Environment International 179 (2023) 108155

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Full length article

A data-derived reference mixture representative of European wastewater treatment plant effluents to complement mixture assessment

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ARTICLE INFO

Handling Editor: Olga Kalantzi

Keywords:
Mixture risk assessment
Cell assays
Organism-based assays
Organic micropollutants
Effect data
Background pollution

ABSTRACT

Aquatic environments are polluted with a multitude of organic micropollutants, which challenges risk assessment due the complexity and diversity of pollutant mixtures. The recognition that certain source-specific background pollution occurs ubiquitously in the aquatic environment might be one way forward to approach mixture risk assessment. To investigate this hypothesis, we prepared one typical and representative WWTP effluent mixture of organic micropollutants (EWERBmix) comprised of 81 compounds selected according to their high frequency of occurrence and toxic potential. Toxicological relevant effects of this reference mixture were measured in eight organism- and cell-based bioassays and compared with predicted mixture effects, which were calculated based on effect data of single chemicals retrieved from literature or different databases, and via quantitative structure-activity relationships (QSARs). The results show that the EWERBmix supports the identification of substances which should be considered in future monitoring efforts. It provides measures to estimate wastewater background concentrations in rivers under consideration of respective dilution factors, and to assess the extent of mixture risks to be expected from European WWTP effluents. The EWERBmix presents a reasonable proxy for regulatory authorities to develop and implement assessment approaches and regulatory measures to address mixture risks. The highlighted data gaps should be considered for prioritization of effect testing of most prevalent and relevant individual organic micropollutants of WWTP effluent background pollution. The here provided approach and EWERBmix are available for authorities and scientists for further investigations. The approach presented can furthermore serve as a roadmap guiding the development of archetypic background mixtures for other sources, geographical settings and chemical compounds, e.g. inorganic pollutants.

1. Introduction

Aquatic environments are polluted with complex mixtures of inorganic and organic compounds emitted from a variety of different anthropogenic sources such as agricultural and urban runoff or effluents of wastewater treatment plants (WWTPs). Especially for organic micropollutants, the number and in many cases also the identity of organic micropollutants is unknown. Efforts to characterize chemical mixtures in the environment, by means of large-scale monitoring studies applying wide-scope chemical target screening, revealed a large and variable number of compounds (Finckh et al., 2022, Halbach et al., 2021, Kostich et al., 2014, Loos et al., 2013, Moschet et al., 2014). Next

Abbreviations: **ACC**, activity measured above a respective threshold in a cell assay; **CRT**, chronic risk threshold; **EAR**, exposure-activity-ratios; **EU**, effect units; **EWERBmix**, European WWTP effluent reference background mixture; **HTS**, high-throughput screening; **IC**, induction ratio; **MCR**, maximum cumulative risk ratio; **mMEC**, median measured environmental concentration; **REF**, relative enrichment factor.

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https://doi.org/10.1016/j.envint.2023.108155
Received 5 June 2023; Received in revised form 6 August 2023; Accepted 16 August 2023
Available online 17 August 2023

to advances in wide-scope screening technologies including non-target approaches which aim to grasp the complexity of chemical pollution (Hollender et al., 2017), there is a lack of suitable assessment tools for predicting ecotoxicological effects of mixtures prevalent in aquatic systems and for determining the role of individual components in the mixtures (Altenburger et al., 2019).

Joint exposure (e.g., Malaj et al., 2014, Rorije et al., 2022) and combined effects of chemicals in environmental samples (e.g., Escher et al., 2020b, Tang and Escher, 2014) and respective potential risks for aquatic species have been shown in numerous studies and are a matter of recent debates on amendments in predictive assessment, regulation, and management of chemical mixtures in the environment (Bopp et al., 2019, Drakvik et al., 2020, Kortenkamp et al., 2019; European Commission, 2019). Although requirements for assessment of intentional mixtures, i.e., formulations and products, are in place in some regulations (e.g., Plant Protection Products Regulation (European Commission, 2009) and Biocidal Products Regulation (European Commission, 2012)), provisions to assess and regulate the risks of unintentional mixtures in the environment at large are still missing (Hassold et al., 2021). Component-based predictive mixture risk assessment requires knowledge on exposure and effects of individual mixture components as well as appropriate mixture risk models (Bopp et al., 2019).

In order to approach unintentional mixtures in the environment, it is necessary to acknowledge temporal and spatial variations. The assumption that certain background pollution may be characteristic for certain types of emission sources (e.g., municipal or industrial WWTPs) or environments (e.g., creeks in urban landscapes) might be one way forward to account for mixtures in the assessment of individual chemicals (Burton et al., 2015). Such reference mixtures may be derived from frequently occurring compounds for which effect and exposure data should ideally be available. This approach would allow for a component-based mixture risk assessment for what might be considered a typical background burden.

The aim of this study was aimed to define and investigate one representative WWTP effluent mixture of organic micropollutants that could be suggested as a reference for testing and assessment of a realistic and typical WWTP effluent pollution. For this, exposure data was used from a recent study by Finckh et al. (2022), who characterized chemical mixtures emitted by WWTP effluents across Europe by measuring 499 chemicals in 56 effluent samples from 52 European WWTPs in 15 countries. We evaluated to which extent a prediction of mixture effect was possible for this mixture using existing knowledge extracted from databases containing measured *in vivo* and *in vitro* effect data, such as the ECOTOXDB (U.S. EPA, 2019a) and the InvitroDB (U.S. EPA, 2019b), as well as effect data predicted with quantitative structure–activity relationships (QSARs) and measured assay-specific single substance effect data. The combined effect predictions were compared with measurements of mixture effects using a selection of three organism-based and five cell-based bioassays.

The approach and composition of the reference mixture provided here are available (see Supporting information (SI), Table S6) for authorities and scientists for further investigations. Furthermore, this approach can be used as a case study guiding the development of additional background mixtures characterizing other emission and exposure scenarios. In addition, our study highlights current data gaps and provides guidance for prioritization of effect testing and monitoring for some of the most prevalent and relevant individual organic micropollutants occurring in European freshwaters due to WWTP effluents.

2. Materials and methods

2.1. Data retrieval

2.1.1. Chemical data on organic micropollutants in WWTP effluents

To obtain a representative mixture of organic micropollutants emitted from WWTPs, a data set obtained from wide scope multi-target screening by Finckh et al. (2022) was used. This data set is available on PANGAEA (https://doi.pangaea.de/10.1594/PANGAEA.940755). In the study by Finckh et al. (2022), 56 WWTP effluent samples from 52 WWTPs were taken by large volume solid phase extraction LVSPE (Schulze et al., 2017) and extracted according to Välitalo et al. (2017). Chemical analysis was performed by liquid chromatography and high-resolution mass spectrometry (LC-HRMS, Thermo QExactive Plus instrument) and chemical concentrations were quantified with Tracefinder (version 4.1, Thermo Scientific) and respective R scripts (Finckh et al., 2022). For the identification and preparation of a WWTP effluent reference mixture, these data were reanalyzed regarding the following criteria: i) Replicate samples, i.e., resulting from repeated sampling campaigns on different days, were omitted to avoid bias to certain sampling sites. This was applied to one WWTP which was sampled twice. Thus, only included 55 WWTP effluent samples were included in our data analysis. ii) Only compounds which were detected above their respective method detection limit in at least 90 % of all investigated effluent samples were considered. This resulted in 110 chemicals out of 499 that were further considered.

2.1.2. Effect data for organic micropollutants in WWTP effluents

ECOTOXDB: For these 110 chemicals, available data on effect concentrations (EC) were retrieved as described in the following sections and summarized in Fig. 1. Experimentally determined ECs for fish, crustaceans, and algae were retrieved from the ECOTOX knowledgebase (ECOTOXDB, release 12.12.2019; U.S. EPA (2019a)) as reported in Busch et al. (2016). Briefly, the 5th percentile was taken from all reported EC_{10} to EC_{90} values as well as lowest observed effect levels and concentrations (LOEL/LOEC) if more than one EC was reported for one compound and the respective species group.

InvitroDB: ToxCast™ MySQL-database version 3.2 (InvitroDB; U.S. EPA (2019b)) was queried for the selected 110 compounds and all available and curated activity concentrations at cutoff (ACC) values for all available bioassays were extracted (U.S. EPA, 2019b). The ACC value represents the concentration at which the regression model first reaches the cutoff value for a data-series to be considered active (Filer et al., 2016). Assay-compound matches were further checked for quality issues and were marked accordingly. If several ACC values were available for one compound, the lowest ACC value was selected to represent the most sensitive assay and a worst-case scenario (Fig. 1).

QSARs: In case of missing experimentally determined ECs in the ECOTOXDB for the selected compounds and species groups, baseline toxicity was determined using QSARs according to Busch et al. (2016) for fish, algae and daphnia and according to Kluver et al. (2019) for zebrafish embryo test (ZFET). All equations and details are provided in SI (SI 1.1.).

2.2. Calculation of toxic units (TU) and exposure-activity-ratios (EAR)

Hazards posed by measured individual compound concentrations in WWTP effluents were expressed by toxic units (TUs) or exposure-activity ratios (EARs) (Blackwell et al., 2017), respectively. TUs and EARs were calculated by dividing the median concentration detected in the WWTP effluent samples for a chemical, further called the median measured environmental concentration (mMEC), with the experimentally determined or modelled ECs for TU or ACC for EAR values, respectively (Eq. 1a and b). If a TU value or an EAR value equals or is higher than 1, an acute effect or activity is induced in the respective assays by the measured environmental concentration. Values lower than 1 indicate that an enrichment of environmental concentration is required to induce acute effects or activities in the respective assays.

Equation (1): Calculation of toxic units (a) and exposure-activity-ratios (b).

$$\begin{aligned} &(a) \quad TU_i = mMEC_i / EC_i \\ &(b) \quad EAR_i = mMEC_i / ACC_i \end{aligned} \tag{1}$$

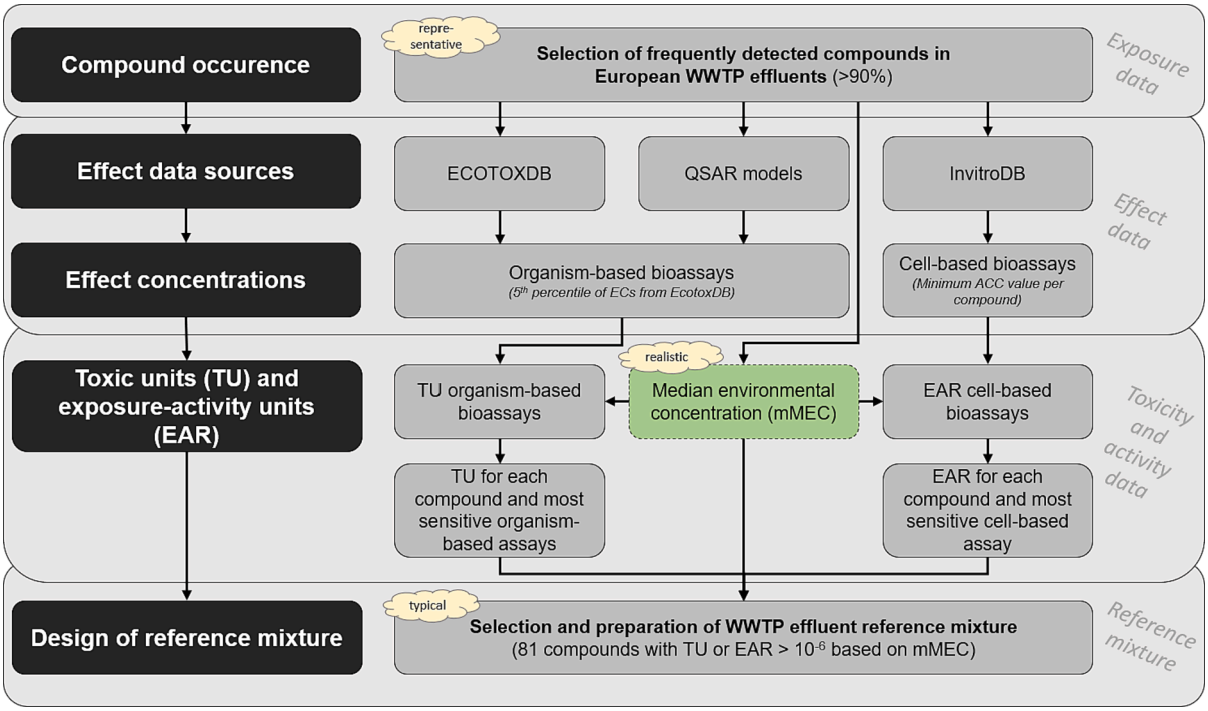

Fig. 1. Flow chart of the process of identification and design of the European wastewater treatment plant (WWTP) effluent reference background mixture (EWERBmix). ACC = activity measured above a respective threshold in an assay; QSAR = quantitative structure–activity relationships; ECs = effect concentrations.

2.3. Design and preparation of the European WWTP effluent reference background mixture (EWERBmix)

2.3.1. Selection of organic micropollutants for the WWTP effluent reference mixture

For cell-based assays, the maximum EAR per compound was determined across all EARs calculated with effect data from InvitroDB. For the organism-based assays and environmentally relevant species groups, the maximum TU was determined for each compound across all TUs calculated with effect data from the ECOTOXDB or QSARs in case no measured data was available. The TUs/EARs for all compounds and assays were pooled and the highest value (i.e., the most sensitive assay) for each compound was recorded to obtain a summarized list of the most potentially bioactive compounds (Table S6).

2.3.2. Preparation of the WWTP effluent reference mixtures of organic micropollutants

The EWERBmix was prepared in methanol from single substance stock solutions (approximately 1 mg/ml). The concentration in the mixture corresponded to the mMEC enriched by a factor of 100′000 (Table S6).

2.3.3. Chemical analysis of test medium

The prepared reference mixture and the concentration of mixture components in organism-based assays were analyzed by LC-HRMS (see Section 2.1.1). Samples were analyzed by direct injection (100 µL) and prepared in different dilution (1:100 and 1:500) depending on enrichment factors in bioassays.

2.4. Toxicity testing of the WWTP effluent reference mixture with organism-based assays

2.4.1. Preparation of control and treatment solutions

The mixture stock solutions were stored at −20 °C. One day prior exposure, the desired amount of mixture stock solutions was transferred in adequately sized GC vials (VWR International) and evaporated to drought under flowing nitrogen. The fully dried residue of mixture stock solutions was recovered in a methanol fraction to obtain a thousandfold higher concentration as needed for effect measurement. The respective solution was stored at 4 °C until exposure. At the day of exposure, the concentrated mixture sample was brought to room temperature and diluted to desired exposure concentrations using the respective growth medium, i.e. ISO standard dilution water for the Zebrafish Embryo Acute Toxicity Assay (DIN ISO 7346–3; 79.99 mM CaCl2··2H2O, 20.00 mM $MgSO_4 \cdot 7H_2O$, 30.83 mM $NaHCO_3$, 3.09 mM KCl, pH = 7.4 ± 0.1, O_2 saturation > 80 %), Grimm and Boardmann medium (GB medium) for the Algae Growth Inhibition Test, and ADaM for the *Daphnia magna* Immobilization Test. All exposure as well as control solutions contained 0.1 % (v/v percent) of methanol.

2.4.2. Organism-based bioassays

The organism-based assays were performed according to OECD guideline. The algae growth inhibition test was conducted according to OECD TG 201 (OECD, 2011) with adaptations as described in Faust et al. (2001), the *Daphnia magna* Immobilization Test according to OECD TG 202 (OECD, 2004), and the Zebrafish Embryo Acute Toxicity Test according to OECD TG 236 (OECD, 2013). Further details can be found in the SI (see section SI 1.2.).

2.4.3. Activity testing of the EWERBmix with cell-based assays

The reference mixture was tested in five cell-based assays including the aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor gamma (PPARγ), oxidative stress response (AREc32), pregnane × receptor (PXR) and the estrogen receptor (ERα) assays. AhR CALUX and AREc32 were performed according to Neale et al. (2017). PXR, PPARγ and ERα CALUX reporter gene assays (BioDetection Systems B.V., Amsterdam) were performed as described in Völker et al. (2022) with minor modifications. In brief, cells were cultured in growth medium (DEMEM/F-12 with phenol red supplemented with 8 % foetal bovine serum (FBS), non-essential amino acids, 1 % penicillin/streptomycin and 0.4 % genitizine) at 37 °C and 5 % CO_2. To remove steroids, present in the growth medium in the ERα assay, the cell culture medium was

changed to assays medium (DEMEM/F-12 without phenol red supplemented with 5 % charcoal-stripped FBS, non-essential amino acids and 1 % penicillin/streptomycin) two days prior to the experiment. 24 ± 1 h before to exposure cells were seeded in 384-well plates (CellStar 781098, Greiner Bio-One) at a concentration of 5000 cells per well in assay medium. The wells at the plate edges contained medium without cells. For the exposure, the dried reference mixture was re-dissolved in assay medium and used within 48 h. Cells were exposed to the reference mixture for 23 h in 20 concentrations serially diluted 1:1.5 (relative enrichment factor (REF) 9–20000). On each plate negative controls (cells in assay medium), vehicle controls (cells in assay medium with 0.05 % dimethylsulfoxide) and concentration series of the positive controls were included; Nicardipine (CAS 54527-84-3, 0.005–5 μM) for PXR, Rosiglitazone (CAS: 122320-73-4, 0.0003–3 μM) for PPARγ and 17-beta-estradiol (CAS 50-28-2, 0.2–100 pM) for ERα. High content imaging (Cytation 5 Cell Imaging Multimode reader, BioTek) was used to count stained nuclei (Nucblue, Thermo Fisher Scientific), to normalize the reporter gene response and assess cytotoxicity. The open-source software CellProfiler (Carpenter et al., 2006) was used for cell counting. Receptor activation was analysed after cell imaging by measuring luminescence (Cytation 5) of the lysed cells for 1 sec following substrate injection. The sample was analysed in three to four independent experiments per assay, each with four technical replicates.

2.4.4. Determination of effect concentrations from measured data

Dose-response curves for measured effect data were modeled using a maximum-likelihood approach. Log-logistic and Weibull models were fitted to the experimental data and best fitting models were selected based on the Akaike Information Criterion. Fitting was done using R (version 4.2.1; R Core Team (2021)) and the package drc (version 3.0.1; Ritz et al. (2016)). Determined EC_{10}, EC_{50} and LC_{50} values are expressed in μmol/L and converted to REFs by multiplication with the original concentration of the reference mixture. The conversion of EC_{10}, EC_{50} or LC_{50} values, respectively, in units of REF to TU is performed according to equation 2a. The reporter gene activation is not a toxicity and therefore the inverse of the EC_{10} for reporter gene activation are termed effect units (EU; Eq. 2b). Cytotoxicity is expressed as induction concentration (IC). In case of the AREc32 assay, the concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) was determined.

Equation (2): Calculation of toxic units (a) and effect units (b).

$$\begin{aligned} &(a) \quad TU = 1/E/LC_{50} \text{ or } 1/IC_{10} \\ &(b) \quad EU = 1/EC_{10} \text{ or } 1/EC_{IR1.5} \end{aligned} \tag{2}$$

2.5. Comparison of measured and predicted toxic units and effect units

The measured effects were compared to the respective predicted toxicities for the mixture, which were calculated based on the principle of concentration addition (CA) as the more conservative and precautionary component-based assessment measure (Backhaus and Faust, 2012). The predicted mixture toxicity was obtained by summing up TU_i or EU_i values for those of the 81 individual mixture components (i) for which data were available (sumTU, sumEU; Eq. (3). TU_i were calculated for a) ECs retrieved from the ECOTOXDB, b) ECs predicted by QSARs and c) assay-specific ECs. The latter were retrieved from single substance tests performed in the own respective laboratories. For ECs predicted by QSARs, logD_{lipw}-based QSARs for algae and daphnia identified after the selection and preparation of the EWERBmix were applied (Escher et al., 2020b). Measured TUs of cytotoxicity in cell-based assays were compared to predicted baseline toxicity based on QSAR models according to Lee et al. (2021). Details on all applied QSARs are shown in the SI and Table S9-S11. Additional EC data from literature was included for the algae, daphnia, and the ZFET (Table S7) as well as for the AREc32 and the AhR CALUX assay (Table S8) (Lee et al., 2022). SumTU were calculated for all these different datasets according to equation (3). D_{lipw} values were calculated according to Eq. 7 in SI 1.1.

Equation (3): Calculation of sumTU (a) and sumEU (b).

$$\begin{aligned} &(a) \quad \text{sumTU} = \Sigma TU_i \\ &(b) \quad \text{sumEU} = \Sigma EU_i \end{aligned} \tag{3}$$

2.5.1. Retrieval of predicted no effect concentrations

Predicted no effect concentrations (PNECs) were retrieved from NORMAN database (https://www.norman-network.com/nds/ecotox/lowestPnecsIndex.php (07.10.2022)). For 50 out of 81 compounds of the EWERBmix, lowest PNEC for freshwater were retrieved from the database and used for a PNEC-based risk assessment of the mixture. For comparisons of sumTU and sumRQ (Eq. (5) as shown in Fig. 6, ECs and PNECs provided by Finckh et al. (2022) were used.

Equation (4): Calculation of risk quotient of individual compounds (RQ_i).

$$RQ_i = mMEC_i/PNEC_i \tag{4}$$

Equation (5): Calculation of sumRQ.

$$\text{sumRQ} = \Sigma RQ_i \tag{5}$$

2.5.2. Contribution of risk driving compounds in the EWERBmix

The contribution of the potential most risk driving compound at each WWTP effluent site was identified by the ratio of the maximum TU of an individual compound ($maxTU_i$) to the sumTU at the respective WWTP effluent site (Eq. (6)). The ratio is called maximum cumulative risk ratio (MCR) adapted from Price and Han (2011). TU data for the 81 EWERBmix compounds was taken from Finckh et al. (2022). MCRs are shown in Table S15.

Equation (6): Calculation of MCR

$$MCR = maxTU_i/\text{sumTU} \tag{6}$$

3. Results and discussion

3.1. Concept and approach for the data-driven identification of a *European WWTP effluent reference background mixture (EWERBmix)*

The identification and selection of chemicals and their concentrations for a representative, realistic and typical reference mixture simulating background exposure by WWTP effluents sites in Europe was performed employing different steps. These steps include data retrieval for exposure data of 55 WWTP effluent samples (Finckh et al., 2022) ("*representative*") and subsequent determination of the mMEC ("*realistic*") and calculation and ranking of hazard ratios based on effect data for organism-based and cell-based assays (see materials and methods for details). Due to high variations of compound concentrations potentially resulting from site-specific or time-specific inputs, the mMEC as a more realistic exposure scenario was preferred over the maximum compound concentration representing a worst-case scenario. Concerning organism-based assays, effect data for fish, crustaceans and daphnia were selected as these groups of species are considered environmentally relevant and are considered as representative taxa for surface water under substance-oriented regulatory frameworks, e.g., for the definition of biological quality elements (BQE) under the Water Framework Directive (WFD) (European Commission, 2000). The whole process of identification and selection of the components for the EWERBmix is schematically illustrated in Fig. 1 and maybe adapted to other geographical settings, emission scenarios or pollutants ("*typical*"). Results of each selection step are presented and discussed in the following sections.

3.2. Commonly detected micropollutants in European WWTP effluents

To design and build a reference mixture representative for European WWTP effluents, compounds, which occurred in 90 % of all measured effluents (Finckh et al., 2022), were selected for further investigations. This cutoff was chosen to allow for consideration of analytical

uncertainties (i.e., false negatives due to failed peak integration or levels below the method detection limits, which depends on the sensitivity for a given compound and the background noise) as well as natural variations of compound concentrations (e.g., due to local and temporal variations in consumption and emissions) in individual WWTP effluents samples. The selection resulted in 110 chemicals with median concentrations between 3 ng/L (isoproturon, herbicide) and 15 μg/L (sucralose, artificial sweetener) (Table S1, Figure S1). There is a considerable variance in the detected concentrations of individual compounds across the considered sites, with maximum and minimum values several orders of magnitude above and below the median concentration. For example, 5-methyl-1H-benzotriazole, a transformation product of the corrosion inhibitor benzotriazole, was detected at all sites in concentrations ranging from 55 ng/L up to 22 μg/L and a median concentration of 18 μg/L.

The 110 compounds comprise parent compounds as well as transformation products of: i) food additives, such as the artificial sweeteners sucralose and acesulfame (median concentration of 1.8 μg/L); and ii) pharmaceuticals, which represent the dominant use group among the 110 ubiquitous WWTP effluent compounds. Their occurrence is in line with their production and consumption in high amounts and throughout the year. Pharmaceutical compounds included beta-blockers, sartans, antihistamines, antidepressants, antibiotics, nonsteroidal anti-inflammatory drugs, anesthetics, and anticonvulsants. Hydrochlorothiazide, diclofenac, and metformin were found in the highest median concentrations (i.e., 2.3, 1.4, 1.6 μg/L, respectively). Furthermore, iii) pesticides, mostly legacy pesticides which are partially also applied as biocides, for example, for protection of facades and wood (e.g., diuron, terbutryn, fipronil, tebuconazole, isoproturon, mecoprop, and carbendazim), as well as pesticides for private (e.g., 2,4-D) and professional use (e.g., ethofumesate, lenacil) were detected. In addition, iv) industrial chemicals, such as benzothiazole rubber additives and their degradation products, perfluorosulfonic acids (PFOS), both with median concentrations > 100 ng/L, and phosphorous-containing flame retardants as well as 1,3-diphenylguanidine and hexamethoxymethylmelamine were found. Recently, the latter two have received increasing attention as they are linked to tire wear leachates and urban runoff (Johannessen et al., 2021, Krauss et al., 2019, Peter et al., 2018).

3.3. Availability of measured effect concentrations in databases for commonly found WWTP effluent compounds

Aiming at ranking the compound list according to their potential hazard, ECs for the identified 110 WWTP effluent compounds were retrieved and analyzed. Major data gaps were identified which resulted in a sparse data set for measured ECs. Fig. 2A shows the number of compounds, for which data were available from the ECOTOXDB for the respective aquatic BQE. Measured ECs were available only for about 25 % of the compounds (Table S2). ECs were mostly available for pharmaceuticals (17 compounds) followed by pesticides (10 compounds) and industrial compounds (10 compounds). Furthermore, ECs were retrieved for four biocides as well as for piperine (food additive) and DEET (insect repellent).

Fig. 2B shows a heatmap indicating for each of the 110 compounds if it was expected to be active, inactive or not measured in any of the 1210 assay components recorded in the InvitroDB. Here, unclear measurement outcomes, e.g. noisy data, or only one or few data points above the baseline resulting in a non-fit of the model, were flagged and also considered as inactive. For 34 compounds, which were mostly transformation products of pharmaceuticals and industrial compounds, no entries were available at all in the InvitroDB. Owing to their ubiquitous presence in the aquatic environment (Pan et al., 2018), closing data gaps for those two groups should be a of high priority in the future. Most compounds (72 of 110) were found to be analyzed in several assays from the ToxCast/Tox21 program, with most compounds not showing any activity in these assays up to the highest tested concentration, which was typically 100 μM. ECs, i.e., activity measured above a respective threshold in an assay (ACC) (Filer et al., 2016), were available for 53 compounds in 377 different assays (Table S3). The highest number of active compounds were found for three assays detecting stress-related endpoint components, namely ATG_PXRE_CIS_up (16 active compounds out of 47 tested), ATG_AhR_CIS_up (9 active / 47 tested), and ATG_NRF2_ARE_CIS_up (11 active / 47 tested). Fig. 2C displays the data availability and data quality for these three assays, as well as for two additional assays with endocrine-related endpoints (ATG_PPARg_TRANS_up, 7 active / 47 tested and ATG_ERa_TRANS up, 3 active / 47 tested). These five endpoints were considered for mixture testing in this study.

The coverage of InvitroDB data for detected chemicals is in the same range as other studies have reported which used this database for screening and prioritization of environmental chemicals (Alvarez et al., 2021, Blackwell et al., 2017, Corsi et al., 2019). These studies also reported a better coverage with high-throughput screening (HTS) data in comparison with organismal toxicity data. In this sense, our study provides also an example of the potential of HTS data for effect prediction. In comparison to organism-based assays, HTS data are able to provide a more comprehensive picture of potential specific molecular effects due to the inclusion of many different endpoints which may arise from environmental chemicals. Naturally there are also limitations to this approach. Within HTS data retrieved from the InvitroDB, chemicals are generally tested in a standardized concentration range up to 100 μM, which may not represent a suitable range for effect modeling. This was discussed by Blackwell et al. (2017) who reported that ACCs might in some cases be below the lowest tested concentration leading to inaccurate estimates of ACC. Furthermore, assays included in the InvitroDB database exclusively focus on mammalian molecular targets and will miss other potentially important ecotoxicologically relevant effects.

Overall, the sparse effect data available for some organism-based assays (ECOTOXDB) and HTS data (InvitroDB) highlights the great need for a systematic testing strategy focusing on common WWTP effluent chemicals.

3.4. Toxic units and exposure-activity ratios for the most common European WWTP effluents compounds

Based on the derived effect data sets, TUs or EARs, which provide the ratio between environmental concentrations of a compound and its respective EC for a specific species group, endpoint or bioassay, were calculated. Fig. 3 illustrates the top 10 compounds with highest median TUs or EARs based on the concentrations measured across all WWTP effluents. Fig. 3A-C shows highest median TUs for the three aquatic BQE species groups and Fig. 3D-H the highest median EARs for the five selected cell-based bioassays. Thirty-four different compounds of the 110 compounds were among the top 10 for the considered species groups and cell-based assays (Table S4, Fig. 3). The TUs and EARs ranged from $4.2*10^{-2}$ (clarithromycin/algae) to $2.3*10^{-7}$ (perfluorooctanesulfonic acid/ ATG_ERa_TRANS_up). The latter, however, might represent a cytotoxicity burst artefact rather than a specific reporter gene activation (Escher et al., 2020c).

For the three BQE, the top 10 compounds mostly belong to the groups of pharmaceuticals (8 compounds) and biocides (7 compounds). For the selected cell-based assays, most of the top 10 compounds are used in biocides and in industrial applications.

Despite a considerable overlap of compounds across the different assays and species, compounds rank differently according to TU or EAR among the considered assays and species groups, indicating specific action of a compound in a specific assay or organism. An example is diuron, a well-investigated herbicide with the highest TU for algae (TU $= 1.2*10^{-2}$) and the lowest for fish (TU $= 5.6*10^{-7}$). It is reported to be active in 35 cell-based assays and inactive in 679 cell-based assays. This compound represents one of the most broadly investigated chemicals. For many other compounds the comparison between assays is more

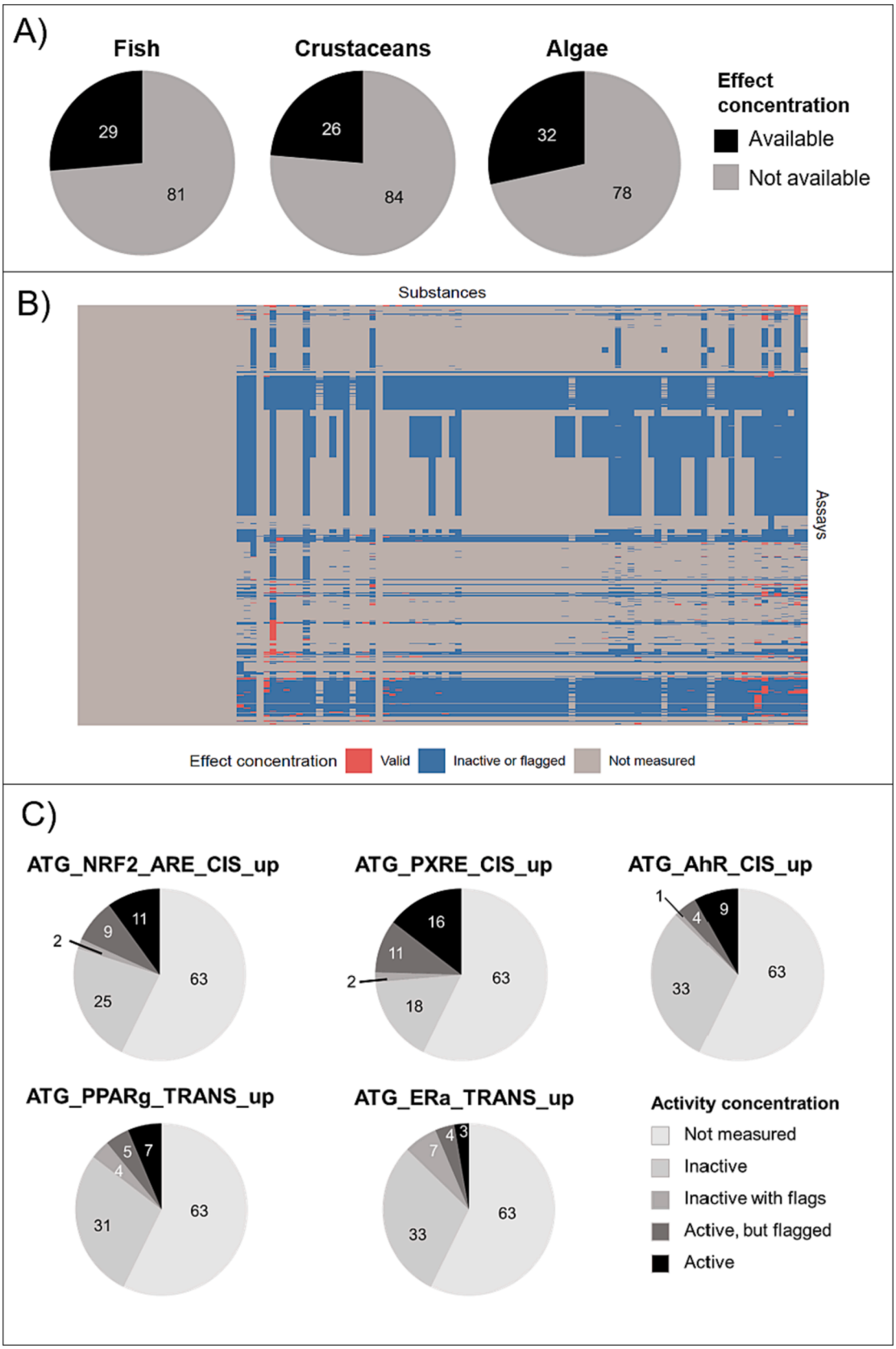

Fig. 2. Availability and quality of effect concentration data for the 110 most common European WWTP effluent chemicals. A) number of compounds with available and non-available effect concentrations from the ECOTOXDB for the respective BQE species groups fish, crustaceans, and algae; B) heatmap displaying data availability and quality for assays listed in the InvitroDB; C) numbers of compounds, which were either not measured, or determined to be active or inactive in respective assays, data derived from the InvitroDB.

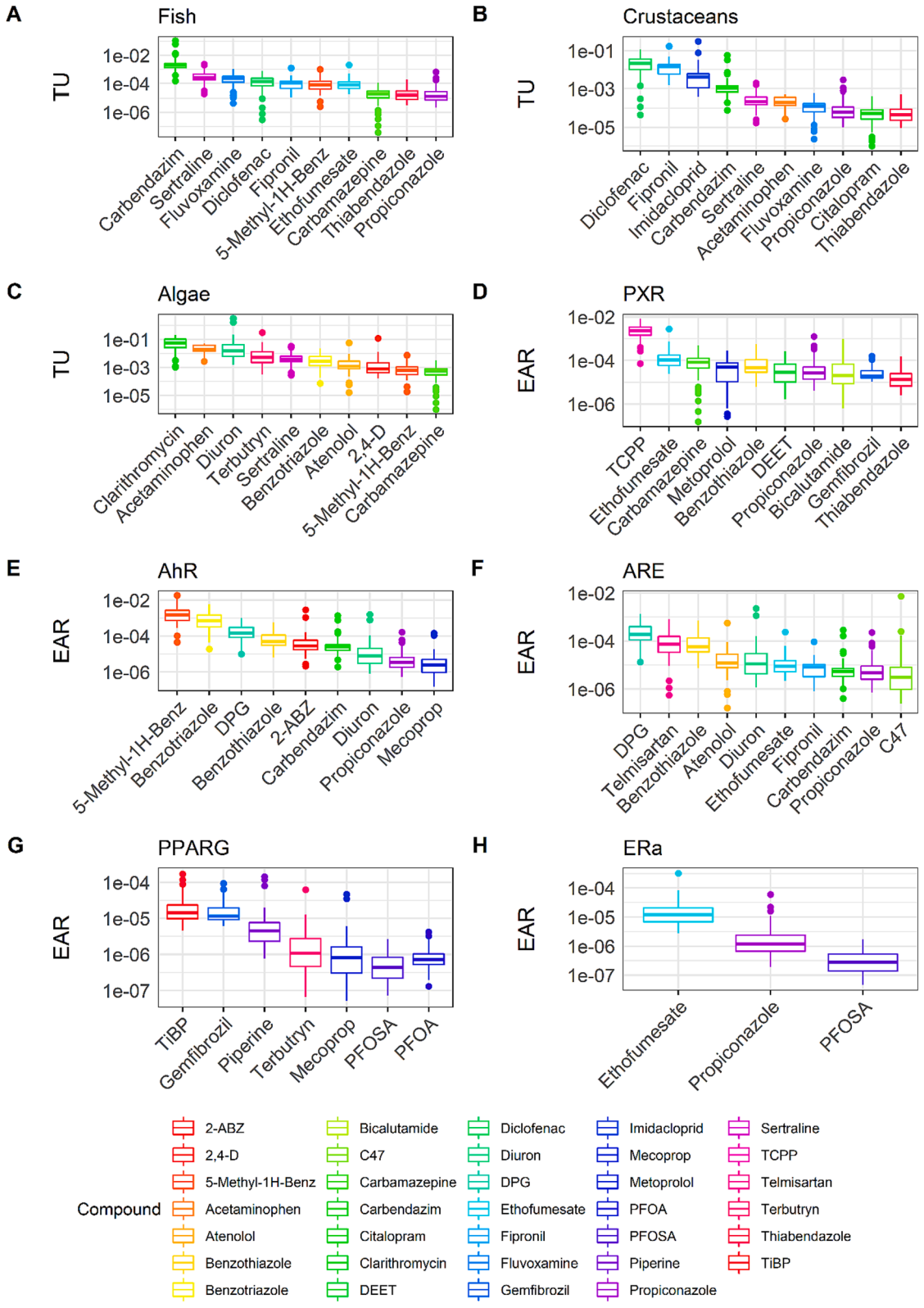

Fig. 3. Boxplots of top 10 compounds with highest median TUs/EARs across the 52 EU WWTP effluents. Data shown for three aquatic species representing different trophic levels A) fish, B) crustaceans, C) algae (based on effect data from ECOTOXDB); and five cell-based assays D) ATG_PXRE_CIS_up, E) ATG_AhR_CIS_up, F) ATG_NRF2_ARE_CIS_up, G) ATG_PPARG_TRANS_up and H) ATG_ERa_TRANS_up (based on data from the InvitroDB). 5-Methyl-1H-Benz = 5-Methyl-1H-benzotriazole, 2-ABZ = 2-Aminobenzimidazole, 2,4-D = 2,4-Dichlorophenoxyacetic acid, Benzotriazole = 1H-Benzotriazole, C47 = 7-Diethylamino-4-methylcoumarin, DPG = 1,3-Diphenylguanidine, PFOAS = Perfluorooctanesulfonic acid, PFOA = Perfluorooctanoic acid, TCPP = Tris(1-chloro-2-propyl)phosphate, TiBP = Tri-iso-butylphosphate. Each box indicates the lower (25 %) and upper (75 %) quantile and the horizontal line in the box indicates the median. Whiskers represent the most extreme, non-outlier data points.

difficult as the data sets are incomplete.

In this ranking (Fig. 3), we omitted compounds for which no measured effect data were available. To obtain more comprehensive data, existing data gaps were filled with QSAR-derived baseline toxicity ECs for the organism-based endpoints algae, daphnia, and fish. QSARs were applied for 71 % (78 of 110) compounds concerning estimated minimum bioactivity in algae, 76 % (84 of 110 compounds) for daphnia, and 74 % (81 of 110 compounds) for fish (Fig. 2A, Table S2). When TUs were ordered resulting from QSAR-based ECs, the potentially most active compounds of the 110 common WWTP effluent compounds changed and some rather lipophilic compounds with high logD_{ow} values, e.g., the pharmaceutical telmisartan (logD_{ow} = 4.9), the vulcanization accelerator TP 2-(methylthio)-benzothiazole (logD_{ow} = 3.4), and the flame retardants tris(1-chloro-2-propyl)phosphate (TCPP) and tri-isobutylphosphate (TiBP) (logD_{ow} = 3.3 and 3.9, respectively) ranked high (Table S4). As the applied QSAR models for baseline toxicity are based on a linear correlation of logD_{ow} and toxicity, this finding was to be expected (Figure S2). Additionally, baseline toxicity values were retrieved for the zebrafish embryo model system with a logD_{lipw}-based QSAR model (SI, Table S5). Here, the ranking of potentially most active compounds for the zebrafish embryo test (ZFET) was dominated again by telmisartan (logD_{lipw} = 4.05), TCPP (logD_{lipw} = 3.51) and TiBP (logD_{lipw} = 4.01), 2-(methylthio)-benzothiazole (logD_{lipw} = 3.58) as well as antifungal agent climbazole (logD_{lipw} = 4.50) and the pharmaceutical diclofenac (logD_{lipw} = 2.64). For each of these compounds a TU of $1*10^{-5}$ to $8*10^{-5}$ was calculated.

It must be noted that the QSARs applied in this study predict baseline toxicity, which is the minimal toxicity any compound can elicit. Thus, toxicity based on specific mechanisms and effects will be underestimated for compounds, for which no measured ECs are available. Furthermore, QSAR-based methods for effect prediction depend on physico-chemical properties and might lead to false predictions especially for compounds with structure-based predicted high logK_{ow} values. Furthermore, logK_{ow}-based QSARs for baseline toxicity have been demonstrated to be only valid for non-polar neutral chemicals (Vaes et al., 1998). Considering the impacts of pH-dependent speciation on membrane partitioning can reduce these prediction errors. Therefore, logD_{lipw}-based QSARs might be applied for ionizable organic chemicals for algae, daphnia and fish in the future (Escher et al., 2020b).

3.5. Selection of compounds for the EWERBmix

Only components with high hazard potential based on measured or QSAR-based ECs were considered in the EWERBmix. The selection was based on the maximum TU or EAR per compound across all bioassays. In doing so, the bias towards chemical concentration was reduced. For example, the food additive sucralose, which had the highest median concentration of all compounds across all investigated WWTP effluents was not included in the reference mixture due to a low predicted toxicity across biosystems.

Fig. 4A shows the distributions of TUs and EARs. EARs peak at 10^{-5} and TUs obtained with effect data for aquatic organisms peak slightly above 10^{-6}. TUs at 10^{-6} were mostly based on QSAR predictions for baseline toxicity. QSAR-based TUs $< 10^{-6}$ increasingly resulted from predicted ECs above the compounds' calculated water solubility and thus need to be considered with caution (Table S2). Fig. 4B highlights the number of compounds with TUs or EARs for different orders of magnitude of the hazard potential. This number of compounds dropped considerably below TU or EAR = 10^{-6} (Fig. 4B). Based on this distribution, we defined a general TU and EAR threshold at 10^{-6} and included all compounds above this threshold as components for the reference mixture. This resulted in 81 compounds to be included in the mixture (Fig. 4B, Table S6). Here, lenacil was included in the final reference mixture due to an initially high TU for ZFET ($9.8*10^{-2}$) which resulted from a predicted high logD_{lipw} (8.71) based on linear solvation energy relationship (LSER) descriptors. Subsequently, the logD_{lipw} value was identified to be incorrect (corrected logD_{lipw} = 2.37). Thus, the TU is predicted to be below our threshold of 10^{-6}. Consequently, lenacil should be omitted from the EWERBmix in future studies. All data on TU for ZFET are reported in Table S5.

3.6. Measured effects of the EWERBmix

The EWERBmix was prepared according to the mMEC determined for each of the 81 compounds across the analyzed WWTP effluent samples resulting in a total concentration of 154 nmol/L (Table S6). Subsequently, the mixture was tested for effects in eight different bioassays (Table 1). Concentration-response curves for the effects measured with all bioassays and the reference mixture are provided in Figures S3-S10. The derived ECs are summarized in Table 1 in three different units, i.e., sum concentration in μmol/L, REFs and TU or effect units (EU) in case of receptor activation in cell-based assays.

The highest toxicity in organism-based assays was observed for the algae assay with an EC_{50} value at 39 μmol/L which corresponds to a REF of 255 and a TU of $3.9*10^{-3}$ (Table 1, Fig. 5). The strongest receptor activation was determined with the PXR assay with an EC_{10} value of 34 μmol/L (REF = 217, and TU = $4.6*10^{-3}$), closely followed by the PPARγ assay with an EC_{10} of 36 μmol/L (REF = 231.6, TU = $4.3*10^{-3}$) and AhR

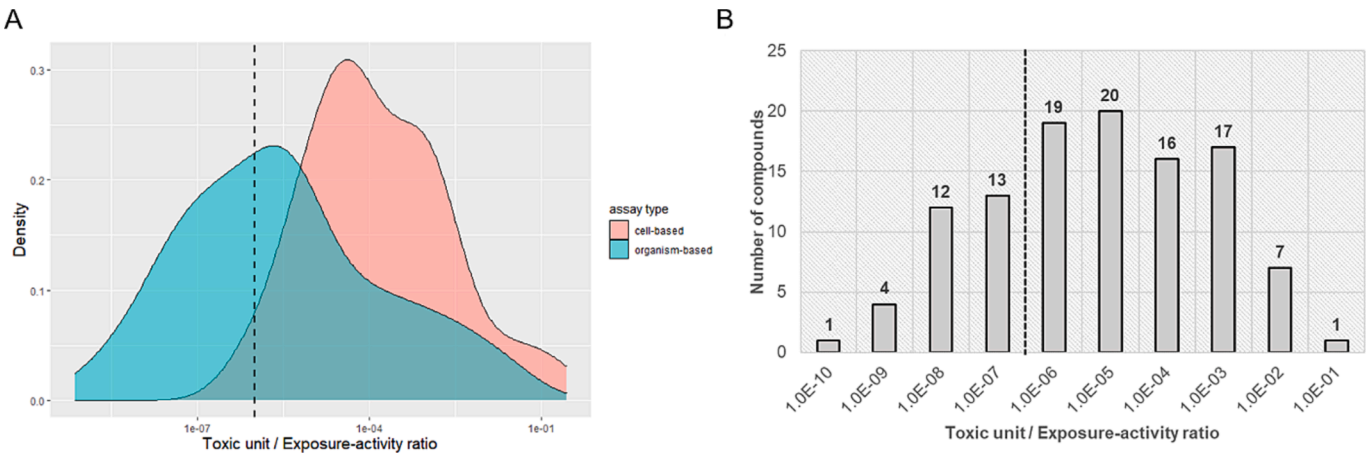

Fig. 4. Summary of maximum TU and EAR values. Distributions (A) and histogram (B) of maximum TUs and EARs of 110 common WWTP effluent chemicals across orders of magnitude based on median measured water concentrations and respective maximum effect concentrations across all available data from the InvitroDB and data for algae, crustaceans, and fish derived from ECOTOXDB. All compounds right of the vertical line at TU/EAR = 10^{-6} were considered in the EWERBmix.

Table 1
Effect concentrations (±standard errors (SE)) determined for the EWERBmix. REF 1 = 154 nmol/L. IC = Induction concentration, IR1.5 = induction ratio of 1.5. *no specific effect detectable below cytotoxicity cutoff.

Assay	Endpoint	Effect concentration measure	EC in units of μmol/L	EC in units of relative enrichment factor (REF)	Toxic unit (TU) or effect unit (EU); range according to SE	Assay Reference
Algae	Growth inhibition	EC_{50}	39(±4.8)	255(±31)	$3.9*10^{-3}(3.5*10^{-3} - 4.5*10^{-3})$	OECD (2011)
Daphnia	Immobi-lization	EC_{50}	360 (±36)	2329 (±235)	$4.3*10^{-4}(3.9*10^{-4} - 4.8*10^{-4})$	OECD (2004)
Zebrafish embryo	Lethality	LC_{50}	270 (±10)	1748 (±65)	$5.7*10^{-4}(5.5*10^{-4} - 5.9*10^{-4})$	OECD (2013)
AREc32 Agonist	Receptor activation	$EC_{IR1.5}$	n.d.*	n.d.*	–	Neale et al. (2017)
AREc32 Agonist	Cytotoxicity	IC_{10}	440 (±77)	2851 (±497)	$3.5*10^{-4}(3.0*10^{-4} - 4.2*10^{-4})$	Escher et al. (2019)
AhRAgonist	Receptor activation	EC_{10}	95 (±24)	613 (±350)	$1.6*10^{-3}(1.3*10^{-3} - 2.2*10^{-3})$	Neale et al. (2017)
AhR Agonist	Cytotoxicity	IC_{10}	617 (±28)	3993 (±182)	$2.5*10^{-4}(2.4*10^{-4} - 2.6*10^{-4})$	Escher et al. (2019)
PXRAgonist	Receptor activation	EC_{10}	34 (±2)	217.(±15)	$4.6*10^{-3}(4.3*10^{-3} - 5.0*10^{-3})$	Piersma et al. (2013)
PXRAgonist	Cytotoxicity	IC_{10}	673 (±38)	4356 (±247)	$2.3*10^{-4}(2.2*10^{-4} - 2.4*10^{-4})$	
PPARγ Agonist	Receptor activation	EC_{10}	36 (±5)	232 (±35)	$4.3*10^{-3}(3.7*10^{-3} - 5.1*10^{-3})$	Gijsbers et al. (2011)
PPARγ Agonist	Cytotoxicity	IC_{10}	701 (±83)	4537 (±537)	$2.2*10^{-4}(2.0*10^{-4} - 2.5*10^{-4})$	Escher et al. (2019)
ERα Agonist	Receptor activation	EC_{10}	249 (±14)	1609 (±91)	$6.2*10^{-4}(5.9*10^{-4} - 6.6*10^{-4})$	Sonneveld et al. (2004)
ERα Agonist	Cytotoxicity	IC_{10}	569 (±58)	3684 (±378)	$2.7*10^{-4}(2.5*10^{-4} - 3.0*10^{-4})$	

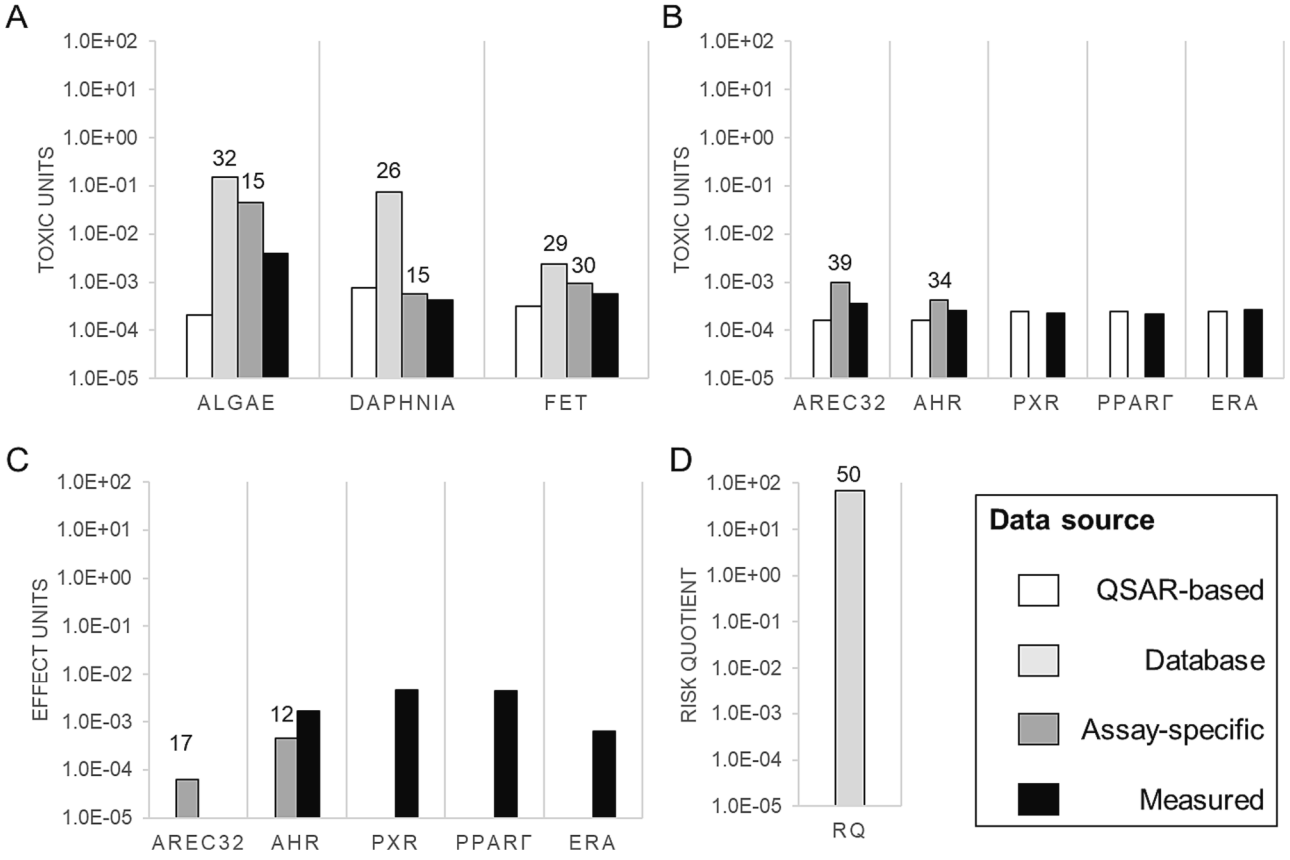

Fig. 5. Measured and predicted toxic units, effect units and risk quotient for the EWERBmix. A) Toxic units determined for EWERBmix in organism-based assays. B) Cytotoxicity measured in cell-based assays by EWERBmix. C) Effect units for EWERBmix determined for cell-based-assays. D) PNEC-based risk quotient of EWERBmix. Numbers at bars indicate number of compounds on which calculations were based. Effect concentrations for individual compounds were derived from literature data and data from involved laboratories (assay-specific), the ECOTOXDB (organism-based assays), QSAR models (QSAR based) and from measurements of the whole mixture containing all 81 compounds as well as a PNEC-based risk quotient. Data is shown in Table S13.

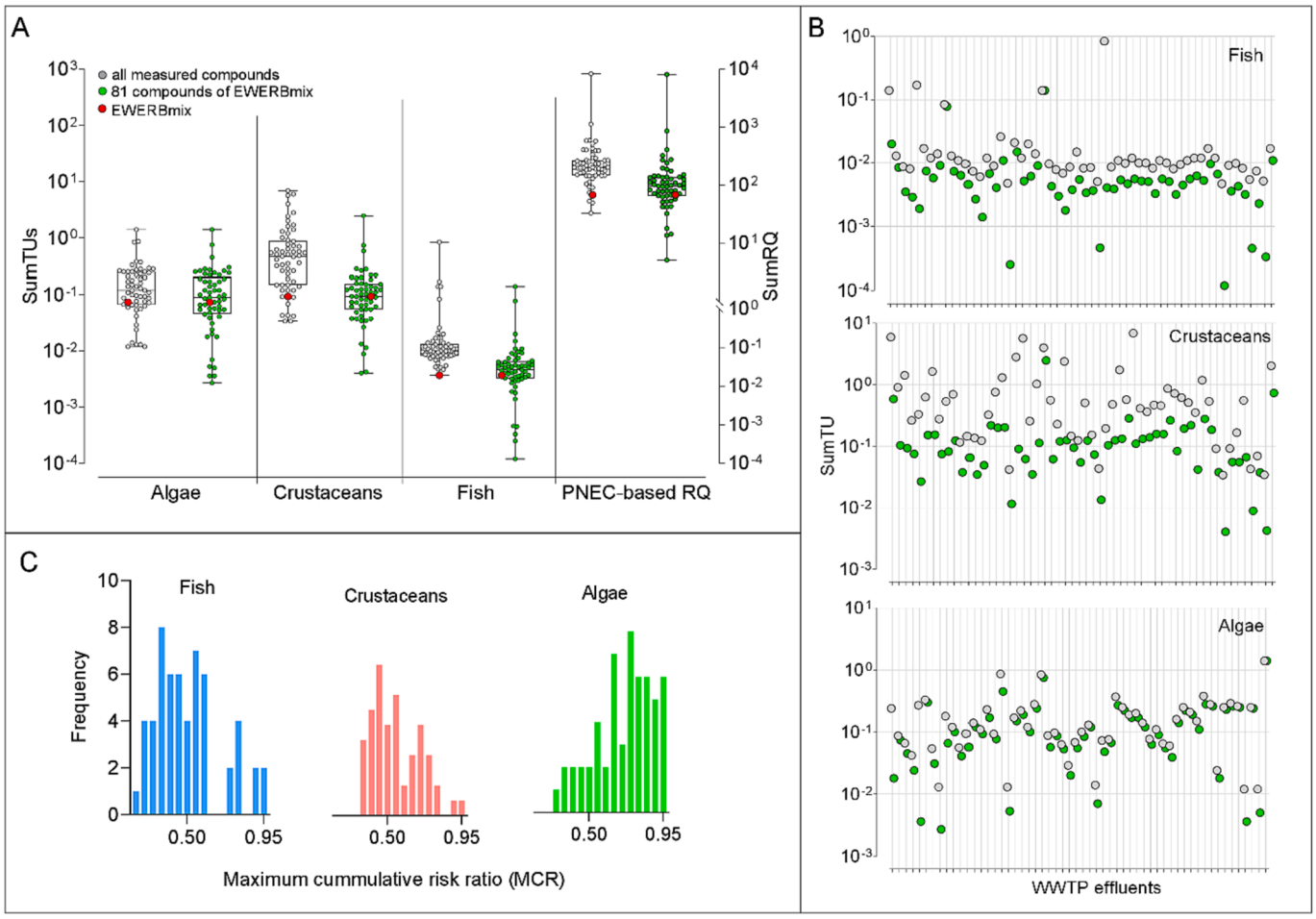

Fig. 6. Representation of EWERBmix in greater dataset and in individual WWTP effluents. sumTU and sumRQ of all WWTP effluents analyzed in Finckh et al. (2022) for algae, crustaceans and fish, calculated with sample-specific concentrations for i) all detected compounds (grey points), ii) and for the 81 compounds of the EWERBmix (green points). The red dots indicate sumTUs/sumRQs determined for the EWERBmix. A) Boxplots considering all WWTP effluent samples plus the EWERBmix. Each box indicates the lower (25 %) and upper (75 %) quantile and the horizontal line in the box indicates the median. Whiskers represent the most extreme, non-outlier data points. B) sumTUs per WWTP effluent sample for fish, crustaceans, and algae. C) Distribution of maximum cumulative risk ratio (MCR) for fish, crustacean and algae across all WWTP effluents. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CALUX (EC_{10} = 95 µmol/L; REF = 613, TU = $1.6*10^{-3}$). For the ERα, daphnia and ZFET, the measured ECs were above 240 µmol/L. This corresponds to a REF of > 1500. For these assays, this background reference mixture is three orders of magnitude below an acute effect ratio of 1. The specific effect of AREc32 assay was below the cytotoxicity cutoff and, thus, cannot be further evaluated.

3.7. Predicted versus observed toxicity and activity of the EWERBmix

To evaluate to which extent the mixture hazard can be predicted, the measured TU and EU of the EWERBmix (Table 1) were compared to predicted TU and EU based on simple addition of TUs for individual mixture components using the mixture toxicity concept of concentration addition (CA) (Backhaus and Faust, 2012). Fig. 5 shows the sumTU and sumEU for the EWERBmix a) calculated from assay-specific measured ECs of individual compounds derived from literature and provided by the laboratories where the assays were conducted (Table S7; Table S8, (Lee et al., 2022)), b) calculated from reported ECs for the three species groups (ECOTOXDB) and c) calculated with QSAR-based ECs for the respective species. For the algae and daphnia assay, we applied logD_{lipw}-based QSARs according to Escher et al. (2020b) (see SI and Tables S9, S10). In addition, QSARs predicting baseline cytotoxicity for the AREc32 and AhR CALUX as well as a generalized QSAR for baseline cytotoxicity in cell-based assays for the ERα, PPARγ CALUX and PXR assay were used (Lee et al., 2021) (see SI and Table S11). All predicted sumTU and sumEUs are compared to d) the measured results for the EWERBmix for each assay. The numbers at the end of each bar indicate the respective number of compounds - if different from 81 - that were considered for the respective mixture effect calculations. Concentrations of mixture compounds were analytically confirmed in the prepared reference mixture (Table S12). Predictions were corrected according to concentrations in the spiking solution.

Whole organism effects. QSAR-based predictions for the EWERBmix for the three BQE were rather low with sumTU of $2.1*10^{-4}$ for algae, $3.2*10^{-4}$ for ZFET, and $7.8*10^{-4}$ for daphnia and fell within one order of magnitude across the species (Fig. 5A, QSAR-based). Although all mixture compounds were considered for the sumTU calculations based on QSAR predictions, specific action of chemicals are not considered in the baseline QSAR model. This effect is shown by sumTU predicted based on reported effect data derived from the ECOTOXDB, which were at least one order of magnitude higher than QSAR predictions, even though ECs for fewer compounds were available, i.e., sumTU algae = $1.5*10^{-1}$ (32 compounds), fish = $2.3*10^{-3}$ (29 compounds), crustaceans = $7.3*10^{-2}$ (26 compounds) (Fig. 5A, database). These database-derived sumTUs were also based on ECs for species of algae, fish and crustaceans other than the ones considered in the

bioassays used here in order to obtain a larger and more representative data set. For all three organism-based assays, sumTU predicted by database-derived ECs were higher than measured effects. This could have resulted from including more sensitive species and assays in the ECOTOXDB data sets leading to a higher predicted sumTUs. These database-retrieved toxicity assessments may thus serve as valuable precautionary measures. In case of crustaceans and algae, these sumTUs were higher than 0.01 exceeding the chronic risk threshold (CRT) for crustaceans (TU = 0.001) and are close to the CRT for algae (TU = 0.02) (Malaj et al., 2014). Even though dilution in the receiving surface water body needs to be considered, this may be alarming regarding this low concentrated and constant background pollution from WWTP effluents to which further pollution from other sources as well as site-specific pollution at the respective WWTP effluents may be added in surface waters.

Unexpected results were obtained when the measurements for the EWERBmix were compared to CA mixture effect prediction based on available data for individual mixture components for the respective specific assays (Fig. 5A, assay-specific). For the daphnia immobilization assay and the algae assay, respectively, the mixture effect calculation could be based on ECs for 15 out of the 81 compounds. For the ZFET, it was based on data for 30 compounds. While the measured EWERBmix effect was similar to the CA predictions for daphnia and the ZFET, the predictions were higher for the algae assay by a factor of 7. As much fewer compounds were considered in the component-based predictions, it seems that the mixture effect for these acute assays is overestimated in a component-based CA assessment. Several studies have been performed showing that CA provides a conservative estimate for joint effects, e.g., Coors et al. (2018) and studies of multicomponent mixtures performed with realistic concentration ratios obeyed CA for algal toxicity (Tang and Escher, 2014) as well as bacterial toxicity (Tang et al., 2013). For sublethal effects, mixtures of low concentrated components showed more than additive effects in the ZFET (Jakobs et al., 2020). The observed discrepancies might be related to compound interactions potentially also on the toxicokinetic level, which should be considered and investigated in (short-term) assays. So far, interactive effects of complex pollutant mixture are difficult to predict as these assessments require a lot of data and knowledge on these interactions, which are mostly missing (Bopp et al., 2019). Once these data for mixture components are generated, further mixture assessment can be performed to unravel potential interactive (antagonistic) effects or the effects of independently acting compounds. The components of the EWERBmix should be prioritized for filling these data gaps. Still, the measured toxicity of the EWERBmix (Fig. 5A, measured) in the ZFET and daphnia test was very similar to the mixture predictions, where the individual components' toxicity was predicted with the baseline toxicity QSARs (Table S5, Table S9, Table S10). This supports the assumption of an underlying joint additive baseline toxicity of the EWERBmix.

Cytotoxicity. Measured cytotoxicity of the EWERBmix in cell-based assays was compared to measured cytotoxicity of individual mixture components in the same assay (Lee et al., 2022) (Table S8) as well as compared to baseline toxicity predicted by QSAR models (Lee et al., 2021) (Table S11). The measured cytotoxicity in all five cell lines agreed well with the QSAR-predicted baseline toxicity; yet was slightly overestimated by component-based CA assessment based on assay-specific ICs (Fig. 5B). As described by Drescher and Boedeker (1995) and shown by Escher et al. (2020a), the difference between CA assessments and predictions based on independent action (IA) increases with increasing number of mixture components. However, experimental variability has to be taken into account, which exceeded deviations among CA and IA predictions in the study by Escher et al. (2020a).

Specific effects quantified with reporter gene assays. Assay-specific (i.e., identical cell line) determined ECs for single substances included in the EWERBmix were only available for the AREc32 and the AhR CALUX assay (Table S8). The numbers in Fig. 5C (assay-specific) indicate the number of compounds, which triggered a response in the respective cell assay. CA predictions based on 17 single substance ECs for the AREc32 assay and 12 for the AhR assay resulted in sumEU of $6.2*10^{-5}$ and $4.6*10^{-4}$, respectively. For the AhR, the sumEU based on single substance ECs was one order of magnitude lower than the sumEU measured for the whole mixture. Due to the cytotoxicity cutoff, the specific effect in the AREc32 assay could not be evaluated. Even though the number of single substance ECs seems rather low for comparison, the data by Lee et al. (2022) also indicates that 53 out of 81 mixture compounds for the AREc32 assay and 57 for the AhR assay were considered as non-active when tested individually in the respective assay. The remaining data gaps (12 compounds of the EWERBmix for AhR CALUX assay) should be closed in the future to further enhance component-based CA predictions. Moreover, experimental single substance ECs are needed for the PXR and PPARγ CALUX assay since both assays were also clearly induced by the EWERBmix.

Overall, the EWERBmix can provide a starting point for further measurements of mixture components, deriving ECs from such assays and calculating mixture effects in order to close data gaps for certain substances. Furthermore, the EWERBmix should be tested in different test systems to obtain a more holistic picture on the potential impacts of mixtures on specific endpoints, organism groups or populations and communities (e.g., including mesocosm studies) in the environment. The experimental assessment of the EWERBmix as well as the mixture predictions indicated no acute toxicity, which was to be expected for a mixture which occurs ubiquitously in European WWTP effluents with components present at very low concentrations, without a specific enrichment of individual specifically acting or effect driving compounds. Since the WWTP effluent mixture will be further diluted in the respective surface water body, the inclusion of chronic tests in bioassay batteries and tests covering several generations or species are also recommended to better capture potential effects of such WWTP effluent background exposures (Brack et al., 2019, Coors et al., 2018).

3.8. Risk assessment for the EWERBmix

Environmental risk assessment is based on measured or predicted hazard and exposure data for individual compounds. To end up with one hazard value being protective for different ecosystems (e.g., surface or marine waters) and the diversity of species, available ECs for the most sensitive representative species are considered to determine predicted no effect concentrations (PNEC). Based on the quality and availability of ECs for different taxa, assessment factors ranging from 5 to 1000 are considered for the establishment of PNECs. PNECs are derived within different substance-oriented legislations and can deviate according to the protection targets and thus across regulations. Here, we extracted PNEC values from the NORMAN database for 50 out of 81 compounds (Table S14). The risk quotients (RQ_i, Eq. (4), i.e., the ratio of measured exposure concentration divided by the PNEC, were summed up for all component to derive a cumulative risk quotient (sumRQ). The sumRQ (Eq.5) for the EWERBmix based on PNECs for 50 compounds out of 81 was 68. Thus, it clearly exceeds the threshold of 1 indicating that the mixture cannot be considered as "safe" (Fig. 5D). The sumRQ is based on PNECs with partially high assessment factors and follows the precautionary principle and highlights need for management as well as the need for improving data quality of PNEC values and thus lowering assessment factors. Furthermore, the approach can trigger more detailed investigations of high-risk samples, mixtures or individual chemicals (Bopp et al., 2019).

3.9. Suitability of the EWERBmix as European WWTP effluent reference

To put the EWERBmix into relation with hazards and risks identified for individual WWTP effluents, we compared the respective sumTUs and sumRQs to sumTUs and sumRQs of the effluents of all individual WWTPs were derived in Finckh et al. (2022) (Fig. 6). In Fig. 6A, the sumTUs of the EWERBmix (red dots) for the three BQE were located close to the

median in boxplots of sumTUs derived from the concentrations of the 81 EWERBmix compounds in the samples (green dots). However, the sumTUs derived from all detected compounds per sample (gray dots) were substantially higher than the sumTUs of the EWERBmix (red dots). If only the 81 compounds of the EWERBmix were considered, the EWERBmix accounted well for the overall combined effect. This was true for all three species groups as it was intended. Comparing gray and green boxplots, it becomes obvious that the EWERBmix compounds allow to assess whole WWTP effluent effects on algae very well. This is also underlined by Fig. 6B which shows the sumTU for each WWTP effluent sample in grey (all compounds) and green (i.e., 81 EWERBmix compounds), respectively. In most cases, both sumTU values for algae are similar or close to each other with a median deviation ratio of 1.3 while discrepancies are seen especially when sumTUs are generally low. Individual compounds responsible for some of these deviations are, for example, the quaternary ammonium compound didecyldimethylammonium, which was detected in two WWTP effluents, or the biocide cybutryne, which was detected in six WWTP effluents.

The picture is different for crustaceans and fish, where the sumTUs based on the EWERBmix compounds were lower than those based on all measured compounds per WWTP effluent sample (Fig. 6A-B). While there seems to be a rather systematic underestimation with the EWERBmix compared to the respective more comprehensive exposure of fish with a median deviation ratio of 2.3, there were larger variations and less systematic patterns observed for crustacean sumTU although the median deviation ratio of 3.8 still indicated a solid representation of WWTP effluent hazards based on EWERBmix compounds. Relevant contributions to the calculated hazards here could be assigned for example to the biocide diazinon, which was detected in 33 WWTP effluents, and the insecticide metabolite 3,5,6-Trichloro-2-pyridinol, detected in 46 WWTP effluents. Such compounds are candidates to be added to the EWERBmix in the future.

The importance of individual drivers in case of algae or in case of crustaceans and fish the increasing contribution of several mixture components to the overall risk in the EWERBmix is highlighted in Fig. 6C. Here, the distribution of the maximum cumulative risk ratio (MCR) across all sites is depicted. The MCR was adapted from (Price and Han, 2011) and describes the ratio of the highest TU posed by an individual substance to the sumTU at the respective site (Eq. (6); Table S15). Thus, the lower the MCR, the more compounds contribute to the sumTU and less clearly risk driving compounds can be identified. The MCR allows for quick identification of compounds contributing significantly to the overall risk and may support goal-oriented monitoring and environmental management. The distribution of the MCR is increasingly skewed to the left from algae to fish indicating a decreasing number of risk driving compounds from algae to fish.

For fish and crustaceans, the EWERBmix represents the lower end of the sumTU distributions of all WWTP effluents (Fig. 6A). The same can be concluded for the distributions of sumRQ indicating that the EWERBmix represents the baseline background hazard and risk to be expected for European WWTP effluents under consideration of dilution in surface waters. Moreover, WWTP- specific compounds or site-specific elevated concentrations may lead to additional toxic pressure in individual WWTP effluents.

4. Conclusions

The awareness for combined exposures, effects and risks has been increasing over the past years (Drakvik et al., 2020). Despite the diversity of exposure in time and space, the characterization of “typical” reference mixtures allows for a better understanding of the composition of co-exposures (e.g., quantity and quality of substances) and their possible risks and impacts on ecosystems. In this study, we derived a reference mixture of organic chemical pollutants for European WWTP effluents, which we call the EWERBmix. The EWERBmix can be reproduced based on Table S6. This reference mixture could serve as a background mixture proxy and supports the identification of co-exposures for chemical monitoring and surveillance in WWTP effluents and aquatic environments within the EU. Furthermore, it provides measures for baseline mixture exposures to be expected from WWTP effluents, e.g., by estimating environmental exposure concentrations under consideration of a defined dilution factor (Finckh et al., 2022). The EWERBmix presents a reference mixture for regulatory authorities for the development and use of mixture risk assessment approaches that might be driven from both sides, the prospective assessment (and possible regulation) of mixture components within different regulations as well as from the retrospective site-specific water quality assurance under the WFD. Moreover, the knowledge on measured environmental concentrations and the magnitude of potential effects and risks is also important in the context of the implementation of a mixture assessment factor in REACH (Hassold et al., 2021), which is proposed within the European Commissions’ Chemicals Strategy for Sustainability. A possible estimation of its magnitude on the basis of monitoring data is currently discussed by Backhaus (2022).

Our approach was inspired by a SETAC Pellston workshop in 2015 (Burton et al., 2015), where exposure-scenario-based mixture risk assessment approaches were discussed and investigated based on generic considerations (Diamond et al., 2018). While actual mixtures occurring in aquatic environments in the context of agricultural landscapes strongly vary according to respective land uses, a representative background pollution could be confirmed across European WWTP effluents. The studied WWTPs reflected different treatment technologies and catchment characteristics. Thus, monitoring should be continued for further WWTPs including industrial WWTPs and supplemented by scientific studies documenting spatial–temporal co-occurrences of substances while ensuring common quality assurance and standards. The proposed workflow for the selection of the reference mixture should be extended to other geographical settings and chemical compounds, e.g. inorganic pollutants, highly hydrophobic or hydrophilic compounds, which were outside the range of the applied chemical analysis by Finckh et al. (2022).

Open access to a large set of high-quality monitoring data and evidence for joint exposures and effects would support regulatory measures that can be taken via the substance-oriented frameworks addressing pesticides, biocides, industrial chemicals or pharmaceuticals. The European IPChem and NORMAN provide central EU wide data platforms for documentation accessible to all actors. Besides measured environmental exposure, the composition of the EWERBmix was selected based on predicted or measured data on effects of the individual mixture components. While the data availability of the certain reporter gene assays is excellent, single chemical effect data is sparse especially for *in vivo* bioassays and should be extended. Information on compounds assessed as inactive compounds is valuable for mixture risk assessment. While this information is provided in the InvitroDB for the cell-based assays, it is not available for organism-based bioassays in most cases. For data-driven approaches, in particular read-across-based hazard assessment as performed in REACH (European Commission, 2006), or the development of green chemicals and substitutions of hazardous substances, the documentation on structures and substances considered inactive on the basis of limit tests (e.g., up to 1 mg/L), QSARs or *in vitro* studies is as important as the information on toxicants are.

Consequently, single substance testing and monitoring should be systematically performed and established, possibly based on the presented compound ranking of at least the 80 reference mixture components selected in this study (omitting lenacil due low TU) and the data gaps highlighted for the different assays. The monitoring and toxicity testing could further be extended to compounds identified as non-ubiquitous risk drivers in monitoring studies, such as diazinon (Finckh et al., 2022). Moreover, the mixture effects may be investigated in combination with other pollutant mixture, e.g. heavy metals, or in the presence of abiotic stressors, e.g. nutrients or temperature extremes, to allow for an even more comprehensive risk assessment in a multiple

stressor context.

CRediT authorship contribution statement

Liza-Marie Beckers: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Rolf Altenburger:** Conceptualization, Funding acquisition, Writing – review & editing. **Werner Brack:** Writing – review & editing. **Beate I. Escher:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Jörg Hackermüller:** Data curation, Formal analysis, Writing – review & editing. **Enken Hassold:** Writing – review & editing. **Gianina Illing:** Formal analysis, Methodology. **Martin Krauss:** Formal analysis, Methodology, Writing – review & editing. **Janet Krüger:** Formal analysis, Methodology. **Paul Michaelis:** Formal analysis, Visualization, Writing – review & editing. **Andreas Schüttler:** Data curation, Formal analysis, Writing – review & editing. **Sarah Stevens:** Formal analysis, Methodology, Writing – review & editing. **Wibke Busch:** Conceptualization, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

The data is available in the extensive tables within the supplementary materials.

Acknowledgements

The authors thank Aleksandra Piotrowska and Roman Gunold for the preparation and chemical analysis of the EWERBmix. The QExactive Plus LC-HRMS used and the robotic HTS systems are part of the major infrastructure initiative CITEPro (Chemicals in the Terrestrial Environment Profiler) funded by the Helmholtz Association. This work was carried out in the framework of the European Partnership for the Assessment of Risks from Chemicals (PARC) and has received funding from the European Union's Horizon Europe research and innovation programme [grant agreement number 101057014]. Views and opinions expressed are however those of the authors only and do not necessarily reflect those of the European Union or the Health and Digital Executive Agency. Neither the European Union nor the granting authority can be held responsible for them.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.108155.

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