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# Micropollutant biotransformation and bioaccumulation in natural stream biofilms

Werner L. Desiante[a,b], Nora S. Minas[a,b], Kathrin Fenner[a,b,c,*]

[a] Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland
[b] Institute of Biogeochemistry and Pollutant Dynamics, ETH Zürich, 8092 Zürich, Switzerland
[c] Department of Chemistry, University of Zürich, 8057 Zürich, Switzerland

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ABSTRACT

Micropollutants are ubiquitously found in natural surface waters and pose a potential risk to aquatic organisms. Stream biofilms, consisting of bacteria, algae and other microorganisms potentially contribute to bioremediating aquatic environments by biotransforming xenobiotic substances. When investigating the potential of stream biofilms to remove micropollutants from the water column, it is important to distinguish between different fate processes, such as biotransformation, passive sorption and active bioaccumulation. However, due to the complex nature of the biofilm community and its extracellular matrix, this task is often difficult. In this study, we combined biotransformation experiments involving natural stream biofilms collected up- and downstream of wastewater treatment plant outfalls with the QuEChERS extraction method to distinguish between the different fate processes. The QuEChERS extraction proved to be a suitable method for a broad range of micropollutants (> 80% of the investigated compounds). We found that 31 out of 63 compounds were biotransformed by the biofilms, with the majority being substitution-type biotransformations, and that downstream biofilms have an increased biotransformation potential towards specific wastewater-relevant micropollutants. Overall, using the experimental and analytical strategy developed, stream biofilms were demonstrated to have a broad inherent micropollutant biotransformation potential, and to thus contribute to bioremediation and improving ecosystem health.



## 1. Introduction

Worldwide, vast amounts of micropollutants (MPs) enter into natural aquatic environments through various pathways, including run-off from agricultural streams and wastewater treatment plant (WWTP) effluents (Kreuger 1998; Schwarzenbach et al. 2006; Margot et al. 2015; aus der Beek et al. 2016). Thus, MPs such as pharmaceuticals, personal care products, artificial sweeteners and pesticides end up in complex mixtures in streams and rivers (Buerge et al. 2009). These MPs can pose a risk to human and environmental health (Gavrilescu et al. 2015). Amongst other effects, they may exert a variety of undesired effects on non-target organisms, such as invertebrates, fish or microorganisms, which ultimately can lead to alterations in the structure and function of ecosystems (Caliman and Gavrilescu 2009; Tlili et al. 2017; Gad et al. 2020; Li, Sharp, and Drewes 2016).

Microbial stream biofilms are the most dominant form of life in natural streams (Battin et al. 2003). Stream biofilms, also referred to as periphyton, consist of complex communities of algae, bacteria, fungi and other microorganisms, which are embedded in an extracellular polymeric matrix (EPM) and can be attached to any surface in a stream. Stream biofilms play a key role in biogeochemical cycles and ecosystem respiration and, as primary producers, they form the basis of the food web (Battin et al. 2016). It has been suggested that stream biofilms can also be used to monitor the effects of MPs on river ecosystems (Sabater et al. 2007). Specifically, the concept of pollution-induced community tolerance (PICT) can offer insights into whether the presence of MPs modulates community responses to chemical stress. Since it is likely that different species within a community will exhibit different extents of tolerance to MPs, it is conceivable that, upon MP exposure, tolerant species within the community are favored, while sensitive ones disappear (Tlili et al. 2016). Several studies have shown that MPs, such as diuron, ibuprofen and diclofenac can induce PICT (Pesce, Margoum, and Montuelle 2010; Corcoll et al. 2014). This raises the following questions: What are the mechanisms under-

* Corresponding author.
*E-mail address:* kathrin.fenner@eawag.ch (K. Fenner).

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lying PICT? - and - How does PICT establish in biofilms? One conceivable mechanism leading to PICT in biofilms is the removal of MPs through biotransformation.

Several studies have demonstrated that stream biofilms can remove MPs from the aquatic environment by acting as a natural sink (Schorer and Eisele 1997; Lawrence et al. 2001; Winkler, Lawrence, and Neu 2001; Gantzer et al. 1988). However, there is rarely any differentiation between possible removal mechanisms. On the one hand, MPs can passively sorb to the EPM or cell surfaces within the biofilms. On the other hand, active biological processes, such as bioaccumulation or biotransformation, can remove compounds from the water phase. However, it has to be noted that only biotransformation leads to permanent removal of organic compounds, while the other processes are potentially reversible and re-release of MPs into the water might occur. It has been shown that, due to the heterogeneous composition of biofilms, pesticides with very diverse physico-chemical properties can accumulate in biofilms and, thus, that biofilms could potentially serve as natural passive samplers to better estimate ecological risks (Mahler et al. 2020). A recent review on the role of biofilms in contaminant bioaccumulation revealed the wealth of studies that have been conducted on this topic (Bonnineau et al. 2020). In contrast, biotransformation studies in stream biofilms remain limited. For example, the biotransformation of three carbamate pesticides (Tien et al. 2013), two sulfonamide antibiotics (Vila-Costa et al. 2017), five endocrine disruptors (Writer et al. 2011), and several single compounds, such as venlafaxine (Rozman, Acuna, and Petrovic 2018), alachlor (Paule et al. 2015) and diuron (Pesce et al. 2009) has been investigated. However, those studies often focus on single substances or small groups of compounds only. Thus knowledge on the fate of complex mixtures of polar organic MPs in stream biofilms is still very limited.

When investigating biotransformation in natural stream biofilms, it is important to be able to distinguish it from other fate processes such as sorption and active bioaccumulation. However, it is often difficult to reliably quantify accumulated contaminants in biofilms due to the complex nature of the EPM (Bonnineau et al. 2020). EPM consists mainly of hydrated polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender 2010), and intercalated into the EPM, a great variety of different microorganisms is usually found. When targeting a wide range of MPs with solvent extraction methods, those polymeric substances, as well as intracellular substances released during extraction steps, often lead to analytical interferences and large matrix effects (i.e., ion suppression or enhancement) (Huerta et al. 2016). Moreover, so far, we are not aware of any experimental approach that allows distinguishing passive sorption from active bioaccumulation. However, the two mechanisms are likely to lead to different extents of exposure of the microorganisms, and hence need to be distinguished. As a consequence of the analytical challenges mentioned and the lack of a robust experimental approach, quantification of the different MP fate processes in biofilms, including biotransformation, has remained challenging.

In this study, we therefore aimed to develop a coherent methodological approach to investigate the fate of complex mixtures of MPs at environmentally relevant concentrations in biofilms, with a particular focus on distinguishing between biotransformation, passive sorption and active bioaccumulation. We used this method to investigate the biotransformation potential of natural stream biofilms collected up- and downstream of WWTP outfalls towards a large set of structurally diverse, environmentally relevant MPs. With this work, we provide valuable methodological guidance and interesting first insights on how biotransformation varies as a function of chemical structure and biofilm exposure history that will help guide future efforts to elucidate the fate of MPs in streams.

## 2. Methods

A summary of the methods including an illustrated overview of the methodological workflow is provided in the SI section S1.

### 2.1. Compounds

A complete list of the 63 target substances used in our experiments can be found in Table S1. They were chosen based on their occurrence in treated wastewater and surface water and hence expected relevance to surface waters, and to cover multiple use classes (i.e., pesticides, pharmaceuticals, artificial sweeteners and other compounds originating from human activity). Furthermore, we chose substances to cover a broad range of potential initial biotransformation reactions and functional groups, including primary, secondary and tertiary amides and amines, carbamates, carboxylic acids, chloroacetanilides, esters, nitriles, sulfonamides, triazoles and halogenated compounds. HPLC grade chemicals were purchased from Sigma-Aldrich GmbH, Honeywell Specialty Chemicals, HPC Standards GmbH, Dr. Ehrenstorfer GmbH, and Toronto Research Chemicals. The 63 substances were combined in a spiking mix in EtOH (1 mg/L). For analysis, labelled internal standards were spiked to the samples and assigned to the respective target compounds (Table S1).

### 2.2. Study sites

Two WWTPs located in central Switzerland (Buttisholz (B) and Hochdorf (H)) were selected as study sites. Buttisholz is designed to serve 3500 person equivalents (PE) and has a mechanical-biological treatment, while Hochdorf serves 50'000 PE and has a mechanical-biological treatment with advanced P-elimination and nitrification. Further information on the WWTPs, the land use and results from a screening of 389 organic MPs and 57 prioritized organic MPs conducted in the years 2013 and 2014, respectively, can be found in Munz et al. (2017). Biofilms were cultivated in the adjacent rivers up- and downstream of the WWTP (BUp, BDown, HUp, HDown). The locations were chosen based on previous studies where PICT towards MPs extracted from passive samplers deployed in the respective wastewater discharges had been demonstrated in both of the respective downstream communities (Tlili et al. 2017). Notably, at both sites, there are no WWTPs emitting into the river section upstream of the study site.

### 2.3. Biofilm colonization

The biofilm colonization period was between 11$^{th}$ of September and 8$^{th}$ of October 2018. A similar system was used as described in Tlili et al. (2017). Shortly, three glass slides (approximately 910 cm$^2$ per slide), which served as growth substrate for the biofilms, were mounted in plastic boxes, allowing for water flow through the boxes along the slides. Three biological replicates, each consisting of one box with three glass slides, were installed at each sampling site, such that the top of the substrates was at a depth of 20 – 30 cm below the water surface. After four weeks of colonization, the glass slides were removed from the river, separately stored in zip bags containing the respective stream water and transported to the laboratory in cooling boxes. During the colonization period, water quality parameters (pH, conductivity, dissolved $O_2$) were measured in situ on a weekly basis (Table S2). In addition, 1 L weekly grab samples were taken and analyzed in the laboratory for 18 additional water quality parameters according to standard methods (Table S3).

### 2.4. Biotransformation experiments

The biofilms contained in one box were scraped of the glass slides and suspended in approximately 400 mL of artificial river water, i.e., periquil (Stewart, Behra, and Sigg 2015). Standardization to similar biomass concentrations was achieved by means of OD measurements at 685 nm, and by further diluting samples with higher biomass concentration to a target absorbance of 0.32 ± 0.05 (sample dilution of 4:1 periquil/biofilm suspension). From these stock suspensions, subsamples of 100 mL were taken for the biotransformation experiment (BT) and a sorption control (SC). Additionally, abiotic controls (AC) were prepared, containing only artificial river water. Samples for SC and AC experiments were autoclaved (121°C, 2 bar, 20 min) before starting the experiment. The experiments were conducted in 250 mL Schott bottles, in a temperature-controlled room at 16°C. BT batches were exposed to a 12 h light-dark cycle (Philips 10NC MAS LEDtube Value HF 1200 mm 16.5W865; 1600lm each; 865 Cool daylight; PPFD: 40 – 50 $\mu mols^{-1}m^{-2}$ at the height of the reactors). A green foil (121 LEE Green, LEE filters UK) was installed between the light source and the experimental vessels, in order to simulate a water depth of approximately 1 m and to prevent UV light to penetrate the vessels. To start the experiment, 100 µL of spiking mix was added to each BT, SC and AC experimental vessel, resulting in a final concentration of approximately 1 µg/L of each substance (Table S1). On the one hand, this spiking concentration was chosen based on the study conducted by Munz et al. (2017), where it was shown that MPs from several categories (plant protection products, food additives, corrosion inhibitors, and others) reached concentrations of several hundered ng/L. On the other hand, we chose the spiking concentration such that concentrations could be followed by means of our analytical methods, without further enrichment, down to about 95% loss of the initially spiked concentration. All experimental vessels were placed on laboratory shakers and shaken circularly at 150 rpm. Aqueous samples (1.5 mL) of the BT batches were taken before and at approximately 0, 1, 2, 4, 8, 24, 48, 72 and 96 hours after the start of the experiment, while samples of the SC and AC batches were taken before and at approximately 0, 4, 24, 48 and 72 hours after the start of the experiment. Samples were centrifuged (15 min, 4°C, 21'130 g), the supernatant (1 mL) was transferred into an amber HPLC vial, spiked with 20 µL internal standard mix to a concentration of 0.5 µg/L and then immediately frozen. Biofilm samples for determining solid phase concentrations were taken at 72 and 96 h after the start of the experiment for the SC and BT batches, respectively. To do so, 10 mL of the suspension were sampled, centrifuged (15 min, 23°C, 3'800 g), the supernatant discarded and the pellet frozen (-80°C) until further extraction.

### 2.5. Biofilm extraction

In order to determine solid phase concentrations, a QuEChERS extraction method was used (Munz et al. 2018). Biofilm samples were freeze-dried, spiked with 30 µL internal standard mix (25 µg/L) and stored overnight at room temperature. After addition of 500 mg of 1 mm zirconia/silica beads (BioSpec Products, Inc., U.S.A.), extraction in ACN/ultrapure $H_2O$ (1000 µL, 1:1) was performed in a Fast Prep bead beater (MP Biomedicals, Switzerland) in two cycles of 15 s at 6 m/s with cooling the samples on ice between the cycles. Then, samples were centrifuged (6 min, 20°C, 20'000 g) and 800 µL of the supernatant was added into a vial containing 300 mg QuEChERS salts (4:1, MgSO4:NaCl, Agilent Technologies), shaken immediately and vortexed for approximately 1 min, leading to phase separation. The samples were then centrifuged (6 min, 20°C, 20'000 g) and 200 µL of the upper organic phase was added into a third vial. An additional 500 µL ACN was added to the vial containing the biomass and extraction as described above was repeated. Another 600 µL of the organic phase was added to the third vial and the combined ACN phase was then cleaned twice by extraction with heptane (2×500 µL). Then, 700 µL of the ACN phase was transferred into an amber HPLC vial and stored at -20°C until evaporation under a gentle $N_2$ stream. The residue was redissolved in 700 µL ultrapure $H_2O$ and stored at -20°C until analysis.

### 2.6. Recovery experiment

In order to validate the performance of the QuEChERS extraction method with stream biofilm samples, recovery experiments were performed. Freeze-dried biofilm samples (100 mg) were prepared in triplicate and used in 5 spiking schemes (for more details see SI section S4) where 40 µL standard (20 µg/L) and 30 µL internal standard mix (25 µg/L) were spiked before and/or after extraction, respectively. From these spiking schemes, absolute and relative recoveries could be determined, as well as the matrix factors. The magnitude of these three factors can further be used to estimate the proportional systematic error of the method for every single analyte and could potentially be used to correct, e.g., biases in the measured concentrations of analytes that are due to analytical interferences.

### 2.7. Chemical analysis

Chemical analysis was performed by means of reverse phase liquid chromatography coupled to a QExactive Plus (Thermo Scientific). Phase separation was achieved using an Atlantis T3 column (particle size 3 µm, 3.0×150 mm, Waters). The mobile phase consisted of ultrapure water (Barnstead Nanopure, Thermo Scientific) and MeOH (HPLC-grade, Thermo Fisher Scientific, Waltham, MA, USA), with 0.1% added formic acid (98 – 100%, Merck KGaA, Darmstadt, Germany), respectively. Positive and negative full-scan MS spectra in the range of 50 – 750 m/z were recorded at a resolution of 140.000 at 200 m/z. Calibration standards for the biotransformation experiments were prepared in periquil and for the QuEChERS experiments in ultrapure $H_2O$ ranging from 5 – 2000 ng/L.

### 2.8. Data processing

Target quantification for the 63 investigated chemicals was done using the software Tracefinder 4.1 (Thermo Scientific). Further data evaluation of experimental data was conducted in RStudio (Version 1.2.5001). Figures of concentration-time series were produced using the package ggplot2 (Wickham 2016). Concentration-time series were used to calculate first-order rate constants ($k_{bio}$) over all replicates simultaneously, assuming pseudo first-order kinetics. $R^2$ was used as quality indicator of the fit. In order to compare biotransformation trends across the investigated chemicals and the four biofilm communities, we level-normalized the $k_{bio}$ for each chemical (van den Berg et al. 2006) and created a heatmap using the R package pheatmap (Kolde 2015). The data used in this manuscript and the corresponding R-scripts are freely accessible at https://doi.org/10.25678/0002J8.

### 2.9. Bacterial biomass quantification by flow cytometry

During the biotransformation experiment, 5 mL samples of the BT batches were taken right before spiking and after 96 h, fixated with 5 mL buffer solution (4% paraformaldehyde + 0.2 % sodium pyrophosphate) in glass vials and stored at 4°C until measurement. Before measuring, the samples were sonicated (4×20 s) by means of a sonication probe, while cooling on ice between cycles. The

samples were diluted with filtered (0.22 µm; Millex-GP, Millipore) EVIAN water to a final dilution of 1000x, stained with 1% SYBR® Green (1:100 dilution in DMSO) and incubated at 37°C for 10 min before measurement. An amount of 50 µL of a 1000 µL sample was then measured on a BD Accuri C6® flow cytometer (BD Accuri, San Jose CA, USA) with the fluidics set on fast and a lower threshold on the green fluorescence (FL1) set at 800. Data processing was done with the BD Accuri™ C6 Analysis software.

## 3. Results and Discussion

### 3.1. Analytical performance

Our selection of MPs for this study included 63 compounds containing a variety of different functional groups and covering different hypothesized initial biotransformation reactions. Five compounds (cetirizine, caffeine, fenofibrate, gemfibrozil, paracetamol) exhibited analytical problems across all sample types and were therefore excluded from further data evaluation. Cetirizine underwent ethylation to form the corresponding carboxylic acid ester in the EtOH spike solution, and was later backtransformed to cetirizine during the course of the experiment. Thus it was not possible to quantify the actual removal of cetirizine. Caffeine, fenofibrate, gemfibrozil and paracetamol all exhibited high matrix suppression, as recognized from low intensities and bad peak shapes in matrix samples compared to signals from calibration rows. In addition, we did not have an isotopically labelled analogue of the analyte gemfibrozil, which made it difficult to distinguish it from multiple isobars with similar retention times. Levetiracetam, ethofumesate and oxcarbazepine showed high matrix suppression exclusively in the sorption controls. These substances were still considered for further data evaluation since evidence from biotransformation experiments was sufficiently clear to be evaluated. Finally, diclofenac had a high LOQ of 20% of the initially spiked concentration, and exhibited considerable noise in the respective chromatograms, but was also considered further.

### 3.2. QuEChERS recoveries

To evaluate the performance of the QuEChERS method for biofilms, absolute and relative recoveries were determined as described in SI section S4. For 50% of the 58 evaluated compounds we found relative recoveries between 80% and 120% (absolute recovery $\geq$ 29%). For another 35% of the compounds, the recoveries were still in an acceptable range (relative recovery between 70% and 130%, absolute recovery $\geq$ 19%; Table S4). A number of compounds showed worse performance in the recovery experiments (i.e., capecitabine, cilastatin, diclofenac, fenhexamid, gabapentin, indomethacin, kresoxim-methyl, mianserin and pheniramine). Recoveries for cilastatin and indomethacin could not be determined because no peaks were detected for those compounds in the QuEChERS extracts. Diclofenac, fenhexamid, gabapentin and kresoxim-methyl displayed very low absolute recoveries, i.e., $<$ 15%, and except for gabapentin, all of them displayed very low matrix factors (i.e., $<$ 20%). Pheniramine was the only compound for which ion enhancement was relevant as can be seen from the high matrix factor (228%) and high absolute recovery (127%). Overall, evaluating the QuEChERS procedure for a large number (n=58) of chemically diverse MPs in biofilm yielded average relative recoveries of 108% ($\pm$ 14% standard deviation) for $>$ 80% of the substances, demonstrating that the QuEChERS method is a broadly applicable extraction method for diverse mixtures of MPs from biofilms. In the following, we employed the QuEChERS extraction method to evaluate mass balances for individual MPs in the biotransformation and sorption control experiments.

### 3.3. Distinguishing biotransformation from sorption and other biological processes

For the 58 compounds, results from abiotic (AC), sorption (SC) and biotransformation (BT) experiments were compared to classify them according to the fate processes they underwent. In order to distinguish biotransformation from any kind of accumulation in the biofilm flocs, we extracted the biomass from the BT and SC experiments at the end of the experiment using QuEChERS.

#### 3.3.1. Abiotically removed compounds

Three compounds, all belonging to the class of strobilurin fungicides, showed $>$ 30% removal in the abiotic controls (AC), i.e., azoxystrobin, pyraclostrobin and trifloxystrobin. Interestingly, these compounds showed less removal in the sorption controls (SC) over the same time period. The three compounds were excluded from further data evaluation, as it could not definitely be concluded whether these compounds underwent abiotic transformations or not. Two additional compounds (i.e. kresoxim-methyl and valsartan) exhibited minor abiotic removal ($<$ 15%), which, however, did not interfere with further analysis, as biological removal in the BT batches was considerably faster than abiotic removal. These two compounds were therefore considered further.

#### 3.3.2. Persistent compounds

Thirteen compounds (2,6-dichlorobenzamide, acesulfame, amisulpride, bromoxynil, candesartan, carbamazepine, carbendazim, clofibric acid, ethofumesate, hydrochlorothiazide, imidacloprid, indomethacin, and lamotrigine) showed a concentration decrease in the aqueous phase of the BT vessels of $<$ 20% in all four of the investigated biofilm communities (Figure S3). Removal of these compounds was too low to yield a statistically significant dissipation signal although in individual cases (e.g., acesulfame in HDown) dissipation seemed observable visually. Lack of statistical significance in those cases was a result of procedural and analytical uncertainties in the order of 10–20% relative standard deviation as obvious from relative standard deviations between experimental replicates at time 0 (FigureS3). Overall, these compounds were therefore considered recalcitrant to biotransformation by the biofilm communities and under the conditions investigated here. In fact, these findings are mostly in line with literature. Acesulfame and carbamazepine, for instance, are considered conservative tracers and have been shown to be persistent in surface waters (Bergheim et al. 2015; Segura et al. 2011; Tixier et al. 2003; Buerge et al. 2009; Li, Sobek, and Radke 2016). The pharmaceuticals candesartan and clofibric acid have been shown to be persistent in streams and lake water, respectively (Schaper et al. 2018; Tixier et al. 2003). The half-lives of hydrochlorothiazide have been shown to be subject to seasonal effects in surface waters, pointing towards phototransformation as the main removal mechanism with biotransformation a minor contributor only (Zou et al. 2015; Baena-Nogueras, Gonzalez-Mazo, and Lara-Martin 2017). As phototransformation was negligible in our experiments, those findings do not contradict the persistence of hydrochlorothiazide in our biofilm experiments. Recently, imidacloprid has been shown to persist in sediment suspensions prepared with lake and river water inocula (Seller et al. 2020) and, for indomethacin, biological degradation in river water systems was shown to be negligible (Jimenez et al. 2017). The fungicide carbendazim was shown to be biotransformed in WWTPs and by complete ammonia-oxidizing bacteria (Kern et al. 2010; Han et al. 2019), but was persistent in freshwater microcosms (Cuppen et al. 2000). The pesticide metabolite 2,6-dichlorobenzamide has been shown to persist in aquatic environments and is regularly found in groundwater samples exceeding maximum allowed concentrations of pesticides and metabolites as defined by the European Commission

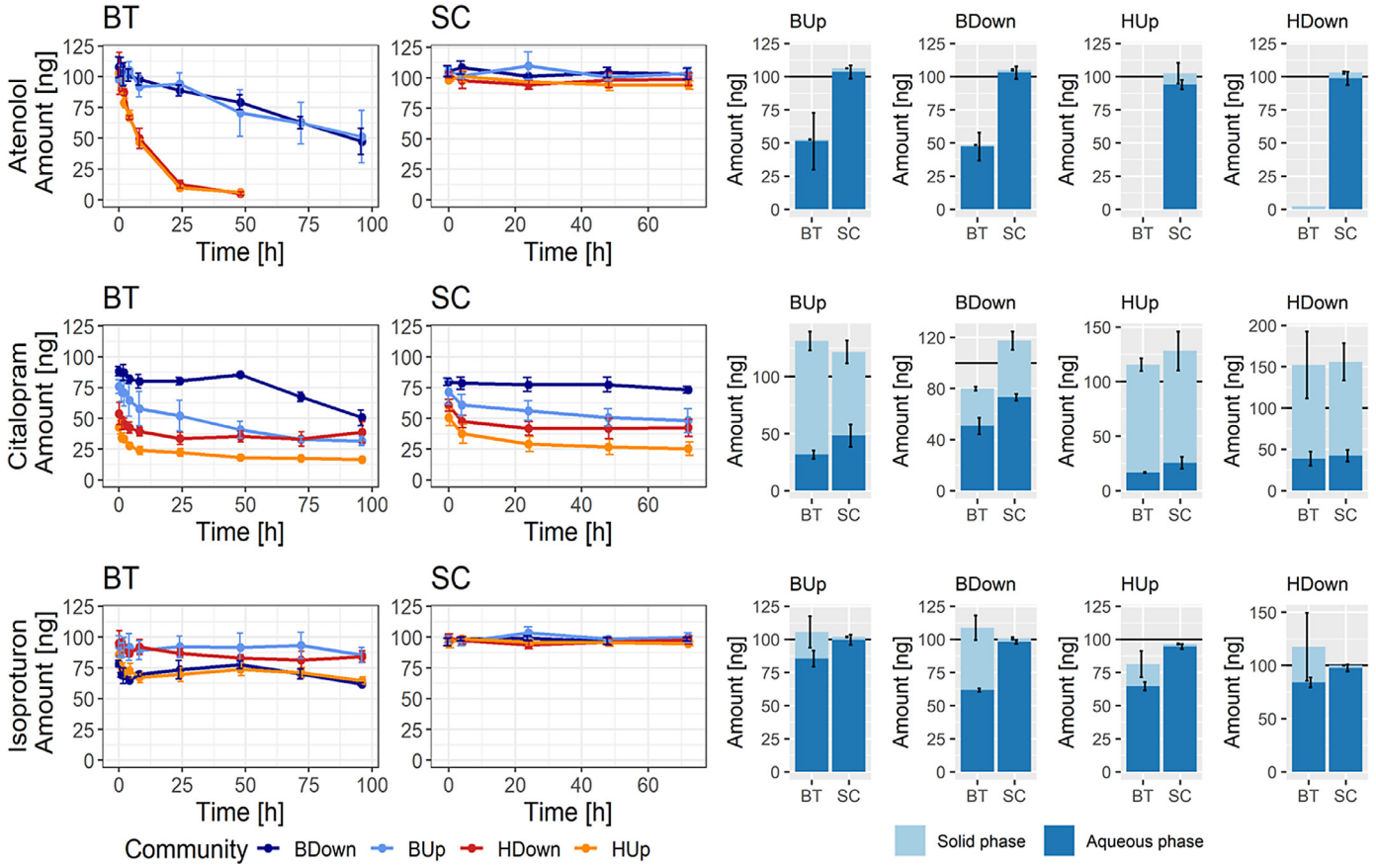


**Fig. 1.** Aqueous phase concentration-time series of the compounds atenolol, citalopram and isoproturon in BT (left) and SC batches (middle), and the corresponding mass balance plots (right) including data from the QuEChERS extraction of the solid phase at the end of the experiment. The black line in the mass balance plots corresponds to the theoretical spiked amount of 100 ng. The error bars in all plots represent standard deviations from triplicate experiments.

(Bjorklund et al., 2011a,b). Likewise, amisulpride and lamotrigine were detected in groundwater and no significant removal was observed in WWTPs (Bollmann et al. 2016), thus it is conceivable that they also persist in surface waters. While the herbicides bromoxynil and ethofumesate have been demonstrated to be biotransformed in WWTPs and wetlands (Achermann, Falas, et al. 2018; Helbling et al. 2012; Degenhardt et al. 2011), but ethofumesate was found to be persistent in arable soils (Laitinen et al. 2006), their fate in rivers remains unknown.

### *3.3.3. Qualitative distinction between biotransformation, sorption and active bioaccumulation*

For the remaining 42 substances, which were not removed abiotically and for which we found a concentration decrease in the aqueous phase of ≥ 20% in at least one of the BT batches, we considered all experimental evidence to qualitatively differentiate between biotransformation, sorption and bioaccumulation (i.e. an active process that can only take place in live biofilms). To this end, we compared the removal in the BT batches with that of the SC batches and took into consideration the extracted amounts from the QuEChERS extraction. These data are compiled exemplarily for three compounds (i.e., atenolol, citalopram and isoproturon) in Fig. 1 and for all other compounds in Figure S4. Atenolol represents the group of compounds that were solely biotransformed and neither sorbed to nor actively accumulated in the biomass as evidenced by (i) a clear concentration decrease in the concentration-time series of the BT batches (48 – 100% removal within 96 h), but not in the SC batches (<5% removal) and (ii) hardly any amount found in the QuEChERS extracts of the solid phase at the end of both the BT (<2%) and SC (<5%) experiments. Citalopram was chosen to represent the group of mostly sorbing compounds. While we found a concentration decrease in the aqueous phase of the BT batches (43 – 61% removal within 96 h), the compound was also attenuated in the SC batches to a similar extent (8 – 50% removal). We were able to recover the amounts removed from the aqueous phase from both the BT and the SC batches in the QuEChERS extracts of the solid phase for all but the BDown community, as is evident from the mass balance plots in Fig. 1 (right panels). This indicates that citalopram was biotransformed in the BDown community, while it only sorbed to the biomass in the other communities. Isoproturon was chosen as an example of a compound that did not sorb in the autoclaved sorption controls, but rather seemed to be removed through accumulation in the live biofilm. Whereas we observed a concentration decrease in the BT batches (8 – 25% removal), no corresponding decrease in the aqueous phase concentrations of the SC batches was visible. However, at the end of the BT experiment, we found that we could recover the removed amounts of isoproturon (29 ± 18%) in the QuEChERS extracts of the solid phase of the BT batches. This indicates that this compound, rather than having been passively sorbed, must have been actively taken up into components of the biofilm. Using the same considerations as discussed in detail above for the three compounds in Fig. 1, we qualitatively assessed each of the 42 substances with respect to the fate processes they underwent. These findings are summarized in Table 1.

As can be seen in Table 1, we identified six other compounds besides citalopram (i.e., fipronil, kresoxim-methyl, mianserin, pheniramine, propranolol and venlafaxine) for which removal of the compound was attributable to sorption, at least in part. Interestingly, with the exception of fipronil and kresoxim-

**Table 1**
Main observed fate processes in biofilms and calculated biotransformation rate constants.

| Compound[a] | Fate process[b] | $k_{bio,BUp}$ [$day^{-1}$] ($R^2$) | $k_{bio,BDown}$ [$day^{-1}$] ($R^2$) | $k_{bio,HUp}$ [$day^{-1}$] ($R^2$) | $k_{bio,HDown}$ [$day^{-1}$] ($R^2$) |
|---|---|---|---|---|---|
| 5-Methyl-benzotriazole | Acc, BT | | | | |
| Acetamiprid | BT | 0.006 (0.01) | 0.013 (0.07) | 0.050 (0.49) | 0.059 (0.57) |
| **Atenolol** | BT | 0.185 (0.65) | 0.191 (0.89) | 1.718 (0.90) | 1.604 (0.95) |
| Benzotriazole | Acc, BT | | | | |
| **Capecitabine** | BT | 0.083 (0.69) | 0.130 (0.81) | 2.555 (0.95) | 1.194 (0.93) |
| **Cilastatin** | BT | 0.386 (0.66) | 0.482 (0.88) | 14.864 (0.97) | 32.951 (0.99) |
| Citalopram | S, BT | | | | |
| Cyclamate | BT | -0.003 (0.01) | 0.006 (0.05) | 0.019 (0.19) | 0.243 (0.88) |
| DEET | BT | 0.003 (0.01) | 0.007 (0.09) | 0.020 (0.22) | 0.240 (0.89) |
| Diclofenac | BT | 0.017 (0.11) | 0.041 (0.29) | 0.111 (0.84) | 0.134 (0.85) |
| Dimethenamid | BT | 0.025 (0.35) | 0.203 (0.77) | 0.062 (0.71) | 0.035 (0.45) |
| Eprosartan | BT* | 0.008 (0.03) | 0.009 (0.05) | 0.069 (0.50) | 0.132 (0.75) |
| **Fenhexamid** | BT | 0.170 (0.83) | 0.265 (0.90) | 0.348 (0.90) | 0.288 (0.93) |
| Fipronil | S, BT | | | | |
| Furosemide | BT | 0.035 (0.50) | 0.049 (0.73) | 0.068 (0.51) | 0.048 (0.45) |
| Gabapentin | BT* | 0.004 (0.01) | 0.015 (0.20) | 0.045 (0.42) | 0.071 (0.60) |
| Iprovalicarb | BT | 0.028 (0.41) | 0.038 (0.39) | 0.05 (0.041) | 0.095 (0.59) |
| Isoproturon | Acc | | | | |
| Ketoprofen | BT | -0.017 (0.07) | -0.025 (0.12) | 0.072 (0.64) | 0.079 (0.61) |
| Kresoxim-methyl | S, BT | | | | |
| Levamisole | BT* | 0.143 (0.53) | 0.286 (0.78) | 0.186 (0.69) | 0.099 (0.67) |
| Levetiracetam | BT§ | 0.018 (0.12) | 0.005 (0.01) | 0.044 (0.35) | 0.224 (0.86) |
| Lidocaine | Acc, BT | | | | |
| Mecoprop | BT* | 0.004 (0.01) | 0.011 (0.06) | 0.044 (0.64) | 0.062 (0.69) |
| Metoprolol | BT | 0.036 (0.29) | 0.125 (0.76) | 0.562 (0.86) | 0.340 (0.90) |
| Mianserin | S, BT | | | | |
| **Oxcarbazepine** | BT§ | 1.357 (0.92) | 2.548 (0.94) | 6.327 (0.96) | 7.999 (0.98) |
| Pheniramine | S, BT | | | | |
| **Propachlor** | BT | 0.817 (0.95) | 2.696 (0.90) | 1.888 (0.92) | 1.147 (0.98) |
| Propranolol | S, BT | | | | |
| **Ranitidine** | BT* | 0.121 (0.85) | 0.122 (0.92) | 0.382 (0.95) | 0.296 (0.91) |
| Rufinamide | BT | 0.029 (0.20) | 0.025 (0.18) | 1.293 (0.98) | 2.561 (0.99) |
| Saccharin | BT | 0.004 (0.02) | 0.020 (0.33) | 0.003 (0.00) | 0.153 (0.91) |
| **Sulfadiazine** | BT | 0.689 (0.89) | 0.794 (0.93) | 0.692 (0.81) | 0.271 (0.70) |
| **Sulfamethazine** | BT | 0.365 (0.56) | 0.637 (0.88) | 0.420 (0.94) | 0.310 (0.80) |
| **Sulfamethoxazole** | BT | 0.738 (0.93) | 1.140 (0.95) | 0.585 (0.94) | 0.384 (0.80) |
| **Sulfapyridine** | BT | 0.399 (0.84) | 0.892 (0.93) | 0.577 (0.95) | 0.372 (0.74) |
| **Sulfathiazole** | BT | 0.576 (0.94) | 0.943 (0.96) | 0.497 (0.96) | 0.429 (0.86) |
| Thiacloprid | BT | 0.005 (0.01) | 0.001 (0.00) | 0.013 (0.02) | 0.057 (0.49) |
| **Trinexapac-ethyl** | BT | 0.226 (0.82) | 0.394 (0.88) | 0.916 (0.94) | 0.666 (0.89) |
| Valsartan | BT | -0.008 (0.01) | 0.031 (0.19) | 0.103 (0.53) | 0.259 (0.80) |
| Venlafaxine | S, BT | | | | |

[a] Compounds in bold were biotransformed in all four of the investigated microbial communities.
[b] Acc = bioaccumulation; BT = Biotransformation; S = Sorption;
* = no QuEChERS data;
§ = no sorption control and QuEChERS data.

methyl, all of these compounds were amine-containing substances. It is also notable that all of these compounds exhibit rather high logP values (>3; Table S1), thus indicating that strong sorption to the biofilms correlates at least in part with hydrophobicity and positive charge of the compounds. A similar correlation between hydrophobicity and the extent of MP accumulation in stream biofilms has been shown before (Bonnineau et al. 2020). For all other compounds, sorption as evident from sorption controls was minor and the majority of removal from the aqueous phase could be attributed to an active biological process. By only considering evidence from the aqueous phase, one might have concluded that all these compounds were biotransformed. However, when comparing amounts recovered in the QuEChERS extracts of the solid phase from BT experiments with those from SC experiments, we found evidence that besides isoproturon, three other compounds (5-methyl-benzotriazol (5.2 ± 4.1% in the BT extracts vs. 3.0 ± 0.7% in the SC extracts), benzotriazol (7.2 ± 4.1% vs. 2.4 ± 0.4%), and lidocaine (7.7 ± 4.1% vs. 2.4 ± 0.5%)) also showed signs of being actively bioaccumulated in the biofilms, yet to a lower extent than isoproturon (29.3 ± 18.3% vs. 2.3 ± 0.6%). In all three cases, and in contrast to the phenylurea herbicide isoproturon, active bioaccumulation seemed to occur in combination with biotransformation. Phenylurea herbicides, e.g., diuron and isoproturon, have been shown before to accumulate in pond biofilms (Chaumet et al. 2019) and microbial biofilms from river sediments (Trinh, Hiscock, and Reid 2012). For the remaining 31 compounds, biotransformation seemed to be the major removal mechanism. However, it needs to be noted that for seven of these compounds (eprosartan, gabapentin, levamisole, levetiracetam, mecoprop, oxcarbazepine, ranitidine) active bioaccumulation could not be excluded, as we were not able to quantify amounts extracted from the solid phase, and for levetiracetam and oxcarbazepine we also cannot rule out sorption as a removal mechanism because we could not acquire aqueous phase concentrations from the sorption controls either.

### 3.3.4. Delayed onset of biotransformation

Another distinct pattern that we observed involved the amine-containing compounds citalopram, levamisol, lidocaine, metoprolol, pheniramine, propranolol and venlafaxine. In Fig. 2a, example concentration-time series from the BT batches for this group are displayed. All of these compounds showed a lag phase of about two days in the BDown community, followed by a concentration decrease that could be attributed to biotransformation as described above. For most of these compounds, ion-trapping in acidic vesicles of protozoa has been shown to be a relevant removal mechanisms in activated sludge (Gulde et al. 2018). As we could not recover the removed amounts from the solid phase of BDown at the end of the biotransformation experiment, we doubt that these compounds have been trapped as previously described, but they rather were biotransformed.

## 3.4. Patterns in biotransformation rate constants

For the 31 compounds that we hypothesize to mostly undergo biotransformation based on the above considerations, we used the aqueous phase concentration-time series of the BT batches to obtain pseudo first-order degradation rate constants. Calculated biotransformation rate constants ($k_{bio}$) for the investigated compounds and biofilm communities and coefficients of determination ($R^2$) of the fits are listed in Table 1. Since $R^2$ describes the extent of variability in concentration between sampling points that can be explained by the factor time, low $R^2$ values coincided with low $k_{bio}$ values, corresponding to low removal (< 20 - 30%) in the respective batches. We identified 13 compounds (i.e., atenolol, capecitabine, cilastatin, fenhexamid, oxcar-

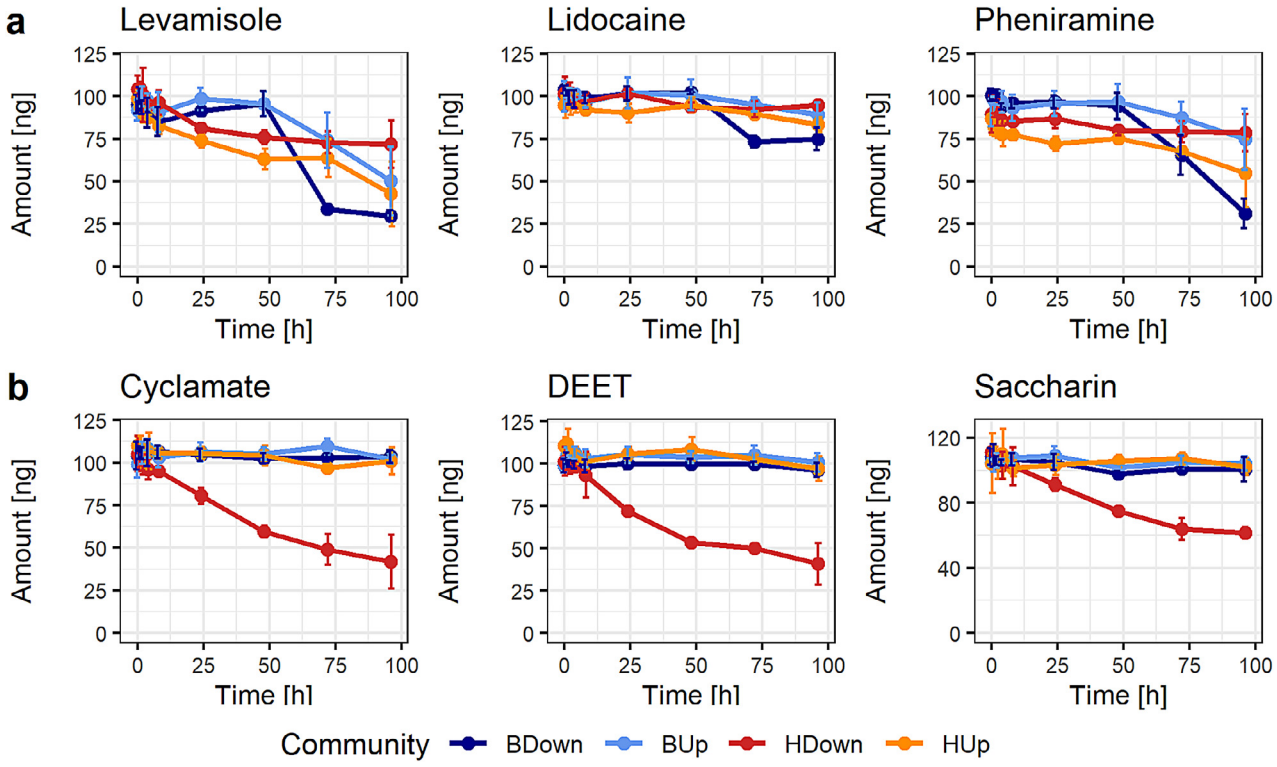


**Fig. 2.** Concentration-time series of a) the amine-containing compounds levamisole, lidocaine and pheniramine that all had a lag phase in the BDown community, followed by biotransformation and b) three selected compounds (cyclamate, DEET, saccharin) that were exclusively biotransformed by the HDown community. Error bars represent the standard deviation of three biological replicates.

bazepine, propachlor, ranitidine, sulfadiazine, sulfamethazine, sulfamethoxazole, sulfapyridine, sulfathiazole, trinexapac-ethyl) that were clearly biotransformed, i.e., $k_{bio}$ values $> 0.055$ day$^{-1}$ (corresponding to a 20% concentration decrease after 4 days), in all four biofilm communities. When comparing with information on biotransformation reactions in activated sludge, it is noteworthy that most of these compounds (with the exception of fenhexamid, ranitidine and oxcarbazepine) had been observed to undergo nucleophilic substitution-type primary biotransformation reactions (i.e., hydrolysis reactions, conjugations). For instance, the sulfonamide antibiotics investigated here have been shown to undergo a variety of substitution reactions in cobble biofilms (Vila-Costa et al. 2017) and pterin-conjugations in activated sludge (Achermann, Bianco, et al. 2018). The primary and secondary amides atenolol and cilastatin, respectively, and the ester trinexapac-ethyl have been found to undergo hydrolysis biotransformation reactions in activated sludge, while the chloroacetanilide propachlor underwent substitution at the C–Cl-moiety, most likely through substitution by glutathione (Achermann, Falas, et al. 2018). The enzymes catalyzing these reactions (i.e., dihydropteroate synthases, amidohydrolases, esterases, glutathione-S-transferases) are key enzymes supporting assimilation, growth and defense processes and are therefore thought to be ubiquitous among different types of bacteria (Johnson et al. 2015). This most likely explains why all of the investigated biofilms seemed to harbor the potential to biotransform these still structurally diverse MPs.

Next, we looked for trends in biotransformation potential across the 31 biotransformed chemicals and the four different biofilm communities. To do so, we clustered the level-normalized rate constants according to their relative transformation behavior across the four sites, resulting in three main clusters across the investigated MPs (Fig. 3). One very distinct cluster contained the two artificial sweeteners cyclamate and saccharin, the insect repellent DEET, the insecticide thiacloprid, and the pharmaceutical levetiracetam. Those five compounds were all exclusively biotransformed by the HDown biofilms, but remained persistent in the other communities (examples shown in Fig. 2b). This finding is consistent with results from pre-experiments (SI section S6) where we even found exclusive biotransformation in both downstream communities (HDown and BDown) for those same compounds. Also, in those pre-experiments, we observed a clear dissipation signal for the artificial sweetener acesulfame in the HDown community, whereas in the data presented here dissipation was visible but not statistically significant. A similar pattern of biotransformation being exclusively observed downstream of WWTP outfalls has recently been reported for acesulfame in sediments (Coll et al., 2020).

Two possible mechanisms might explain why certain compounds are exclusively biotransformed in communities that are impacted to some extent by treated wastewater. First, it is feasible that the microorganisms within the downstream biofilms responded to the selective pressure from the WWTP effluent, leading to changes in the community composition and function, which in turn lead to the observed increased biotransformation potential. Alternatively, wastewater bacteria capable of biotransforming these compounds may be transferred via the WWTP effluents into the downstream communities, leading to the increased biotransformation potential as observed in HDown. Artificial sweeteners, particularly acesulfame, are well-known to be present in high concentrations up to several µg/L in downstream surface waters (Munz et al. 2017; Bergheim et al. 2015), thus potentially serving the downstream biofilms as a carbon source. However, efficient removal of acesulfame, cyclamate and saccharin in WWTPs has also been shown (Buerge et al. 2009; Castronovo et al. 2017; Li et al. 2018), suggesting that WWTP bacteria are well capable to biotransform these compounds. It has already been demonstrated that antimicrobial resistance genes can be transferred from WWTPs into stream biofilms (Marti, Jofre, and Balcazar 2013;

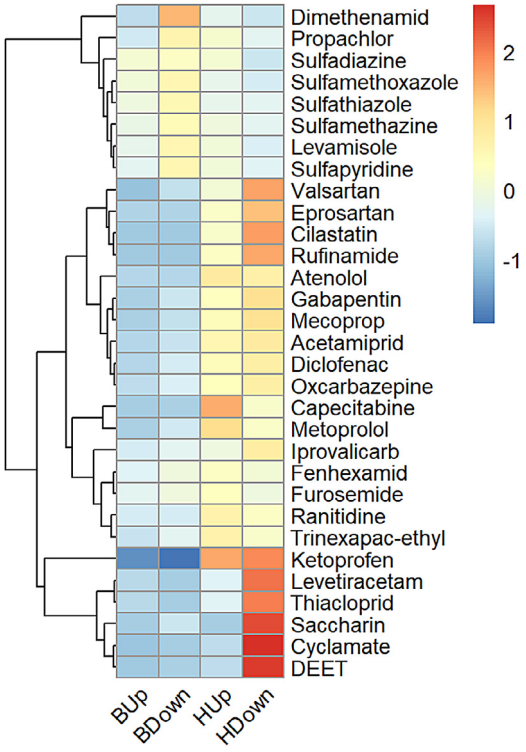


**Fig. 3.** Heatmap displaying the biotransformation rate constants of the 31 compounds. Rate constants have been level-scaled. Clustering was done by rows. Blue represents slower, while red represents relatively faster biotransformation.

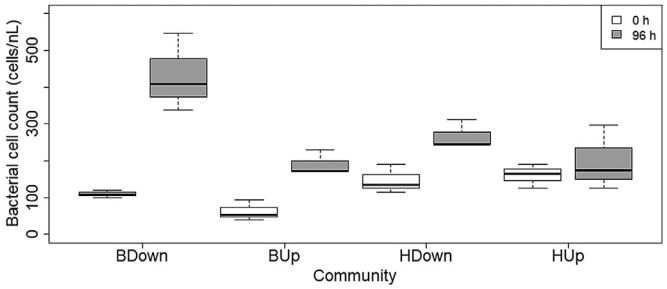


**Fig. 4.** Bacterial cell counts for the four investigated microbial communities from the BT batches (n = 3) at the beginning (0 h) and at the end of the experiment (96 h). Box sizes correspond to the first and third quartiles (25% and 75% quartiles); whiskers extend to ± 1.5 times the interquartile range.

Proia et al. 2016; Subirats et al. 2017), and it is thus also conceivable that bacteria capable to biotransform MPs or their genes can be released from WWTPs and manifest in downstream biofilms.

We also note that the phenomenon of increased biotransformation potential at downstream sites was not fully consistent across our experiments. While it was observed at both downstream sites in the pre-experiment, it was only observed at HDown in the main experiment. Also, one compound, acesulfame, only showed a clear signature in the pre-experiments, but not in the main experiments. The variability in these observations could be due to various factors, including different environmental conditions (e.g., temperature, solar irradiation or discharge) or changes in operational conditions in the WWTP. The operators of WWTP Buttisholz confirmed that no fundamental changes in the operation of the WWTP were made between the two experimental periods. Hence, this possibility can be ruled out. Further research will be needed to understand the variability of the observed downstream phenomenon and the underlying mechanisms. In particular, information on the biotransformation potential of the activated sludge microbial community of the respective WWTPs and the load of microorganisms released by the WWTP in combination with taxonomic information, e.g., from 16S rRNA amplicon sequencing, could possibly help to elucidate this phenomenon.

The second and largest cluster contained 18 compounds (e.g., atenolol, trinexapac-ethyl, rufinamide) that showed generally faster biotransformation in the Hochdorf communities (HUp and HDown) compared to the Buttisholz communities (BUp and BDown). Even though contamination patterns between up- and downstream sites have been shown to differ (Munz et al. 2017), no significant differences in contamination patterns were found between Hochdorf and Buttisholz that would explain this finding. Thus, pre-exposure to higher concentrations may not be the cause of this phenomenon. However, one distinguishing feature between the communities from those two sites is that bacterial cell counts at the beginning of the experiment were higher by a factor of 2-3 in the Hochdorf communities compared to the Buttisholz communities (Fig. 4). Higher biomass might explain the increased biotransformation potential at Hochdorf to some extent, considering that we have evidence from previous work with activated sludge that the transformation potential for most of these compounds is widespread among bacteria (Achermann, Falas, et al. 2018). It may also be the case that higher initial cell numbers lead to an overall higher bacterial diversity (Jaeger et al. 2019), thus increasing the likelihood of rarer species to be present, and adding to the biotransformation potential of the biofilms (Johnson et al. 2015).

The third cluster that we identified contained eight compounds (i.e., dimethenamid, propachlor, sulfadiazine, sulfamethoxazole, sulfathiazole, sulfamethazine, sulfapyridine, levamisol) that were biotransformed fastest, but not exclusively, in the BDown community. Interestingly, all five sulfonamide antibiotics and both chloroacetanilides that we investigated were found in this cluster. The sulfonamide antibiotics have been previously described to follow a pterin-sulfonamide conjugation pathway in activated sludge (Achermann, Bianco, et al. 2018), which is catalyzed by an enzyme (i.e., dihydropteroate synthase) that is part of the folate synthesis pathway. As folic acid is essential for protein and nucleic acid synthesis, the enzymes involved in this pathway are ubiquitous among bacteria and inherently linked to growth. Initial biotransformation reactions of the chloroacetanilides (i.e., dimethenamid, propachlor) have been shown to mostly occur via glutathione-S-transferase-mediated conjugations (Singh and Singh 2016; Achermann, Falas, et al. 2018). The primary role of these enzymes is detoxification and they are upregulated in response to oxidative stress. From Fig. 4 it can be seen that bacterial numbers increased most strongly between 0 and 96 hours in the BDown community, resulting in highest cell numbers at 96 hours. Neglecting potential differences in cell decay rates, it can therefore be assumed that bacterial growth rates were highest in BDown. While we do not fully understand the reasons behind the high growth rates at BDown, this site has the highest native nutrient and COD contents (Table S3). It could thus be speculated that the BDown community was best adapted to the nutrient rich medium used in the biotransformation experiments. This would potentially explain the increased biotransformation potential for the sulfonamides and chloroacetanilides for which the enzymes assumed to be responsible for their initial biotransformation reaction are known to be intimately linked to cell growth and defense against oxidative stress (Achermann, Bianco, et al. 2018; Roland et al. 1979; Achermann, Falas, et al. 2018; Singh and Singh 2016). However, to further support these conclusions, additional experiments with varying amounts of initial biomass and analysis of transformation product formation would be required.

### 3.5. Environmental relevance and implications

Overall, we presented in this study, for the first time, a complete mass balance procedure that can be used to study the fate of a large number of structurally diverse MPs in biofilms. Particularly, we demonstrated that the QuEChERS extraction procedure allowed satisfactory recovery for >80% of the MPs investigated. This not only allows assessing the role of river biofilms in decontaminating surface waters from xenobiotic substances, but also provides new evidence and opportunities to further investigate the potential use of river biofilms for effect-based monitoring and as bioassay in chemical risk assessment.

We found that the extent of passive sorption to biofilms mostly depends on the compounds' hydrophobicity and charge, with a strong preference for sorption of positively charged compounds (i.e., amine-containing compounds). This is qualitatively consistent with sorption to other sediment organic materials, but would require more quantitative studies to, e.g., evaluate the suitability of river biofilms as "natural" passive samplers (Writer, Ryan, and Barber 2011). This is even more so since we also observed signs of active bioaccumulation of specific substances. Further exploration of this phenomenon would require a more spatially and temporally resolved analysis of individual biofilm components to understand the dynamics and define compartments where the active accumulation takes place. For the herbicide isoproturon, specifically, it would be particularly important to see whether it accumulates into the photoautotrophic members, where it might exert specific toxic effects.

This was the first study investigating the biotransformation potential of stream biofilms involving a large number of MPs at environmentally relevant concentrations. We found that roughly half of the 63 compounds investigated were biotransformed to some extent by the four biofilm microbial communities. However, observed biotransformation reactions mostly corresponded to substitution-type reactions most likely catalyzed by a limited number of enzymes that are ubiquitous among different types of bacteria because they support central metabolic processes. The biotransformation rate constants of MPs undergoing those transformations accordingly scaled with either bacterial biomass or biomass growth as quantified with flow cytometry. More specialized transformations of a number of MPs, including the artificial sweeteners studied, was only observed for one of the two downstream communities in this study (with some evidence from pre-experiments that transformation of the same compounds takes place more generally in downstream communities). This suggests that the treated effluents of wastewater treatment plants tend to increase not only the pollution-induced community tolerance (PICT) of downstream biofilms, as demonstrated previously (Tlili et al. 2017), but also their biotransformation capacity for certain MPs. While, in the case of PICT and contaminant transformation, these effects might be considered positive as they point towards lowered risk from chemical exposure, the findings also raise the question what other biofilm functions are affected by the treated effluents and whether all of these effects can be considered positive from an ecosystem health perspective. To address this ecologically highly relevant question, more controlled experiments are warranted that allow differentiating the underlying mechanisms (i.e., in-stream adaptation to chemical exposure or import of biological material from WWTPs) and to clarify how far downstream of the WWTP the observed effects actually extend.

## 4. Conclusion

In this study, we collected biofilms at two field study sites and used a combination of batch experiments, chemical-analytical and biological methods methods to investigate the micropollutant biotransformation potential of natural stream biofilms. The major conclusions of this study are:

- The QuEChERS extraction method proved to be a broadly applicable extraction method for a diverse set of micropollutants, even for a highly complex and biologically heterogeneous matrix such as biofilm
- Natural stream biofilms were found to possess a broad inherent micropollutant biotransformation potential (i.e. 26 out of 59 investigated MPs were biotransformed in several biofilm communities), thus contributing to bioremediation and improving ecosystem health of natural surface waters
- Through complete mass balance analysis, active bioaccumulation into biofilms could be demonstrated for a number of compounds
- Input of treated wastewater clearly changed the micropollutant biotransformation potential of downstream biofilms, and hence their ecological function, resulting in increased biotransformation for a number of compounds, particularly artificial sweeteners

## Declaration of Competing Interest

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

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.116846.

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