20 | 1.0 | 0 | 1.2 |
| fipronil | 0.70 | AA-EQS proposal | **2.5** | **3.2** | **3.2** | **1.1** | **4.3** | **3.0** | **2.4** | **2.6** | **1.9** | 0.10 | 1.0 | 0 | 1.1 |
| thiacloprid | 10 | AA-EQS | 0.85 | 0.78 | **1.5** | 0.88 | **1.2** | **1.3** | **1.6** | **4.8** | **1.7** | 0.10 | 0.67 | 0.17 | 0.93 |
| nicosulfuron | 8.7 | AA-EQS | NC | NC | NC | **1.6** | **3.0** | **3.4** | NC | **3.3** | **3.4** | 0.10 | 0.56 | 0 | 0.66 |
| tri(butoxyethyl)phosphate | 142 | P-PNEC pred | **1.3** | **1.3** | **1.1** | 0.35 | 0.46 | 0.98 | 0.74 | 0.59 | 0.20 | 0.10 | 0.33 | 0.17 | 0.60 |
| dimethenamid | 130 | JG-MKN (totaal) | 0.55 | 0.35 | 0.45 | 0.45 | 0.55 | 0.98 | **1.2** | **1.3** | 0.48 | 0.10 | 0.22 | 0.17 | 0.49 |
| venlafaxine | 38 | AA-EQS-proposal | **3.7** | **1.2** | 0.96 | 0.83 | 0.02 | 0.16 | 0.04 | 0.02 | <0.01 | 0.10 | 0.22 | 0.11 | 0.43 |
| chlorpyrifos | 0.46 | AA-EQS | NC | NC | NC | NC | 0.73 | **1.02** | 0.68 | **1.7** | 0.23 | 0.10 | 0.22 | 0.11 | 0.43 |
| 17α-ethinylestradiol | $3.2 \times 10^{-3}$ | AA-QSfw,eco | **24** | NA | NC | NC | NC | NA | NA | NA | NA | 0.20 | 0.11 | 0 | 0.31 |
| terbuthylazine-2-hydroxy | 7.3 | PNEC chronic | 0.38 | 0.41 | 0.55 | 0.47 | 0.49 | 0.90 | 0.74 | 0.66 | 0.38 | 0 | 0 | 0.22 | 0.22 |
| clotrimazole | 30 | P-PNEC pred | NC | **2.8** | NC | NC | NC | 0.23 | NC | 0.20 | NC | 0.10 | 0.11 | 0 | 0.21 |
| N,N-dimethyltetradecylamine | 26 | PNEC | NC | NC | NC | NC | **1.3** | NC | NC | NC | NC | 0.10 | 0.11 | 0 | 0.21 |
| metolachlor | 200 | JD-UQN | 0.18 | 0.15 | 0.14 | 0.17 | 0.14 | 0.76 | 0.59 | 0.81 | 0.23 | 0 | 0 | 0.17 | 0.17 |
| flufenacet | 40 | AA-EQS | 0.70 | 0.71 | 0.63 | 0.27 | 0.26 | 0.44 | 0.45 | 0.21 | 0.10 | 0 | 0 | 0.17 | 0.17 |
| imidacloprid | 8.3 | PNEC | 0.22 | 0.17 | 0.29 | 0.28 | 0.61 | 0.48 | 0.37 | 0.56 | 0.78 | 0 | 0 | 0.17 | 0.17 |
| triclocarban | 1.1 | PNEC chronic | NC | NC | NC | NC | NC | NC | 0.99 | NC | NC | 0 | 0 | 0.06 | 0.06 |
| diflubenzuron | 4.0 | CQC ad hoc | NC | NC | NC | NC | 0.80 | NC | NC | NC | NC | 0 | 0 | 0.06 | 0.06 |
| cumulative risk at sites (RQ sum)[a] | | | 127 | 79 | 116 | 65 | 91 | 108 | 110 | 91 | 90 | | | | |
| number of compounds with RQ > 1 | | | 6 | 6 | 5 | 4 | 6 | 5 | 5 | 7 | 5 | | | | |

| Compound | *PNEC* (ng/L) | *PNEC* type | SR samplers | | | | | | | | | *EoE* score | *FoE* | *MRC* | Final score[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *RQ* (*EEC*/*PNEC* ratio) | | | | | | | | | | | | |
| | | | site 1 | site 2 | site 3 | site 4 | site 5 | site 6 | site 7 | site 8 | site 9 | | | | |
| heptachlor epoxide | $2.0 \times 10^{-4}$ | AA-EQS | **21** | **5.3** | **7.1** | **13** | **5.9** | **5.2** | **6.3** | **22** | **10** | 0.20 | 1.0 | 0 | 1.2 |
| heptachlor | $2.0 \times 10^{-4}$ | AA-EQS | 0.14 | 0.08 | 0.06 | NC | 0.15 | 0.12 | 0.39 | **12** | **2.1** | 0.20 | 0.22 | 0 | 0.42 |
| alpha endosulfan | $5.0 \times 10^{-3}$ | AA-QSfw,eco | 0.61 | 0.23 | 0.15 | 0.41 | 0.25 | 0.12 | 0.17 | NC | 0.04 | 0 | 0 | 0.06 | 0.06 |
| cumulative risk at sites (*RQ* sum)[a] | | | 22 | 5.7 | 7.3 | 13 | 6.3 | 5.4 | 6.8 | 35 | 12 | | | | |
| number of compounds with *RQ* > 1 | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | | | | |

**Abbreviations and description:** [a] – rounded values of *EoE*, *FoE*, *MRC* and *RQ* are presented, the compounds exceeding the acceptable *RQ* of 1 are marked in bold. *EEC* – estimated environmental concentration, *PNEC* – predicted no-effect concentration, *NA* – not analyzed, *NC* – not calculated because the concentration of the compound was below limit of quantification (the respective limits of quantification are given in Supplementary material 2), *EoE* – extent of exceedance sub-score, *FoE* – frequency of exceedance sub-score, *MRC* – mixture risk contribution sub-score, the Final score is calculated as the sum of *EoE*, *FoE* and *MRC*, for details see section 2.5.

AA-EQS – annual average environmental quality standard, AA-QSfw-eco – annual average quality standard for ecological integrity of freshwater. P-PNEC pred – provisional Predicted No-Effect Concentration based on QSAR predictions, AA-EQS proposal by the Swiss Ökotox Zentrum, JG-MKN (totaal) – Dutch annual average quality standard for freshwater derived by RIVM, JD-UQN – German annual average quality standard for freshwater derived by UBA, PNEC chronic – PNEC based on chronic data derived by NORMAN, CQC ad hoc – preliminary chronic Quality Criteria derived by the Swiss Ökotox Zentrum. The sources of PNECs of the prioritized compounds are given in SM 2, A12 and for the remaining compounds in NORMAN Ecotoxicology Database (accessed 14.07.2022), see section 2.5.

**Table 5**
Bioanalytical Equivalent Concentrations (BEQ) per sampler and converted to aqueous BEQ (based on estimated sampled volumes of water provided in Table 1 with details of the calculation in section 3.1. and SM 2, A3).

| Site | Type of sampler | AhR TCDD-EQs ± SE | | PPARγ GeneBLAzer Rosiglitazone-EQ ± SE | | ARE c32B(a) P-EQ ± SE | | ERα GeneBLAzer E2 EQs ± SE | | AR MDA-kB2 DHT EQ ± SE | | Anti-AR MDA-kB2 FLU EQ ± SE | | GR _GeneBLAzer DEX-EQ ± SE | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ng/sampler | pg/L | µg/sampler | ng/L | µg/sampler | ng/L | ng/sampler | pg/L | ng/sampler | pg/L | µg/sampler | µg/L | µg/sampler | ng/L |
| 1 | HLB | **2.6 ± 0.4** | **36 ± 6** | **0.45 ± 0.03** | **6.3 ± 0.4** | <58 | <799 | <6.5 | <89 | <10 | <139 | **215 ± 69** | **3.0 ± 0.9** | <0.36 | <4.9 |
| 2 | HLB | **2.6 ± 0.7** | **35 ± 9.1** | **0.73 ± 0.07** | **10 ± 1** | <80 | <1098 | **8.6 ± 1.5** | **119 ± 22** | <14 | <187 | **353 ± 150** | **4.9 ± 2.1** | <0.24 | <3.4 |
| 3 | HLB | **2.2 ± 0.4** | **31 ± 5.3** | **0.68 ± 0.05** | **9.4 ± 0.6** | <94 | <1293 | <11 | <144 | <7.6 | <105 | **290 ± 70** | **4.0 ± 1.0** | <0.58 | <7.9 |
| 4 | HLB | **1.2 ± 0.1** | **16 ± 1.5** | **0.84 ± 0.10** | **12 ± 1** | <58 | <799 | **18 ± 2** | **244 ± 26** | <4.8 | <66 | **225 ± 74** | **3.1 ± 1.0** | <0.36 | <4.9 |
| 5 | HLB | **2.0 ± 0.1** | **27 ± 0.8** | **2.2 ± 0.3** | **30 ± 4** | <93 | <1285 | **41 ± 2** | **558 ± 34** | <27 | <376 | **1,031 ± 269** | **14 ± 3.7** | <0.52 | <7.1 |
| 6 | HLB | **3.0 ± 0.3** | **41 ± 4.1** | **3.6 ± 0.5** | **50 ± 7** | <139 | <1910 | <15 | <213 | <23 | <321 | **920 ± 358** | **13 ± 4.9** | <0.85 | <12 |
| 7 | HLB | **2.1 ± 0.2** | **29 ± 2.2** | **1.8 ± 0.2** | **25 ± 3** | <91 | <1258 | <10 | <140 | <37 | <505 | **440 ± 150** | **6.0 ± 2.1** | <0.28 | <3.8 |
| 8 | HLB | **2.4 ± 0.7** | **33 ± 9.8** | **1.4 ± 0.1** | **19 ± 1** | <133 | <1834 | <15 | <204 | <28 | <379 | **583 ± 194** | **8.0 ± 2.7** | <0.82 | <11 |
| 9 | HLB | **1.1 ± 0.3** | **16 ± 4.2** | **0.64 ± 0.05** | **8.8 ± 0.6** | <139 | <1910 | <7.7 | <106 | <7.1 | <98 | **659 ± 334** | **9.1 ± 4.6** | <0.43 | <5.9 |
| 1 | SR | **3.6 ± 0.5** | **1.6 ± 0.2** | <0.34 | <0.15 | **102 ± 7** | **45 ± 3** | <10 | <4.6 | <10 | <4.3 | **203 ± 88** | **0.09 ± 0.04** | <0.57 | <0.25 |
| 2 | SR | **17 ± 2** | **1.4 ± 0.2** | <1.6 | <0.14 | <236 | <20 | <49 | <4.3 | <14 | <1.2 | **241 ± 100** | **0.02 ± 0.01** | <2.7 | <0.24 |
| 3 | SR | **4.5 ± 0.1** | **0.8 ± 0.02** | <0.85 | <0.14 | **195 ± 14** | **33 ± 2** | <26 | <4.4 | <7.2 | <1.2 | **164 ± 11** | **0.03 ± 0.002** | <1.4 | <0.24 |
| 4 | SR | **5.5 ± 0.4** | **2.7 ± 0.2** | <0.29 | <0.14 | **73 ± 3** | **36 ± 2** | <8.9 | <4.4 | <13 | <6.5 | **148 ± 42** | **0.07 ± 0.02** | <0.49 | <0.24 |
| 5 | SR | **2.7 ± 0.6** | **0.9 ± 0.2** | <0.12 | <0.04 | **55 ± 3** | **19 ± 1** | <13 | <4.5 | **18 ± 4** | **6.1 ± 1.3** | <137 | <0.05 | <0.72 | <0.25 |
| 6 | SR | **6.2 ± 2.2** | **1.3 ± 0.5** | <0.72 | <0.15 | **115 ± 5** | **23 ± 1** | <22 | <4.4 | <28 | <5.7 | <233 | <0.05 | <1.2 | <0.24 |
| 7 | SR | **4.6 ± 0.6** | **1.5 ± 0.2** | <0.46 | <0.15 | **82 ± 4** | **26 ± 1** | <14 | <4.6 | <16 | <5.2 | <161 | <0.05 | <0.79 | <0.25 |
| 8 | SR | **7.5 ± 1.0** | **1.6 ± 0.2** | **0.72 ± 0.06** | **9.9 ± 0.8** | **149 ± 8** | **32 ± 2** | <21 | <4.5 | <6.9 | <1.5 | **448 ± 210** | **0.10 ± 0.04** | <1.2 | <0.25 |
| 9 | SR | **7.6 ± 1.3** | **1.5 ± 0.3** | <0.76 | <0.15 | <102 | <20 | <23 | <4.6 | **14 ± 7** | **2.8 ± 1.3** | <260 | <0.02 | <1.3 | <0.26 |

**Abbreviations and description:** the values with the symbol "less than" are the limits of quantifiable effects from *in vitro* bioassays calculated as the ratio of EC and the highest non-cytotoxic concentration. AhR – activation of the aryl hydrocarbon receptor, PPARγ - activation of the gamma isoform of the peroxisome proliferator-activated receptor, ARE – adaptive stress response to oxidative stress, ERα – activation of the alpha isoform of estrogen receptor, AR – activation of androgen receptor, Anti-AR – antagonism to the androgen receptor, GR – activation of the glucocorticoid receptor, HLB – AttractSPE$^{TM}$ HLB, SR – silicone rubber, IR – induction ratio, LOQs were calculated as $EC_{10}$ or $EC_{20}$ or $EC_{IR1.5}$ of the reference compound divided by the highest tested non-cytotoxic concentration of the sample, TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, ROS – rosiglitazone, B(a)P – benzo(a)pyrene, E2 – 17β-estradiol, DHT – dihydrotestosterone, FLU – flutamide, DEX – dexamethasone. Positive detections are marked in bold. Values are presented as mean ± standard error of the mean.

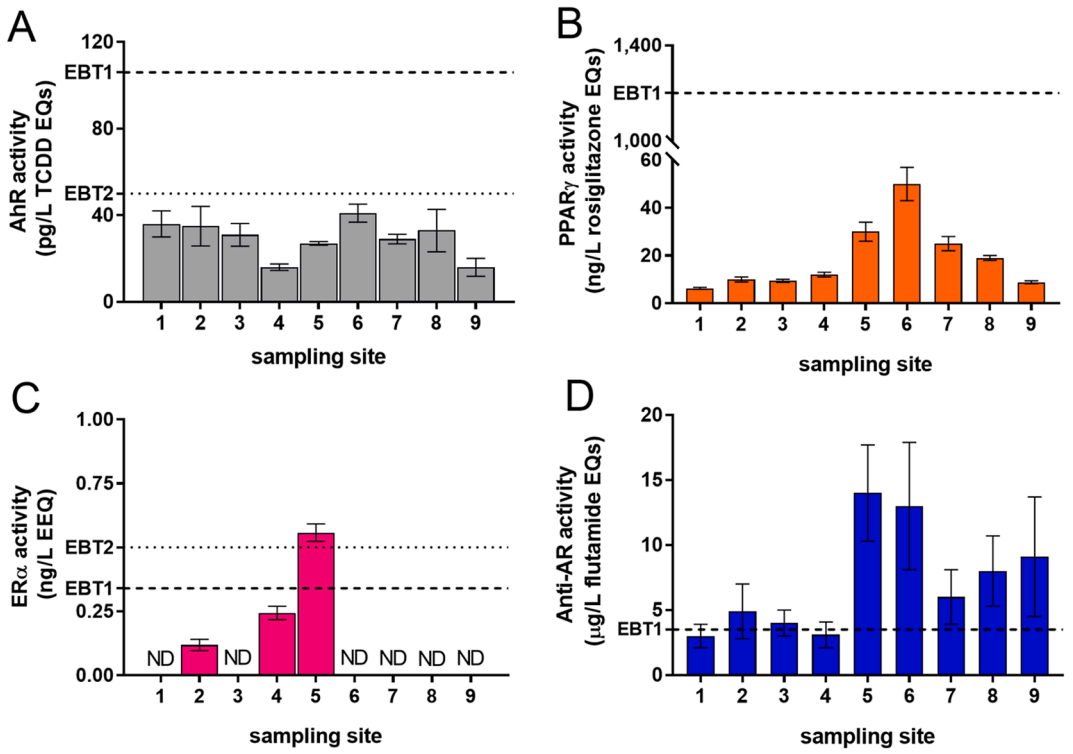


**Fig. 3.** Comparison of available effect-based trigger values (EBTs) and detected *in vitro* biological activities in HLB samplers. (A) AhR-mediated activity, (B) PPARγ-mediated activity, (C) estrogenic activity, (D) anti-androgenic activity. Values are given as mean ± standard error of the mean. The information on the applied EBT1s can be found in Table 2. EBT2s are from the study by van der Oost et al., 2017b. EEQ = 17ß-estradiol equivalents. ND = not detected.

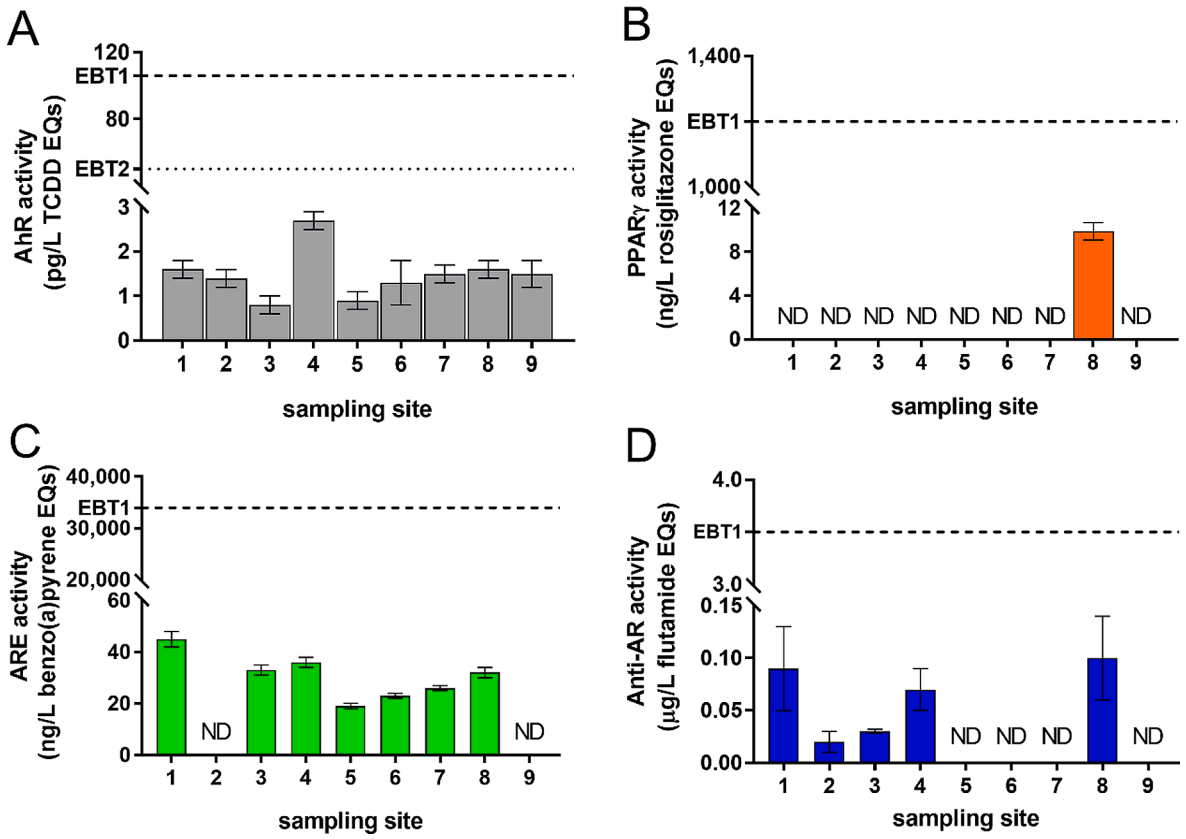


**Fig. 4.** Comparison of available effect-based trigger values (EBTs) and detected *in vitro* biological activities in SR samplers. (A) AhR-mediated activity, (B) PPARγ-mediated activity, (C) adaptive stress response to oxidative stress, (D) anti-androgenic activity. Values are given as mean ± standard error of the mean. The information on the applied EBT1 can be found in Table 2. EBT2 is from the study by van der Oost et al. (2017b). ND = not detected.

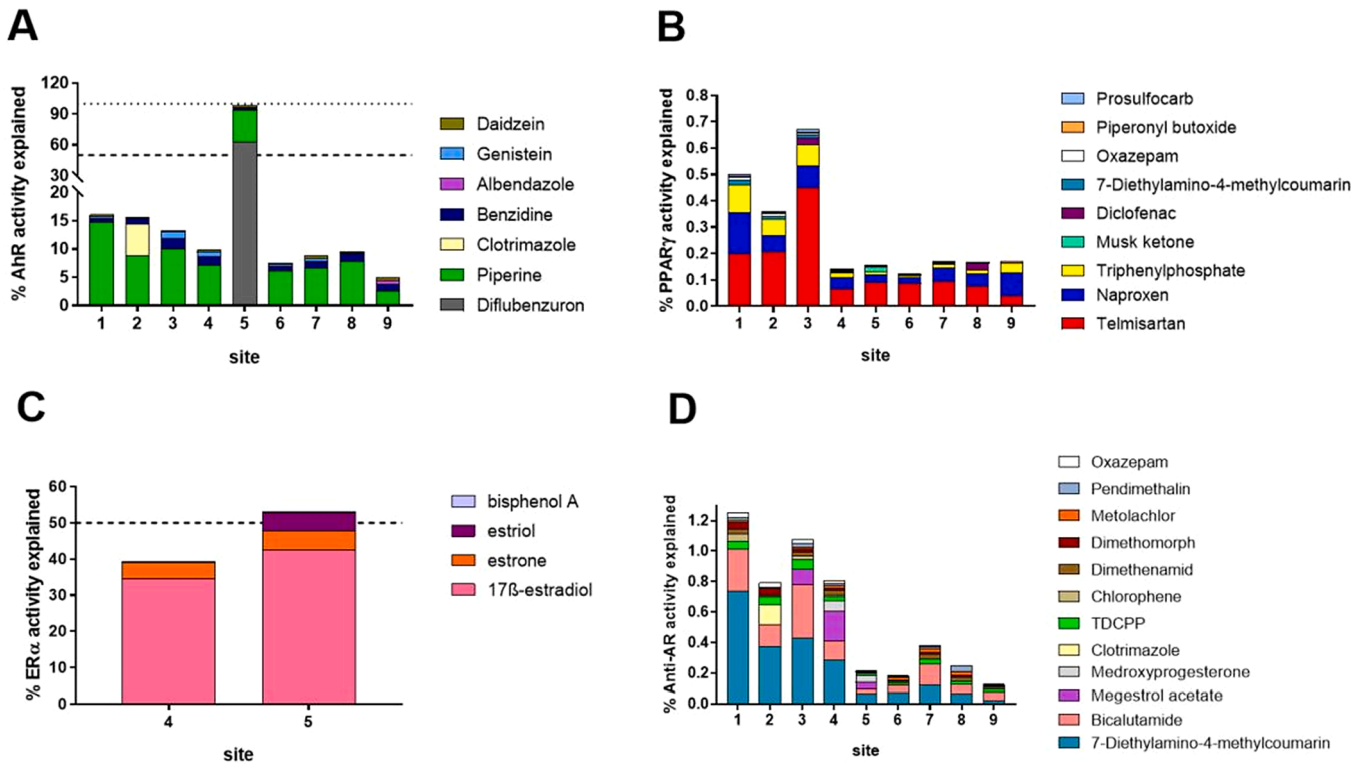


**Fig. 5.** Top known contributors to the *in vitro* biological activities detected in HLB samplers. The dotted and dashed lines indicate the 100% and 50% contribution of detected compounds to the measured effect, respectively. The contribution to the following mixture effects is shown: (A) AhR-mediated activity, (B) PPARγ-mediated activity, (C) estrogenic activity, (D) anti-androgenic activity.

potential risk in a recent study by Alygizakis et al. (2019), who analyzed WWTP effluents in the Danube River basin. Noteworthy, all three compounds with a *FoE* score above 1 in HLB samplers, i.e., exceeding the *RQ* of 1 at all sites, namely PFOS, hexa(methoxymethyl)melamine, and fipronil (Table 4) were also among the compounds contributing the most to the total risk in European WWTP effluents (Finckh et al., 2022).

### 3.4. *In vitro* bioassays and contribution of compounds to mixture effects

A battery of *in vitro* reporter gene bioassays was used to characterize the effects of organic micropollutants mixtures in extracts of two types of passive samplers from the nine sites in the Danube and to assess the contribution of the detected compounds to the observed effects. Six out of seven studied endpoints showed responses in bioassays in at least one sample (Table 5). In HLB samplers, anti-androgenic, AhR-mediated, and PPARγ activities were found at all nine monitored sites and estrogenic activity was detected at three sites. The following activities were found in SR samplers: AhR-mediated activity (all sites), adaptive stress response to oxidative stress (seven sites), anti-androgenic activity (five sites), androgenic activity (two sites), and PPARγ (one site) (Table 5). The detection frequency of individual activities in both types of samplers (in total 18 samples) was as follows: AhR-mediated activity (89%), anti-androgenicity (61%), PPARγ activity (56%), adaptive stress response to oxidative stress (39%), estrogenic activity (17%), and androgenic activity (6%). Glucocorticoid activity was not detected in any sample. Cytotoxicity was never observed in the case of SR extracts but appeared after exposure to most extracts from HLB disks (Table S5), where it could mask a portion of specific effects. The contribution of detected compounds to the observed *in vitro* biological activities of each sample is shown in Supplementary Materials (SM 2, A13 – A21).

#### 3.4.1. *Activation of AhR*

Out of the 671 compounds analyzed in HLB samplers in this study, 66 compounds were known to be active in the *in vitro* assay for transactivation of aryl hydrocarbon receptor (AhR), 359 compounds were inactive, and for 248 compounds there was no information about their activity (SM 2, A13). Out of the 76 compounds analyzed in SR samplers, REPs (ratio of effective concentration of the reference compound and effective concentration of the compound at a given efficacy) were available for ten compounds, 32 compounds were inactive, and for 34 compounds REPs were not available (SM 2, A13). In the present study, transactivation of aryl hydrocarbon receptor was detected in HLB as well as SR samplers at all nine sites (Fig. 3A and 4A, SM 2, A13, A17). The AhR-mediated activity in HLB samplers from 3 month-lasting sampling was rather comparable across the sites (Fig. 3A, Table 5). The highest TCDD-EQ in the HLB samplers was found in the lower part of the Danube (3.0 ng/sampler that is estimated to correspond to 41 pg/L) downstream the Iron Gate dam at site 6. Similarly, TCDD-EQs in SR samples recalculated per pg/L were comparable among most sites, with site 4 showing greatest TCDD-EQ of 2.7 pg/L (Table 5). The estimated effect-based trigger value for AhR activation (EBT-TCDD-EQ) of 106 pg/L (Table 2) was not exceeded in any of the samples in this study (Fig. 3A and 4A). This EBT-TCDD-EQ was re-calculated from EBT-B(a)P-EQ based on AhR-CALUX assay derived by Escher and Neale (2021), since the EBT specific to the CAFLUX assay is not available. As AhR CALUX (H4L7.5c2 cell line) established by Brennan et al. (2015) and AhR CAFLUX (cell line H4G1.1c2) established by Nagy et al. (2002) employ rat AhR and mouse DRE inserts which show high homology, they are similar enough to perform this recalculation and comparison. However, the existing EBT or any proposed EQS values for AhR activity need to be considered with caution in the context of inter-species variability in the activation of AhR among vertebrates (Xu et al., 2021). The EBT-TCDD-EQ of 50 pg/L for the DR CALUX (van der Oost et al., 2017b) was also not exceeded by any of the samples (Fig. 3A).

21 to 29 compounds were identified as contributors to AhR activation in HLB samplers. The sum contribution of these compounds to AhR activation in HLB samplers ranged from 6.3 to 101 % (median 13%, SM 2, A13), which is higher than the explicability of AhR activation by

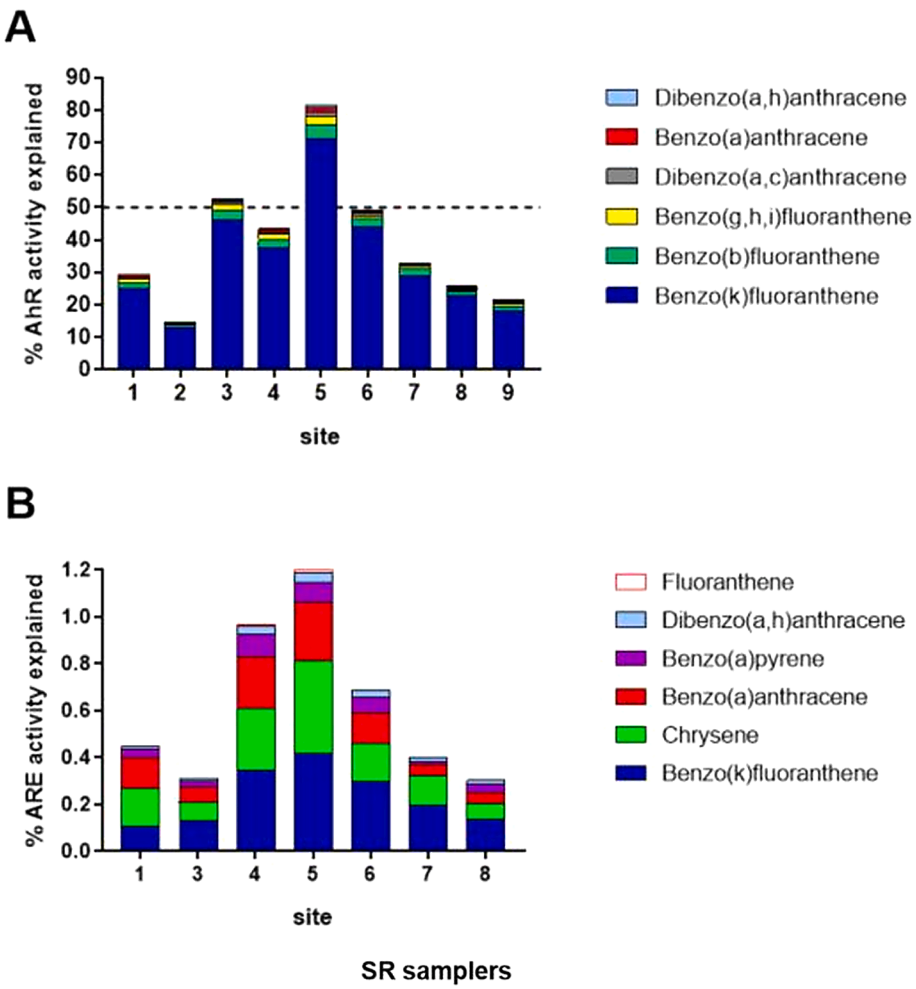


**Fig. 6.** Top known contributors to the *in vitro* biological activities detected in SR samplers. The dashed line indicates the 50% contribution of detected compounds to the measured effect. The contribution to the following mixture effects is shown: (A) AhR-mediated activity, (B) adaptive stress response to oxidative stress. The other activities (androgenic activity and anti-androgenic activity, and transactivation of peroxisome proliferator-activated receptor), where the contribution of detected compounds with known relative potencies was negligible, can be found in Supporting Information 1, Fig. S39.

detected chemicals (1%) in a nationwide US study of bioactivities that also analyzed over 700 chemicals in surface waters (Blackwell et al., 2019). The top contributors to AhR-mediated activity in our study are shown in Fig. 5A. The highest observed contribution was indicated for insecticide diflubenzuron, explaining as much as 63% of the observed effect at site 5, which was the only site with its detection. The alkaloid piperine, present in black pepper, displayed the highest mean contribution to AhR-mediated activity (11%) throughout all sites in the HLB samplers (SM2, A13) with *EEC*s ranging from 1.9 to 39 ng/L. These top contributors (piperine and diflubenzuron) identified in our study were not analyzed in Blackwell et al. (2019), which might be related to the lower percentage of AhR activation explained in their study. Recently, piperine was also reported in other European rivers up to concentration of 338 ng/L, which is higher than the Threshold for Toxicological Concern of 100 ng/L (Nanusha et al., 2020,2021). Zwart et al. (2018) and Gonzalez-Marino et al. (2012) reported detection of piperine in wastewater, explaining its presence by its use in food, food supplements, care products and as pesticide. This indicates that piperine deserves further attention in environmental monitoring and ecotoxicological research. The other contributors that explained some part of the AhR-mediated activity in HLB samplers were the antifungal compound clotrimazole (5.5%, site 2), and industrial compound benzidine (mean contribution 1.2%, found at all sites). Another contributor to this bioactivity, daidzein, was also among the effect drivers in LVSPE samples in a study from JDS3 by Neale et al. (2015), where it was responsible for a higher proportion of the activity (>50% at some sites) than in the present study.

In SR samplers, 15 to 82 % (median 39%), of the measured AhR activation was explained by the 10 detected compounds, for which REPs were available. The major driver was namely benzo(k)fluoranthene contributing up to 71% (Fig. 6A). In agreement with our results, benzo(k)fluoranthene and benzo(b)fluroanthene were also among the main drivers of the AhR activation in passive samplers in the Danube in JDS3 and a secondary tributary of the Danube, the Bosna River (Novák et al., 2018; Toušová et al., 2019).

Activation of the AhR triggers the expression of genes encoding enzymes involved in the metabolism of potentially toxic compounds (e.g., enzymes of the cytochromes P450 superfamily). It is a relevant, responsive endpoint in bioanalytical assessment of organic micropollutants in surface waters (Blackwell et al., 2019; Neale et al., 2017b; Rosenmai et al., 2018). Various hydrophobic and, to some extent, hydrophilic compounds are ligands of the AhR. The occurrence of AhR-mediated activity is often associated with PAHs, polychlorinated and polybrominated dibenzo-*p*-dioxins and biphenyls (Escher et al., 2021; Kinani et al., 2010) and was associated with WWTP effluents discharges (Dagnino et al., 2010). In our study, significant proportion of the AhR-mediated activity in SR samplers was explained by PAHs (Fig. 6A), while the main identified contributors in HLB samplers were diflubenzuron (63%) and piperine (31%) (Fig. 5A). This indicates that there are diverse sources of both hydrophilic and hydrophobic compounds that activate AhR, and these compounds co-occur in environmental mixtures. The SR samplers were also used in the previous short-term passive sampling of Danube in JDS3 performed by Novák et al. (2018), where the known contributors explained only up to 7.9% of AhR-mediated activity. The percentage of AhR activation explained was considerably higher in this longer-term study (median 39% explained in

JDS4 SR samplers). Nevertheless, four out of five most significant contributors to AhR-mediated activity in SR samplers in the present study were also among the major contributors in JDS3 (Novák et al., 2018), indicating stable levels of these chemicals in the Danube.

### 3.4.2. Peroxisome proliferator-activated receptor isoform gamma-mediated activity

All extracts from HLB disks exhibited PPARγ-mediated activity with rosiglitazone-EQs ranging from 0.45 to 3.6 μg/sampler (corresponding to 6.3 – 50 ng/L; Table 5). Levels of PPARγ-mediated activity were generally greater in the middle part of the Danube (Site 5–8). The number of detected chemicals with available REPs for the PPARγ activation in samples from HLB disks ranged from 14 to 18 compounds at various sites. Their joint contribution to the PPARγ activity in HLB disks was minor and ranged from 0.11 to 0.75 %. The most significant identified contributors to PPARγ activity in HLB disks are given in Fig. 5B. The four compounds contributing most to the observed PPARγ activity were telmisartan, naproxen, triphenyl phosphate (in all HLB samplers) and diclofenac (in 56% samples) (SM 2, A1). The high potency of telmisartan and diclofenac played an important role in their resulting $BEQ_{chem}$. In contrast, the other contributors (naproxen, triphenylphosphate, and musk ketone) had a high $BEQ_{chem}$ instead due to the combination of somewhat lower potency and high concentrations at which they occurred.

Only one SR sampler (at site 8) displayed detectable PPARγ-mediated activity (Table 5). A few compounds (fluoranthene, benzo(k)fluoranthene, phenanthrene, PCB-28, and BDE-47) were found to contribute to its PPARγ activity, but with a negligible percentage of $BEQ_{bio}$ explained (Fig. S39A, SM 1, Text S8).

PPARγ-mediated activity has been frequently detected in surface waters (Blackwell et al., 2019; De Baat et al., 2019). van der Oost et al. (2017b) observed PPARγ activation by SR samples from Dutch surface waters with much higher frequency than it was found in the present study indicating a lower burden of Danube with hydrophobic compounds activating PPARγ. This bioactivity in aquatic environment often remains largely unexplained, which corresponds to its low explicability in our study (SM 2, A14, A19). For example, Neale et al. (2020) similarly to our study revealed only up to 1.7% of the contributors to PPARγ-mediated activity in small agricultural streams in Germany in 2018. The compounds most contributing to PPARγ activity in this study, telmisartan and diclofenac, were also among the 17 prioritized compounds according to their potential ecotoxicity based on environmental concentrations and *PNEC* among assessed 280 compounds in WWTP effluents in the Danube River basin (Alygizakis et al., 2019).

PPARγ is an essential regulator of adipogenesis, energy balance and lipid biosynthesis, and it also affects lipoprotein metabolism and insulin sensitivity (Grygiel-Gorniak 2014). PPARγ is activated by multiple endogenous agents, including prostaglandins, polyunsaturated fatty acids, and oxidized lipids (Willson et al., 2000). The effect-based trigger (EBT) value of 1.2 μg/L rosiglitazone-EQs, the exceedance of which indicates poor water quality (Escher and Neale 2021), was not exceeded in any JDS4 passive sample (Fig. 3B and 4B). However, the frequent detection of this activity in the Danube indicates omnipresent burden of the Danube River with PPARγ agonists. Thus, PPARγ activation deserves further monitoring due to the potential to induce unwanted effects on the normal PPARγ signaling in exposed biota.

### 3.4.3. Oxidative stress response

Oxidative stress response was detectable only in SR samplers. It was detected at seven out of nine sites and the benzo(a)pyrene equivalent concentrations (B(a)P-EQ) ranged from 55 to 195 μg/sampler (median 102 μg/sampler B(a)P-EQs) that corresponds to the estimated range of 19–45 ng/L B(a)P-EQs (Table 5, SM 2, A18). Nevertheless, after converting the adaptive stress response to oxidative stress to estimated concentrations per volume of river water, the differences among sites over the longer sampling period were only minor (maximum two-fold difference between sites). It appears that oxidative stress response elicited by extracts from SR samplers was caused by more hydrophilic compounds rather than B(a)P (SM2, A18), which occurs in Danube at low freely dissolved concentrations (in tens of pg/L; Novak et al., 2018). No specific spatial pattern of this response occurrence was observed among the sampling sites. The top known contributors to adaptive stress response to oxidative stress in SR samplers, which jointly explained only 0.3–1.2% of the total activity (median 0.45%), are shown in Fig. 6B. These include benzo(k)fluoranthene, chrysene, benzo(a)anthracene, benzo(a)pyrene, and dibenzo(a,h)anthracene.

AREc32 is an *in vitro* reporter gene assay based on activating nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway. The Nrf2/ARE-regulated genes have a cytoprotective function and contribute to cellular antioxidant defense systems. The detection of oxidative stress response in the Danube River in our study indicates a widespread presence of unknown chemicals inducing expression of cytoprotective genes. In agreement with our results, the occurrence of compounds causing oxidative stress response (measured by Nrf2-CALUX *in vitro* bioassay) was recently reported by Alygizakis et al. (2019) in 83% of the investigated WWTP effluents along the Danube River. Similar to our findings, the effect drivers of adaptive stress response to oxidative stress remained largely unexplained also in other studies (Escher et al., 2013; Tang et al., 2014). The greatest contributor to oxidative stress response identified in a study by König et al. (2017) was caffeine, even though it was responsible for only 9% of the activity at one site downstream of a wastewater discharge to the Danube River, where it was present at a high concentration (4 μg/L). The bioassays for adaptive stress response to oxidative stress as well as PPARγ-mediated activity and anti-androgenic activity are the bioassay 2 type, where many low-potency compounds can contribute to the effect due to their low specificity ratio (Escher and Neale 2021). Correspondingly, the oxidative stress response in the Danube River samples appears to be a result of combined action of many compounds. The recently revised EBT-dichlorvos-EQ of 1.4 mg/L (Escher and Neale 2021) corresponds to a B(a)P-EQ of 34 μg/L (Table 2), which was not exceeded at any studied site in the present study (Fig. 4C).

### 3.4.4. Estrogen receptor alpha-mediated activity

Cytotoxicity complicated detectability of the estrogenic activity in most of the HLB samplers (at sites 1, 3, 6, 7, 8, and 9; Tables S5 and 5). Thus, estrogenic activity was detectable only at sites 2, 4, and 5, reaching estradiol equivalent concentrations (*EEQ*) of 8.6, 18, and 41 ng/sampler in HLB samplers, respectively (corresponding to 119, 244, and 558 pg/L; Table 5). The EBT of 0.34 ng/L EEQ derived by Escher et al. (2018) as well as SIMONI-EBT of 0.5 ng/L EEQ proposed by van der Oost et al. (2017b) were exceeded at site 5 (Fig. 3C). In addition to target screening and target chemical analyses performed in HLB samplers (SM 2, A1), target analysis of steroids, bisphenols, and parabens was carried out for selected samples. Sites 1 and 3 were selected as control, as estrogenic activity was not detected there, while sites 4 and 5 were chosen because estrogenic activity was the most pronounced there (Table 5, SM 2, A15). In total, seven and eleven chemicals contributed to the estrogenic activities at sites 4 and 5, respectively. Three compounds, 17β-estradiol, estrone, and bisphenol A, were responsible for 39.5% of estrogenic activity at site 4 with a contribution of 34.5, 4.5, and 0.5 %, respectively (Fig. 5C), while the rest of the compounds explained only 0.02% of the estrogenic activity (SM 2, A15). At site 5, the natural estrogens 17β-estradiol, estrone, and estriol explained most of the estrogenicity (52.8%) with a contribution of 42.4, 5.5, and 4.9%, respectively (Fig. 5C), while other compounds contributed only by 0.03% (SM 2, A15).

Bioassays indicative of estrogenic activity are so-called type 1 bioassays, where a small number of chemicals is responsible for most of the observed effect in environmental samples. However, several low potency estrogens were revealed to have low specificity ratio typical rather for category 2 bioassays (Escher and Neale 2021). Therefore, other

unidentified contributors to estrogenic activity in our samples might be multiple low potency compounds. Jarosova et al. (2014) reviewed the data on contribution of environmental chemicals to estrogenic activities. They summarized that the natural estrogens (estrone, 17β-estradiol, and estriol) are together with a synthetic estrogen 17α-ethinylestradiol, the main drivers of the estrogenic activities in WWTP effluents. The three high-potency estrogens (estriol, 17β-estradiol, and 17α-ethinylestradiol) and one low-potency estrogen (estrone) were shown to contribute to the estrogenic activity in environmental waters to much greater extent than various low potency estrogenic compounds (such as bisphenol A) that might be present at very high concentrations (Escher and Neale 2021). The combined concentration addition effects of estrogenic steroids through estrogen receptor activation (manifested as estrogenic activity) were demonstrated in multiple studies (Brian et al., 2005; Hashmi et al., 2018). Our observations of dominance of estriol, 17β-estradiol, and estrone over the estrogenic mixture effect confirm this finding for passive samples from surface water of Danube (Fig. 5C, SM 2, A15). Despite the considerable contribution of these estrogens to the effect, the results also indicate the presence of other unknown likely hydrophilic compounds contributing to estrogenicity in HLB samplers. Our results confirmed that despite bisphenol A often occurs in surface waters at high concentrations, such as at sites 4 and 5 (902 and 1324 ng/sampler) compared to natural estrogens, its contribution to estrogenic activities is relatively low (0.5 and 0.3%, SM 2, A15) due to its weak estrogenic potency (relative effect potency of $7.6 \times 10^{-5}$). In agreement with our results from passive samples, the recent scientific report from JDS4 by Vrana et al. (2021a) concluded that most of the estrogenic activity in LVSPE samples from the Danube River could be explained by estrone and 17β-estradiol at some sites. Similarly, Neale et al. (2015) explained up to 80% of the estrogenic activity in the Danube within JDS3 by the natural hormone estrone and by the phytoestrogen genistein. Ankley et al. (2017) recently reported that the potency and role of estrone in the overall observed estrogenicity in surface waters is likely underestimated, as they described its feminizing effects in male fish elicited by environmentally relevant concentrations, which are usually higher than those of the other potent estrogens.

### 3.4.5. *(Anti-)androgenic activities*

Androgenic compounds have the potential of causing masculinization in aquatic organisms (Gale et al., 1999; Morthorst et al., 2010). Some androgenic compounds have been detected in HLB (e.g. androstenedione and benzophenone-4) and SR (benzo(a)anthracene and dibenzo(a,h)anthracene) samplers in our study (SM 2, A1 and A20). However, androgenic activity was only detected in two SR samples from sites 5 and 9, where the dihydrotestosterone equivalent concentrations (DHT-EQs) reached 18 and 14 ng/sampler, respectively (these correspond to estimated DHT-EQ of 6.1 and 2.8 pg/L; Table 5, SM 2, A20). However, no EBTs for androgenic activity are currently available (Escher et al., 2018), so it was not possible to assess whether this level of androgenic activity poses a risk to the aquatic ecosystem. Only two very weak known contributors to the androgenic activity, benzo(a)anthracene and dibenzo(a,h)anthracene, were detected (Fig. S39B, SM 1, Text S8, and SM 2, A20). Similarly, no androgenic activity was found in a previous report from short-term monitoring of the Danube within the JDS3 (Liška et al., 2015) or in passive samples from other surface waters (Liscio et al., 2014; Tapie et al., 2011).

The anti-androgenic activity was observed in all HLB samplers with flutamide equivalent concentrations (FLU-EQs) in the range of 215–1,031 μg/sampler (corresponding to 3.0–14 μg/L; Table 5). Out of the 671 compounds analyzed in HLB samplers, info on activity/REPs was available for 425 compounds, and there were 86 known anti-androgenic compounds detected at least once. The number of detected known contributors to the anti-androgenic activity at a site ranged from 51 to 74 compounds in HLB samplers (SM 2, A16). All these compounds jointly contributed to the anti-androgenic activity in HLB samplers from 0.2 to 1.4% showing that a large portion of the activity remained unexplained (top contributors shown in Fig. 5D). The highest contributor to anti-androgenic activity was 7-diethylamino-4-methylcoumarin, used as a dye, contributing up to 0.74% of this effect at site 1 in HLB samplers (SM 2, A16). This compound explained 32% of the overall anti-androgenic activity at a hot spot in the German river Holtemme as revealed by effect-directed analysis in a study by Muschket et al. (2018). The other significant contributors to anti-androgenic activity in HLB in our study were bicalutamide (anti-cancer drug, synthetic anti-androgen), megestrol acetate (synthetic progestin), medroxyprogesterone (synthetic progestin metabolite), and clotrimazole (antifungal pharmaceutical; SM 2, A16). Two fungicides, tebuconazole and propiconazole, which were previously identified as anti-androgens in effect-directed analyses (Houtman et al., 2020; Zwart et al., 2018), have also slightly contributed to anti-androgenic activity in Danube HLB samplers.

In the SR samplers for hydrophobic compounds, anti-androgenic activity was found at five out of nine sites. The FLU-EQs were relatively comparable and ranged from 148 to 448 μg/sampler (corresponding to 0.07–0.10 μg/L; Table 5, SM 2, A21). Only three known contributors to anti-androgenic activity were identified in SR samplers and their contribution was negligible (Fig. S39C, SM 1, Text S8, SM 2, A21). It is worth noting that Novák et al. (2018) detected anti-androgenic activity with the mobile passive sampling at all sampled stretches in the previous JDS (in both SR samplers and Empore disks). This difference could have been caused either by sampling of more anti-androgenic compounds or less androgenic compounds by short-term mobile passive samplers in the JDS3 (Novák et al., 2018).

The anti-androgenic effects on exposed organisms are related to demasculinization/feminization (Hua et al., 2015; Jobling et al., 2009). Anti-androgenic activity has been frequently detected in the aquatic environment (König et al., 2017; Leusch et al., 2014; Macikova et al., 2014b; Šauer et al., 2018; Thomas et al., 2009; Urbatzka et al., 2007). Anti-androgenic activity has been found even in bile of fish exposed to WWTP effluents (Hill et al., 2010), indicating a bioaccumulative potential of compounds causing this activity. A wide range of environmental pollutants is known to exhibit anti-androgenic activity, such as pesticides (Aït-Aïssa et al., 2010), PAHs (Thomas et al., 2009; Weiss et al., 2009), and pharmaceuticals (Zwart et al., 2018). These compounds are typically of low potency. Two high potency progestins (megestrol acetate and medroxyprogesterone) were among the main contributors to anti-androgenic activity in extracts from HLB samplers but explained only a negligible portion of the overall sample effect (Fig. 5D, SM 2, A16). In agreement with our results, various other studies reported that a considerable part of anti-androgenic activity remained unexplained in aquatic environment matrices (Chen and Chou 2016; Kinani et al., 2010; Šauer et al., 2018; Urbatzka et al., 2007; Zwart et al., 2018). Given the wide range of analyzed compounds with known relative effect potencies, this observation is in line with the classification of bioassays for anti-androgenic activity as category 2 bioassays, where many chemicals contribute to the observed mixture effect (Escher et al., 2018).

To the best of our knowledge, only a few other studies analyzed anti-androgenic activity in passive samplers in wastewater and surface water (Creusot et al., 2013; Elkayar et al., 2022; Hamers et al., 2018; Jálová et al., 2013; Liscio et al., 2014; Nguyen et al., 2021; Toušová et al., 2019; van der Oost et al., 2017a,b). Liscio et al. (2014) found much higher anti-androgenic activity (FLU-EQ of 1 mg/sampler) using yeast androgen screen *in vitro* assay in SR samplers that were deployed for two weeks in surface water in the United Kingdom, compared to the anti-androgenic activity detected in SR samplers in the present study. Escher et al. (2018) estimated the EBT-FLU-EQ for anti-androgenic activity of 3.5 μg/L which was exceeded by the activity observed in HLB extracts from seven sites (Fig. 3D). No exceedance of the EBT-FLU-EQ for anti-androgenic activity was observed in SR samplers (Fig. 4D). Alygizakis et al. (2019) reported exceedance of EBTs for anti-androgenic activity in about 25% of WWTP effluents along the Danube the, which indicates potential impact of other sources of anti-androgenic compounds next to those coming from WWTPs, such as surface run-off from agricultural

areas.

#### 3.4.6. Glucocorticoid activity

In the present study, glucocorticoid activities were found neither in HLB samplers nor in samples from SR samplers. Similarly, König et al. (2017) did not find glucocorticoid activity in the middle part of the Danube, which is in line with our results. Previous studies detected glucocorticoid activities expressed as dexamethasone equivalent concentrations (DEX-EQs) in actively sampled European surface waters in the range of 0.30–31 ng/L DEX EQs (Macikova et al., 2014a; Schriks et al., 2013; Tousova et al., 2017; van der Linden et al., 2008) and up to approximately 350 ng/L DEX EQs in passive samplers deployed in European surface waters (Nguyen et al., 2021; van der Oost et al., 2017a, b). Alygizakis et al. (2019) and Tousova et al. (2017) observed glucocorticoid activity in the WWTP effluents in the Danube River basin. Therefore, the non-detected glucocorticoid activity in the present study might have been attributed to high dilution of glucocorticoid-like compounds in the Danube River.

## 4. Conclusions

We present one of the most comprehensive analytical and bioanalytical assessments of organic micropollutants in the major European river, the Danube, to date, performed on extracts of long-term exposed passive samplers. Deployment of continuously operating passive samplers over an extended period of three months provided more representative samples of seasonal contamination patterns and spatial trends along the river than conventionally used spot sampling. Besides the well-established sampling of non-polar compounds using SR, long-term deployment of HLB adsorbent disks was for the first time successfully demonstrated for the monitoring of polar organic contaminants.

The time-integrative sampling with SR enables the accumulation of sufficient amounts of hydrophobic compounds with log $K_{ow} > 5$ from an equivalent of several thousands of liters of water, quantitatively reflecting the time-weighted average of freely dissolved aqueous concentrations of compounds that fluctuate over time. This makes passive sampling with SR an excellent method for investigative monitoring of hydrophobic waterborne pollutants. The potential underestimation of BEQ aqueous concentrations of partially or completely equilibrating compounds with log $K_{ow} < 5$, caused by improper application of integrative uptake model to those compounds, can in future be dealt with by fractionating the SR extracts according to the compounds' hydrophobicity.

The equilibrium sampling with HLB enabled to obtain representative samples of polar Danube contaminants, which allows the identification of patterns and spatial profiles of individual compounds without the need of data conversion to concentrations in water. Although such conversion provides semiquantitative aqueous concentrations, the resulting uncertainty is compensated by the sample representativity. As long as the uncertainty of HLB-derived aqueous concentration is lower than the variability of contaminant levels in water, passive sampling is better suited for representative monitoring than infrequent spot sampling.

At least five compounds with an *RQ* higher than 1 were present at each of the nine sites. It is worth noting that among the observed compounds in HLB samplers, some had exceedances of *RQ* of even more than ten-fold (PFOS, hexa(methoxymethyl)melamine, and 17α-ethinylestradiol). In SR samplers, only heptachlor epoxide exceeded the *RQ* of 1 at all sites, with maximum *RQ*s of >20 at sites 1 and 8.

Various *in vitro* biological activities were found (AhR activation, adaptive stress response to oxidative stress, PPARγ-mediated activity, estrogenic, and (anti-)androgenic activities), for which some effect drivers were revealed. However, despite the very high number of analyzed chemicals, a large proportion of the activities remain unexplained by the analytes for which relative effect potencies were available. The EBTs exceeded in this study were the thresholds for estrogenic activity at site 5, and for anti-androgenic activity at seven sites in HLB samplers.

The levels of pollutants, potential environmental risks, and *in vitro* biological activities derived from the innovative long-term application of passive sampling technology may serve as a baseline for further water quality monitoring in the Danube River basin and the developed monitoring methodology can be applied also in other freshwater bodies.

## CRediT authorship contribution statement

**Pavel Šauer:** Investigation, Writing – original draft, Formal analysis, Visualization. **Branislav Vrana:** Conceptualization, Methodology, Investigation, Writing – original draft. **Beate I. Escher:** Supervision, Writing – review & editing, Resources. **Roman Grabic:** Investigation, Funding acquisition. **Zuzana Toušová:** Investigation, Data curation, Visualization. **Martin Krauss:** Investigation. **Peter C. von der Ohe:** Investigation. **Maria König:** Investigation. **Kateřina Grabicová:** Investigation. **Petra Mikušová:** Investigation. **Roman Prokeš:** Investigation. **Jaromír Sobotka:** Investigation. **Pavla Fialová:** Investigation. **Jiří Novák:** Investigation, Data curation. **Werner Brack:** Investigation, Resources, Writing – review & editing. **Klára Hilscherová:** Conceptualization, Supervision, Formal analysis, Writing – review & editing, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.107957.

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