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Influence of Emission Sources and Tributaries on the Spatial and Temporal Patterns of Micropollutant Mixtures and Associated Effects in a Small River

Maximilian E. Müller,[a] Martina Werneburg,[a] Clarissa Glaser,[a] Marc Schwientek,[a] Christiane Zarfl,[a] Beate I. Escher,[a,b] and Christian Zwiener[a,*]

[a]Center for Applied Geoscience, Eberhard Karls University of Tübingen, Tübingen, Germany
[b]UFZ–Helmholtz Centre for Environmental Research, Leipzig, Germany

Abstract: Organic micropollutants of anthropogenic origin in river waters may impair aquatic ecosystem health and drinking water quality. To evaluate micropollutant fate and turnover on a catchment scale, information on input source characteristics as well as spatial and temporal variability is required. The influence of tributaries from agricultural and urban areas and the input of wastewater were investigated by grab and Lagrangian sampling under base flow conditions within a 7.7-km-long stretch of the Ammer River (southwest Germany) using target screening for 83 organic micropollutants and 4 in vitro bioassays with environmentally relevant modes of action. In total, 9 pesticides and transformation products, 13 pharmaceuticals, and 6 industrial and household chemicals were detected. Further, aryl hydrocarbon receptor induction, peroxisome proliferator–activated receptor activity, estrogenicity, and oxidative stress response were measured in the river. The vast majority of the compounds and mixture effects were introduced by the effluent of a wastewater-treatment plant, which contributed 50% of the total flow rate of the river on the sampling day. The tributaries contributed little to the overall load of organic micropollutants and mixture effects because of their relatively low discharge but showed a different chemical and toxicological pattern from the Ammer River, though a comparison to effect-based trigger values pointed toward unacceptable surface water quality in the main stem and in some of the tributaries. Chemical analysis and in vitro bioassays covered different windows of analyte properties but reflected the same picture. *Environ Toxicol Chem* 2020;39:1382–1391.

Keywords: Chemical analysis; Bioassays; Catchment scale; Micropollutant mixtures; Lagrangian sampling

INTRODUCTION

Intentionally and unintentionally released anthropogenic organic micropollutants may enter the aquatic environment via different pathways. Most household applications of compounds, such as pharmaceuticals, protection agents, personal care products, and flame retardants, are discharged to municipal sewerage systems. A substantial part of these pollutants is designed to be rather stable (e.g., pharmaceuticals, flame retardants) and in many cases highly mobile in water, thus facilitating the passage of pollutants through wastewater-treatment plants (WWTPs) into surface waters without complete removal (Schwarzenbach et al. 2016; Munz et al. 2017). In densely populated countries of Europe like Germany the impact of treated wastewater to rivers and small streams is quite common and dominates pollution input, especially during dry summer periods (Loos et al. 2009; Bueno et al. 2012; Englert et al. 2013). Other compounds such as pesticides are introduced directly into the environment. Thus, runoff and leachate from agricultural but also urban areas can lead to high micropollutant concentrations in surface and ground waters (Loos et al. 2009; Masoner et al. 2014; Kuzmanović et al. 2015; Szöcs et al. 2017; Lee et al. 2019). These mixtures of organic micropollutants may pose a risk to environmental organisms, reduce biodiversity, and affect drinking water quality and environmental services (Alpizar et al. 2019; European Environment Agency 2019).

The overarching aim of the present study was to identify the emission sources and the influence of tributaries on the contaminant concentrations and loads in a small river, the Ammer

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* Address correspondence to christian.zwiener@uni-tuebingen.de
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River in southwest Germany. Chemical analysis and in vitro bioassays are complementary screening approaches which capture a broad range of organic micropollutants and have previously been used to assess the quality of treated and untreated wastewater and their impact on river water quality (Farré and Barceló 2003; König et al. 2017; Neale et al. 2017b) or to track pesticides in surface water from agricultural areas (Lundqvist et al. 2019). Whereas chemical analysis provides information on the identity and quantity of individual micropollutants (Gago-Ferrero et al. 2019), in vitro bioassays give information on the combined effect of all bioactive compounds present in a sample and on their mode of action (Neale et al. 2017b; Müller et al. 2019). In the present study a battery of 4 in vitro bioassays was applied that covered the environmentally relevant modes of action, aryl hydrocarbon receptor induction (Brennan et al. 2015), peroxisome proliferator–activated receptor activity (Neale et al. 2017a), estrogenicity (König et al. 2017), and oxidative stress response (Escher et al. 2012, 2013b). Because each compound present in a sample can potentially affect the viability of a cell, the cytotoxicity measured in the bioassays can reflect the total chemical load of a sample.

Our hypotheses are that 1) inputs from agricultural and urban areas show different compound and effect patterns, and 2) organic micropollutant and effect patterns and their temporal variability change with increasing distance from their input sources. To address hypothesis 1), each tributary and the main stem were investigated by grab sampling. For hypothesis 2), we followed individual water parcels using a Lagrangian sampling technique (Writer et al. 2011; Schwientek et al. 2016).

MATERIALS AND METHODS

Study site and sampling

The present study was conducted on 19 June 2018 on a 7.7-km-long section of the Ammer River, near Tübingen, southwest Germany. In this section the Ammer River receives input from 10 tributaries out of 15 (data on discharge are given in Table 1) and 2 WWTPs. This allowed comparison of the micropollutant burden of river water unaffected and affected by treated wastewater and river water affected by tributaries from agricultural and/or urban areas (Figure 1). Until the most downstream sampling site (autosampler 3 [AS3]) the Ammer catchment integrates an area of 134 km² with agricultural (71%), urban (17%), and forestry (12%) land use (Grathwohl et al. 2013). The large WWTP1 with 80 000 person equivalents is located upstream of Altingen approximately 250 m upstream of AS1 and contributed 50% of the discharge of the Ammer downstream of the WWTP1 on 19 June 2018 (Table 1). The much smaller WWTP2 with 9000 person equivalents is located in Hailfingen, discharging into Kochhart Creek (T8). One-liter grab samples were collected from each tributary and the Ammer River closely upstream of each confluence. All samples were taken from the middle of the water body 5 cm beneath the water surface, assuming well-mixed conditions. Moreover, 1-h composite samples (250 mL every 15 min) were collected over 24 h at 3 sampling sites, AS1, AS2, and AS3, which define reaches 1 and 2 (Figure 1). To sample the same water parcel at downstream sites AS2 and AS3, the travel time of water was considered for sampling similar water parcels and estimated as described (C. Glaser et al., Center for Applied Geoscience, University of Tübingen, Germany, unpublished manuscript, 2019a). Travel times were 135 min for reach 1 between AS1 and AS2 and 165 min for reach 2 between AS2 and AS3. All samples were taken in glass bottles, stored at 4 °C, and processed within 48 h after sampling.

TABLE 1: Discharges, in liters per second (Ls^{-1}), of the Ammer River and its tributaries[a]

Tributary/sampling site		Q (Ls^{-1})
Ammer (main stem)	Am-T0	270 ± 20
	AS1	540 ± 34
	AS2	577 ± 30
	AS3	743 ± 23
WWTP1 effluent		274 ± 38
Tributaries	T1	2
	T2	16
	T3	6
	T4	35
	T8	8
	T9	2
	T10	120[b]
	T11	1
	T12	1
	T15	12

[a]Discharges were determined as described in C. Glaser et al. (Center for Applied Geoscience, University of Tübingen, Tübingen, Germany, unpublished manuscript, 2019a). Discharges at autosamplers AS1, AS2, and AS3 were averaged over the 24 h of sampling at each site, thus leading to standard deviations (±) because of the daily discharge fluctuation of the wastewater-treatment plant (WWTP). The 24-h measurement of the WWTP effluent was performed with an offset of 22 min to account for the water travel time to AS1.
[b]C. Glaser et al. (Center for Applied Geoscience, University of Tübingen, Tübingen, Germany, unpublished manuscript, 2019b).
Q = discharge; T = tributary; WWTP = wastewater-treatment plant.

Chemicals and reagents

Methanol, acetonitrile, water, acetic acid, and ammonium acetate were all liquid chromatography/mass spectrometry (LC/MS) grade and purchased from Thermo Fisher Scientific. Ethyl acetate was purchased from Acros Organics, Thermo Fisher Scientific. The 83 monitored target analytes as well as their usages and vendors are listed in Supplemental Data, Table S1.

Sample preparation

Water samples were passed through a pleated cellulose filter (Whatman 595 1/2, pore size 4–7 μm) to remove suspended particulate matter. Filtrates were enriched by solid-phase extraction (SPE) using Waters Oasis HLB, 6CC, 200-mg cartridges preconditioned with 5 mL methanol, 5 mL ethyl acetate, and 5 mL ultrapure water. One liter of filtered water sample was passed through the extraction cartridges using a vacuum manifold. After this, extraction cartridges were flushed with 5 mL ultrapure water and aspirated to dryness by vacuum. Subsequently, they were eluted with 5 mL methanol, followed

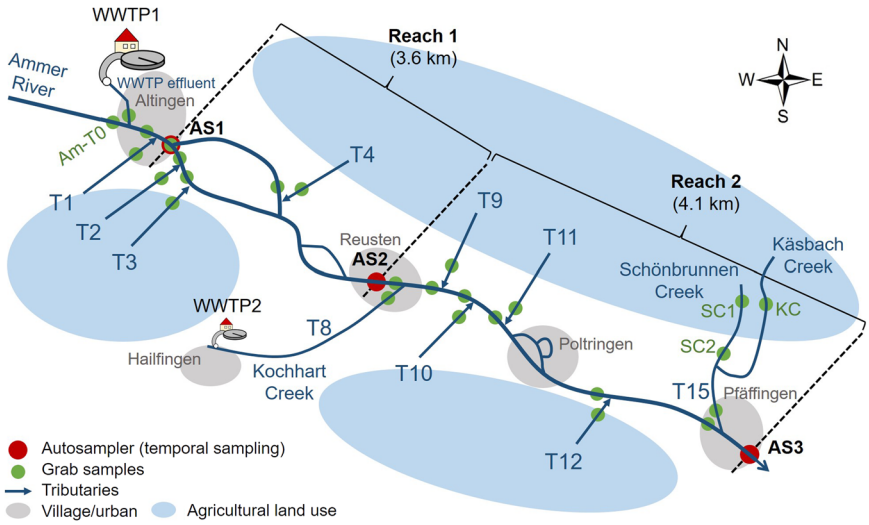

FIGURE 1: Conceptual map of the study site. Autosamplers AS1, AS2, and AS3 were located at the beginning, the center, and the end of the study site for 24-h sampling (red dots). Grab samples from the tributaries are marked green and named as the tributary (T2–T4, T8–T12, T15, SC1, SC2, and KC). Sample names of the grab samples taken from the Ammer River are named according to the adjacent downstream tributary (Am-T1, Am-T4, Am-T8–Am-12, Am-T15), except for Am-T0 upstream of the wastewater-treatment plant effluent and AS1. WWTP = wastewater-treatment plant.

by 5 mL ethyl acetate. Eluates were combined, evaporated under a gentle stream of nitrogen at 40 °C, and dissolved in methanol to achieve an enrichment factor of 1000. Final extracts were filtered (Agilent Captiva Premium Syringe filter, polyethersulfone, 0.2 µm) and stored at –20 °C until measurement. Blanks were prepared by SPE of 1 L MilliQ (GenPure Pro UV-TOC; Thermo Fisher Scientific). Prior to measurements, extracts were diluted 1:100, to minimize matrix effects (Villagrasa et al. 2007; Kruve et al. 2009), in water/acetonitrile (98/2 v/v) at room temperature. All volumes for reuptake and dilution were determined gravimetrically.

Chemical analysis

Eighty-three organic micropollutants were quantified by liquid chromatography (1260 Infinity HP-LC; Agilent Technologies) coupled to tandem mass spectrometry (6490 iFunnel Triple Quadrupole; Agilent Technologies). Separation was achieved on an Agilent Poroshell 120 EC-C18 column (2.7 µm particle size, 4.6 × 150 mm) at 40 °C with a gradient elution program using water with 0.1 mM ammonium acetate and acetonitrile, both with 0.1% formic acid. Positive ionization and negative ionization of target compounds were achieved by electrospray ionization. An external calibration with standard solutions was used for quantification of the target compounds, for which generally 2 mass transitions (qualifier, quantifier) were acquired. The lowest external calibration level within ±20% deviation from the method calibration curve was defined as the limit of quantification (LOQ; signal-to-noise ratio ≥10, quantifier/qualifier ion ratio within ±20% of the average of calibration standards from the same sequence and target compound). If analytes were detected in the SPE blank, the LOQ was defined as the mean blank concentration plus 10 times its standard deviation. Measurement uncertainty was assessed based on repeated standard solution measurements (coefficient of variance, $n = 10$, 5.0 µg L^{-1} of each analyte). Matrix effects caused by the WWTP1 effluent were investigated by standard addition of all target compounds to samples from all 3 sampling sites of the Ammer (AS1, AS2, AS3) at 3 sampling times (AS1_1, AS1_12, AS1_24, AS2_1, AS2_12, AS2_24, AS3_1, AS3_12, and AS_24) each at 2 different spiking levels (0.24 and 1 µg L^{-1}). Results of target compounds exceeding 20% signal suppression or enhancement were corrected according to the following scheme: the mean matrix effect of samples AS1_1, AS1_12, and AS1_24 was considered for samples WWTP effluent, Am-T1, and all samples of AS1, Am-T2, Am-T3, Am-T4; the mean matrix effect of samples AS2_1, AS2_12, and AS2_24 was considered for all samples of AS2, Am-T8, Am-T9, Am-T10, Am-T11, Am-T12, and T15; the mean matrix effect of samples AS3_1, AS3_12, and AS3_24 was considered for all samples of AS3. Recoveries during the SPE procedure of the target compounds were assessed by extracting 1 L MilliQ water spiked with standard solution mix, including all target compounds, to a final concentration of 100 ng L^{-1} ($n = 2$). Results of target compounds with <70% recovery were corrected. Information on mass transitions, collision energies, LOQs, relative standard deviation, recoveries during the SPE, and matrix effects of the target compounds is given in Supplemental Data, Tables S2 and S3.

In vitro bioassays

The in vitro bioassays AhR-CALUX (Brennan et al. 2015), AREc32 (Escher et al. 2012, 2013b), PPARγ-GeneBLAzer (Neale et al. 2017a), and ER-GeneBLAzer (König et al. 2017) covered 4 different environmentally relevant endpoints. For each

bioassay cytotoxicity was measured. More information on their mode of action, environmental relevance, and experimental procedures can be found in König et al. (2017) and Neale et al. (2017a). All effect concentrations (ECs) and cytotoxic inhibition concentrations (ICs) were expressed in units of relative enrichment factors, taking the enrichment during the extraction procedure and the dilution in the assay into consideration. A detailed description on the EC and IC derivation is provided in the Supplemental Data. The derived concentrations causing a 10% effect (EC10) in AhR-CALUX, PPARγ-GeneBLAzer, and ER-GeneBLAzer and concentrations causing an induction ratio of 1.5 (ECIR1.5) in AREc32 of each sample were then converted to bioanalytical equivalent concentration (BEQ; Equation 1) and effect units (Equation 2). The concentrations causing 10% cell growth inhibition (IC10) were converted to toxic units (TUs; Equation 3). This serves for a better visualization because high BEQ and toxic unit relate to high effects and cytotoxicity and allow comparison to other surface water case studies.

$$\text{BEQ} = \frac{\text{EC10 (reference)}}{\text{EC10 (sample)}} \text{ or } \frac{\text{ECIR1.5 (reference)}}{\text{ECIR1.5 (sample)}} \quad (1)$$

$$\text{Effect unit} = \frac{1}{\text{EC10}} \text{ or } \frac{1}{\text{IR1.5}} \quad (2)$$

$$\text{Toxic unit} = \frac{1}{\text{IC10}} \quad (3)$$

A list of the reference chemicals in each assay and their EC10 values can be found in Supplemental Data, Table S4. The errors of the BEQ, effect unit, and toxic unit were calculated by error propagation as described (Escher et al. 2018b).

Calculation of the influence of tributaries on the spatial effect and micropollutant dynamics in the main stem

The concentrations ($C_{downstream}$) and effects ($BEQ_{downstream}$) in the Ammer downstream of the respective tributary were obtained using a mass balance approach including concentrations and effects in the Ammer upstream of the tributary and in the tributary (Equations 4 and 5). Discharges (Q in liters per second, Ls^{-1}) are listed in Table 1, and effects (BEQ in nanograms of reference compound per liter) or concentrations (C, in nanograms per liter) from the Ammer and the tributaries are in Supplemental Data, Tables S5 and S6.

$$C_{downstream} = \frac{C_{upstream} \times Q_{upstream} + C_{tributary} \times Q_{tributary}}{Q_{upstream} + Q_{tributary}} \quad (4)$$

$$BEQ_{downstream} = \frac{BEQ_{upstream} \times Q_{upstream} + BEQ_{tributary} \times Q_{tributary}}{Q_{upstream} + Q_{tributary}} \quad (5)$$

Because the Ammer River splits into 2 smaller branches downstream of AS1 which reunite after approximately 900 m, discharges, effects, and concentrations of tributaries T2, T3, and T4 of both branches were combined and considered as one unit in reach 1. Consequently, the effects and concentrations of target compounds measured in the grab sample at AS1 were considered as the input of that unit. For each tributary confluence, the river discharge $Q_{upstream}$ was determined as the sum of all upstream tributary inputs and the discharge of the Ammer River Am-T0 (according to Table 1). To derive $Q_{downstream}$, the respective $Q_{tributary}$ was added. The mass fluxes J_i of target compounds (i) and the effect fluxes E_a measured in the bioassays (a) upstream and downstream of the tributary inlets were calculated with Equations 6 and 7.

$$J_i = Q \times C_i \quad (6)$$

$$E_a = Q \times BEQ_a \quad (7)$$

The mass flux increases of target compounds (ΔJ) and effects (ΔE) in the Ammer main stem downstream of the tributaries were calculated with Equations 8 and 9.

$$\Delta J_{i,Tx} = \frac{J_{i,downstream}}{J_{i,upstream}} - 1 \quad (8)$$

$$\Delta E_{a,Tx} = \frac{E_{a,downstream}}{E_{a,upstream}} - 1 \quad (9)$$

Similarity of either compound or effect profiles among the grab samples from the Ammer and the tributaries was assessed by a hierarchical cluster analysis with the unweighted pair-group method using arithmetic averages.

To give an equal weight to each individual target compound, their mass fluxes in all hourly water parcels at AS1, AS2, and AS3 were normalized each to the corresponding mass flux of water parcel 10 sampled at AS1 (if a target compound was not detected in AS1_10 the calculation referred to AS1_8 or AS1_9) according to Equation 10. Sample AS1_10 was used as a reference because it contained a high number of detected target compounds.

$$\text{Overall chemical burden} = \sum \frac{J_i}{J_{i,(AS1\_10)}} \quad (10)$$

RESULTS AND DISCUSSION

Impact of tributaries on the micropollutant and effect patterns in the river

The WWTP effluent had a mean discharge of $274 \pm 38\ Ls^{-1}$, which was on average 51% of the Ammer discharge during the 24 h of sampling at AS1 ($540 \pm 34\ Ls^{-1}$). Sample extracts were screened for 83 target compounds relevant for surface water, which included 38 pesticides, 28 pharmaceuticals and antibiotics, 6 transformation products, and 11 industrial, household and personal care products (Supplemental Data, Table S1). This includes pesticides currently used in the Ammer catchment (personal communication with local farmers), for example, strobilurine (pyraclostrobin) and triazole fungicides (epoxiconazole) or the sulfonyl urea herbicide nicosulfuron. In total, 28 compounds were detected in water samples, mostly in the nanograms per liter range (Supplemental Data, Table S5).

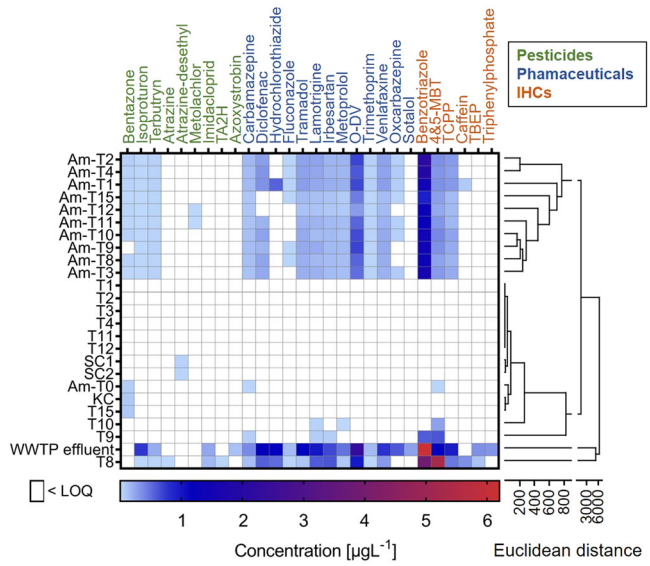

FIGURE 2: Target compounds in the Ammer main stem, the tributaries, the wastewater-treatment plant effluent, and Schönbrunnen Creek and Käsbach Creek, the tributaries of T15. Pesticides are highlighted in green, pharmaceuticals and antibiotics in blue, and industrial and household chemicals in orange. Similarity of compound profiles among samples is represented in a hierarchical cluster analysis with the unweighted pair-group method using arithmetic averages. Differences between samples and groups are expressed by the Euclidean distance. Am-T = Ammer tributary; IHCs = industrial and household chemicals; KC = Käsbach Creek; LOQ = limit of quantification; 4&5-MBT = 4&5-methylbenzotriazole; O-DV = O-desmethylvenlafaxine; SC = Schönbrunnen Creek; TA2H = terbuthylazine-2-hydroxy; TBEP = tris(2-butoxyethyl)phosphate; TCPP = tris(1-chloro-2-propyl)phosphate; WWTP = wastewater-treatment plant.

Among them were 9 pesticides and transformation products thereof, 13 pharmaceuticals, and 6 industrial, household and personal care products. The samples from the main stem downstream of the WWTP and from the tributaries clustered differently in a hierarchical cluster analysis, indicating clearly different compound profiles (see Figure 2). The compound profiles of the WWTP1 effluent and T8 impacted by WWTP2 appeared to be rather unique. Organic micropollutant concentrations in the Ammer main stem were considerably impacted by the input of WWTP1. Upstream of WWTP1 only the wastewater indicators carbamazepine and 4&5-methylbenzotriazole and the herbicide bentazone, which was previously detected in the Ammer source (Müller et al. 2018), occurred at approximately 20 to 60 ng L^{-1}. In the WWTP effluent 4 pesticides (azoxystrobin, imidacloprid, isoproturon, and terbutryn), 5 industrial, household and personal care products (benzotriazole, 4&5-methylbenzotriazole, tris[2-butoxyethyl]phosphate [TBEP], tris[1-chloro-2-propyl]phosphate [TCPP], and triphenylphosphate [TPP]), and 13 pharmaceuticals (e.g., carbamazepine, diclofenac, fluconazole, hydrochlorothiazide, metoprolol, tramadol) were detected. Concentrations were high for benzotriazole at 6 µg L^{-1}, O-demethylvenlafaxine at 2.3 µg L^{-1}, for 4&5-methylbenzotriazole at 1 µg L^{-1}, hydrochlorothiazide at 1 µg L^{-1}, diclofenac at 1 µg L^{-1}, and tramadol at 1 µg L^{-1}; pesticides ranged between 100 and 750 ng L^{-1} and other pharmaceuticals between 100 and 900 ng L^{-1}. Only caffeine and the 5 pesticides atrazine, atrazine-desethyl, bentazone, metolachlor, and terbuthylazine-hydroxy were absent in the WWTP effluent.

Farther downstream 5 pollutants decreased by >70% to concentrations below LOQ and did not occur any more at site Am-T1: azoxystrobin, imidacloprid, sotalol, TBEP, and TPP. Biodegradation, however, is not a reasonable process because of the short travel time of approximately 5 min between the WWTP1 input and Am-T1. Therefore, we speculate that sorption to suspended particulate matter (SPM) in the WWTP effluent and sedimentation may be considerable removal processes. This is supported by the relatively high octanol–water partition constant (log K_{OW}) of 2.5 for azoxystrobin (European Food Safety Authority 2010), 3.65 for TBEP (Van der Veen and de Boer 2012), and 4.59 for TPP (Hansch et al. 1995). Sotalol and imidacloprid have log K_{OW} values of the neutral species below 1 (ChemSpider 2020), and they are positively charged at neutral pH and therefore cannot be assessed by the K_{OW}. Also, charge interaction with negatively charged SPM and organic matter has to be considered in this case. Dilution and incomplete mixing can be ruled out based on the results of other pollutants (e.g., carbamazepine). The antihypertensive drug hydrochlorothiazide was detected in the river only once above the LOQ and was therefore not further considered. Other pollutants were transported throughout the river stretch and still appeared at Am-T15, the farthest downstream site: 3 pesticides (terbutryn at 27 ng L^{-1}, bentazone at 13 ng L^{-1}, isoproturon at 13 ng L^{-1}), 3 industrial, household and personal care products (benzotriazole at 850 ng L^{-1}, 4&5-methylbenzotriazole at 270 ng L^{-1}, TCPP at 140 ng L^{-1}), and 10 pharmaceuticals (e.g., O-desmethylvenlafaxine at 430 ng L^{-1}, tramadol at 150 ng L^{-1}, venlafaxine at 140 ng L^{-1}, lamotrigine at 120 ng L^{-1}, carbamazepine at 90 ng L^{-1}; see Supplemental Data, Table S5).

In tributaries T1, T2, T3, T4, T11, and T12 none of the pollutants could be detected. In T15, which is dominated by agricultural activity, only the herbicide bentazone was detected. A tributary of T15 (Schönbrunnen Creek) also contained atrazine-desethyl, the metabolite of the legacy herbicide atrazine. In T8, 5 pesticides (atrazine, imidacloprid, isoproturon, terbutryn, and terbuthalazine-2-hydroxy), 11 pharmaceuticals (e.g., carbamazepine, diclofenac, fluconazole, hydrochlorothiazide, tramadol), and 5 industrial, household and personal care products (e.g., caffeine, benzotriazole, 4&5-methylbenzotriazole, TCPP) were found; T8 receives WWTP effluents and inputs from agricultural land. The highest concentrations were measured for 4&5-methylbenzotriazole at 5.2 µg L^{-1} and benzotriazole at 3.8 µg L^{-1}. Caffeine, an indicator for untreated wastewater (Buerge et al. 2003), reached 336 ng L^{-1}. It appears that tributaries T9 and T10 were also impacted by WWTP2 effluent because the typical wastewater pollutants occurred: in T9, carbamazepine, irbesartan, lamotrigine, and benzotriazole; in T10, only lamotrigine, metoprolol, and 4&5-methylbenzotriazole. A likely explanation is wastewater from T8 percolating into the karstic groundwater, which enters farther downstream T9, T10, and the Ammer River, as shown in an earlier study (Harreß 1973). This is supported by the input of additional 22 Ls^{-1} water at AS3 that

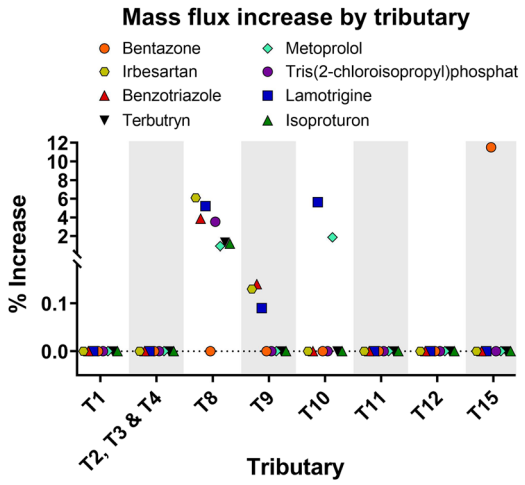

FIGURE 3: Mass flux increases in the Ammer main stem downstream of the tributaries (ΔJi, increase in percentage) of bentazone, metoprolol, irbesartan, tris(2-chloroisopropyl)phosphate, benzotriazole, lamotrigine, terbutryn, and isoproturon.

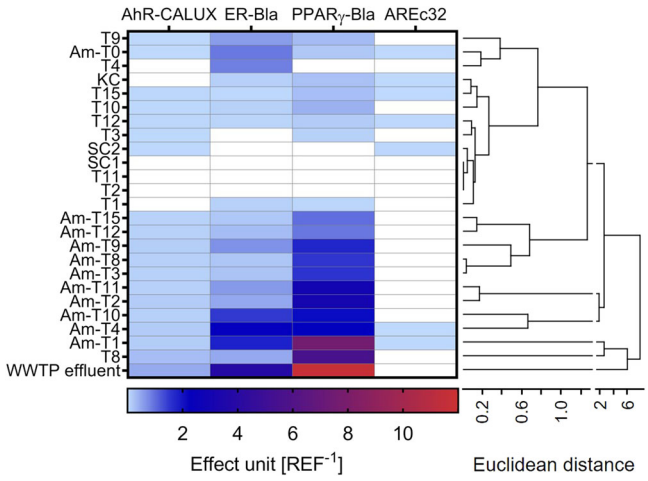

FIGURE 4: Effect units of the extracts from the Ammer main stem, the tributaries, the wastewater-treatment plant effluent, and the tributaries of T15, Schönbrunnen Creek and Käsbach Creek, measured in the in vitro bioassays AhR-CALUX, PPARγ-GeneBLAzer, ER-GeneBLAzer, and AREc32. Similarities of compound profiles among samples are represented in a hierarchical cluster analysis with the unweighted pair-group method using arithmetic averages. Differences between samples and groups are expressed by the Euclidean distance. Am-T = Ammer tributary; ER-Bla = ER-GeneBLAzer; KC = Käsbach Creek; PPARγ-Bla = PPARγ-GeneBLAzer; SC = Schönbrunnen Creek; WWTP = wastewater-treatment plant.

could not be attributed to any tributaries between AS2 and AS3 (see discharges in Table 1). Pollutants are subjected to dilution and attenuation processes during seepage passages, leading to reduced concentrations and the disappearance of compounds in T9 and T10 compared to T8 (e.g., diclofenac, irbesartan, lamotrigine, metoprolol, venlafaxine, 4&5-methylbenzotriazole, and TCPP).

To assess the influence of the tributaries on the compound pattern and abundance in the main stem, mass flux increases in the main stem caused by tributaries were calculated based on Equation 8 exemplarily for 8 compounds: the herbicides bentazone, isoproturon, and terbutryn; the pharmaceuticals metoprolol, lamotrigine, and irbesartan; the flame retardant tris (2-chloroisopropyl)phosphate; and the corrosion inhibitor benzotriazole (Figure 3). These chemicals represent different applications and thus diffusive (e.g., pesticides) and point source (via treated wastewater) inputs to the river. The influence of the tributaries on the compound profile and abundance in the main stem was generally insignificant apart from T8, T9, T10, and T15, with the highest mass flux increase for bentazone (10.4%) in T15. Most of the target compounds in Figure 3 were introduced by T8 (see Figure 2), though overall T8 had a rather low influence on the main stem because of its relatively low discharge (see Table 1). In contrast, T10 had a relatively high discharge but low compound concentrations except for lamotrigine and thus had little influence on the main stem as well.

The samples of the tributaries and the Ammer itself were further analyzed by in vitro bioassays AhR-CALUX, PPARγ-Bla, ER-Bla, and AREc32 (see Figure 4; Supplemental Data, Table S6. Similar to the compound profiles, the effect profiles of the tributaries clustered in a hierarchical cluster analysis as well as the samples of the Ammer main stem downstream of WWTP1. The samples that were most similar to the WWTP1 effluent were Am-T1 and T8. This finding is different from the chemical analysis but can be rationalized because Am-T1 and T8 are strongly influenced by WWTP effluents. The effect profiles of the Ammer clustered in a similar way as for the chemical analysis. The influence of the tributaries on the effect fluxes in the Ammer were generally significant (Figure 5). The highest effect flux increase in the Ammer was observed in ER-Bla, with 6.4% in reach 1 by T2, T3, and T4 together. Effect fluxes in the Ammer, measured in AhR-CALUX, PPARγ-Bla, and ER-Bla, increased approximately 1 to 5% by input from T8 and T10.

Although the chemical analysis and the in vitro bioassays covered different analytes and compound classes, they revealed similar pollution patterns and uncovered the tributaries as minor input sources of organic micropollutants and

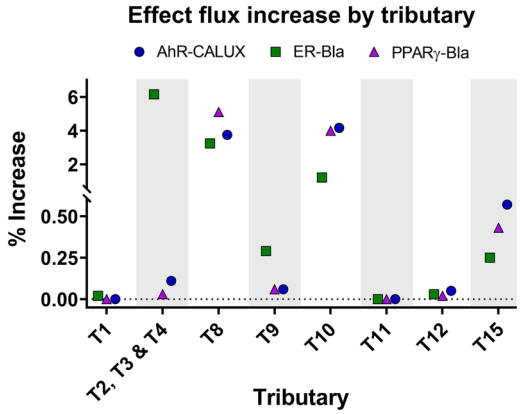

FIGURE 5: Effect flux increases in the Ammer main stem downstream of the tributaries measured in the bioassays AhR-CALUX, PPARγ-GeneBLAzer, and ER-GeneBLAzer. ER-Bla = ER-GeneBLAzer; PPARγ-Bla = PPARγ-GeneBLAzer.

associated effects. The tributaries had little impact on the Ammer because of their low discharge.

Temporal and spatial effects, cytotoxicity, and micropollutant dynamics in the main stem

In hourly composite water samples taken at the autosamplers, 23 of the target compounds were detected at AS1, AS2, and AS3 (Supplemental Data, Table S7). A different pollution profile at AS1 was found compared to AS2 and AS3, each of which clustered in a principal component analysis (PCA; see Supplemental Data, Figure S1A). The separation of the 3 groups occurred along principal component 1 (PC1), which explained 63.6% of the total variance. The compounds benzotriazole, 4-&5-methylbenzotriazole, and TCPP showed dominant negative loadings (Supplemental Data, Figure S1B). All are highly mobile in water, are rather stable toward degradation processes, and do not sorb considerably (Hem et al. 2003; Hart et al. 2004; Meyer and Bester 2004). Therefore, their concentrations did not decrease. O-Desmethylvenlafaxine and diclofenac showed dominant positive loadings (see Supplemental Data, Figure S1B) and consequently dissipated from the water phase more readily (Supplemental Data, Table S7). Both compounds are photodegradable under solar irradiation (Rúa-Gómez and Püttmann 2013; Baena-Nogueras et al. 2017). Principal component 2 explained 13.8% of the total variance and was mostly affected by the compounds diclofenac and mesotrione (Supplemental Data, Figure S1C). All of these compounds usually find their way into the environment by treated wastewater. Hydrochlorothiazide was not considered in the PCA because it was barely detected above the relatively high LOQ.

To consider the overall chemical burden of the Ammer River, normalized mass fluxes of the samples obtained over the course of 24 h at sampling sites AS1, AS2, and AS3 were summed up using Equation 10 (Figure 6A; named as "overall chemical burden"). The WWTP effluent had a high contribution to the Ammer main stem (51% of the Ammer discharge at AS1), and the WWTP discharge showed a temporal variability that cross-correlated moderately with the overall chemical burden at AS1 ($r_s = 0.52$). Hence, temporal dynamics of compounds from WWTPs can partially be explained by the effluent discharge, but the diurnal pattern of each individual target compound is still unknown. The coefficient of variation of the overall chemical burden over time was 18.7, 15.9 and 7.8% at AS1, AS2, and AS3, respectively, indicating a damping of temporal variability with increasing distance from the WWTP. The average cytotoxicity, $TU_{average}$, acquired from the in vitro bioassays AhR-CALUX, PPARγ-Bla, ER-Bla, and AREc32 (Supplemental Data, Table S8), was derived by Equation 3 and can reflect the total chemical load of a sample (Figure 6B). The average cytotoxicity was declining along the river flow with a slightly decreasing standard deviation and therewith a slightly declining variability over time (on average 0.040 ± 0.006 at AS1, 0.035 ± 0.006 at AS2, and 0.029 ± 0.004 toxic unit at AS3). The average attenuation of the mean $TU_{average}$ was 12.3 and 17.8%, which is mainly a result of dilution (10.3% in reach 1 and 22.7% in reach 2, based on data in Table 1). Similar to the overall chemical burden at AS1, the discharge of the WWTP effluent over time cross-correlated moderately ($r_s = 0.50$) with the $TU_{average}$ at AS1. However, no cross-correlation was observed with the $TU_{average}$ at AS2 and AS3.

Similar to the target compounds, the effects measured in the in vitro bioassays AhR-CALUX, PPARγ-Bla, ER-Bla, and AREc32 (Supplemental Data, Table S9) showed a different effect profile at AS1 compared to AS3 in a PCA (Supplemental Data, Figure S2A). The effect profiles of all individual samples appeared to cluster according to sampling sites, and separation between AS1 and AS3 occurred along the PC2 axis; PC2 explained 24.9% of the total variance, with strong loadings by AhR-CALUX (negative loading) and ER-Bla (positive loading) and weak loadings by PPARγ-Bla (positive loading) and AREc32 (negative loading; see Supplemental Data, Figure S2C). The differences between the effect profiles at AS1, AS2, and AS3 were mainly driven by the effects measured in AhR-CALUX and ER-Bla, which is in accordance with the data depicted in

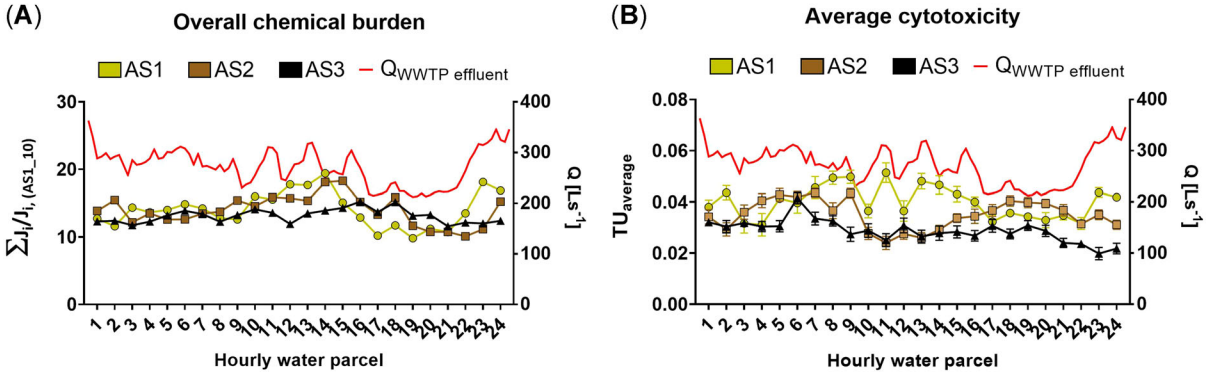

FIGURE 6: Overall chemical burden and average cytotoxicity in the Ammer main stem at autosamplers AS1, AS2, and AS3 over 24 h. (**A**) Sum of normalized mass fluxes of pollutants (chemical burden) measured by liquid chromatography tandem mass spectrometry. (**B**) Average cytotoxicity measured in the assays AhR-CALUX, PPARγ-GeneBLAzer, ER-GeneBLAzer, and AREc32. The discharge of the WWTP1 effluent is displayed on the second *y*-axis. Average cytotoxicity is expressed as toxic unit (see Equation 3), and the overall mass flux is expressed as the sum of the mass fluxes of each detected compound, normalized to the corresponding mass flux of water parcel 10 at AS1 ($\sum J_i/J_{i,(AS1\_10)}$). Error bars in (**B**) indicate standard errors calculated by error propagation. Q = discharge; TU = toxic unit; WWTP = wastewater-treatment plant.

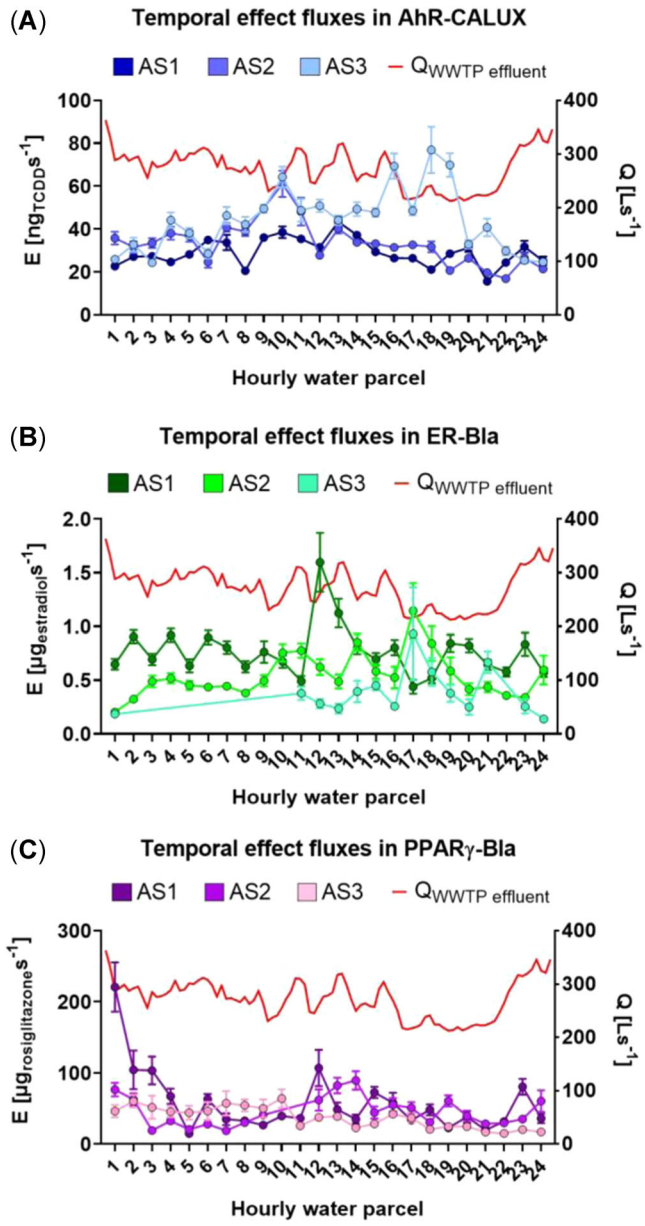

FIGURE 7: Effect fluxes expressed as amount of reference compound per second, in the Ammer main stem at autosamplers AS1, AS2, and AS3 over 24 h measured by the assays AhR-CALUX (**A**), ER-GeneBLAzer (**B**), and PPARγ-GeneBLAzer (**C**). Discharge Q of the WWTP1 effluent, expressed as Ls^{-1}, is displayed as a red line on the right vertical axis. Error bars in indicate standard errors. E = effect flux; ER-Bla = ER-GeneBLAzer; PPARγ-Bla = PPARγ-GeneBLAzer; Q = discharge; WWTP = wastewater-treatment plant.

Figure 7A and B. The effect fluxes averaged over 24 h increased in AhR-CALUX and decreased in ER-Bla along the river flow. The effect fluxes measured in AhR-CALUX, PPARγ-Bla, and ER-Bla (Figure 7A–C) showed temporal dynamics not following the discharge of the WWTP. Overall dynamics over 24 h were not large under these base flow conditions for the day of sampling.

The effect-based data were compared to tentative effect-based trigger values (EBT-BEQ) proposed by (Escher et al. 2018a), representing effect threshold levels for unacceptable surface water quality. These EBT-BEQs were derived from environmental quality standards of the European Union and help in estimating environmental risks of organic micropollutants. The EBT-EQ for benzo[*a*]pyrene is 6.4 $ng_{benzo[a]pyrene}L^{-1}$ for AhR CALUX, the EBT-EQ for 17ß-estradiol is 0.34 $ng_{17ß\text{-}estradiol}L^{-1}$ for ER-Bla, the EBT-rosiglitazone-EQ is 36 $ng_{rosiglitazone}L^{-1}$ for PPARγ-Bla, and the EBT-dichlorvos-EQ is 156 $\mu g_{dichlorvos}L^{-1}$ for AREc32 (Escher et al. 2018a). At AS1, AS2, and AS3 these proposed EBT-BEQs were exceeded in all bioassays except AREc32, where also in many samples the effects were masked by cytotoxicity (Supplemental Data, Tables S10 and S11). Compliant with the EBTs were many of the tributaries and the Ammer at Am-T0 (except for the EBT-EQ for 17ß-estradiol) prior to the WWTP effluent coming in. Aside from the wastewater-contaminated T8, the proposed EBT-BEQs were also exceeded by tributaries T1, T2, T9, T10, and KC in ER-Bla; T9 and T10 in PPARγ-Bla; and T12, T15, KC, and SC2 in AREc32 (see Supplemental Data, Table S6). Although only a few or no target compounds were detected in most tributaries, the measured effects point toward potential problems with the water quality stemming from other sources. One year earlier, in July 2017, the chemical and effect profiles of the Ammer catchment were investigated (Müller et al. 2018). The proportion of treated wastewater deriving from WWTP1 was higher in 2017 with 81% compared to 50% in 2018, and therefore, the effects measured in AhR-CALUX, ER-Bla, and AREc32 were slightly higher in 2017 than in 2018. Although the EBT-BEQs exceeded the measured effects, they were still in the same range as those derived for similar surface water samples by other research groups (Escher et al. 2012, 2013a; Scott et al. 2018).

CONCLUSION

The characterization of input sources and pathways of organic micropollutants in the aquatic environment is important to assess water quality but also a challenging task. The study site is representative of other small river systems in densely populated countries. The present study revealed treated wastewater as the primary input source of organic micropollutants and associated effects in rivers during dry weather periods. Tributaries from agricultural and urban areas carried only a few of the monitored target compounds but exceeded effect-based trigger values, pointing toward unacceptable water quality. However, the contribution of tributaries to the mass and effect fluxes in the main river were negligible under base flow conditions. Increased discharge during rain events may change this situation. Increased runoff from agricultural and urban areas may change the pollution profile, mass and effect fluxes, and therefore the contribution of tributaries to the main river. We therefore recommend further catchment-scale studies to reveal the role of rain events for the water quality of small rivers.

Supplemental Data—The Supplemental Data are available at the Wiley Online Library at https://doi.org/10.1002/etc.4726.

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Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (christian.zwiener@uni-tuebingen.de).

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