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Characterization of wastewater effluents in the Danube River Basin with chemical screening, *in vitro* bioassays and antibiotic resistant genes analysis

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ABSTRACT

Averaged 7-day composite effluent wastewater samples from twelve wastewater treatment plants (WWTPs) in nine countries (Romania, Serbia, Hungary, Slovenia, Croatia, Slovakia, Czechia, Austria, Germany) in the Danube River Basin were collected. WWTPs' selection was based on countries' dominant technology and a number of served population with the aim to get a representative holistic view of the pollution status. Samples were analyzed for 2248 chemicals of emerging concern (CECs) by wide-scope target screening employing LC-ESI-QTOF-MS. 280 compounds were detected at least in one sample and quantified. Spatial differences in the concentrations and distribution of the compounds classes were discussed. Additionally, samples were analyzed for the possible agonistic/antagonistic potencies using a panel of in vitro transactivation reporter gene CALUX® bioassays including ERα (estrogenics), anti-AR (anti-androgens), GR (glucocorticoids), anti-PR (anti-progestins), PPARα and PPARγ (peroxisome proliferators) and PAH assays. The potency of the wastewater samples to cause oxidative stress and induce xenobiotic metabolism was determined using the Nrf2 and PXR CALUX® bioassays, respectively. The signals from each of the bioassays were compared with the recently developed effect-based trigger values (EBTs) and thus allowed for allocating the wastewater effluents into four categories based on their measured toxicity, proposing a putative action plan for wastewater operators. Moreover, samples were analyzed for antibiotics and 13 antibiotic-resistant genes (ARGs) and one mobile genetic element (*intl1*) with the aim to assess the potential for antibiotic resistance. All data collected from these various types of analysis were stored in an on-line database and can be viewed via interactive map at https://norman-data.eu/EWW_DANUBE.

1. Introduction

The Danube River Basin (DRB) is the world's most international river basin covering a total area of 801,463 km^2, including territories from 19 countries. DRB is the Europe's second largest river basin and serves > 80 million people by providing drinking water, industrial and agricultural water supply, hydroelectric power generation, tourism and fisheries among others (Liska, 2015). Therefore, careful management of DRB's water resources is needed, including control over chemical pollution. European Union (EU) environmental legislation aims to protect all European water bodies by achieving their good chemical and ecological status (European Commission, 2013). In the content of chemical status, the EU Water Framework Directive (WFD) established a list of 45 priority substances (European Commission, 2013), supplemented by a set of additional 15 compounds (European Commission, 2018) in the recently revised Watch list, which are required to be monitored by Member States and benchmark their concentration against the Environmental Quality Standards (EQS).

Despite the regulatory efforts, many toxic anthropogenic chemicals are released into the environment that may have an adverse effects on the human health, ecosystem and diminish the quality of the aquatic resources (Altenburger et al., 2015). Despite large investments into

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WWTP technology, point source discharges from sewage plants of big cities in the DRB (e.g., Vienna, Bratislava, Budapest, Belgrade and Bucharest) still represent a significant route of input of numerous contaminants into the river (Heeb et al., 2012; Terzic et al., 2008). The introduction of untreated or partially treated wastewater generates complex chemical mixtures, which may impact severely the ecosystem of the receiving surface water, as shown recently in the case of Novi Sad (Konig et al., 2017). To assess such complex chemical mixtures, it is necessary to investigate their overall toxic potency and prioritize frequently occurring compounds based on their ecotoxicity (Konig et al., 2017).

There are many studies at regional level investigating the occurrence of specific classes of emerging substances in wastewater, such as psychoactive substances (Mackulak et al., 2015), benzodiazepines (Fick et al., 2017; Kosjek et al., 2012), opioid analgesics (Krizman-Matasic et al., 2018), and perfluorinated substances (Clara et al., 2009). There are considerably fewer studies focusing on bioassay applications (Smital et al., 2011; Tousova et al., 2018) and on antibiotic resistance (Birosova et al., 2014; Lupan et al., 2017), whereas only a very limited number of studies are dealing with combined wide-scope chemical and bioassays screening to assess the quality of wastewater (Konig et al., 2017; Smital et al., 2011; Tousova et al., 2018). Also, the DRB was assigned as a 'reservoir of antibiotic resistance' in the Joint Danube Survey 3 (Liska et al., 2015), one of the most serious threats to human health (WHO). It has been clearly recognized that more information is needed to define the composition of typical chemical mixtures, their fate and adverse effects on the environment, and to establish a comprehensive risk assessment scheme allowing the regulators to define preventive action plans within the Programs of Measures (European Commission, 2013) at a local, national or river basin scale. Here, bioassays covering a range of the ecotoxicity spectrum, as wide as possible, are considered as the key instrument in assessing the mixture toxicity (Brack et al., 2015; Fatta-Kassinos et al., 2015).

To facilitate an overview of effluent wastewater released into the Danube River and its tributaries, twelve WWTP effluent samples of various size and using different treatment technologies from nine countries were collected in cooperation with the International Commission for the Protection of the Danube River (ICPDR; 14 European countries and European Commission). The objectives of this study were to: (i) evaluate the occurrence of CECs using the state-of-the art wide-scope chemical screening techniques; (ii) apply NORMAN prioritization framework (Dulio and von der Ohe, 2013; von der Ohe et al., 2011) to prioritize the detected substances; (iii) apply a battery of bioassays to assess the adverse effects of mixtures of pollutants (exceedance of effect-based trigger values associated with various modes of action); (iv) test the feasibility of the newly proposed risk assessment scheme based on bioassays responses, and (v) assess the occurrence of antibiotics and antibiotic resistant genes (A&ARGs) in the collected wastewater effluents.

2. Materials and methods

2.1. Study area and sampling

The WWTPs in the DRB were selected in a way representing each country's predominant wastewater treatment technology including large plants in country capitals (e.g. Budapest, Ljubljana, Bucharest and Zagreb), large cities (e.g. Brno, Cluj-Napoca, Žilina and Augsburg) and towns (e.g., Amstetten and Varaždin). All plants reported to receive both municipal and industrial wastewater. Wastewater from Augsburg and Vipap consisted mainly of industrial wastewater (65% and 73% respectively). An overview of the sampling stations, their location, the population equivalent, the annual average daily wastewater discharge, the sampling collection type, the treatment type, the weather condition and the conductivity can be found in Section S1 of the Supplementary material (SI). Composite effluent wastewater samples were collected during dry weather and under normal operating conditions. Samples for analyses of organic substances remained in the freezer at −20 °C in the WWTP and frozen during transport. Each WWTP collected 7 L of composite wastewater (1 L for every day for a week). The 7 L samples from each WWTP were mixed to form the weekly averaged sample. 2 L from the weekly averaged samples were processed for chemical analysis, 0.5 L were sent to KWR (Nieuwegein, Netherlands) for analysis of antibiotic resistant genes (ARGs) and 0.5 L were sent to BDS (Amsterdam, Netherlands) for application of bioassays. All samples were processed immediately after arrival to the laboratory.

2.2. Chemical analysis

Details for chemicals and reagents used for chemical analysis are given in Section S1-2 (SI). Samples were cleaned up and preconcentrated 4000 times on Atlantic HLB-M Disk using HORIZON SPEDEX 4790 (USA) with 47 mm disk holder according to the extraction program described in Section S1-3 (SI). Extracts were evaporated using gentle stream of nitrogen and reconstituted with 500 µL of 50:50 methanol:water for UHPLC-ESI-QTOF and UHPLC-ESI-QqQ analysis. Before instrumental analysis extracts were filtered through RC syringe filters of 4 mm diameter and 0.2 µm pore size (Phenomenex, USA).

UHPLC-ESI-QqQ instrumental analysis was performed for the highly-sensitive determination of 158 pharmaceuticals, drugs of abuse and their transformation products (Alygizakis et al., 2016; Thomaidis et al., 2016). A Thermo UHPLC Accela system connected to a TSQ Quantum Access triple quadrupole mass spectrometer from Thermo Electron Corporation (San Jose, CA, USA) equipped with an electrospray ionization source (Thermo IonMAX) in both positive and negative mode. Chromatographic separation was performed on an Atlantis T3 C18 (100 mm × 2.1 mm, 3 µm) column from Waters (Milford, MS, USA) at a constant flow rate of 100 µL min^{-1}. The mobile phase, the gradient elution program and the ESI parameters are presented in Section S1-4 (SI) and the optimised ionization mode, fragmentation voltages, collision energies and chromatographic retention times for each analyte are summarized in Table S1-4B (SI).

UHPLC-ESI-QTOF analysis was performed using a UHPLC apparatus (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany), coupled to the QTOF-MS mass analyzer (Maxis Impact, Bruker Daltonics, Bremen, Germany). Chromatographic separation was performed on an Acclaim RSLC C18 column (2.1 × 100 mm, 2.2 µm) from Thermo Fisher Scientific preceded by a guard column of the same packaging material, kept at 30 °C. Gradient program, ESI parameters and mobile phases are summarized in Table S1-5 (SI). Samples were subjected to wide-scope target screening of 2248 compounds (list of compounds "UATHTARGETS" (NORMAN Suspect List Exchange) and screening method (Gago-Ferrero et al., 2019)). A compound was successfully detected if the mass error of the molecular ion was below 2 mDa, retention time deviation was below 0.30 min and at least a qualifier fragment ion was detected.

2.3. Bioassays

Detailed sample preparation protocol using fully validated methods and standard operational procedures are described in Section S2-1 (SI). The CALUX® bioassays (Chemical Activated Luciferase eXpression; BioDetection Systems BV, Amsterdam, the Netherlands) applied in the present study utilise cell lines, incorporating the firefly luciferase gene coupled to Responsive Elements (REs) as a reporter gene for the presence of compounds able to activate the respective REs. Cell culture information for the applied CALUX® bioassays (cell type, cell type species, % DMSO, fold dilution, % CO_2, exposure time, confluence, medium and additions to medium) can be found on Table S2-1 (SI). Cells that were exposed to compounds of interest not only express proteins that are under normal circumstances associated to RE, but also luciferase. By addition of the appropriate substrate for luciferase, light

is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compounds (17β-estradiol, Flutamide, Dexamethasone, Ru486, GW7647, Roziglitazone, B[*a*]P, Curcumine and Nicardipine for the ERα, anti-AR, GR, anti-PR, PPARa, PPARg, PAH, Nrf2 and PXR CALUX®, respectively). In this way, the CALUX® bioassays report 17β-estradiol, Flutamide, Dexamethasone, Ru486, GW7647, Roziglitazone, B[*a*]P, Curcumine and Nicardipine equivalents, respectively. To test for possible cytotoxic effects of the sample extracts, the cytotox CALUX® activity was also determined. Cytotox CALUX® cells constitutively express luciferase. In case cytotox CALUX® cells are exposed to sample extracts causing cytotoxicity, a decrease in luminescence is observed. A reduction of 20% in luminescence is considered as a cytotoxic response. To facilitate water quality assessment, effect-based trigger values (EBTs) have been established. Bioassay responses above EBTs indicated potential risk of adverse effects to the ecosystem. EBTs for the used CALUX® bioassays were retrieved from the literature (Escher et al., 2018; van der Oost et al., 2017) and are presented in Table S2-2 (SI). The signal from each of the bioassays was compared with the EBT and thus allowed for ranking of the toxicity of wastewater effluents.

2.4. Multiplex qPCR assays

Filtration, DNA extraction and gene quantification via multiplex qPCR was performed as previously described (Paulus et al., 2019). qPCR assays were performed for a total of 13 ARGs, for one mobile genetic element (*intI*1) as proxy for the anthropogenic pollution (Gillings et al., 2015) and for the internal control (IC) using multiplex qPCR assays. 16S rRNA was quantified using a SYBR Green II qPCR assay. The following ARGs were quantified by qPCR: *aph*(III)a, bla_{KPC}, bla_{OXA}, bla_{SHV}, *erm*B, *erm*F, *mec*A, *qnr*S, *sul*1, *tet*B, *tet*M, *van*A and *van*B. Primer and probe sequences and conditions of multiplex qPCR assays are described in Section S3-1 (SI). Multiplex standards consisted of gBlock fragments containing relevant gene sequences for the three genes within a multiplex qPCR assay. The 16S rRNA standard consisted of a plasmid containing the relevant 16S rRNA gene sequence. In both cases, standards were made up of 5 subsequent dilutions with concentrations ranging from 2.5E + 04 to 2.5E + 00 gene copies/μL. Multiplex qPCR assays were performed using the iQ™ Multiplex Powermix (Bio Rad, Munich, Germany) and the qPCR reaction was performed using a CFX96™ Real-Time PCR Detection System (Bio Rad, Munich, Germany). CFX96™ Real-Time PCR Detection System were interpreted by CFX Manager v.3.1.1517.0823. Multiplex qPCR data analysis is described in Section S3-2 (SI).

2.5. Quality assurance and quality control

The chemical method used in the present work was evaluated in terms of linearity, accuracy, sensitivity, repeatability and matrix effects. Seven-point calibration curves (0.5–100 ng mL^{-1}) were generated using linear regression analysis. The linearity was qualified by linear correlation coefficient (r^2). Accuracy of the method was assessed with recovery experiments in effluent wastewater samples at two concentration levels (10.0 and 100.0 ng L^{-1}). Extraction recovery was calculated by dividing the peak area of the spiked samples by the peak area of the matrix-matched samples (extracts spiked at the end of the sample preparation). As real samples may already contain target compounds, wastewater samples were analyzed to determine their concentrations, which afterwards were subtracted from the spiked and the matrix-matched samples. Method repeatability was evaluated with calculation of intermediate precision and was expressed in terms of relative standard deviation (% RSD) at the same concentration levels (10.0 and 100.0 ng L^{-1}). Matrix effect was expressed as percentage of suppression or enhancement was calculated using the following equation: %Matrix Effect = (Matrix Factor − 1) × 100, in which matrix factor was the fraction of the peak area of the matrix-matched samples divided by the peak area of the standard solutions. More details about quality assurance and quality control can be found in the Section S4-1 (SI). All samples were spiked for 31 internal standards (Table S1-2). Quantification was based on standard additions, and isotopically labelled compounds were used only for the quantification or those compounds in which isotopically analogues compounds were available. A field blank and a laboratory procedural blank were used to detect any unwanted contamination. The blank samples accompanied the wastewater samples for all types of analysis (chemical, bioassays and ARGs analysis). The signals observed in blank samples were subtracted. Octocrylene was the only case in which the signal of the blank samples exceeded the signal in the wastewater sample and thus was excluded from the results.

For bioassays testing wastewater samples (0.5 L) were extracted by means of Solid Phase Extraction (SPE) according to the fully-validated BDS protocol (p-bds-096). To test for possible cytotoxic effects of the samples analyzed, the cytotox CALUX activity was determined. For the determination of the various CALUX activities, CALUX cells were seeded in 96 wells plates in assay medium. Following exposure of the CALUX cells to serial dilutions of the sample extracts in triplicate, the induction of luciferase production was quantified by measuring luminescence following addition of the substrate luciferin. On each 96-well plate, a complete calibration curve for each respective bioassays is also analyzed using the relevant reference compounds. Analysis result of the test samples are intrapolated in the calibration curve for quantitative determination of (ant)agonistic potential of the test samples. Only dilutions that did not show any signs of cytotoxicity (relative induction in the cytotox CALUX bioassay > 80%) were used for final evaluation of CALUX analysis results. The bioassays were performed according to standard BDS standard operating procedures and protocols for culturing U2OS CALUX cells (p-bds-083), for analysis of luciferase activity in the PAH CALUX bioassay (p-bds-066), for analyzing samples with U2OS CALUX bioassays using sigmoidal dose response curves (with 0.1% or 1% DMSO; p-bds-085), for harvesting the cells and measurement (p-bds-070), and for calculating U2OS CALUX results using sigmoidal dose response curves (p-bds-084).

To assess ARG extraction 10 μL of IC at a concentration of 2.5E + 04 gene copies μL^{-1} were added in order to quantify DNA loss during the extraction process and detect potential qPCR reaction inhibition. All samples were processed within 12 h of their arrival. DNA extracts were stored at −20 °C prior to qPCR analysis. qPCR analysis was performed within 2 weeks of DNA extraction. All qPCR assays were performed in triplicates. Each qPCR assay was performed with undiluted DNA extract, in an initial first qPCR run, and with 1:10 diluted DNA extract, in subsequent runs. This was done to detect potential inhibitors and inhibition of the qPCR reactions for each sample. A positive control and a negative control were included in every assay to ensure multiplex qPCR quality.

2.6. Prioritization of chemicals

Risk assessment of the detected target compounds was based on the prioritization methodology developed by the NORMAN network (Dulio and von der Ohe, 2013; von der Ohe et al., 2011). The method is based primarily on comparing the measured concentrations of detected substances against their Provisional No Effect Concentration (PNEC), which represent their ecotoxicological threshold values. In cases when no experimental data on the toxicity of detected substances were available, predicted PNECs (P-PNECs) were derived by QSTR models (Aalizadeh et al., 2017). All PNEC values used in this study (experimental PNECs for 100 CECs and P-PNECs for 180 CECs) were extracted from the NORMAN ECOTOX database (https://www.norman-network.com/nds/ecotox/). For risk assessment purposes, the lowest PNEC was selected in the order of (a) EQS values; (b) experimental PNEC values from reference laboratories; (c) in silico predicted PNEC. The priority

was evaluated based on three indicators: (i) Frequency of Appearance (FoA); (ii) Frequency of PNEC exceedance (FoE), and (iii) extent of PNEC exceedance (EoE). The first indicator expresses in how many sites the compound was detected above the limit of detection (LOD). The second indicator considers the frequency of monitoring sites with observations of a compound above a certain effect threshold. For the calculation of this indicator, a compound's maximum observed concentration at each site (MEC_{site}) is compared to the lowest PNEC. Subsequently, the number of sites where the threshold was exceeded was divided by the total number of sites where the respective compound was monitored. The third indicator ranks compounds with regard to the extent of the effects expected. It is defined as the 95th percentile of all MEC_{site} values per compound (MEC_{95}) divided to the PNEC. The resulting hazard ration was then scaled from 0 to 1. The Risk Score is the linear combination of the indicators scaled from 0 to 1. In the end, only compounds with a priority ranking value of > 1.01 were listed. For the remaining substances, risk was assumed to be negligible. More details about the prioritization scheme used can be found in the study of Slobodnik et al. (2012) and in NORMAN network (Dulio and von der Ohe, 2013).

3. Results and discussion

3.1. Occurrence and spatial distribution of chemicals

Compound names, category, molecular formula and SMILES chemical identifier for all (280) compounds detected in at least one sample are summarized in Table S4-2 (SI). The detected CECs were grouped in seven categories: Pharmaceuticals (100), Pesticides (42), Psychoactive drugs (40), Industrial chemicals (34), Antibiotics (32) and Drugs of abuse, tobacco ingredients and steroids (26), artificial sweeteners (6). The sum of the concentration of all compounds (indicated as "cumulative concentration") corresponded to 6600–27,000 ng L^{-1} depending on the sampling location (Table S4-2, SI). Effluent wastewater from large WWTPs of capital cities showed the highest cumulative concentration; Ljubljana (27,000 ng L^{-1}), Bucharest (21,200 ng L^{-1}), Budapest (20,300 ng L^{-1}), Zagreb (17,000 ng L^{-1}). The lowest cumulative concentration was observed for Varazdin (7400 ng L^{-1}) and Augsburg (6600 ng L^{-1}).

Pharmaceuticals were not only the most often detected (100 compounds), but also the most ubiquitous class of substances (in terms of concentration) in all samples (Fig. 1). They represented 25–67% of the total concentration of the target substances. The highest concentration for pharmaceuticals was observed for plants serving the capital cities (53–67%). Industrial chemicals (5–30%) and pesticides (3–21%) proved to be the second and third most abundant compound classes. In cases in which concentrations of pharmaceuticals did not exceed 50% of the total concentration (Augsburg, Amstetten, Zilina, Varazdin and Brno), an elevated concentrations were observed for pesticides and industrial chemicals. Psychoactive drugs (3–23%) and antibiotics (2–17%) proved to occur in lower concentration than industrial chemicals and pesticides. The highest antibiotic composition was observed for Ljubljana and Sabac (17 and 16% respectively). Despite of its size, Sabac showed elevated concentrations of antibiotics and ARGs, which can be partially attributed to the pharmaceutical industry (Sabac; production of erythromycin, sulfamethoxazole and ciprofloxacin). Drugs of abuse (2–9%) showed similar occurrence levels in all samples. A detailed description of the occurrence of the individual substances and their TPs is discussed in detail in the SI at Section S5.

3.2. Occurrence of ARGs in wastewater effluents

11 out of the 13 genes, and *intl1* were detected in at least one sample (Section S6-1, SI). Six ARGs (*aph*(III)a, bla_{OXA}, *erm*B, *erm*F, *sul*1 and *tet*M), and *intl*1, seemed to have wide-spread occurrence, since they were detected in all samples. Five ARGs (bla_{SHV}, *mec*A, *qnr*S, *tet*B and *van*A) were detected sporadically, while bla_{KPC} and *van*B remained undetectable (Fig. 2 and Fig. 6-1A at SI). Relative concentration levels of *erm*B, *sul*1 and *tet*M seemed to be constant in all the investigated samples (Welch's ANOVA, $p > 0.05$), with concentrations ranging from 1.8E−05 to 4.9E−03 gene copies normalized to 16S rRNA. *Aph* (III)a, *intl*1, *erm*F and bla_{OXA} fluctuated widely with relative concentrations ranging from 2.4E−08 to 3.1E−02 gene copies per 16S rRNA copy, respectively. *Van*A was detected in two sampling stations (Varazdin and Bucharest). *Mec*A, *qnr*S and *tet*B were detected in relative concentrations ranging from 2.39E−08 to 2.35E−04 gene copies per 16S rRNA.

The most polluted sampling locations by ARG presence and abundance were Varazdin, Bucharest and Sabac. Varazdin and Bucharest were the locations at which 12 out of 14 ARGs were detected. High concentration of ARGs in Varazdin can be attributed to the extensive agriculture (e.g. poultry farming) in the region. The minimum number of detected ARGs was observed at the WWTPs Amstetten and Augsburg (seven genes). WWTPs Bucharest and Varazdin were also the sampling stations with the highest cumulative ARG concentration in gene copies/mL, whereas WWTPs Amstetten and Augsburg were the locations with the lowest cumulative ARG concentration (Fig. 2). The highest absolute concentrations (gene copies/mL) were observed for four ARGs (*aph*(III)a, *tet*M, *van*A, *mec*A) at the WWTP Bucharest, four ARGs (bla_{OXA}, bla_{SHV}, *qnr*S, *tet*B) at the WWTP Sabac, two ARGs (*erm*B, *sul*1) at the WWTP Varazdin and one ARG (*erm*F) at the WWTP Brno. Three of the detected ARGs (bla_{SHV}, *tet*B and *van*A) were found in 50% or less samples. From the investigated genes, *intl*1 and *sul*1 were the most abundant in absolute and relative concentration. *Intl*1 has previously been suggested as an indicator for ARG pollution (Gillings et al., 2015). The present findings further affirm this suggestion, as the three most polluted sampling location coincided with the highest measured *intl*1 concentrations. In most of the cases, the concentration of the antibiotics and the ARGs did not correlate. Exception to this trend was *qnr*S, which correlated significantly with quinolones, having a correlation coefficient of 0.77 (Hollander and Wolfe test, p-value = 0.009). It has previously been shown that elevated ARG concentrations from point sources, such as WWTPs, can have a significant and lasting impact on downstream water bodies (Berglund et al., 2015; Marti et al., 2013; Proia et al., 2016; Rodriguez-Mozaz et al., 2015), which makes information on ARG concentration in WWTP effluent necessary for risk assessment. WWTP effluents are of special interest as treatment has shown the potential to increase the frequency of antibiotic resistant bacteria (Makowska et al., 2016).

3.3. Risk assessment

Table 1 shows the 17 out of the 280 compounds which were prioritized. PFOS was prioritized first, exceeding the established EQS PNEC in all wastewater samples. The compound received the attention of other researchers in the past, who reported the compound in concentrations higher that EQS limit set by WFD (1 ng L^{-1}); medium concentration 7 ng L^{-1} in Danube JDS2 (Loos et al., 2010) samples, 5.9 ng L^{-1} in Danube JDS3 samples (Loos et al., 2017), up to 33 ng L^{-1} in wastewater and river water from Slovenia (Clara et al., 2009) and 95th percentile measured experimental concentration (MEC_{95}) 31 ng L^{-1} in four European catchments (Tousova et al., 2017). The second prioritized compound was the antibiotic ofloxacin with a risk score 2.58, followed by telmisartan with a risk score 2.57. There are not many reports for the occurrence of ofloxacin and telmisartan in wastewater from the DRB region. However, previous reports for ofloxacin in Europe showed ecotoxicological important concentration levels (up to 507 ng L^{-1} in Spain (Biel-Maeso et al., 2018) and 55.5 ng L^{-1} for United Kingdom (Castrignano et al., 2018)). Ofloxacin requires the attention of regulators and researchers for further monitoring of the compound in the catchment and conclusion whether it should be included in the legislation. Telmisartan exceeded P-PNEC value for 83%

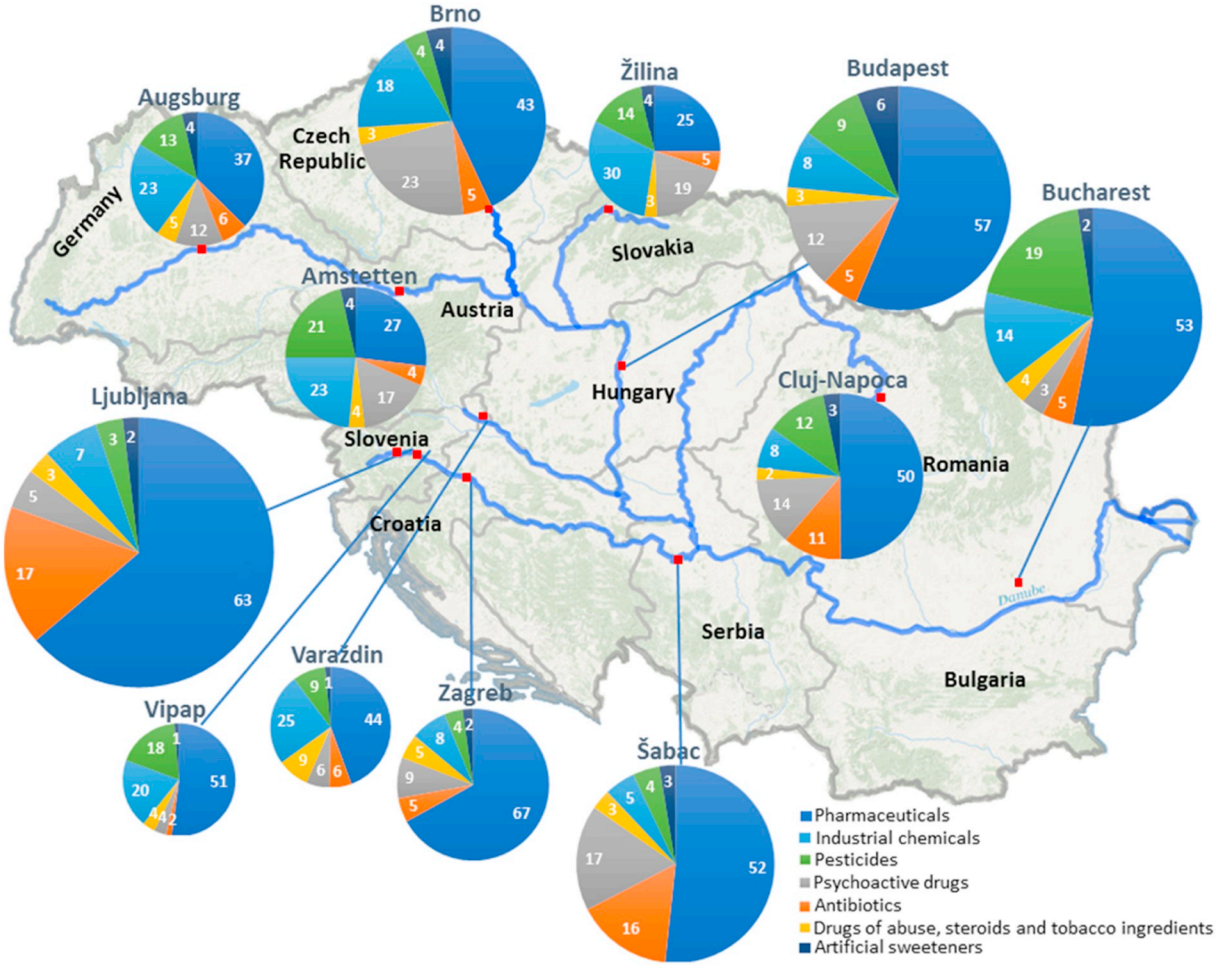

Fig. 1. Composition of chemicals in the effluent wastewater samples collected in the Danube River Basin. Pie plots for each sampling station (marked in red) represent the percentage (%) of each class of target compounds, whereas size of the pie plots is proportional to the cumulative concentration of all detected target compounds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

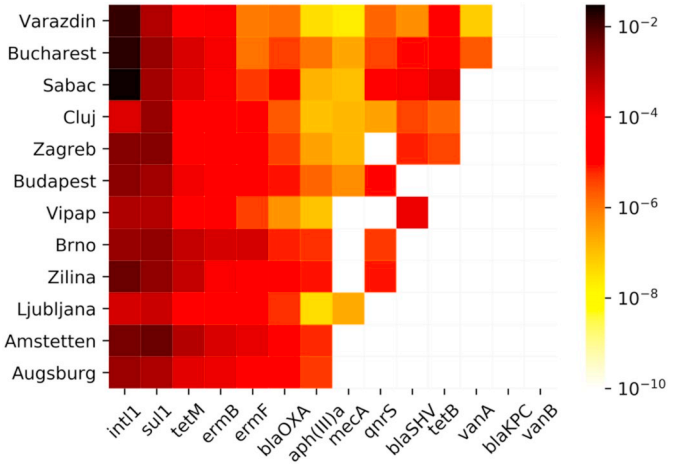

Fig. 2. Gene presence and concentration across the different sampling sites (WWTPs) in the Danube River Basin. Shown are relative gene copy numbers (normalized to 16S rRNA).

of the samples and has not been monitored adequately in the DRB (only a report in Hungarian wastewater at concentration up to 4800 ng L^{-1} (Diuzheva et al., 2018)). It worth noticing that telmisartan reported to exceed P-PNEC in seawater samples from Black Sea in Joint Black Sea Surveys (JBSS; EU/UNDP EMBLAS-II project; 2016) (Slobodnik et al., 2018). Further ecotoxicological experiments are required to verify P-PNEC value, so that robust conclusions are reached whether occurrence of telmisartan in the samples is of concern for the ecosystem. Other similar cases where P-PNEC verification by experimental data is needed to assess the hazard of compounds were the surfactant C12-LAS, the NSAID meclofenamic acid, the insecticide fipronil, the metabolite of methadone (EDDP) and of omeprazole (4-hydroxy-omeprazole). C12-LAS was detected above P-PNEC for 83% of the samples and its exceedance may be alarming for rest LAS surfactants, which even though detected in the samples could not be reliably quantified, because of lack of standards. Meclofenamic acid exceeded P-PNEC at 67% of the samples, whereas fipronil at 58% of the samples. Fipronil was reported by previous studies at lower concentration levels (0.4–4.5 ng L^{-1} in Danube in Novi Sad (Konig et al., 2017) and MEC_{95} 1.51 ng L^{-1} in EU catchments (Tousova et al., 2017)). The high concentration reported here can be attributed to the fact that collected samples were wastewater and not surface water, which indicates high proximity to the source of pollution. Fipronil needs to be monitored in more samples to reach safe conclusions. Same observations were valid for carbendazim, which exceeded PNEC for 67% of the samples, but it was previously reported at lower concentration levels in literature (4.0–12.4 ng L^{-1} in wastewater from Novi Sad (Konig et al., 2017) and MEC_{95} 2.8 ng L^{-1} in EU catchments (Tousova et al., 2017)). Carbendazim and fipronil screening in more samples and in surface water samples is further needed to conclude whether their occurrence induces hazards to ecosystem.

Table 1
Compounds prioritized based on the risk score, which is the sum of Frequency of Appearance (FoA), Frequency of Exceedance (FoE) and Extent of Exceedance (EoE). Table presents only compounds with risk score more than one.

Name	LOQ [μg/L]	PNEC [μg/L]	PNEC type	MEC [μg/L]	FoA	FoE	EoE	Risk score
PFOS	0.0003	0.001	EQS WFD	0.05	1.00	1.00	1.00	3.00
Ofloxacin	0.005	0.021	P-PNEC exp. Aquire 80421	3.1	1.00	0.75	0.83	2.58
Telmisartan	0.003	0.042	P-PNEC	2.3	1.00	0.83	0.74	2.57
Diclofenac	0.005	0.050	EQS-proposal	1.4	1.00	0.92	0.45	2.37
Dodecyl-benzenesulfonate (C12-LAS)	0.010	0.086	P-PNEC	1.8	1.00	0.83	0.24	2.07
Carbamazepine	0.0002	0.050	PNEC chronic Aquire 152195	0.7	1.00	0.83	0.17	2.01
Ibuprofen	0.001	0.010	EQS	1.0	0.50	0.50	0.92	1.92
4-tert-Octylphenol	0.010	0.100	EQS	0.3	1.00	0.67	0.06	1.72
Meclofenamic Acid	0.009	0.097	P-PNEC	0.3	1.00	0.67	0.05	1.72
Fipronil	0.003	0.023	P-PNEC	0.4	1.00	0.58	0.13	1.71
Carbendazim	0.026	0.150	AA-QS water eco INERIS (2017)	1.1	0.92	0.67	0.10	1.68
Venlafaxine	0.006	0.038	EQS-proposal	0.1	0.92	0.58	0.05	1.55
Clarithromycin	0.001	0.120	EQS-proposal	0.7	1.00	0.42	0.05	1.47
4-Hydroxy-Omeprazole	0.002	0.263	P-PNEC	8.5	0.92	0.08	0.13	1.13
EDDP	0.0001	0.137	P-PNEC	0.2	0.83	0.25	0.02	1.10
Temazepam	0.004	0.071	PNEC chronic Aquire 175030	0.2	0.83	0.17	0.02	1.02
Sertraline	0.0003	0.091	PNEC exp. Aquire 107936	0.1	0.92	0.08	0.01	1.01

Other prioritized substances were the NSAIDs diclofenac and ibuprofen, carbamazepine, 4-tert-octylphenol and venlafaxine. All these substances are known to be widespread in the environment and are well-studied (EQS PNEC available and occurrence levels) in the DRB. Their concentration exceeded EQS PNEC for 92%, 83%, 67%, 58% and 50% of the samples for diclofenac, carbamazepine, 4-tert-octylphenol, venlafaxine and ibuprofen respectively. The NSAIDs diclofenac and ibuprofen have been reported to exceed PNEC in several studies (Farre et al., 2008; Loos et al., 2017; Moldovan et al., 2009; Terzic et al., 2008). Both compounds are suggested for regular monitoring in the DRB catchment. Carbamazepine has been detected at concentrations levels 120–1550 ng L^{-1} in wastewater from Balkan region (Terzic et al., 2008), in wastewater from Romania at concentration ranges 213–774 ng L^{-1} (Moldovan et al., 2007) and in surface water of Danube river (average concentration of 33 ng L^{-1} for JDS2 samples (Loos et al., 2010) and 25 ng L^{-1} for JDS3 samples (Loos et al., 2017)). Average concentration in diluted matrices such as Danube water may dropped below PNEC. However, the introduction of large quantity of this drug in the ecosystem may have negative effects at least in regional level.

Venlafaxine and 4-tert-octylphenol were detected in similar concentration as in previous studies (e.g. venlafaxine previously detected as high as 259 ng L^{-1} (Mackulak et al., 2015) and up to 272 ng L^{-1} for 4-tert-octylphenol (Terzic et al., 2008)), whereas clarithromycin measured at higher concentration when comparing to other studies (e.g. as high as 59.7 ng L^{-1} (Konig et al., 2017; Tousova et al., 2017)) Last compounds to be prioritized because of the low FoE and EoE scores were temazepam and sertraline, which exceed PNEC for 17 and 8% of the samples respectively.

3.4. Application of in vitro bioassays

The results acquired by application of CALUX® bioassays expressed per liter of water can be found in Section S7-1A (SI). To facilitate the visualization and discussion, results were expressed as fold-induction relative to the LOQ of the respective CALUX® analysis (Table 2). Results below LOQ were assigned a fold-induction of 0.5. This visualization allows the comparison of the responses of each CALUX® bioassay among sample locations, but also between different CALUX® bioassays.

All samples were proved to be positive for PAH activity and for xenobiotic metabolism (PXR). Highest signal for both PAH and PXR activity was observed for the industrial plant Vipap in Krsko. The next most frequently detected effect was Estrogenic activity (ERα), with Cluj being the only sample that ERα was not detected. Ljubljana, Bucharest and Varazdin exhibited the highest ERα response than the other samples. Oxidative stress (Nrf2) was detected in 83% of the samples (not detected in Cluj and Zagreb) at similar equivalents in the samples. Anti-androgenic (anti-AR) and anti-progestin activity (anti-PR) proved to be effects with medium to high FoA, whereas medium detection occurred for glucocorticoid activity (GR). Peroxisome proliferators (PPARα and PPARγ) were scarcely detected.

As happens in case of detected chemicals, not all the detected effects in the samples are harmful to the environment. This happens because bioassays have become sensitive with low limits of detection. The solution is the application of EBTs on CALUX® bioanalysis results as determined for the WWTPs effluent water samples along the Danube River which resulted in the heat map presented in Table 3.

EBT values are as critical for assessing the importance of the observed effects as PNEC values for assessing the ecotoxicity of detected chemicals. Thus, the establishment of robust and reliable EBT values is of crucial importance, because large variations in proposed EBT values may result in misleading conclusions. In context of the presented study, EBTs of PAH CALUX® (6.2 (Escher et al., 2018) and 150 ng B[*a*]P eq./l sample (van der Oost et al., 2017)) and PXR CALUX® (54 (Escher et al., 2018) and 3 μg Nicardipine eq./l sample (van der Oost et al., 2017)) were considered to deviate enough to prevent consolidated conclusions for the importance of the observed effects of the aforementioned bioassays. In such cases, European or global-wide collaborative trials to establish commonly-agreed EBTs and achieve harmonized EQS is needed. Close EBTs for ERα, Nrf2 and anti-PR CALUX® led to consolidated conclusions. Responses by ERα, oxidative stress and anti-PR CALUX® exceeded the EBT for 92%, 83% and 75% respectively of the investigated samples, while anti-AR® CALUX exceeded the EBT for < 25% of the samples.

3.5. Putative action plan based on in vitro bioassays results

Based on the exceedance of the EBT values, a different response plan for WWTP operators was developed. The sample location and frequency for these bioassays should be linked to specified monitoring requirements for such indications for CECs in these water treatment plant effluents (e.g. sample collection with frequency every six months). An exceedance of the above proposed trigger values could initiate the following actions:

(i) If the result is below EBT or LOQ of bioassay (White): No further action required
(ii) If the result is 1-times < EBT < 3-times (Blue): Quality check of data, continue to monitor every three months, until 1 year and the EBT < 1
(iii) If the result is 3-times < EBT < 10-times (Green): Data check,

Table 2
Heat map of CALUX® analysis results for the various WWTPs effluent water sample sites along the Danube river. Values represent the fold-induction of each analysis relative to its respective LOQ. For analysis results that are below the LOQ, the result is represented as 0.5 times the LOQ (0.5). Low activity is marked with green and high activity with red.

	Cytotox CALUX®	anti-AR CALUX®	ERα CALUX®	GR CALUX®	anti-PR CALUX®	PPARα CALUX®	PPARγ CALUX®	PAH CALUX®	PXR CALUX®	Nrf2 CALUX®
Varazdin	4.5	0.5	49	0.5	4.9	2.3	0.6	40	2.3	1.2
Amstetten	0.5	1.9	10	0.5	5.3	0.5	0.5	68	3.6	1.4
Cluj	2.3	2.7	0.5	0.7	12	0.5	0.5	28	2.3	0.5
Augsburg	0.5	0.9	8.8	1.5	1.7	0.5	0.5	38	3.6	1.4
Vipap	0.8	2.7	5.6	0.5	7.1	0.5	0.5	159	9.2	2.1
Budapest	0.5	1.0	5.3	0.5	3.1	0.5	0.5	46	3.0	1.5
Ljubljana	0.5	0.7	60	3.8	3.6	0.5	0.5	17	2.7	1.5
Bucharest	2.0	0.5	69	1.3	6.6	0.5	0.5	22	2.9	3.9
Zilina	0.5	0.8	20	1.0	0.5	0.5	0.5	57	1.1	1.8
Sabac	0.5	1.2	9.5	0.5	0.5	0.5	0.5	57	0.8	1.4
Brno	0.5	1.1	10	1.1	0.9	1.2	0.5	80	1.7	2.4
Zagreb	0.5	0.5	15	0.5	0.5	0.5	0.5	34	1.6	0.5

immediate re-sampling and analysis to confirm EBT exceedance. It is also required to quantify specific target compounds which are known to cause the effects observed in the respective bioassay. Continue to monitor every three months, until 1 year and the EBT < 1

(iv) If the result is 10-times < EBT < 100-times (Orange): All the above actions and enhance source identification program. Also monitoring in the distribution system closer to the point of exposure to confirm attenuation of CEC is occurring and to confirm the magnitude of assumed safety factors associated with removal efficiency, dilution and post-treatment.

(v) If the results is EBT > 100-times (Red): All the above actions. Immediately confer with the local environmental authorities to determine the required response action. Confirm plant corrective actions through additional monitoring that indicates the CEC levels are below at least an EBT of 100.

The detailed action category table with EBT values for the applied bioassays can be found in Section S7-2 (SI). Application of the described action plan to the WWTP samples collected from Danube catchment resulted in Table 4.

The ERα CALUX® activity observed in in three WWTPs (Varazdin, Ljubljana and Bucharest) would lead to data check, immediate re-sampling to confirm EBT exceedance and chemical analysis of known estrogenicity drivers and to the distribution system to verify attenuation of the drivers. Same response would be in case of PAH CALUX® for ten WWTPs (all wastewater samples with the exception of Cluj and Zagreb). Lower response action (re-sampling, re-analysis to confirm EBT exceedance, chemical analysis of drivers) would be required for ERα CALUX® in four WWTPs (Amstetten, Augsburg, Zilina and Sabac), anti-AR CALUX® in seven WWTPs (Amstetten, Cluj, Augsburg, Vipap, Budapest, Sabac, Brno), PAH CALUX® in two WWTPs (Cluj, Zagreb) and Nrf2 CALUX® in two WWTPs (Bucharest and Brno). Finally, quality check of data and continuation of bioassay monitoring on a regular basis (every three months) for a year would be required for ERα in four WWTPs (Vipap, Budapest, Brno and Zagreb). Same action would be required for anti-AR in 5 WWTPs (Varazdin, Ljubljana, Bucharest, Zilina and Zagreb), for GR in five WWTPs (Cluj, Augsburg, Ljubljana, Bucharest, Zilina and Brno), for PPARγ in one WWTP (Varazdin) and for Nrf2 in eight WWTPs (Varazdin, Amstetten, Augsburg, Vipap, Budapest, Ljubljana, Zilina and Sabac).

4. Conclusions

Representative effluent wastewater samples were collected from nine countries of DRB. The samples were analyzed with the aim to get a holistic overview of the occurrence of chemicals in effluent wastewater using wide-scope target screening methods by LC-QTOFMS and LC-MS/MS. 280 compounds were detected and were subjected in ecotoxicological risk assessment to rank them based on their potential ecotoxicity. 17 out of the 280 compounds were prioritized. The occurrence of PFOS, diclofenac, carbamazepine, ofloxacin and ibuprofen proved to be of concern. Concentration of telmisartan, C12-LAS, meclofenamic acid, EDDP and 4-hydroxy-omeprazole exceeded P-PNEC, but experimental verification of P-PNEC is needed. More occurrence data points were needed for carbendazim and finopril to verify their occurrence and concentration levels. Moreover, the samples were analyzed for a battery of twelve CALUX® bioassays to investigate the effects that chemicals trigger. For this purpose, a set of CALUX® bioassays with a wide-range of mode of actions and established EBT threshold values were selected. The signals obtained by the bioassays were benchmarked against their EBT. Cases with exceedance of EBT were prioritized and a putative action plan was proposed based on the extent of exceedance. The proposed action plan translates the signals from CALUX® bioassays to actions for the WWTP operators. The study highlighted the need for commonly-agreed EBT values, which are needed for the correct translation of the signals from bioassays. Moreover, the lack of relative effect potency (REP) values for the detected chemicals prevent the connection between chemicals and bioassays and their establishment is crucial towards a better understanding of the pollution. Finally, antibiotic prevalence and abundance of 13 ARGs and one antibiotic resistance mobile element were assessed in the collected samples. Correlation of the concentration of ARGs with antibiotics was investigated. In most of the cases, the concentration of the antibiotics and the ARGs did not correlate with the exception of *qnr*S, which correlated significantly with quinolones. All data collected from these various types of analysis contribute towards a better understanding of the environmental problems caused by organic micropollutants.

Table 3
Application of a typical response plan published effect-based trigger values on CALUX® analysis results of WWTPs effluent water sample from various sites along the Danube river. Colour-coding indicates bioactivities above or below the published EBTs

Sampling stations	Escher et al. (2018)								van der Oost et al. (2017)							
	ERα CALUX®	anti-AR CALUX®	GR CALUX®	anti-PR CALUX®	PPARγ CALUX®	PAH CALUX®	Nrf2 CALUX®	PXR CALUX®	ERα CALUX®	anti-AR CALUX®	GR CALUX®	anti-PR CALUX®	PPARγ CALUX®	PAH CALUX®	Nrf2 CALUX®	PXR CALUX®
Varazdin			a		a							a				
Amstetten			a		a							a	b			
Cluj			a		a		b					a	b		b	
Augsburg			a		a							a	b			
Vipap			a		a							a	b			
Budapest			a		a							a	b			
Ljubljana			a		a							a	b			
Bucharest			a		a							a	b			
Zilina			a		a							a	b			
Sabac			a		a							a	b			
Brno			a		a							a	b			
Zagreb			a		a		b					a	b		b	

	ERα CALUX®	anti-AR CALUX®	GR CALUX®	PPARγ CALUX®	PAH CALUX®	Nrf2 CALUX®
Varazdin	5	5.7	<19	640	72	51
Amstetten	1.1	22	<20	<520	122	57
Cluj	<0.06	31	34	<420	52	<63
Augsburg	1	10	72	<410	72	57
Vipap	0.65	32	<25	<460	242	92
Budapest	0.56	11	<23	<430	62	58
Ljubljana	6.6	8.4	120	<350	62	62
Bucharest	7.4	5.7	38	<340	82	162
Zilina	2.2	8.9	78	<480	72	75
Sabac	1.1	14	<41	<490	72	57
Brno	0.54	13	47	<1100	122	100
Zagreb	0.8	6	<42	<1100	52	<21

a: no trigger value available; b: LOQ of bioassay exceeding EBT.

Table 4
Typical application of such a response plan of actions for operators of such WWTPs on CALUX® analysis results of effluent-water sample from various sites along the Danube River.

< trigger values > trigger values

Anti-AR CALUX®: μg Flutamide eq/L; ERα CALUX®: ng 17β Estradiol eq/L; GR CALUX®: ng Dexamethasone eq/L; PPARγ CALUX®: ng Rosiglitazone eq/L; PAH CALUX®: ng Benzo[*a*]pyrene eq/L; Nrf2 CALUX®: μg Curcumin eq/L.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

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