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 Zwienera,*

aCenter for Applied Geoscience, Eberhard Karls University of Tübingen, Tübingen, Germany
bUFZ-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. © 2020 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals LLC on behalf of SETAC.

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; Szőcs 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.
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.

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

| TABLE 1: Discharges, in liters per second (Ls⁻¹), of the Ammer River and its tributaries* |
|---------------------------------|--------------------------|
| Tributary/sampling site         | Q (Ls⁻¹) |
| Ammer (main stem)              |            |
| Am-T0                          | 270 ± 20   |
| AS1                            | 540 ± 34   |
| AS2                            | 577 ± 30   |
| AS3                            | 743 ± 23   |
| WWTP1 effluent                  |            |
| Tributaries                     |            |
| T1                              | 2          |
| T2                              | 16         |
| T3                              | 6          |
| T4                              | 35         |
| T8                              | 8          |
| T9                              | 2          |
| T10                             | 120b       |
| T11                             | 1          |
| T12                             | 1          |
| T15                             | 12         |

*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.

bC. Glaser et al. (Center for Applied Geoscience, University of Tübingen, Tübingen, Germany, unpublished manuscript, 2019b).

Q = discharge; T = tributary; 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; Kruev 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 x 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 AS2_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), PPARy-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 (ERIR1.5) 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.

\[
\text{BEQ} = \frac{\text{EC10 (reference)}}{\text{EC10 (sample)}} \quad \text{or} \quad \frac{\text{ERIR1.5 (reference)}}{\text{ERIR1.5 (sample)}}
\]

\[
\text{Effect unit} = \frac{1}{\text{EC10}} \quad \text{or} \quad \frac{1}{\text{IR1.5}}
\]

\[
\text{Toxic unit} = \frac{1}{\text{IC10}}
\]

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 \( C_{\text{downstream}} \) and effects (\( \text{BEQ}_{\text{downstream}} \)) 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, \( \text{Ls}^{-1} \)) 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.

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

\[
\text{BEQ}_{\text{downstream}} = \frac{\text{BEQ}_{\text{upstream}} \times Q_{\text{upstream}} + \text{BEQ}_{\text{tributary}} \times Q_{\text{tributary}}}{Q_{\text{upstream}} + Q_{\text{tributary}}} \quad \text{(5)}
\]

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 \( Q_{\text{upstream}} \) 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 \( Q_{\text{downstream}} \), the respective \( Q_{\text{tributary}} \) 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.

\[
J_i = Q \times C_i \quad \text{(6)}
\]

\[
E_a = Q \times \text{BEQ}_{a} \quad \text{(7)}
\]

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

\[
\Delta J_{a,Tx} = \frac{J_{a,\text{downstream}}}{J_{a,\text{upstream}}} - 1 \quad \text{(8)}
\]

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

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.

\[
\text{Overall chemical burden} = \sum \frac{J_i}{J_i(\text{AS1}_10)} \quad \text{(10)}
\]

**RESULTS AND DISCUSSION**

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

The WWTP effluent had a mean discharge of 274 ± 38 Ls\(^{-1}\), which was on average 51% of the Ammer discharge during the 24 h of sampling at AS1 (540 ± 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).
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 bentazon, 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 [TCP], 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-demethylenalaxine 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, bentazon, 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 KOW) 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 KOW values of the neutral species below 1 (ChemSpider 2020), and they are positively charged at neutral pH and therefore cannot be assessed by the KOW. 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, bentazon 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-desmethylenalaxine 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 bentazon 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-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, ibersartan, 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
The samples of the tributaries and the Ammer itself were further analyzed by in vitro bioassays AhR-CALUX, PPARy-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, PPARy-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...
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, 4-85-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 (Hern et al. 2003; Hart et al. 2004; Meyer and Bester 2004). Therefore, their concentrations did not decrease. O-Desmethylenolafaxine 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=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, PPARy-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=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, PPARy-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 PPARy-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, PPARy- 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 (ΣJ/JAS1_10). Error bars in (B) indicate standard errors calculated by error propagation. Q = discharge; TU = toxic unit; WWTP = wastewater-treatment plant.
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 17\(\beta\)-estradiol is 0.34 ng 17\(\beta\)-estradiol L\(^{-1}\) for ER-Bla, the EBT-rosiglitazone-EQ is 36 ng rosiglitazone L\(^{-1}\) for PPARy-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 17\(\beta\)-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 PPARy-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 measured effects 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.

**Acknowledgment**—The authors thank C. Adolphi for her assistance in chemical analysis, M. Ebner for his advice on...
statistical data treatment, S. Novak for her laboratory assistance in Tübingen, and M. König, J. John, and R. Schlichting for performing the in vitro bioassays at UFZ. The present study was supported by the Collaborative Research Centre 1253 CAMPOS (Project P1: Rivers), funded by the German Research Foundation (grant SFB 1253/1 2017).

Disclaimer—All authors have no interest to declare. The views expressed in the present study are solely those of the authors.

Author Contribution Statement—M.E. Müller: conceiving and conducting the sampling, sample preparation, conducting the evaluation and interpretation of chemical analysis and bioassay data, in vitro bioassay data acquisition, writing the manuscript, initial design of all figures except for Figure 1; M. Werner: conceiving the sampling, sample preparation, chemical analysis, data acquisition and evaluation, drafting the manuscript; C. Glaser: sampling, revising the manuscript, making substantial contributions to designing Figure 1, conceiving the sampling; M. Schwientek: conceiving and conducting the sampling, revising the manuscript; C. Zarfl: conceiving the sampling, revising the manuscript; B.I. Escher: conceiving the sampling, making substantial contributions to bioassay measurements, analysis and interpretation of data, and drafting the manuscript; C. Ziwiener: conceiving the sampling and making substantial contributions to chemical analysis, interpretation of data, drafting and revising the manuscript.

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

REFERENCES

Alpizar F, Backhaus T, Decker N, Eilks I, Escobar-Pemberthy N, Fantke P, Geiser K, Ivanova M, Jolliet O, Kim H-S. 2019. Global Chemicals Outlook II: From legacies to innovative solutions. Implementing the 2030 agenda for sustainable development. United Nations Environment Programme, Nairobi, Kenya.

Baena-Nogueras RM, González-Mazo E, Lara-Martín PA. 2017. Degradation kinetics of pharmaceuticals and personal care products in surface waters: Photolysis vs biodegradation. Sci Total Environ 590:643–654.

Brennan JC, He G, Tsutsumi T, Zhao J, Wirth E, Fulton MH, Denison MS. 2015. Development of species-specific Ah receptor-responsive third generation CALUX cell lines with enhanced responsiveness and improved detection limits. Environ Sci Technol 49:11903–11912.

Bueno MM, Gomez M, Herrera S, Hernandez M, Agüera A, Fernández-Rodriguez M. 2012. Occurrence and persistence of organic emerging contaminants and priority pollutants in five sewage treatment plants of Spain: Two years pilot survey monitoring. Environ Pollut 164:267–273.

Buerge IJ, Poiger T, Müller MD, Buser H. 2003. Caffeine, an anthropogenic marker for wastewater contamination of surface waters. Environ Sci Technol 37:691–700.

ChemSpider. 2020. Sotalol, imidacloprid. [cited 2020 April 2]. Available from: http://www.chemspider.com/Chemical-Structure.5063.html

Englert D, Zubrod JP, Schulz R, Bundschuh M. 2013. Effects of municipal wastewater on aquatic ecosystem structure and function in the receiving stream. Sci Total Environ 454:401–410.

Escher BI, Ait-Aissa S, Behnisch PA, Brack W, Brion F, Brouwer A, Buchinger S, Crawford SE, Du Pasquier D, Hamers T. 2018a. Effect-based trigger values for in vitro and in vivo bioassays performed on surface water extracts supporting the environmental quality standards (EQS) of the European Water Framework Directive. Sci Total Environ 628:748–765.

Escher BI, Allison M, Altenburger R, Bain PA, Balaguer P, Busch W, Crago J, Denslow ND, Dopp E, Hilscherova K. 2013a. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays. Environ Sci Technol 48:1940–1956.

Escher BI, Dutt M, Maylin E, Tang JYM, Toze S, Wolf CR, Lang M. 2012. Water quality assessment using the AREC32 reporter gene assay indicative of the oxidative stress response pathway. J Environ Monit 14:2877–2885.

Escher BI, Neale PA, Villeneuve DL. 2018b. The advantages of linear concentration–response curves for in vitro bioassays with environmental samples. Environ Toxicol Chem 37:2273–2280.

Escher BI, van Dalee C, Dutt M, Tang JY, Altenburger R. 2013b. Most oxidative stress response in water samples comes from unknown chemicals: The need for effect-based water quality trigger values. Environ Sci Technol 47:7002–7011.

European Environment Agency. 2019. The European environment—State and outlook 2020: Knowledge for transition to a sustainable Europe. Publications Office of the European Union, Luxembourg.

European Food Safety Authority. 2010. Conclusion on the peer review of the pesticide risk assessment of the active substance aoxynystrobin. EFSA J 8:1542.

Farré M, Barceló D. 2003. Toxicity testing of wastewater and sewage sludge by biosensors, bioassays and chemical analysis. Trends Anal Chem 22:299–310.

Gago-Ferrero P, Bletsou AA, Damalas DE, Aalizadeh R, Alygizakis NA, Singer HP, Hollender J, Thomaidis NS. 2019. Wide-scope target screening of >2000 emerging contaminants in wastewater samples with UPLC-Q-TOF-HRMS/MS and smart evaluation of its performance through the validation of 195 selected representative analytes. J Hazard Mater 387:121712.

Grathwohl P, Rügner H, Wöhling T, Osenbrück K, Schwientek M, Gayler S, Wollschläger U, Selle B, Paus M, Delfs J-O. 2013. Catchments as reactors: A comprehensive approach for water fluxes and solute turnover. Environ Earth Sci 69:317–333.

Hansch C, Leo A, Hoekman D. 1995. Exploring QSAR: Fundamentals and Applications in Chemistry and Biology. American Chemical Society, Washington, DC.

Harreß HM. 1973. Hydrogeologische Untersuchungen im Oberen Gäu. PhD thesis, University of Tübingen, Tübingen, Germany.

Hart D, Davis L, Erickson L, Callender T. 2004. Sorption and partitioning parameters of benzotriazole compounds. Microchem J 77:9–17.

Hem LJ, Hartnik T, Roseth R, Breedveld GD. 2003. Photochemical degradation of benzotriazole. J Environ Sci Health A 38:471–481.

Köng M, Escher BI, Neale PA, Krauss M, Hilscherova K, Novák J, Teodorović I, Schulze T, Seidensticker S, Hashmi MAK, Ahlheim J, Brack W. 2017. Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environ Pollut 220:1220–1230.

Kruve A, Leito I, Herodes K. 2009. Combating matrix effects in LC/ESI/MS: The extrapolative dilution approach. Anal Chim Acta 651:75–80.

Kuzmanović M, Ginebreda A, Petrović M, Barceló D. 2015. Risk assessment based prioritization of 200 organic micropollutants in 4 Iberian rivers. Sci Total Environ 503:289–299.

Lee H-J, Kim KY, Hamm S-Y, Kim M, Kim HK, Oh J-E. 2019. Occurrence and distribution of pharmaceutical and personal care products, artificial sweeteners, and pesticides in groundwater from an agricultural area in Korea. Sci Total Environ 659:168–176.

Loos R, Gawlik BM, Locoro G, Rimaviciute E, Contini S, Bidoglio G. 2009. EU-wide survey of polar organic persistent pollutants in European river waters. Environ Pollut 157:561–568.

Lundqvist J, von Brömssen C, Rosenmai AK, Ohlsson Å, Le Godec T, Jonsson O, Kreuger J, Oskarsson A. 2019. Assessment of pesticides in surface water samples from Swedish agricultural areas by integrated bioanalysis and chemical analysis. Environ Sci Eur 31:53.

Masoner JR, Kolpin DW, Furlong ET, Cozzarelli IM, Gray JL, Schwab EA. 2014. Contaminants of emerging concern in fresh leachate from landfills in the contaminated United States. Environ Sci Process Impacts 16:2335–2354.

Meyer J, Bester K. 2004. Organophosphate flame retardants and plasticizers in wastewater treatment plants. J Environ Monit 6:599–605.

© 2020 The Authors

wileyonlinelibrary.com/ETC
Müller ME, Escher BI, Schwientek M, Werneburg M, Zarf C, Zwiener C. 2018. Combining in vitro reporter gene bioassays with chemical analysis to assess changes in the water quality along the Ammer River, southwestern Germany. *Environ Sci Eur* 30:20.

Müller ME, Vikstrom S, König M, Schlichting R, Zarf C, Zwiener C, Escher BI. 2019. Mitochondrial toxicity of selected micropollutants, their mixtures, and surface water samples measured by the oxygen consumption rate in cells. *Environ Toxicol Chem* 38:1000–1011.

Munz NA, Burdon FJ, De Zwart D, Junghans M, Melo L, Reyes M, Schönenberger U, Singer HP, Spycher B, Hollender J. 2017. Pesticides drive risk of micropollutants in wastewater-impacted streams during low flow conditions. *Water Res* 110:366–377.

Neale PA, Altenburger R, Aït-Aïssa S, Altenburger R, Brion F, Busch W, de Aragão Umbuzeiro G, Denison MS, Du Pasquier D, Hilscherová K, Hollert H. 2017a. Development of a bioanalytical test battery for water quality monitoring: Fingerprinting identified micropollutants and their contribution to effects in surface water. *Water Res* 123:734–750.

Neale PA, Munz NA, Aït-Aïssa S, Altenburger R, Brion F, Busch W, Schlichting R, Zarf C, Zwiener C, Escher BI, Hilscherová K, Kuch B, Grathwohl P. 2016. A high-precision sampling scheme to assess persistence and transport characteristics of micropollutants in rivers. *Sci Total Environ* 540:444–454.

Scott PD, Coleman HM, Khan S, Lim R, McDonald JA, Mondon J, Neale PA, Prochazka E, Tremblay LA, Warne MSJ. 2018. Histopathology, vitellogenin and chemical body burden in mosquitofish (Gambusia holbrooki) sampled from six river sites receiving a gradient of stressors. *Sci Total Environ* 616:1638–1648.

Szocs E, Brinke M, Karaoglan B, Scharer RB. 2017. Large scale risks from agricultural pesticides in small streams. *Environ Sci Technol* 51:7378–7385.

Van der Veen I, de Boer J. 2012. Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. *Chemosphere* 88:1119–1153.

Villagrasa M, Guillamón M, Eljarrat E, Barceló D. 2007. Matrix effect in liquid chromatography-electrospray ionization mass spectrometry analysis of benzoxazinoid derivatives in plant material. *J Chromatogr A* 1157:108–114.

Writer JH, Keefe SK, Ryan JN, Ferrer I, Thurman ME, Barber LB. 2011. Methods for evaluating in-stream attenuation of trace organic compounds. *Appl Geochem* 26(Suppl):S344–S345.