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Combining short-term bioassays using fish and crustacean model organisms with ToxCast *in vitro* data and broad-spectrum chemical analysis for environmental risk assessment of the river water (Sava, Croatia)

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ABSTRACT

This study focused on the short-term whole organism bioassays (WOBs) on fish (*Danio rerio*) and crustaceans (*Gammarus fossarum* and *Daphnia magna*) to assess the negative biological effects of water from the major European River Sava and the comparison of the obtained results with *in vitro* toxicity data (ToxCast database) and Risk Quotient (RQ) methodology. Pollution profiles of five sampling sites along the River Sava were assessed by simultaneous chemical analysis of 562 organic contaminants (OCs) of which 476 were detected. At each sampling site, pharmaceuticals/illicit drugs category was mostly represented by their cumulative concentration, followed by categories industrial chemicals, pesticides and hormones. An exposure-activity ratio (EAR) approach based on ToxCast data highlighted steroidal anti-inflammatory drugs, antibiotics, antiepileptics/neuroleptics, industrial chemicals and hormones as compounds with the highest biological potential. Summed EAR-based prediction of toxicity showed a good correlation with the estimated toxicity of assessed sampling sites using WOBs. WOBs did not exhibit increased mortality but caused various sub-lethal biological responses that were dependant relative to the sampling site pollution intensity as well as species sensitivity. Exposure of *G. fossarum* and *D. magna* to river water-induced lower feeding rates increased GST activity and TBARS levels. Zebrafish *D. rerio* embryo exhibited a significant decrease in heartbeat rate, failure in pigmentation formation, as well as inhibition of ABC transporters. Nuclear receptor activation was indicated as the biological target of greatest concern based on the EAR approach. A combined approach of short-term WOBs, with a special emphasis on sub-lethal endpoints, and chemical characterization of water samples compared against *in vitro* toxicity data from the ToxCast database and RQs can provide a comprehensive insight into the negative effect of pollutants on aquatic organisms.

1. Introduction

Continuous release of industrial and municipal effluents resulting in the presence of complex mixtures of organic contaminants (OCs) in European rivers has the potential to adversely impact exposed ecosystems as well as induce harmful effects on their biota (Amoatey and Baawain, 2019; Bielen et al., 2017). Many of these OCs possess the ability to accumulate in the food chain, and therefore represent a potential risk to different trophic levels, and ultimately to humans (Barceló et al., 2020). Currently, only a tiny fraction of OCs is monitored and available environmental regulations are mostly based on a limited number of single chemicals (Neale et al., 2017). However, recent improvements in effective chromatographic separation methods coupled to high-resolution mass spectrometers have expanded the number of potentially hazardous compounds that can be detected and quantified (Prasse et al., 2015; Stipaničev et al., 2017). The pollution risk assessment is tightly interlinked with the assessment of exposure, however, chemical charac-

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terization alone is still not decisive enough for establishing accurate quantification of the biological effects.

Comprehensive environmental risk assessment of complex mixtures of OCs present in rivers requires active integration of chemical and biological data at the same time revealing the potential priority OCs and mixtures that contribute to the observed effects (Li et al., 2017). In the past decades, whole organism bioassays (WOBs) have become important tools in the monitoring of environmental quality as they provide the most comprehensive information about the joint effect of all bioavailable active chemicals present in a sample on the organism-level response (Neale et al., 2017; Prasse et al., 2015). They are still the most relevant predictor of toxic bio-effects as they encompass toxicokinetic processes of absorption, distribution, metabolism, and excretion. In this study, key aquatic organisms such as crustaceans (daphnids and amphipods) and fish (embryos) were used for WOBs. *Daphnia* and *Gammarus* species play an important role in the food chain of the aquatic ecosystems by transferring the energy between producers and secondary consumers and are highly sensitive to contaminants (Lv et al., 2017). Among fish models, *D. rerio* has emerged as one of the leading vertebrate models due to the extensive homology between fish and humans' genomes (Di Paolo et al., 2015).

The most frequently measured and arguably the most important ecologically relevant individual-level adverse effects include survival, growth/development, and reproduction. Other types of individual-based effects that have high ecological relevance include behavioral changes (*e.g.*, in locomotor and feeding behavior), again because these often directly influence survival, growth, and reproduction (Groh et al., 2015). Therefore, our research focused on survival growth/development changes, biochemical alterations as well as behavioral changes of relevant freshwater organisms (fish and crustaceans).

The exposure to activity ratio (EAR) approach (Blackwell et al., 2019) was used to prioritize chemicals and hazards based on high throughput *in vitro* toxicity screening. It evaluates the potential of detected OCs to interact with specific biological targets based on data from the U.S. EPA's Toxicity Forecaster (ToxCast; Richard et al., 2016). Such a "top-down" assessment of "integrative/apical" effects with WOBs which is complementary to *in vitro* bioeffects data allows insight into the underlying molecular and biochemical reactions and targets responsible for toxicant action.

The results of WOBs are compared with the data on bioeffects extracted from the ToxCast database to: i) determine the magnitude of the mixture effect of chemicals in the sample, ii) identify biological targets, iii) identify potential priority OCs or chemicals category, and iv) compare with *in vitro* predicted toxicity of tested water samples. The overall goal of this research was to provide a comprehensive environmental risk assessment of the Sava River at five sites of different pollution intensity by measuring acute and sub-lethal effects on *G. fossarum*, *D. magna* and *D. rerio,* and to compare these results with results obtained with RQ and EAR approaches relative to 562 analyzed OCs distributed among four major categories (*i.e.*, pharmaceuticals/illicit drugs, pesticides, industrial chemical and hormones). The use of such an integrated approach based on WOBs, combined with chemical characterization and high throughput *in vitro* toxicity screening data provides a valuable insight into the toxicity potential of Sava River, offering a basis for fast and thorough monitoring of freshwaters.

2. Materials and methods

2.1. Chemicals

A detailed list of chemicals used in this work is provided in the Supplementary Information.

2.2. Site description and sampling

Surface water samples (2 liters per sample) were collected in May of 2018 from five sites along the River Sava: Jesenice (JES), Jankomir (JAN), Hruščica (HRU), Rugvica (RUG), and Lukavec (LUK) (Fig. 1). A more detailed description of sampling sites is available in the Supplementary Information. Surface water samples were collected in sterile

Fig. 1. Location of the Sava River basin showing surface water sampling sites. Grey areas represent urban zones. The map is generated with ArcGIS Pro software.

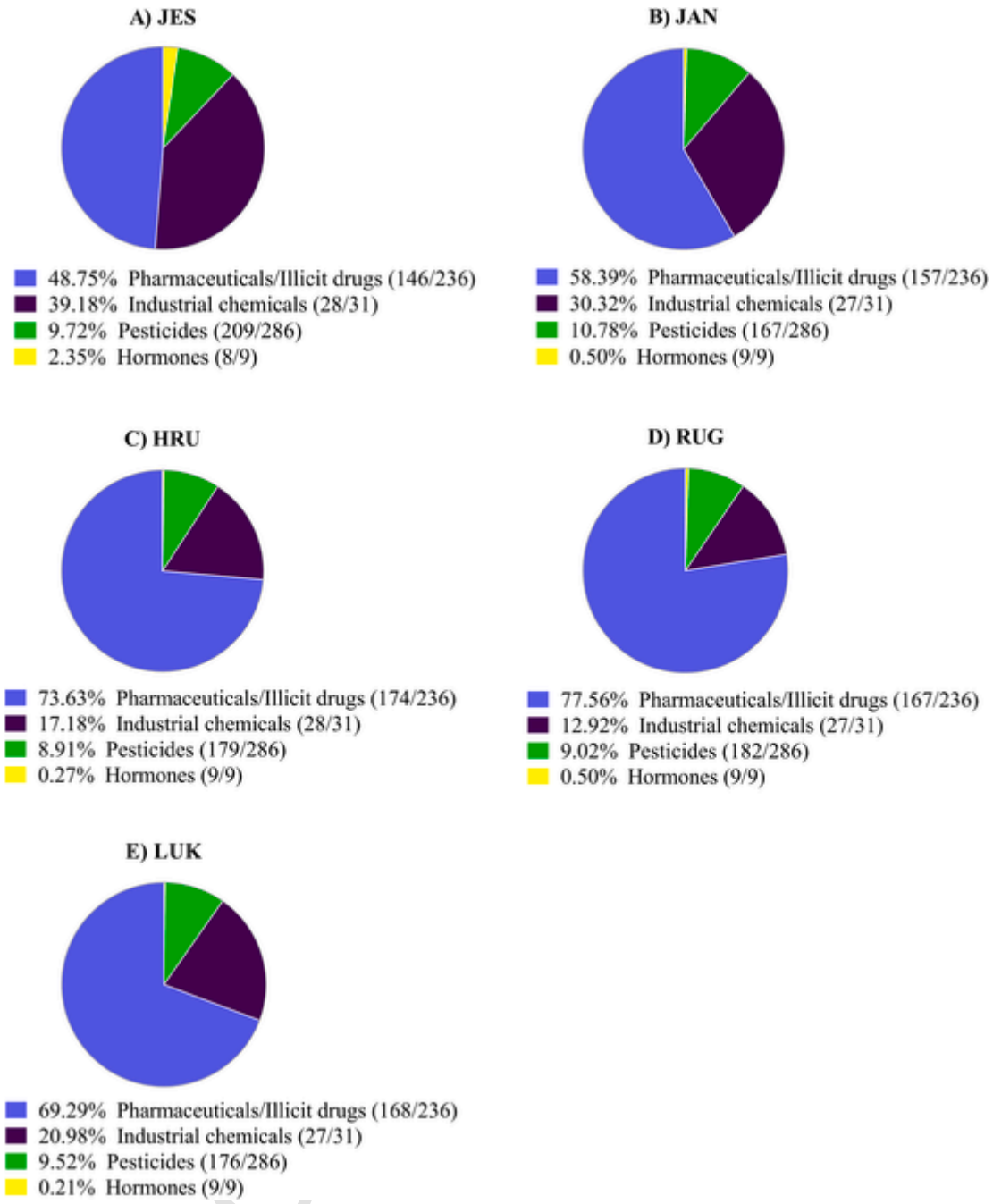

Fig. 2. Distribution of main four OCs categories by their cumulative concentration (expressed in %) in Sava River samples. Numbers specified in brackets represent the number of detected OCs/searched OCs.

polycarbonate flasks as described in Stipaničev et al. (2017) and transported at 4 °C. Immediately upon arrival to the laboratory, samples were filtered using 0.2 μm PTFE filters, and WOBs were conducted.

2.3. Analysis of organic contaminants in surface water samples by UHPLC-qTOF-MS

Analysis of surface water samples by liquid chromatography/time-of-flight mass spectrometry (UHPLC-qTOF-MS) followed the previously reported method (Stipaničev et al., 2017). For details, please see Supplementary information. The list of all analytes, their concentrations, CAS registry numbers, log Kow values is presented in Table S1. The log Kow values were calculated using ChemAxon Marvin (https://chemaxon.com/products/marvin).

All measured organic contaminants (OCs; N = 562) were sorted into 4 major categories: pharmaceuticals/illicit drugs (PhACs/IDrgs); pesticides; industrial chemicals; hormones (Table S1) based on the classification system presented in Babić et al. (2018) and Malev et al. (2020). PhACs/IDrgs and pesticides were divided into sub-categories (Table S1).

2.4. Toxicity testing

2.4.1. Gammarus fossarum: acute toxicity testing

Crustaceans were collected using mesh traps from the upper course of Veliki potok stream at Medvednica Nature Park in May 2018. Gammarids were acclimatized for 7 days in a 20-L glass aquarium. Constantly aerated glass aquaria were maintained in humidity (60%) and

temperature (12 ± 2 °C) controlled chamber with an 8:16 h light:dark photoperiod. All specimens presented a mean total body length of 14.35 ± 0.25 mm and a mean weight of 0.028 ± 0.003 g. Only healthy adults were used in this study (Kunz et al., 2010).

Static exposure was performed according to Environmental Protection Agency (1996) for 48 h, with five replicas of 12 individuals placed in 300 mL of sample. Testing chambers were covered with parafilm to avoid evaporation, aerated, and incubated in darkness at 12 °C. After 48 h of exposure immobility, molting, and mortality were observed. Dead organisms were determined by gentle poking and observing movements of appendages. Inactive/paralyzed animals were identified when only respiration movements were left (Malev et al., 2020). Three replicas were used for measuring biochemical parameters (N = 36), while the other two were used to observe animal locomotion and for the post-exposure feeding assay (N = 24). The control group was exposed to stream water.

2.4.1.1. Swimming and post-exposure feeding behavior analysis. Animal locomotion was recorded after 24 and 48 h of exposure (N = 24) for 10 min with a digital camera (Sony HDR-CX570). Average speed was measured and analyzed using ToxTrac® v2.61; https://toxtrac.sourceforge.io (Rodriguez et al., 2018).

After 48 h of exposure to tested samples feeding assay was performed using *Artemia salina* eggs. Commercially available eggs were left for 30 min in water samples allowing them to settle at the bottom of a container. Floating eggs were removed from the experiment since *G. fossarum* searched the food predominantly at the bottom of the containers (Taylor et al., 1993). Twenty settled *A. salina* eggs were added to each well and the number of consumed eggs in each well was recorded after 3 h.

2.4.1.2. Biochemical biomarkers. At 48 h of exposure, specimens were frozen with liquid nitrogen and individually homogenized (IST 400 mill, 1 min) without a buffer at 30,000 Hz, afterward 400 μL of ice-cold phosphate buffer (pH 6.5) was added and samples were homogenized for 30 s. The homogenate was centrifuged (Sigma 3K18) for 15 min at 20,000 × *g* and 4 °C. The supernatant was collected and kept on ice until further biochemical analysis. Molted amphipods were not used for analyses.

Glutathione-S-transferase (GST) activity was determined according to Habig and Jakoby (1981). Enzyme activity is determined spectrophotometrically at 340 nm (Infinite M200 PRO plate reader, Tecan, Austria) by measuring the formation of the conjugated product dinitrophenyl-thioether produced from CDNB used as artificial substrate and reduced GSH. All values are normalized to total protein content (U mg^{-1} protein). Lipid peroxidation was expressed as content of thiobarbituric acid reactive substances (TBARS) formed during the reaction between malondialdehyde (MDA) and TBA under acidic conditions (Khan et al., 2010). The TBARS content was calculated from the absorbance at 532 nm and normalized to the amphipod fresh weight (FW). Total soluble protein contents of the enzyme extracts were estimated according to Bradford (1976) using bovine serum albumin as a standard and absorbance measurement at 595 nm.

2.4.2. Daphnia magna: acute immobilization testing

The OECD guideline (2014) was followed for measuring *D. magna* acute immobilization in response to the exposure to water samples. Each tested sample utilized a total of 40 neonates (≤24 h old) distributed in four replicates with ten neonates in each vessel containing 30 mL of tested solution (OECD, 2004). Incubation was performed in the dark at 20 ± 2 °C for 48 h. After 24 and 48 h of exposure, the number of immobilized specimens was counted and the overall mortality rate was observed. The animals were considered to be immobilized if they were unable to swim freely for more than 15 s and did not respond to the gentle movement of the vials.

2.4.2.1. Swimming and post-exposure feeding behavior analysis. Analyses of locomotor behavior were based on a video recording of daphnids swimming locomotion using the ToxTrac® program (Rodriguez et al., 2018) after 24 h of exposure. Video tracking analyses were performed directly in the exposure vessels and the video was recorded for 6 min. Average speed was evaluated as the designated locomotion parameter for daphnids. After 48 h of exposure, the feeding response was assessed at 4-h and 20-h exposure (post-exposure feeding assay) to microalga *Chlorella vulgaris* (Barata et al., 2008). Five neonates were exposed to 15 mL of algal suspension (initial density from 2.5 × 10^5 to 3.5 × 10^5 cells mL^{-1}). The experiment was set in 8 replicas (N = 5 specimens per replica) at 20 ± 2 °C in the dark (to avoid algal growth). A control blank (without daphnids) was used to ensure that the initial algal concentration did not significantly increase during the exposure period. Feeding activity was calculated by subtracting the number of algal densities at the beginning of the experiment with their number at the end of feeding analysis (4 and 20 h). Algal cell density was expressed using a standard calibration curve correlating measured absorbance values and cell densities counted using a Bürker-Türk counting chamber.

2.4.2.2. Zebrafish (Danio rerio) embryotoxicity test (ZET). The ZET was conducted following a protocol described in detail by Babić et al. (2019). Daily, mortality and developmental abnormalities (24–96 h post fertilization; hpf), heartbeat rate (at 48, 72, and 96 hpf; beats per 15 s), hatching success (at 72 and 96 hpf) and pigmentation formation (at 48, 72 and 96 hpf; scored 0–3: 0 - no pigmentation, 1 - decreased body and eyes pigmentation, 2 - decreased body pigmentation but normal eye pigmentation, 3 - fully formed pigmentation) were monitored.

All experiments in this study were conducted on the non-protected embryonic zebrafish stages (up to 96 hpf), which do not require permission by animal welfare commissions (Council Directive 2010/63/EU, 2010).

2.4.2.3. The multi-xenobiotic resistance (MXR) efflux activity in zebrafish larvae. The amount of rhodamine B (RB) accumulated in 96 h old zebrafish larvae was measured according to Babić et al. (2017), with slight modifications. At the end of the exposure, larvae were exposed to the mixture of the tested sample and 1 μM of RB. Negative control was run on artificial water containing RB, while the positive control group was exposed to RB and MXR model inhibitor cyclosporine A (10 μM; CYC). After 90 min of incubation in the dark, larvae (N = 10 specimens in 4 replicas) were rinsed and homogenized in 500 μL of distilled water by 30 strokes of micropestles. The homogenate was centrifuged for 10 min at 8603 × *g* (Eppendorf Centrifuge 5804 R). The fluorescence of accumulated RB was measured at excitation/emission: 530/595 nm using a plate reader (Tecan Infinite M200 PRO). The results for RB accumulation were expressed as fluorescent units normalized relative to non-treated controls.

2.5. EARs

Analyzed OCs and their biological targets were prioritized according to the chemical concentration to biological exposure-activity ratios - EARs (Blackwell et al., 2019). Biological activity data for OCs was extracted from the ToxCast database using ToxEval 1.1.0 R Package (http://usgs-r.github.io/toxEval/index.html (Corsi et al., 2019)). The following toxicity information were extracted: the identity of all ToxCast assays and associated biological targets for detected OCs and the minimal concentration of each chemical that induces a response-activity concentration at cut-off in these assays. Since ToxCast includes *in vitro* bioactivity profiles of over 500 high-throughput screening assays, different inferred toxicity pathways, exposure predictions, and chemical properties/descriptors, data were filtered to exclude some assays and endpoints as suggested by the ToxEval package (Corsi et al.,

2019). From these data, EARs were calculated to predict the molecular targets likely to be impacted by the OCs found in the Sava River. Cumulative bioactivity (for each molecular target) of chemical mixtures was evaluated (assuming additivity of effects). This approach is analogous to a toxic unit approach for summing adverse effects for similar-acting chemicals present in complex mixtures as used by Blackwell et al. (2019). To extract a group of most probable biologically active OCs to be compared with results obtained by other methods used in this study a provisory limit of EAR median value was set at 10^{-4}.

2.6. Risk quotients

The risk posed by the detected OCs can be evaluated through the calculation of risk quotients (RQ) (Inam et al., 2015). The RQs for individual compounds were calculated from the measured concentrations (MEC) of the OCs in the water samples and their predicted no-effect concentration (PNEC) using the following Eq. (1):

$$RQ = \frac{MEC}{PNEC} \quad (1)$$

PNEC values were calculated from the lowest no observed effect concentration (NOEC) for each contaminant and the assessment factor of 10 (Eq. (2)):

$$PNEC = \frac{NOEC}{10} \quad (2)$$

NOEC was calculated with VEGA-QSAR (v1.1.5-b48, https://www.vegahub.eu) for three endpoints: fish chronic NOEC, *Daphnia magna* chronic NOEC, and algae chronic NOEC (converted to μg L^{-1}, Table S2). For several compounds, NOEC was not obtainable (a limitation in the VEGA-QSAR models). Zeros and missing values were removed. The top 20 OCs from each site according to their RQs were used for further analyses. Risk index (RI) for each site was calculated as the sum of all RQs at that site (all OCs which is denoted as *i*), following Eq. (3):

$$RI_{site} = \sum_{i=1}^{n(OCs)} RQ_i \quad (3)$$

2.7. Statistical analysis

Data were statistically analyzed using GraphPad Prism 6.0 (GraphPad Software Inc., USA). The results were expressed as means of at least three replicates ± SD, and $p < 0.05$ was used as a cut-off value of statistical significance throughout the manuscript. One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to examine the significance of difference between treatments. When the assumption for normality was violated the Kruskal-Wallis One-way analysis of variance on ranks was performed.

Spearman's rank correlation tests were used for univariate evaluation of the association of toxicological test endpoints with the concentration of OCs detected in water samples. In order to account for multiple testing, the Bonferroni adjustment of p value was applied to maintain the alpha error <0.05. Multivariate analysis was performed using principal component analysis (PCA) of both toxicological and chemical results of the water sites. Spearman's rank correlation tests and PCA were performed using R v.4.1.0 (R Core Team 2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org/.

3. Results

3.1. Chemical characterization

Among 562 simultaneously analyzed OCs, 476 were detected in at least one of 5 sampling sites: JES (391), JAN (360), HRU (390), RUG (385), and LUK (380). Concentrations of all tested chemicals per site summed up as follows: 14.5 μg L^{-1} (HRU) > 13.3 μg L^{-1} (RUG) > 13.1 μg L^{-1} (JES) > 10.6 μg L^{-1} (LUK) > 8.9 μg L^{-1} (JAN). Representatives of OCs that were detected in the highest concentrations varied somewhat at each sampling site (Table S1). Ten OCs that were detected at the highest concentrations at each site are presented in Table 1 showing that PhACs/IDrgs are largely replacing industrial chemicals as top OCs after receiving wastewaters from the central wastewater treatment plant of Zagreb.

Among the detected categories, PhACs/IDrgs was the most represented category at each site according to the cumulative concentrations (expressed in percentage as a share of total content) (Fig. 2, S1), ranging from 48.8% (JES), 58.4% (JAN), 69.3% (LUK), 73.6% (HRU), up to 77.6% (RUG). The second category by percentage content at each site

Table 1
Chemicals detected at the highest concentrations at each sampling site. Concentrations are presented in brackets and expressed as μg L^{-1}. Industrial chemicals are marked grey.

JES	JAN	HRU	RUG	LUK
4-nonylphenol (3.5)	Nicotine (1.6)	Metoprolol (1.2)	Nicotine (2.2)	Phenobarbital (1.3)
Fludrocortisone-acetate (0.8)	NP1EO (0.7)	Nicotine (1.2)	Phenobarbital (1.3)	Caffeine (1.0)
Naproxen (0.6)	OP2EO (0.6)	Sulfapyridine (1.2)	Caffeine (1.1)	Nicotine (0.9)
4-tert-octylphenol (0.5)	Metoprolol (0.6)	Phenobarbital (1.2)	Tetracycline HCl (0.7)	NP1EO (0.7)
Nafcillin sodium monohydrate (0.5)	Amoxicillin (0.6)	Caffeine (1.0)	Sulfapyridine (0.5)	Sulfapyridine (0.7)
Ibuprofen (0.5)	Caffeine (0.5)	Amoxicillin (1.0)	Metoprolol (0.5)	Acetylsalicylic acid (0.6)
Nicotine (0.4)	4-nonylphenol (0.5)	OP2EO (0.8)	Bisphenol A (0.5)	Metoprolol (0.6)
Bisphenol A (0.4)	NP2EO (0.3)	Tetracycline HCl (0.7)	Amoxicillin (0.5)	OP2EO (0.5)
Metoprolol (0.4)	Phenobarbital (0.3)	NP1EO (0.6)	Naproxen (0.4)	4-nonylphenol (0.3)
Etodolac (0.3)	1-H benzotriazole (0.2)	4-nonylphenol (0.4)	4-nonylphenol (0.3)	NP2EO (0.2)

was industrial chemicals, varying from 12.9% (RUG), 17.2% (HRU), 20.9% (LUK), 30.3% (JAN), to 39.2% (JES).

A large number of pesticides have also been detected: JES – 209, JAN – 167, HRU – 179, RUG – 182, and LUK – 176, due to agricultural activities along the Sava River (Fig. S1). The cumulative concentration of pesticides was 5.0–8.6 times lower at each site (JES – 1.3 μg L^{-1}, JAN – 1.0 μg L^{-1}, HRU – 1.3 μg L^{-1}, RUG – 1.2 μg L^{-1} and LUK – 1.0 μg L^{-1}) when compared to PhACs/IDrgs category at the same site. Hormones made less than 1% of the total cumulative concentration of OCs at all sites, aside from the JES site with 0.3 μg L^{-1}; *i.e.*, 2.35% of the total content (Fig. 2, S2; Table S1).

3.2. *Gammarus fossarum* toxicity testing

3.2.1. Survival rate

After 24 and 48 h of exposure, the number of dead amphipods (mortality) and the number of immobile/paralyzed or molted amphipods were monitored. A total of 246 individuals in all exposure groups were analyzed. Mainly male adult specimens were used for laboratory tests (15 individuals out of 246 were females – control (N = 2); JES (N = 2); HRU (N = 8); RUG (N = 3)). The average weight of specimens was 0.020 ± 0.005 g. Individuals for which sex was not possible to determine were classified as juveniles and not used for this research. During the experiment, no mortality was observed. The number of recently molted amphipods among all tested samples was in total 6: JES (N = 2); JAN (N = 3); RUG (N = 1), which were excluded from further analyses. No molted individuals were found in the control group.

3.2.2. Biochemical markers

A statistically significant increase in total proteins, compared to control (19.85 ± 5.38 mg g^{-1} FW), was observed in amphipods exposed to HRU (24.66 ± 5.33 mg g^{-1} FW; $p < 0.001$) and RUG (23.33 ± 5.46 mg g^{-1} FW; $p < 0.01$). Also, compared to control, a significant decrease in total protein content was noticed in the LUK exposure group ($p < 0.01$) (Fig. 3A). GST activity (Fig. 3B) in JAN group was at the highest (0.33 ± 0.09 U mg^{-1} protein) and in LUK group at the lowest (0.25 ± 0.06 U mg^{-1} protein). A significant decrease in lipid peroxidation was observed in JES exposure group (Fig. 3C). Even though not statistically significant, such a decreasing trend in LP values was recorded in all other exposure groups (except JAN). Exposure to JAN induced a significant rise of TBARS in amphipods. This increase was significant ($p < 0.01$) if compared to JES exposure group where was observed the lowest LP value (Fig. 3C).

3.2.3. Swimming behavior

The effect of Sava River samples on the swimming behavior of *G. fossarum* was examined by recording average speed (Fig. 4A). Reduction of average swimming speed was noticed in amphipods after 24-h exposure to HRU and RUG, being statistically significant only at HRU (a decrease of 11.1 ($p = 0.008$) and 16.8%, respectively; Fig. 4A). Amphipods from JES and JAN groups exhibited slightly increased average speed than in control animals. No differences in swimming speed were observed after 48 h. Due to technical issues and a restricted laboratory environment, the experiment consisted of two separate exposure trials (*i.e.*, in the first one we tested JES, JAN, LUK samples, while in the second one HRU and RUG, each trial used with their relative control groups). For that reason, only statistical differences among the same exposure groups were analyzed. In that manner, JES and LUK appeared to be statistically different (Table S3). Within the control group, no alteration in swimming behavior was detected.

3.2.4. Feeding behavior

The post-exposure feeding assay (after 48h) provided a rapid indication of the status of exposure groups based on the analysis of the number of consumed *Artemia salina* eggs during 3 h (Fig. 4B). A tendency of

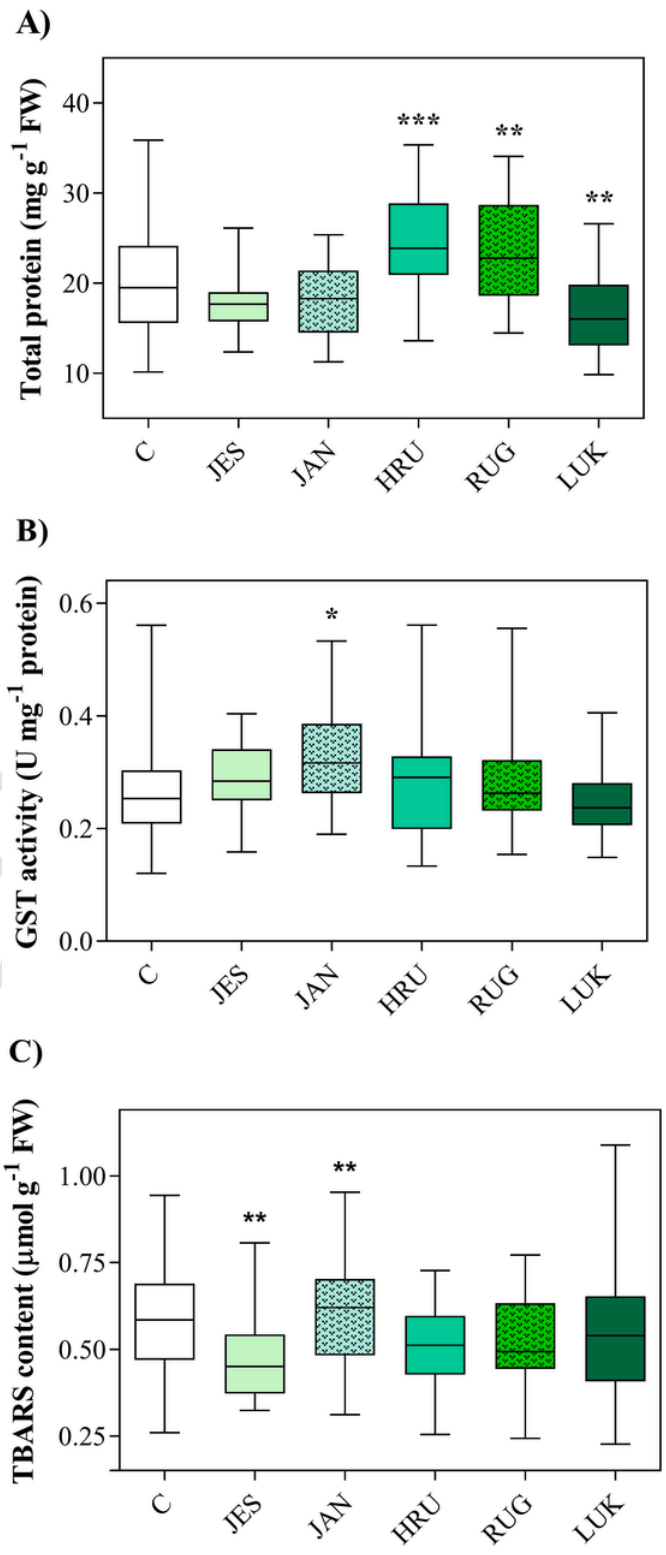

Fig. 3. A) Whole-body total protein content (mg g^{-1} FW), **B)** GST activity (U mg^{-1} protein) and **C)** TBARS content (μmol g^{-1} FW) in *Gammarus fossarum* measured after 48 h of exposure to Sava River samples. The boundaries of boxplot indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers above and below the box indicate 10th and 90th percentiles. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

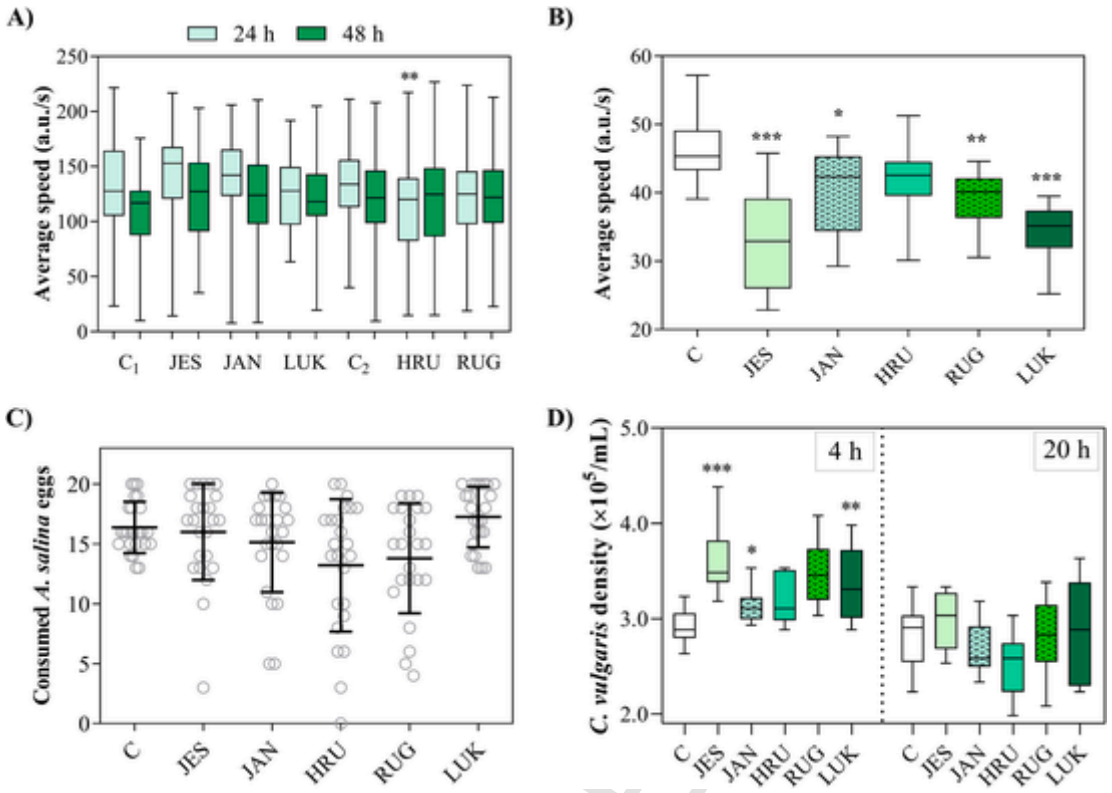

Fig. 4. Changes in locomotor and feeding behavior upon *Gammarus fossarum* (**A** and **C**) and *Daphnia magna* (**B** and **D**) after exposure to Sava River samples. Average speed is expressed as arbitrary pixel readout per second. The boundaries of box-plot indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers above and below the box indicate 10th and 90th percentiles. Results are presented as mean ± SD. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

decrease in feeding activity, although not statistically significant, is observed in all exposure groups (except LUK).

3.3. Daphnia magna toxicity testing

To evaluate the toxicity of Sava River samples towards *D. magna*, neonates were exposed for 24 and 48 h. After 24 h of exposure no change was observed in the percentages of living daphnids, while prolonged exposure (48 h) demonstrated 5% of mortality ($p > 0.05$) on JES and 12.5% ($p < 0.001$) on RUG (Table S4). No mortality was observed in the control group.

3.3.1. Swimming behavior

Average speed was assessed as a measure of locomotor activity of *D. magna* and compared among Sava River samples (Fig. 4C). The most pronounced effect, compared to control (46.4 ± 4.8 a. u./s) was observed in JES and LUK exposure groups ($p < 0.001$) that showed a reduced average speed of daphnids for 28.7% (33.0 ± 7.5 a. u./s) and 26.4% (to 34.10 ± 4.1 a. u./s), respectively (Fig. 4C). Significant differences in swimming behavior were noted between samples: JES and JAN, JES and HRU, and HRU and LUK (Table S3). Within the control group, no alteration in locomotor activity was noticed.

3.3.2. Feeding behavior

The results (Fig. 4D) show that 4-h exposure to JES, JAN, and LUK had a marked effect on feeding rate ($p < 0.05$) by significantly decreasing the rate of algal consumption. The lowest feeding rate (1.3×10^4 of consumed algal cells during 4 h of exposure) was observed at JES being 23.6% lower compared to the control group ($p < 0.001$). Significantly different feeding behavior was observed only between JES and RUG ($p < 0.05$; Table S3) after 4 h of exposure. With prolonged feeding time (20 h) no significant difference among samples was noted.

3.4. Danio rerio toxicity testing

3.4.1. Zebrafish embryotoxicity test (ZET)

Zebrafish exposed to Sava River water displayed no significant lethality and developmental abnormality (<10%). However, pigmentation formation, heartbeat and hatching rates were further evaluated and used as additional sub-lethal endpoints of acute exposure. A notable reduction in heartbeat frequency was observed already at 48 hpf (Fig. 5A), reaching 14.3% and 9.5% lower values in JES and RUG exposure groups, respectively. Prolonged exposure (96 hpf) progressively decreased heartbeat rate, which was statistically significant for all samples with exception of JES and HRU. The strongest decrease of heartbeat rate was noticed on JAN, which reduced the rate up to 77.4% of the control group rate at 72 hpf ($p < 0.001$) (Fig. 5A). Significant differences among JES and JAN groups were noted during all three days of measurements ($p < 0.01$), while heartbeat rates upon 96 h of exposure to JES statistically differ among all four sites ($p < 0.01$; Table S3).

Statistically significant reduction in pigmentation formation was recorded during exposure to JES and RUG (Fig. 5B), whose values statistically differ among other sampling sites [HRU, LUK ($p < 0.01$; Table S3)]. Half of the specimens exposed to both samples were classified as score 2, with several ones being classified as score 1. Compared to the control, tested samples did not significantly affect the hatching rate after 96 h of exposure. Indeed, for all tested samples, more than 97% of all larvae emerged out of their chorions by 72 hpf (data not shown).

3.4.2. MXR transporter activity assessment

The activity of ABC transport proteins was determined by measuring the amount of fluorescent dye RB accumulated in larvae. A significant accumulation of fluorescent substrate in the whole zebrafish specimens was recorded after 90 min of exposure to JES, HRU, and LUK contain-

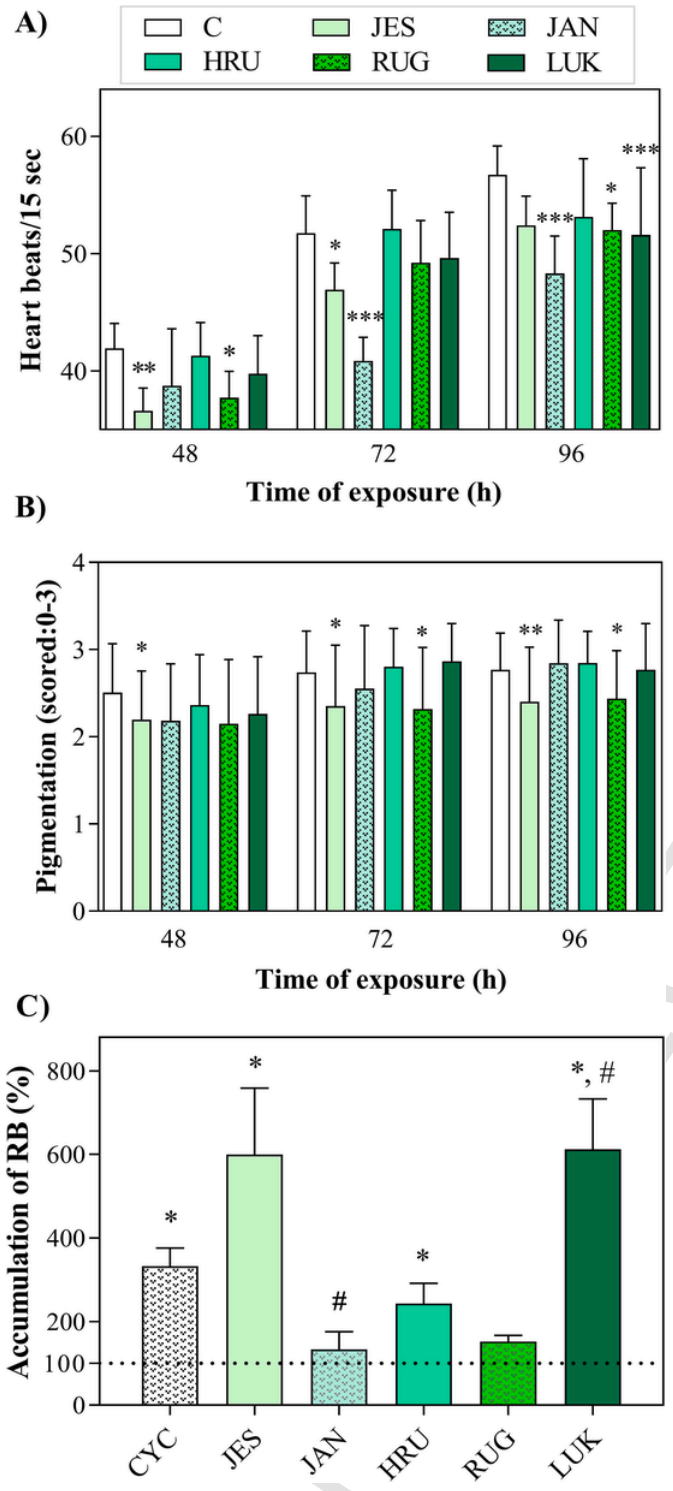

Fig. 5. A) Heartbeat rate (N = 20), **B)** pigmentation formation (N = 40) after *D. rerio* exposure to Sava River samples, and **C)** accumulation of the rhodamine B (RB) in 96 h old larvae upon exposure to cyclosporine A (CYC) and tested samples. Dashed line indicates the control group expressed as 100% value, # indicates a significant difference between the tested sample and positive control (CYC). $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***). Significant differences among the river samples are presented in Table S3.

ing 1 μM of RB dye. The highest accumulation of RB was observed in JES and LUK exposure groups showing an increase of ~83% compared to the control AW group (Fig. 5C). In comparison with the model MXR inhibitor (CYC), RB concentration was significantly enhanced in larvae exposed to LUK samples (an increase of 45.65%; $p < 0.05$).

3.5. Correlation of toxicological endpoints with concentration of OCs

For univariate analysis, Spearman's rank correlation tests of toxicological endpoints and OC concentrations showed a significant association of 256 OCs ($p < 0.05$) of which 80 OCs were significant after applying the conservative Bonferroni adjustment of p values ($p < 0.001$). These significant monotonic relationships between OCs varied between different tests (data available in Table S5). Additionally, for multivariate analysis PCA of all data was performed to explore relationships between all variables and their grouping of field sites. Hierarchical clustering of PCA showed JES as a separate cluster from all other sites on the first principal component/dimension that was mostly correlated with industrial OCs. The second cluster formed three clades with LUK and RUG in one, and JAN and HRU as separate clades; all differentiated by the second dimension (Fig. S3). Both of these analyses only account for the concentration of OCs and not externally available toxicity data.

3.6. EAR based prioritization of OCs

From the total number of detected OCs (N = 467) 367 compounds were available in the ToxCast database. The EAR-based approach indicated steroidal anti-inflammatory drugs (7 out of 10 compounds detected were found in ToxCast – 7/10), antibiotics (49/57), and antiepileptics/neuroleptics (16/16) with the highest cumulative EAR values in a majority of analyzed River Sava water samples (Fig. 6). Categories Hormones (9/9) and Industrial chemicals (13/30) as well as sub-categories: insecticides (83/89) and hallucinogens/stimulants (6/11) represent the group of chemicals with observed lower cumulative EAR values. Furthermore, those cumulative EAR values were higher than those calculated for fungicides (57/63), herbicides (71/86), hypnotics/anticonvulsants/anesthetics (19/27), analgesics (13/14), contrast agents (1/1), antiparasitics and antifungal agents (7/7), antidepressants (8/15), cardiovascular medicals (7/8), cannabinoids/illicit drugs and metabolites (3/15) and opioids (5/17) (Fig. 6).

Among antibiotics, sulfapyridine and enrofloxacin were indicated as having the highest EAR values, while dexamethasone and flumethasone were indicated among steroidal anti-inflammatory drugs with the highest EARs (Fig. S4A-E). Compound with the highest EARs within the antiepileptics/neuroleptics was lamotrigine and within hallucinogens/stimulants, those were cotinine and nicotine (Fig. S4A-E).

According to cumulative EARs for each location, HRU had the highest biological potential followed by RUG, JES, LUK, and JAN (Fig. 6). This is a rather accurate representation of the toxicity estimation for each location, which is also in accordance with the expected toxicity trend (please see Supplementary information, paragraph 1.2). The majority of detected OCs in the Sava River was indicated to activate nuclear receptors (Fig. S5) what was the biological category of greatest concern. Seventeen most probable biologically active OCs according to EAR values (in alphabetical order) are: 4−nonylphenol, bisphenol A (BPA), cotinine, caffeine, carisoprodol, dexamethasone, enrofloxacin, flumethasone, hydrocortisone, lamotrigine, metoprolol, monensin sodium, nicotine, perfluorohexanoic acid, sulfapyridine, sulfamethizole, and valproic acid.

3.7. Risk indexes

Risk indexes (RIs) have shown the following order of potential toxicity within sampled sites JES > HRU > JAN > RUG > LUK (2.59 > 1.54 > 1.32 > 1.27 > 1.26 respectively; Table S2). Top 20 OCs

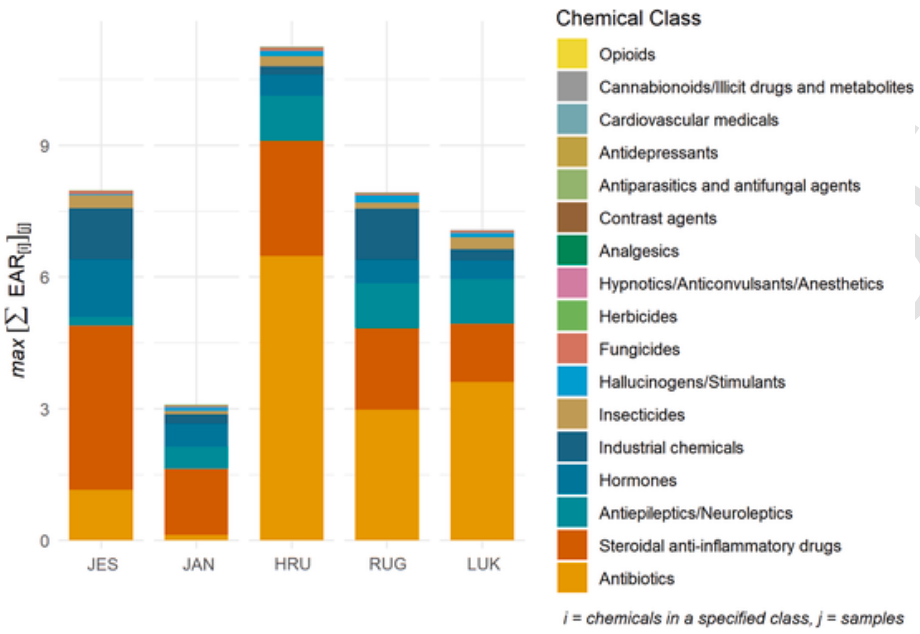

Fig. 6. Summed EAR values of chemical categories and/or sub-categories for each sampling site.

from each site according to their RQs amount to the total of 37 compounds and are shown in Table S2.

3.8. Agreement between different approaches on OCs of the most importance

The investigation of agreement between different approaches in identifying the most important OCs for the results of toxicity assessments showed that only metoprolol was identified by all 3 approaches while 12 OCs were in agreement by at least 2 approaches (Fig. 7). As expected, EAR and RQ approaches identified fewer OCs than the correlation tests because the former two incorporate comprehensive toxicity data from multiple sources whereas correlation tests only evaluate monotonic relationships.

4. Discussion

Chemical analysis pointed out PhACs/IDrgs and pesticides as categories with the greatest number of detected compounds, while PhACs/IDrgs category had overall cumulative concentrations up to 3.5 (RUG) folds higher than pesticides. High concentrations of PhACs/IDrgs at RUG (77.6% of all OCs cumulative concentration) and HRU (73.6%)

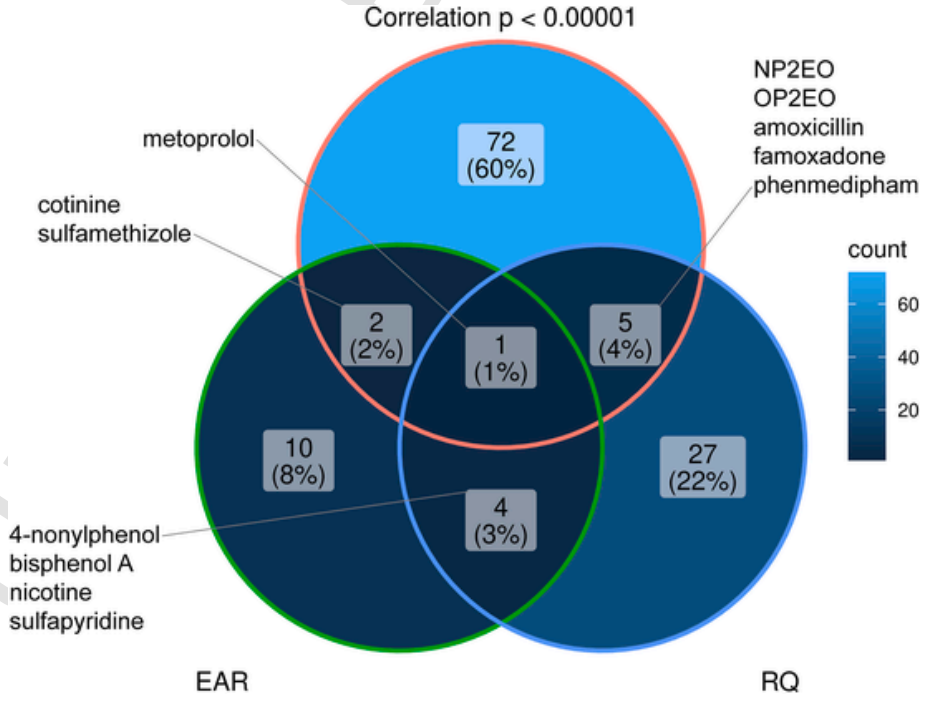

Fig. 7. Venn diagram of the agreement between top 17 most important OCs by EAR and top 20 OCs by RQ analyses and univariate Spearman's rank correlation test with Bonferroni adjusted p values in identifying chemical compounds from 5 Sava River sites significantly associated with toxicological test endpoints from 3 animal model organisms.

were expected since these sites are receiving municipal/industrial wastewaters from the city of Zagreb, whereas concentrations of PhACs/IDrgs at JES and JAN sites found upstream of the central wastewater treatment plant of Zagreb were not as high. However, industrial chemicals were more frequent at JES where they equaled the concentration of PhACs/IDrgs indicating a stronger input from the industry. A relevant influence of urban wastewaters was evident on HRU and RUG sites where stimulants nicotine (1.2 and 2.2 μg L^{-1}) and caffeine (1.0 and 1.1 μg L^{-1}) were among the OCs with the highest concentrations. Similar concentrations of caffeine were reported in rivers of the San Diego regions (0.03–0.7 μg L^{-1}) and the Island of Montréal (0.003–1.5 μg L^{-1}). Based on *Daphnia magna* toxicity testing, nicotine concentration of 0.1 μg L^{-1} was estimated as predicted no-effect concentration - PNEC (Oropesa et al., 2017). Nicotine (and its metabolite cotinine), as well as caffeine, were already indicated in our previous study as OCs of potential concern for fish (Malev et al., 2020).

Having in mind that toxicological behavior of OCs mixtures could be contrasted with cumulative effects of single OCs and/or antagonistic-synergistic interactions it was relevant to integrate chemical characterization with WOBs, RQ and EAR approach. Therefore, the toxicity of sampled water and quantified OCs was additionally determined with WOBs to get insights into the joint effects of all bioavailable pollutants (Neale et al., 2017). Our research focused mainly on sub-lethal endpoints *i.e.*, growth/development changes and biochemical alterations of freshwater organisms as well as behavioral changes, *i.e.*, feeding and locomotor activity of crustaceans. Such individual-based effects that include behavioral alterations have high ecological relevance because they often influence survival, growth, and reproduction (Groh et al., 2015). Behavior responses of different aquatic organisms under contaminant exposure have been reported in crustaceans (Billoir et al., 2007) and fish (Liu et al., 2011), confirming their sensitivity to sub-lethal chemical concentrations.

The slowest gammarids (24 h exposure) with the lowest feeding rate (48 h exposure) were found in test organisms exposed to waters from the most polluted sites (HRU and RUG). In the case of gammarids, a reduction of feeding intensity is often one of the first observed responses to chemical exposure and can be triggered by various chemical classes (Könemann et al., 2019). Among tested sites, HRU and RUG sites contained the highest concentrations of cotinine (0.23 and 0.22 μg L^{-1}, respectively) that negatively correlated ($p < 0.0001$; Table S5) with the speed ratio (normalized to controls) of gammarids. This predominant metabolite of nicotine appears to act as a suppressor of locomotor activity in invertebrates (Pagán, 2019), which could also interfere with the normal behavior of gammarids (e.g., reduced feeding and swimming speed) consequently leading to long-term effects on metabolism, immunity, growth, and reproduction.

Slower-moving daphnids were observed after exposure to all samples while the slowest individuals were detected in water from JES site. Initially, an increased feeding rate in daphnids was found in all samples, probably as the first response to stress induced by contaminants, while after 20 h all daphnids showed only slightly reduced feeding rates (*i.e.*, JAN, HRU). Correlation analysis showed that swimming reduction was most significantly associated ($p < 0.0001$, $rho = -1$) with the presence of 2 pesticides: hexaconazole and quinalphos (Table S5). These two pesticides were the most abundant at JES site that reduced daphnids activity by almost 30%. Sample taken from JES site has also high concentrations of industrial chemicals and hormones in comparison to other sites in the study, thus their negative effects on daphnids cannot be excluded. Campos et al. (2013) reported the hormetic consistent action of industrial nonylphenols at low concentrations (i.e., 3–15 μg L^{-1}), which might contribute to changes in daphnids' metabolic pathways. Taken together, *G. fossarum* and *D. magna* were found as suitable models for the assessment of toxic sub-lethal action. Endpoints such as locomotory behavior (i.e., average speed) and post-exposure feeding correlated in both species and were found as sensitive sub-lethal parameters.

Biochemical biomarkers showed an increased level of total protein contents in *G. fossarum* at sites HRU and RUG while elevated GST activity and TBARS content were observed only at JAN site. A high positive correlation ($p < 0.0001$) was observed between two industrial compounds, NP2EO and OP2EO, and the ratio of the total protein content in gammarids. These two compounds were among the top 20 chemicals with the highest RQs at all sites with the highest value observed at HRU (Table S2). The observed increase in the total protein content at most polluted sites (HRU and RUG) was also noted in other crustaceans (*i.e.*, crayfish) cage-exposed at polluted sites in River Sava downstream of Zagreb confirming the potential stress caused by exposure to overall OCs present at both sites at high concentrations or related to the higher amount of enzymes associated to detoxification processes (Klobučar et al., 2012).

Adverse effects observed in crustaceans (invertebrates) could differ from the ones that occur in zebrafish (vertebrates) due to inherent differences in their physiology, pharmacodynamics, and consequently specific biochemical interactions and toxicity (Besse and Garric, 2008). Although the survival and abnormality rates of zebrafish embryos/larvae were not altered, all Sava River samples induced a decrease in the heartbeat rate. Interestingly, the highest decrease ($p < 0.001$) was observed in water from the JAN site. A negative correlation ($p < 0.001$) with heartbeat ratio was observed with 10 compounds (7 pesticides and 3 pharmaceuticals). Amongst, penconazole and triazophos ($p < 0.0001$, $rho = -1$) have been reported to induce cardiovascular complications (including a decrease of heartbeat rate) during the early development stages of fish *D. rerio* and *Gobiocypris rarus* (Zhu et al., 2014; Jia et al., 2020). Interestingly, among the OCs with the highest concentrations at JAN is β-blocker metoprolol (0.6 μg L^{-1}), with very high RQ values at all sites and considerable EAR value at HRU, for which Sun et al. (2014) reported the ability to decrease the heartbeat rate and survival of the zebrafish embryos. In our study metoprolol was positively correlated with zebrafish heartbeat but it can be assumed that its effect in decreasing heartbeats manifests after prolonged exposure. Delay/absence of pigmentation formation that was most pronounced on JES and RUG negatively correlated ($p < 0.0001$) with 10 compounds among which 9 pesticides and 1 pharmaceutical. Although according to our knowledge no studies associated a lack of zebrafish pigmentation formation and exposure to those compounds, pesticides are already well known for their potential to inhibit melanogenesis during early embryo development (Şişman and Türkez, 2010; Fan et al., 2018). Furthermore, the inhibition of MXR mechanisms on JES, HRU and LUK sites is very concerning as it could lead to increased toxicity effect allowing previously excluded OCs to actively accumulate in the cell reaching higher (toxic) concentrations (Epel et al., 2008). Only 2 compounds were strongly correlated ($p < 0.0001$, $rho = 1$) with the RB accumulation, *i.e.*, hypnotic lorazepam and insecticide phoxim.

Our approach based on WOBs results was compared and complemented with data extracted from the ToxCast database allowing us to prioritize OCs of concern and identify targets of interest. According to cumulative EARs for each site, HRU, JES and RUG had the highest potential for adverse bio-effects followed by LUK and JAN, which is comparable to observed cumulative OCs concentrations. Additionally, standard risk assessment methodology was applied using RQs, where risk indexes were calculated for each site thus pointing out JES and HRU as the samples with the highest toxicity potential followed by JAN, RUG and LUK. In this case, the higher RIs in JES and JAN in comparison to RQ approach were highly influenced by the presence of only two industrial chemicals: 4-nonylphenol and PFDoS. It should be emphasized that PCA analysis showed a majority of industrial compounds as being responsible for the separation of all sites along dimension 1, resulting in positioning JES as a separate cluster from all other sites (a subset of

these OCs that are in agreement with EAR and RQ approaches is shown in Fig. S3).

Using high-throughput screening data from the ToxCast database further categories were additionally highlighted as having potential environmental risk: antibiotics, steroidal anti-inflammatory drugs, antiepileptics/neuroleptics, and hormones. ToxCast EAR-based approach has emphasized synthetic glucocorticoids (SG) like dexamethasone and flumethasone which had higher EAR values among steroidal anti-inflammatory drugs. The same prioritization method was applied in our previous study on the presence of pharmaceuticals in the plasma of fish from Sava River and indicated SGs dexamethasone and prednisolone as compounds with the highest potential biological impact (Malev et al., 2020). Much attention should be given to SGs that can chronically modulate the activity of nuclear receptors (*e.g.* glucocorticoid receptors - GRs), which was indicated within this and our previous study (Malev et al., 2020) as the biological category of greatest concern that could lead to different adverse outcomes at the whole organism level (*e.g.* physiological effects in zebrafish embryos).

Another group of OCs with high cumulative EAR values were antiepileptics/neuroleptics and lamotrigine as the most prominent compounds which are known to target serotonin and dopamine receptors. However, antiepileptics/neuroleptics were not flagged with other used methods. Also, OCs with high cumulative EAR values were hormones, *i.e.*, equilin, 17α-ethinylestradiol and progesterone. Progesterone was 10th OC with the highest RQ out of 391 OCs detected in JES sample. Although the concentrations of hormones in tested samples made less than 1% of total OCs concentration (except for JES - 2.4%), their occurrence, even at low concentrations (ng L^{-1} to μg L^{-1} range) have been linked with adverse effects on the endocrine system of aquatic organisms and humans (Daniels et al., 2018). Furthermore, EAR approach singled out additional chemicals of concern: BPA (industrial chemical) and nicotine, cotinine, and caffeine (hallucinogen/stimulant). BPA and other detected industrial chemicals in the Sava River are known for their estrogen-like or anti-androgenic activity. A persistent and ubiquitous xenoestrogen, 4-nonylphenol highly contributed to a significant industrial load detected in Sava River (especially at JES site). Also, BPA and 4-nonylphenol were among the 20 top OCs with the highest RQs in a majority of water samples from Sava River. Due to their similar MoA, additive action among the co-occurring hormonal disruptors (hormones and industrial chemicals category) should be taken into account when defining the estrogenic potency of polluted river waters.

According to the EAR approach, among detected OCs, the antibiotics sulfapyridine and enrofloxacin contributed the most to the potential bioactivity and potency of the compounds detected in the river water samples. Sulfapyridine was also among the top 20 OCs with the highest RQs in HRU, RUG and LUK sites while antibiotic amoxicillin was within the top 20 in JAN and HRU samples. Sulfapyridine and enrofloxacin are ubiquitous in aquatic ecosystems reaching from 0.140 to 3.2 μg L^{-1} in the rivers of Japan and China (Bojarski et al., 2020). Sulfapyridine was associated with toxic effects including a decrease in zebrafish body weight and increased cardiac activity (20, 40, 80 and 160 μg L^{-1}; Hamid et al., 2020). Authors showed that a binary mixture of sulfapyridine and other sulfonamides, i.e. sulfamethoxazole, sulfameter and sulfaziadine, induced even higher developmental toxicity and significantly perturbed the detoxification pathway in zebrafish when compared to the individual compound exposure. Bielen et al. (2017) reported the presence of enrofloxacin in the treated effluents released from the Croatian antibiotic-producing company and discharged into the Sava River reaching up to 98 μg L^{-1} in winter months. In the study of Dalla Bona et al. (2015) the toxicity of enrofloxacin was associated with transgenerational toxicity in *D. magna*. Zebrafish exposure to enrofloxacin caused oxidative stress, along with changes in catalytic activities of some of the antioxidant enzymes (Sehonova et al., 2019). Although Sehonova et al. (2019) emphasized that zebrafish can adapt to enrofloxacin within a short period, such results should be of concern since behavior of chemicals in a mixture may have a distinct toxic effect on non-target organisms which often do not correspond to the one obtained during single compound toxicity testing.

Overall, it is important to consider that toxicity estimation of detected OCs in Sava River obtained with the ToxCast-based approach may be conditioned by the number of detected OCs which can be found in the ToxCast database. A large part of the detected psychoactive chemicals like opioids, hallucinogens/stimulants, and cannabinoids/illicit drugs were not present in the ToxCast database. Such a lack of data could underestimate the prediction of effects caused by psychoactive compounds which were highlighted as those with high potential biological impact on fish in our previous study using the Fish plasma model approach (Malev et al., 2020). Therefore, a combination of different risk assessment approaches like the calculation of RQs is desirable since it can produce more comprehensive and precise predictions of negative biological effects caused by pollutants.

5. Conclusions

The present study has proved that the extension of river water quality assessment via chemical analysis, EAR approach, calculations of RQs, and WOBs can provide a comprehensive environmental risk assessment of freshwater ecosystems. The responses of model organisms to tested Sava River surface water samples varied. None of the used model organisms alone showed toxicity for all tested samples, but by pooling the data obtained with a battery of WOBs focusing on behavioral and physiological/morphological changes we were able to highlight the contaminated sites and group of chemicals with specific toxic potential. Given the obtained results, special focus should be put on zebrafish embryotoxicity bioassay and integration of toxicity testing with additional sub-lethal endpoints in crustaceans (e.g., behavioral parameters – locomotor and feeding activity). Using this approach, toxicity of environmental samples could be calculated for different taxonomic groups (fish, crustaceans) thus taking into account the differences in their phenotypes that could be responsible for different ecological impact scenarios caused by present contaminants. The bridging of chemical monitoring data and measured biological effects with available *in vitro* platforms allows a credible basis for prioritization of contaminants of greatest concern and a more comprehensive environmental screening of contaminants mixtures.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.118440.

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References

Amoatey, P., Baawain, M.S., 2019. Effects of pollution on freshwater aquatic organisms. Water Environ. Res. https://doi.org/10.1002/wer.1221.

Babić, S., Barišić, J., Stipaničev, D., Repec, S., Lovrić, M., Malev, O., Martinović-Weigelt, D., Čož-Rakovac, R., Klobučar, G., 2018. Assessment of river sediment toxicity: combining empirical zebrafish embryotoxicity testing with *in silico* toxicity characterization. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2018.06.124.

Babić, S., Barišić, J., Višić, H., Sauerborn Klobučar, R., Topić Popović, N., Strunjak-Perović, I., Čož-Rakovac, R., Klobučar, G., 2017. Embryotoxic and genotoxic effects of sewage effluents in zebrafish embryo using multiple endpoint testing. Water Res. https://doi.org/10.1016/j.watres.2017.02.049.

Babić, S., Malev, O., Pflieger, M., Lebedev, A.T., Mazur, D.M., Kužić, A., Čož-Rakovac, R., Trebše, P., 2019. Toxicity evaluation of olive oil mill wastewater and its polar fraction using multiple whole-organism bioassays. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2019.06.046.

Barata, C., Alañon, P., Gutierrez-Alonso, S., Riva, M.C., Fernández, C., Tarazona, J.V., 2008. A *Daphnia magna* feeding bioassay as a cost effective and ecological relevant sublethal toxicity test for environmental risk assessment of toxic effluents. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2008.06.028.

Barceló, D., Žonja, B., Ginebreda, A., 2020. Toxicity tests in wastewater and drinking water treatment processes: a complementary assessment tool to be on your radar. J. Environ. Chem. Eng. https://doi.org/10.1016/j.jece.2020.104262.

Besse, J.P., Garric, J., 2008. Human pharmaceuticals in surface waters. Implementation of a prioritization methodology and application to the French situation. Toxicol. Lett. https://doi.org/10.1016/j.toxlet.2007.10.012.

Bielen, A., Šimatović, A., Kosić-Vukšić, J., Senta, I., Ahel, M., Babić, S., Jurina, T., González Plaza, J.J., Milaković, M., Udiković-Kolić, N., 2017. Negative environmental impacts of antibiotic-contaminated effluents from pharmaceutical industries. Water Res. https://doi.org/10.1016/j.watres.2017.09.019.

Billoir, E., Péry, A.R.R., Charles, S., 2007. Integrating the lethal and sublethal effects of toxic compounds into the population dynamics of *Daphnia magna*: a combination of the DEBtox and matrix population models. Ecol. Model. https://doi.org/10.1016/j.ecolmodel.2006.11.021.

Blackwell, B.R., Ankley, G.T., Bradley, P.M., Houck, K.A., Makarov, S.S., Medvedev, A.V., Swintek, J., Villeneuve, D.L., 2019. Potential toxicity of complex mixtures in surface waters from a nationwide survey of United States streams: identifying *in vitro* Bioactivities and Causative Chemicals. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.8b05304.

Bojarski, B., Kot, B., Witeska, M., 2020. Antibacterials in Aquatic Environment and Their Toxicity to Fish. Pharmaceuticals. https://doi.org/10.3390/ph13080189.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. https://doi.org/10.1016/0003-2697(76)90527-3.

Campos, B., Garcia-Reyero, N., Rivetti, C., Escalon, L., Habib, T., Tauler, R., Tsakovski, S., Piña, B., Barata, C., 2013. Identification of metabolic pathways in *Daphnia magna* explaining hormetic effects of selective serotonin reuptake inhibitors and 4-nonylphenol using transcriptomic and phenotypic responses. Environ. Sci. Technol. https://doi.org/10.1021/es4012299.

Corsi, S.R., De Cicco, L.A., Villeneuve, D.L., Blackwell, B.R., Fay, K.A., Ankley, G.T., Baldwin, A.K., 2019. Prioritizing chemicals of ecological concern in Great Lakes tributaries using high-throughput screening data and adverse outcome pathways. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2019.05.457.

Council Directive 2010/63/EU, 2010. Council Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes. Official Journal of the European Union.

Dalla Bona, M., Zounková, R., Merlanti, R., Blaha, L., De Liguoro, M., 2015. Effects of enrofloxacin, ciprofloxacin, and trimethoprim on two generations of *Daphnia magna*. Ecotoxicol. Environ. Saf. https://doi.org/10.1016/j.ecoenv.2014.11.018.

Daniels, K.D., VanDervort, D., Wu, S., Leusch, F.D.L., van de Merwe, J.P., Jia, A., Snyder, S.A., 2018. Downstream trends of in vitro bioassay responses in a wastewater effluent-dominated river. Chemosphere. https://doi.org/10.1016/j.chemosphere.2018.07.190.

Di Paolo, C., Seiler, T.B., Keiter, S., Hu, M., Muz, M., Brack, W., Hollert, H., 2015. The value of zebrafish as an integrative model in effect-directed analysis - a review. Environ. Sci. Eur. https://doi.org/10.1186/s12302-015-0040-y.

Environmental Protection Agency, 1996. Gammarid Acute Toxicity Test. Ecol. Eff. Test Guideline.

Epel, D., Luckenbach, T., Stevenson, C.N., MacManus-Spencer, L.A., Hamdoun, A., Smital, T., 2008. Efflux transporters: newly appreciated roles in protection against pollutants. Environ. Sci. Technol. https://doi.org/10.1021/es087187v.

Fan, Y., Miao, W., Lai, K., Huang, W., Song, R., Li, Q.X., 2018. Developmental toxicity and inhibition of the fungicide hymexazol to melanin biosynthesis in zebrafish embryos. Pestic. Biochem. Physiol. https://doi.org/10.1016/j.pestbp.2017.10.007.

Groh, K.J., Carvalho, R.N., Chipman, J.K., Denslow, N.D., Halder, M., Murphy, C.A., Roelofs, D., Rolaki, A., Schirmer, K., Watanabe, K.H., 2015. Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: I. Challenges and research needs in ecotoxicology. Chemosphere. https://doi.org/10.1016/j.chemosphere.2014.09.068.

Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases. Methods Enzymol. https://doi.org/10.1016/S0076-6879(81)77053-8.

Hamid, N., Junaid, M., Pei, D.S., 2020. Individual and combined mechanistic toxicity of sulfonamides and their implications for ecological risk assessment in the Three Gorges Reservoir Area (TGRA), China. J. Hazard Mater. https://doi.org/10.1016/j.jhazmat.2019.121106.

Inam, E., Offiong, N.A., Kang, S., Yang, P., Essien, J., 2015. Assessment of the occurrence and risks of emerging organic pollutants (EOPs) in Ikpa River Basin freshwater ecosystem, Niger Delta-Nigeria. Bull. Environ. Contam. Toxicol. https://doi.org/10.1007/s00128-015-1639-9.

Jia, M., Teng, M., Tian, S., Yan, J., Meng, Z., Yan, S., Meng, Z., Yan, S., Li, R., Zhou, Z., Zhu, W., 2020. Developmental toxicity and neurotoxicity of penconazole enantiomers exposure on zebrafish (*Danio rerio*). Environ. Pollut. https://doi.org/10.1016/j.envpol.2020.115450.

Khan, F.R., Bury, N.R., Hogstrand, C., 2010. Differential uptake and oxidative stress response in zebrafish fed a single dose of the principal copper and zinc enriched sub-cellular fractions of *Gammarus pulex*. Aquat. Toxicol. https://doi.org/10.1016/j.aquatox.2010.06.007.

Klobučar, G.I.V., Malev, O., Šrut, M., Štambuk, A., Lorenzon, S., Cvetković, Ž., Ferrero, E.A., Maguire, I., 2012. Genotoxicity monitoring of freshwater environments using caged crayfish (*Astacus leptodactylus*). Chemosphere. https://doi.org/10.1016/j.chemosphere.2011.11.060.

Könemann, S., Müller, Y., Tschentscher, D., Krauss, M., Inostroza, P.A., Brückner, I., Pinnekamp, J., Schiwy, S., Hollert, H., 2019. Combination of in situ feeding rate experiments and chemical body burden analysis to assess the influence of micropollutants in wastewater on *Gammarus pulex*. Int. J. Environ. Res. Publ. Health. https://doi.org/10.3390/ijerph16050883.

Kunz, P.Y., Kienle, C., Gerhardt, A., 2010. Gammarus spp. in aquatic ecotoxicology and water quality assessment: toward integrated multilevel tests. Rev. Environ. Contam. Toxicol. https://doi.org/10.1007/978-1-4419-5623-1_1.

Li, S., Villeneuve, D.L., Berninger, J.P., Blackwell, B.R., Cavallin, J.E., Hughes, M.N., Jensen, K.M., Kahl, M.D., Stevens, K.E., Thomas, L.M., Weberg, M.A., Ankley, G.T., Li, S., Berninger, J.P., Jorgenson, Z., Schroeder, A.L., 2017. An integrated approach for identifying priority contaminant in the great lakes basin – investigations in the lower green Bay/fox River and milwaukee estuary areas of concern. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2016.11.021.

Liu, Y., Lee, S.H., Chon, T.S., 2011. Analysis of behavioral changes of zebrafish (*Danio rerio*) in response to formaldehyde using Self-organizing map and a hidden Markov model. Ecol. Model. https://doi.org/10.1016/j.ecolmodel.2011.02.010.

Lv, X., Huang, B., Zhu, X., Jiang, Y., Chen, B., Tao, Y., Zhou, J., Cai, Z., 2017. Mechanisms Underlying the Acute Toxicity of Fullerene to *Daphnia Magna*: Energy Acquisition Restriction and Oxidative Stress. Water Res. https://doi.org/10.1016/j.watres.2017.07.023.

Malev, O., Lovrić, M., Stipaničev, D., Repec, S., Martinović-Weigelt, D., Zanella, D., Ivanković, T., Sindičić Đuretec, V., Barišić, J., Li, M., Klobučar, G., 2020. Toxicity prediction and effect characterization of 90 pharmaceuticals and illicit drugs measured in plasma of fish from a major European river (Sava, Croatia). Environ. Pollut. https://doi.org/10.1016/j.envpol.2020.115162.

Neale, P.A., Munz, N.A., Aït-Aïssa, S., Altenburger, R., Brion, F., Busch, W., Escher, B.I., Hilscherová, K., Kienle, C., Novák, J., Seiler, T.B., Shao, Y., Stamm, C., Hollender, J., 2017. Integrating chemical analysis and bioanalysis to evaluate the contribution of wastewater effluent on the micropollutant burden in small streams. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2016.10.141.

OECD, 2004. OECD Guideline for Testing of Chemicals. Daphnia Sp., Acute Immobilisation Test.

Oropesa, A.L., Floro, A.M., Palma, P., 2017. Toxic potential of the emerging contaminant nicotine to the aquatic ecosystem. Environ. Sci. Pollut. Res. https://doi.org/10.1007/

s11356-017-9084-4.
Pagán, O.R., 2019. Cotinine as a possible allosteric modulator of nicotine effects in various models. In: Neuroscience of Nicotine. Academic Press, pp. 57–63. https://doi.org/10.1016/B978-0-12-813035-3.00008-3.
Prasse, C., Stalter, D., Schulte-Oehlmann, U., Oehlmann, J., Ternes, T.A., 2015. Spoilt for Choice: A Critical Review on the Chemical and Biological Assessment of Current Wastewater Treatment Technologies. Water Res. https://doi.org/10.1016/j.watres.2015.09.023.
Rodriguez, A., Zhang, H., Klaminder, J., Brodin, T., Andersson, P.L., Andersson, M., 2018. ToxTrac: a fast and robust software for tracking organisms. Methods Ecol. Evol. https://doi.org/10.1111/2041-210X.12874.
Sehonova, P., Tokanova, N., Hodkovicova, N., Kocour Kroupova, H., Tumova, J., Blahova, J., Marsalek, P., Plhalova, L., Doubkova, V., Dobsikova, R., Chloupek, P., Dolezalova, P., Faldyna, M., Svobodova, Z., Faggio, C., 2019. Oxidative stress induced by fluoroquinolone enrofloxacin in zebrafish (*Danio rerio*) can be ameliorated after a prolonged exposure. Environ. Toxicol. Pharmacol. https://doi.org/10.1016/j.etap.2019.02.002.
Şişman, T., Türkez, H., 2010. Toxicologic evaluation of imazalil with particular reference to genotoxic and teratogenic potentials. Toxicol. Ind. Health. https://doi.org/10.1177/0748233710375951.
Stipaničev, D., Dragun, Z., Repec, S., Rebok, K., Jordanova, M., 2017. Broad spectrum screening of 463 organic contaminants in rivers in Macedonia. Ecotoxicol. Environ. Saf. https://doi.org/10.1016/j.ecoenv.2016.09.004.
Sun, L., Xin, L., Peng, Z., Jin, R., Jin, Y., Qian, H., Fu, Z., 2014. Toxicity and enantiospecific differences of two β-blockers, propranolol and metoprolol, in the embryos and larvae of zebrafish (*Danio rerio*). Environ. Toxicol. https://doi.org/10.1002/tox.21867.
Taylor, E.J., Jones, D.P.W., Maund, S.J., Pascoe, D., 1993. A new method for measuring the feeding activity of *Gammarus pulex* (L.). Chemosphere. https://doi.org/10.1016/0045-6535(93)90188-B.
Zhu, B., Gong, Y.X., Liu, L., Li, D.L., Wang, Y., Ling, F., Wang, G.X., 2014. Toxic effects of triazophos on rare minnow (*Gobiocypris rarus*) embryos and larvae. Chemosphere. https://doi.org/10.1016/j.chemosphere.2014.03.036.