Environmental Science & Technology

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Influence of Different Sewer Biofilms on Transformation Rates of Drugs

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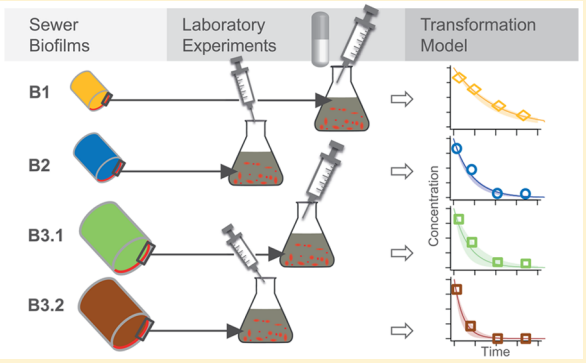
Supporting Information

ABSTRACT: To estimate drug consumption more reliably, wastewater-based epidemiology would benefit from a better understanding of drug residue stability during in-sewer transport. We conducted batch experiments with real, fresh wastewater and sewer biofilms. Experimental conditions mimic small to medium-sized gravity sewers with a relevant ratio of biofilm surface area to wastewater volume (33 m^2 m^{-3}). The influences of biological, chemical, and physical processes on the transformation of 30 illicit drug and pharmaceutical residues were quantified. Rates varied among locations and over time. Three substances were not stable—that is, >20% transformation, mainly due to biological processes—at least for one type of tested biofilm for a residence time ≤2 h: amphetamine, 6-acetylcodeine, and 6-monoacetylmorphine. Cocaine, ecgonine methyl ester, norcocaine, cocaethylene, and mephedrone were mainly transformed by chemical hydrolysis and, hence, also unstable in sewers. In contrast, ketamine, norketamine, *O*-desmethyltramadol, diclofenac, carbamazepine, and methoxetamine were not substantially affected by in-sewer processes under all tested conditions and residence times up to 12 h. Our transformation rates include careful quantification of uncertainty and can be used to identify situations in which specific compounds are not stable. This will improve accuracy and uncertainty estimates of drug consumption when applied to the back-calculation.



INTRODUCTION

Wastewater-based epidemiology (WBE) is an increasingly applied approach to estimate drug consumption at municipality and city levels.[1] Ideally, results are communicated with objective credible intervals to allow meaningful interpretation, for example, illicit drug use compared to other indicators, such as population surveys. Excreted drug residues (subsequently referred to as *biomarkers*) enter the sewer networks through toilets and are transported for periods of minutes to several hours before collection at a wastewater treatment plant, typically in a 24 h composite sample. The average daily per capita drug consumption is estimated from the sample concentration, considering contributing population, wastewater volume, drug-specific pharmacokinetic excretion rates, purity of the drugs, and potential instability of biomarkers.[2,3] Each of these factors contributes to the overall uncertainty of these drug use estimates.[4] Recent research efforts have addressed uncertainties related to population,[5−7] pharmacokinetic correction factors,[8] sampling,[9] and sample preparation.[10] Furthermore, it has been shown that some biomarkers can be transformed in sewers, which requires consideration in the back-calculation.[11,12] The extent of biomarker transformation is potentially variable, since each sewer network is unique.

Sewers not only transport rain and wastewater; they are also biochemical reactors comprising four compartments: (i) bulk

Received: August 21, 2016
Revised: November 14, 2016
Accepted: November 14, 2016
Published: November 14, 2016

DOI: 10.1021/acs.est.6b04200
Environ. Sci. Technol. 2016, 50, 13351−13360

liquid (including suspended particulate matter (SPM)), (ii) sediment, (iii) atmosphere (for gravity sewers), and (iv) biofilm growing on the submerged sewer walls. Many wastewater constituents (e.g., ammonia and volatile fatty acids) that are mostly primary substrates for microorganisms have previously been shown to undergo transformation during in-sewer transport.[13] Sewer conditions such as flow rates, shear forces, and chemical composition of the wastewater (e.g., pH, temperature, dissolved oxygen, and ammonium and nitrite macronutrients) influence these transformations. These conditions are subject to variations both temporally (short-term, diurnal, seasonal, annual) and spatially (within one catchment and among different catchments).[14,15] The physical, chemical, and biological compositions of biofilms tend to adapt to this environmental variability. Biofilms can contribute substantially to in-sewer transformation processes,[13] since they are highly reactive ecosystems composed of mostly heterotrophic biomass that can be as active, and high in ATP content, as activated sludge.[16,17]

Most studies investigating the in-sewer stability of drugs, only considered bulk liquid, including SPM, when quantifying possible transformation.[11] Furthermore, the effect of different redox conditions and sorption to SPM was studied recently.[18] However, two pioneer studies performing measurements in sewers or laboratory experiments *with* biofilm have shown an increased loss of pharmaceuticals and illicit drugs during in-sewer transport.[12,19] Biomarker transformation varied by sewer type, with lower stability in gravity sewers compared to anaerobic raising mains (pressurized pipes).[12] These two studies have been conducted using a single time-point in a specific laboratory reactor[12] and a localized real sewer stretch.[19] From the vast number (>100) of studies on micropollutant removal in wastewater treatment, it is evident that removal rates can vary over a wide range for different substance classes, for different processes, and even for the same individual compound in different studies.[20,21] Based on this evidence we found it important to systematically investigate the effect of spatial and temporal variability of sewer conditions and wastewater composition on the reproducibility of transformation rates for illicit drugs.

Consequently, our main focus was to study *different* sewer biofilms and their effect on transformation of biomarkers. We hypothesized that (a) the presence of different biofilms increases transformation rates of illicit drugs in sewer systems to varying extents and (b) a first-order transformation model, differentiating biological, chemical, and physical processes, can sufficiently describe the observations and predict in-sewer transformations. To evaluate these hypotheses the subsequent approach was followed:

(i) Investigate spatial differences of transformation rates (collect biofilms from different sewers);
(ii) Assess temporal variability of transformation rates (repeat selected experiments at different times of the year);
(iii) Characterize the microbial communities of the different biofilms (perform high-throughput 16s rRNA gene-targeted amplicon sequencing);
(iv) Interpret results in an appropriate and rigorous statistical framework (develop a model which allows deriving objective credible intervals and generalize results).

MATERIALS AND METHODS

Substances. We selected biomarkers for commonly consumed illicit drugs, pharmaceuticals, and metabolites: amphetamine (AMP), cocaine (COC), benzoylecgonine (BE), benzoylecgonine-D3 (BED3), cocaethylene (COE), ecgonine methyl ester (EME), norcocaine (NorCOC), (±)-3,4-methylenedioxymethamphetamine (MDMA), 4-hydroxy-3-methoxymethamphetamine (HMMA), methylenedioxypyrovalerone (MDPV), mephedrone (MEPH), methamphetamine (METH), methiopropamine (MPA), methoxetamine (MTO), 4-methoxyamphetamine (PMA), 4-methoxymethamphetamine (PMMA), 6-monoacetylmorphine (MAM), morphine (MOR), 6-acetylcodeine (AC), codeine (COD), methadone (MTD), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), tramadol (TRA), O-desmethyltramadol (ODMT), zolpidem (ZOL), carbamazepine (CBZ), diclofenac (DCF), caffeine (CAF), ketamine (KET), and norketamine (NorKET). Detailed information on the chemicals (standards and internal standards) and preparation of the mixtures is included in the Supporting Information (SI). The selected biomarkers were spiked at environmentally relevant concentrations (upstream in a catchment they are expected to be higher than further downstream or in a composite sample), which were sufficiently high for analytical quantification. In experiment E1 and E2 the nominal concentration in each reactor was 3000 ng L^{-1} and in experiments E3−E6 2000 ng L^{-1}.

Sewer Biofilms. Our experimental setup included the collection of biofilms from real sewer pipes and investigating their potential to transform biomarkers in batch reactors (in suspension). The advantage of using this approach is its efficiency in accounting for a wide variety of biofilms grown under real conditions. This facilitates the comprehensive investigation of transformation potentials with a focus on the natural variability within single and different sewers. A 50 cm^2 area of biofilm was collected from below the water level in three different gravity sewers at four locations in Zurich, Switzerland (see SI for details about these sewers). Biofilm was scraped off the sewer walls with a plastic spatula directly below the water level. The cohesive biofilm facilitated collection with no substantial losses to the wastewater stream. Samples were transported in plastic containers on ice and suspended to batch reactors within 5 h. Biofilm B1 was taken from a small sewer pipe (diameter 0.5 m) connecting an upstream residential area to a downstream trunk sewer (diameter 1.6 m; total population approximately 25 000) where biofilm B2 was collected. Biofilms B3.1 and B3.2 were taken from the main transport sewer "Glattstollen" with no lateral confluents and neither in- nor exfiltration (B3.1 at the beginning, B3.2 5 km downstream; diameter 1.1 m, for more details see Kaegi et al., 2013[22]). This sewer consists of two parallel pipes that, during dry weather, are operated intermittently (daily switch), implying that the biofilm does not dry out. Biofilm samples were taken from the pipe approximately 8 h after the wastewater flow was diverted to the other pipe.

Laboratory Biofilm. Intact laboratory biofilm (LB_{intact}) grew in a Couette−Taylor reactor (CTR) with a continuous flow through of fresh wastewater, which is pumped out of a real sewer (population in catchment approximately 20 000) to feed the Eawag pilot wastewater treatment plant. The general setup was modified from Derlon et al. (2013),[23] without the recycling loop and aeration chamber. Biofilm was exposed to an average

DOI: 10.1021/acs.est.6b04200
Environ. Sci. Technol. 2016, 50, 13351−13360

shear stress of 1.2 Pa (literature values ranging from 0.33 to 2.86 Pa[24]) and grew in the dark inside the outer wall of the reactor (surface area 0.117 m^2). No inoculum was used, and during the two-month growth phase, oxygen in the bulk liquid varied from 0.2 to 1 mg L^{-1}.

Transformation Test System. The general setup was adapted from the OECD international testing guidelines (test 314) by additionally including biofilms. The biofilms were weighed, homogenized, transferred to 5 L Erlenmeyer flasks, and filled up to 1.5 L with *fresh* wastewater (<1 h after collection). The wastewater was warmed to room temperature (21 ± 1 °C), and all environmental parameters were measured regularly (pH, dissolved oxygen, redox potential, conductivity, macronutrients; see SI). To set up a positive control, 200 mL of activated sludge from the Eawag pilot plant were centrifuged, and solids were resuspended in 1.5 L of wastewater to a final concentration of 0.5 g L^{-1} volatile suspended solids (VSS). As further controls, one reactor with wastewater alone (0.2 g L^{-1} VSS) and abiotic reactors with (i) doubly deionized water (DDI; Millipore, 18.2 MΩ cm), (ii) tap water (no chlorine present in our lab's tap water), or (iii) autoclaved and filtered (GF-5 0.45 μm, MN) wastewater (acWW) were run in parallel. The batch reactors with suspended biofilms (0.5−3.7 g L^{-1} VSS) were operated with a biofilm mass that is equivalent to a ratio of *intact biofilm surface area* to *wastewater volume* (A/V) in a real sewer of approximately 33 m^2 m^{-3}, which is a realistic estimate. In an additional experiment, the influence of different A/V ratios on transformation rates was investigated by testing B2 with A/V ratios of 17 m^2 m^{-3} and 67 m^2 m^{-3} by doubling and halving the collected biofilm area added to the 1.5 L reactor volume.

Since several different biofilms were tested, some repeatedly throughout a year, six experiments were necessary and could not be conducted at one point in time. Therefore, for each experiment, a fresh grab sample of wastewater had to be used. Over 24 h, the reactors were shaken in the dark at 50−90 rpm.

Transformation of LB_{intact} was tested in the CTR similarly to the previously described setup and with an A/V of 76 m^2 m^{-3}. A grab sample of 2.2 L of wastewater was filled into the reactor, and the same shear stress as under the growth conditions was applied. Two days later the same laboratory biofilm was tested suspended in 2.2 L of another fresh wastewater (E6) grab sample.

Sampling of Liquid Phase. Samples for analysis of biomarkers were taken 15 min before spiking (quantification of background concentration), 2−5 min after spiking (start conditions C_0 after full mixing), and 10 times thereafter at 1/4, 1/2, 1, 2, 4, 6, 8, 12, 16, and 24 h. Using a 20 mL plastic pipet, a sample of 5 mL was extracted and transferred to a 15 mL polypropylene centrifuge tube. To preserve the sample and stop biological activity instantly, the samples were immediately flash frozen in liquid nitrogen and subsequently stored at −20 °C until analysis within 10 days.

Chemical Analysis. After thawing, samples were centrifuged (9000 g) for 6 min, and 1.2 mL of supernatant was immediately filtered (0.2 μm Whatman PTFE syringe filter, Primo 1 mL syringe). The first 0.2 mL of filtrate was discarded to equilibrate the filter. Additionally, a filter test was performed with biomarkers spiked in DDI water to a final concentration of 1000 ng L^{-1}. Sample analysis was performed with LC-MS/MS involving a Dionex UltiMate 3000 high-performance liquid chromatography (HPLC) system coupled to an Applied Biosystems 5500 QTrap linear ion trap triple quadrupole mass spectrometer (Sciex, Darmstadt/Germany) with Analyst software (Version 1.6.2). Analytical method accuracy and precision, relative recoveries, method validation, sorption experiments, and further information are listed in the SI.

Estimation of Kinetic Parameters. We adapted a first-order kinetics micropollutant transformation model[25,26] to describe and compare the processes occurring in the sewer. The initial aqueous biomarker concentration C_0 evolves over time t due to chemical (abiotic) and biological transformations:

$$C_{aq}(t) = C_0 \exp\left[-t\left(k_a + k_{WW} + k_{biofilm}\frac{A}{V}\right)\right] \quad (1)$$

where k_a is the abiotic transformation rate constant, k_{WW} is the biotransformation rate constant in wastewater including the effect of suspended particles, and $k_{biofilm}$ is the biotransformation of the biofilm. The biomass in the system is assumed to be in steady state, and growth over the 24 h experiment is negligible (COD balance resulted in less than 10% growth of heterotrophic biomass). The ratio of the biofilm surface area A and the water volume V normalizes $k_{biofilm}$ so that the rate can be applied to different sewer geometries (diameters and fill levels). For every location (biofilms B1, B2, B3.1, B3.2) and the six wastewater grab samples $WW_{i=1\cdots6}$ individual kinetic rates were estimated. Also, the initial concentration C_0 was inferred separately for every single reactor to account for varying spike levels and background concentrations. For experiments without biofilm, the area A was set to zero. A model based on eq 1 (see SI for detailed description) was calibrated involving data from all experiments to allow for simultaneous estimation of all rates despite variability among different experiments.

Data preparation and visualization were performed with R,[27] and the actual model was implemented in JAGS 3.4.0.[28] For inference, five independent Markov chain Monte Carlo chains were generated with 60 000 samples each. The first 10 000 samples were discarded as "burn-in", and every tenth sample was saved for analysis. Each chain was inspected for convergence. Based on the samples, mean and 10% and 90% quantiles were calculated.

Molecular and Numerical Analyses of Microbial Community Compositions. The microbial community compositions of sewer biofilms were analyzed to investigate relationships with the observed transformation rates. Thereto, 16S and 18S rRNA gene-based amplicon sequencing with some adaptations to the MiDAS field guide for activated sludge was used.[29,30] Genomic DNA (gDNA) was extracted from the homogenized biomass samples by bead-beating in four series of 20 s each and purified using the FastDNA SPIN Kit for Soils (MP Biomedicals, USA). The purified gDNA extracts were diluted to 20 ng mL^{-1} and sent to Research and Testing Laboratory (Lubbock, TX, USA) for amplicon sequencing (MiSeq Illumina). Further details about primers, PCR, and applied rarefication procedure can be found in the SI. The multivariate numerical analyses of relationships among biomarker transformation rates and microbial community compositions followed the workflow proposed by Weissbrodt et al., 2014.[30,31] Ordination techniques using nonmetric dimensional scaling (NMDS; accounts for any similarity distance) and principal component analysis (PCA; accounts for Euclidean distances only)[32] were computed in R with adaptations to the ampvis package[30] to represent the extent of dissimilarities between the compositions of the microbial communities measured by amplicon sequencing from each

DOI: 10.1021/acs.est.6b04200
Environ. Sci. Technol. 2016, 50, 13351−13360

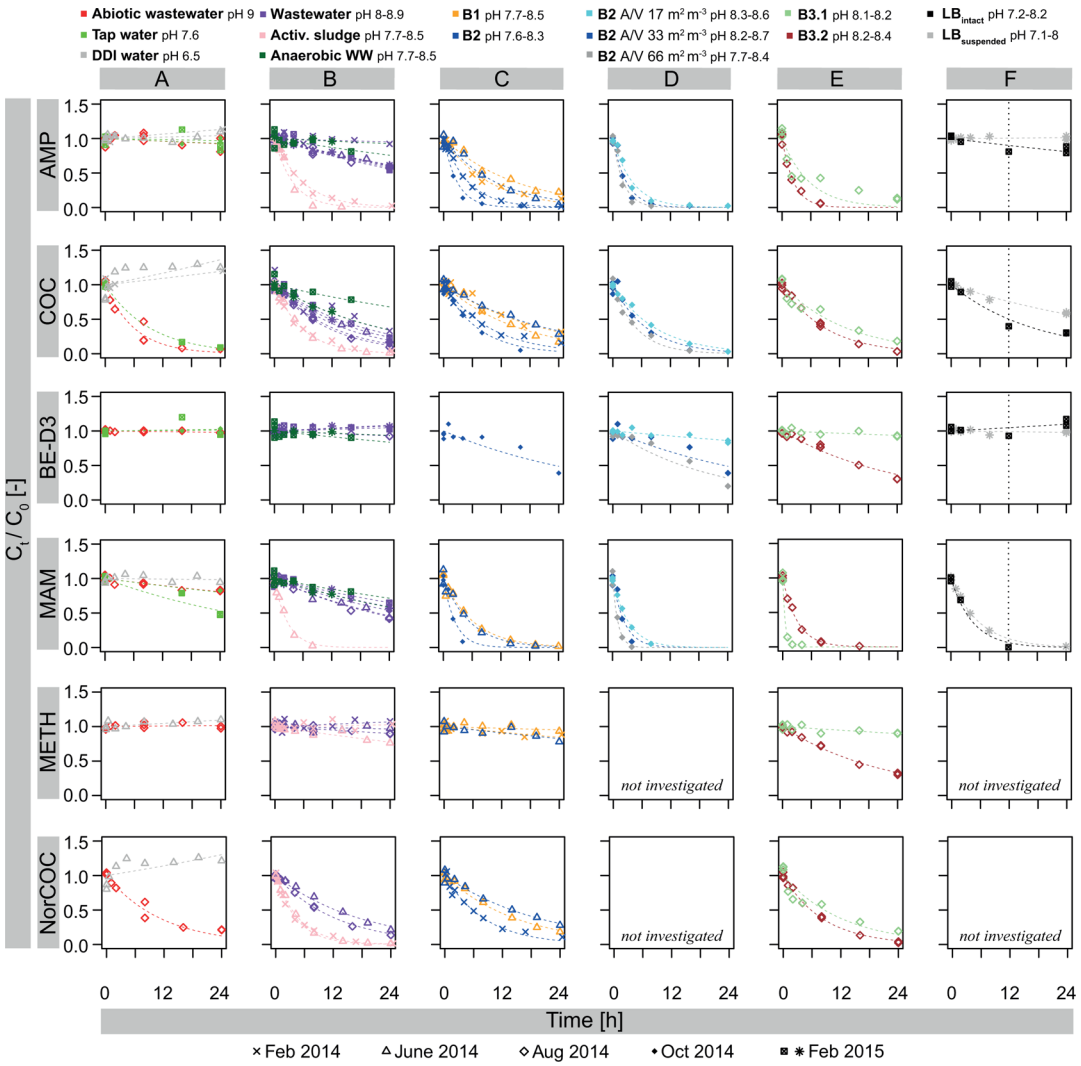


Figure 1. Time series from all experiments with (A) autoclaved and filtered wastewater, tap water, doubly deionized water (DDI), (B) wastewater tested under aerobic and anaerobic conditions, and activated sludge (aerobically tested), and (C–E) different biofilms B1, B2 (A/V ratios 17, 33, and 66 $m^2\ m^{-3}$), B3.1 and B3.2. A/V ratios in the reactors with biofilms were at 33 $m^2\ m^{-3}$, if not otherwise indicated. (F) Laboratory grown biofilm tested intact and in suspended form. Different symbols indicate the different experiments conducted in February, June, August, October 2014, and February 2015. Lines show the fit of a first order model and are added only to visually support readability. Time series for all other biomarkers can be found in the Supporting Information. Not all conditions were tested for all biomarkers. Please see the online edition for a color version.

biofilm and wastewater ecosystem. A PCA biplot was then performed to assess whether differences in biomarker degradation patterns may link to differences in microbial community compositions and their diversity index, as well as in overall aerobic microbial activity (measured as OUR). The base input files consisted of (i) the matrix of operational taxonomic units (OTUs) detected in the amplicon sequencing data sets of each biofilm and wastewater sample and (ii) the matrix of biomarker transformation rates.

RESULTS AND DISCUSSION

Transformation Mechanisms. In the 24 h laboratory batch experiments, the concentrations of biomarkers changed to varying degrees due to chemical, biological, and physical processes that are discussed subsequently. The different configurations with wastewater only and wastewater plus biofilm enabled the differentiation between biological transformation caused by SPM and biofilm. Additional abiotic and sorption experiments allowed distinguishing chemical and physical transformations from biological processes. Physical sorption of the drugs to suspended particles and/or biofilms played a minor role, as literature data and results from the sorption study suggested (see detailed discussion in the SI). Therefore, sorption was assumed negligible for the compounds investigated.

Abiotic Chemical Transformations. Results from the different abiotic control experiments showed chemical transformation in autoclaved wastewater (acWW) and tap water (tap) for MEPH, COC, EME, NorCOC, and COE. No transformation occurred in DDI water, presumably due to lower pH 6.5 in DDI (Figure 1A and Figure S8 in SI for all other biomarkers). The estimated first order constant k_a for COC in DDI water was the same as previously measured in Milli-Q water at pH 5.7 (0.001 h^{-1}).[33] It is known that biomarkers with alkyl esters can hydrolyze.[11,33−35] Chemical hydrolysis is pH-dependent which explains the observed variability in transformation rates. In previous studies with no abiotic controls, this abiotic biomarker loss was erroneously attributed to biological transformations.

DOI: 10.1021/acs.est.6b04200
Environ. Sci. Technol. 2016, 50, 13351−13360

Transformation in Wastewater. Biological transformation in wastewater occurred for AMP, MAM, AC, and ZOL (Figure 1B). Transformation rates in wastewater were very similar under aerobic and anaerobic conditions, which contrasts with one other study.[18] Trying to identify reasons based on the meta data available for this small number of studies seems purely speculative at this stage. Variability of loss of biomarker in the repeated experiments with aerobic wastewater, i.e., different grab samples of wastewater, was within 40%, including measurement uncertainty. Previous studies in wastewater without biofilm also revealed varying degrees of biomarker losses.[11] For comparison, removal rates of micropollutants in activated sludge in different wastewater treatment plants exhibit equally high or even higher biological variability.[36] Biomarkers that transformed under abiotic conditions exhibited no quantifiable additional biotic transformation in wastewater (Figure 1A and B). Interestingly, the transformation of COC, EME, COE, and NorCOC was lower in wastewater than in the abiotic controls.

Influence of Biofilms. The biotransformation of AMP, MAM, and AC increased with biofilms compared to rates in wastewater alone (Figure 1, Figure S8). Rates for biomarkers that were transformed due to hydrolysis (abiotic) were unaffected by the presence of biofilm (e.g., COC, NorCOC).

It is known that an increasing active biomass in a system increases the conversion rates of macromolecules (primary substrate). As hypothesized, transformation rates of biomarkers that are transformed biologically increased when biofilm biomass was added. Similarly, Thai et al. (2014) measured three times higher transformation rates of MAM in their reactor mimicking a gravity sewer with biofilm compared to rates in wastewater without biofilm.[12]

Falas et al. (2013) found an overall increased removal with biofilm grown on carriers compared to suspended particles in aerobic wastewater treatment reactors.[37] Furthermore, they observed removal for DCF only when biofilm carriers were present (>10 days solids retention time;[38] allowing slow growing organisms to establish themselves in the biofilm), concluding that the biofilm biomass composition and redox conditions influenced this biomarker-specific transformation. In our study, some of the biomarkers (BED3, MDMA, HMMA, METH, MPA, PMA, and PMMA) that were stable in wastewater, were not stable over the 24 h time frame when specific biofilms (B2 and/or B3.2) were present (Figure 1C–E). However, biofilms B1 and B3.1 did not lead to additional transformation compared to wastewater alone. Biofilm B3.2 was taken 5 km downstream in the same sewer pipe as B3.1 (no confluents between B3.1 and B3.2), and transformation rates appeared to be already distinctly different [e.g., BED3, METH, MPA, PMA, and PMMA (Figure 1 and Figure S8)]. This was most likely due to different environmental conditions (B3.1 aerobic and B3.2 anaerobic) and altered wastewater compositions at the two locations. This resulted in divergent growth conditions for the biofilms and, subsequently, different transformation potentials (see Table S3 for detailed information about growth conditions).

The observed biotransformation may be driven by the specific biofilm biomass and, therefore, should also raise with increasing amounts (increasing surface area) of that biomass. Biofilm-mediated biotransformation rates indeed amplified with increasing A/V ratios from 17 m^2 m^{-3} to 66 m^2 m^{-3} (Figure 1D). Testing three different A/V ratios with the same biofilm on the same day resulted in very similar transformation rates when normalizing with corresponding biofilm surface areas. Therefore, the rate coefficient for biofilm ($k_{biofilm}$) in the sewer transformation model (eq 1) was normalized with the A/V ratio to account for the varying amounts of biomass present (analogously to different surface areas of collected biofilm). The simultaneous parameter estimation included data from all experiments (E1–E6). Alternative ways to normalize the rates, e.g., using dried biomass (VSS) in activated sludge treatment, seemed inappropriate for the sewer, since some biofilm biomass contained high amounts of inert materials (e.g., sand or cellulose, see SI chapter S2.2 for a detailed investigation of biological activity).

Redox conditions in the bulk liquid and the different layers of the biofilm may influence transformation of organic (primary and secondary) substrates, while some compounds might be degraded under aerobic, anoxic, anaerobic, all conditions, or not at all.[21,39,40] In the conducted experiments over the first 12 h, the conditions were anoxic (nitrate was available). The wastewater was "fresh" at the beginning of the experiment, and the heterotrophic activity decreased after the easily biodegradable primary substrate (soluble COD) was consumed after approximately 12 h, leading to anoxic/anaerobic conditions in the reactors (Figures S2–S6 in SI). Nonetheless, biomarker transformation rates were not affected by this change in redox conditions, since the sewer model fitted the time series well over the 24 h experiment (Figure 1 and Figure S8 in SI). Furthermore, transformation rates in wastewater with suspended particles were unchanged under aerobic and anaerobic conditions (Figure 1B).

Biofilm Structure. We have to differentiate four biofilm situations: (i) growth of intact biofilm in real sewers (approximate thickness ≥2 mm), (ii) real sewer biofilms tested in suspended form in the laboratory, and (iii) intact laboratory biofilm (LB_{intact}; approximately 1 mm thickness) grown and tested intact under realistic conditions in the CTR, and (iv) the CTR biofilm ($LB_{suspended}$) tested in suspension in batch reactors.

Since our hypothesis made testing intact biofilm from different locations not possible, we suspended the biomass and changed the biofilm structure. Smaller diffusion depth and altered redox gradients may have changed the transformation potentials of the biofilm. However, our results from experiments with intact and suspended biofilm (LB) show no difference in transformation rates for biomarkers that were not affected by chemical hydrolysis (Figures 1F and S7).

In general, it is still difficult to compare the performances of intact and suspended biofilms. Transformations can be mass transfer (diffusion) limited and/or limited by the substrate conversion rate. Intact sewer biofilms are so-called deep biofilms, which means that mass transport limitations will affect system performance. In our experimental setup and in real sewers, aerobic growth will be limited by oxygen. Organic substrate will diffuse deeper into the biofilm to be oxidized with other electron acceptors (SO_4^{2-}, NO_3^-). However, in a gravity sewer, the top 1–2 mm of biofilm contained 80% of the measured ATP content—a measure for activity[41]—subsequently indicating that the deeper layers of the biofilm may not be active. Similarly, a study with whole and dispersed activated sludge that looked at the availability of low and high molecular weight substrates to extracellular enzymes, found only a higher activity in the dispersed biomass for some of the investigated enzymes.[42] Since it is still unknown which enzymes

DOI: 10.1021/acs.est.6b04200
Environ. Sci. Technol. 2016, 50, 13351–13360

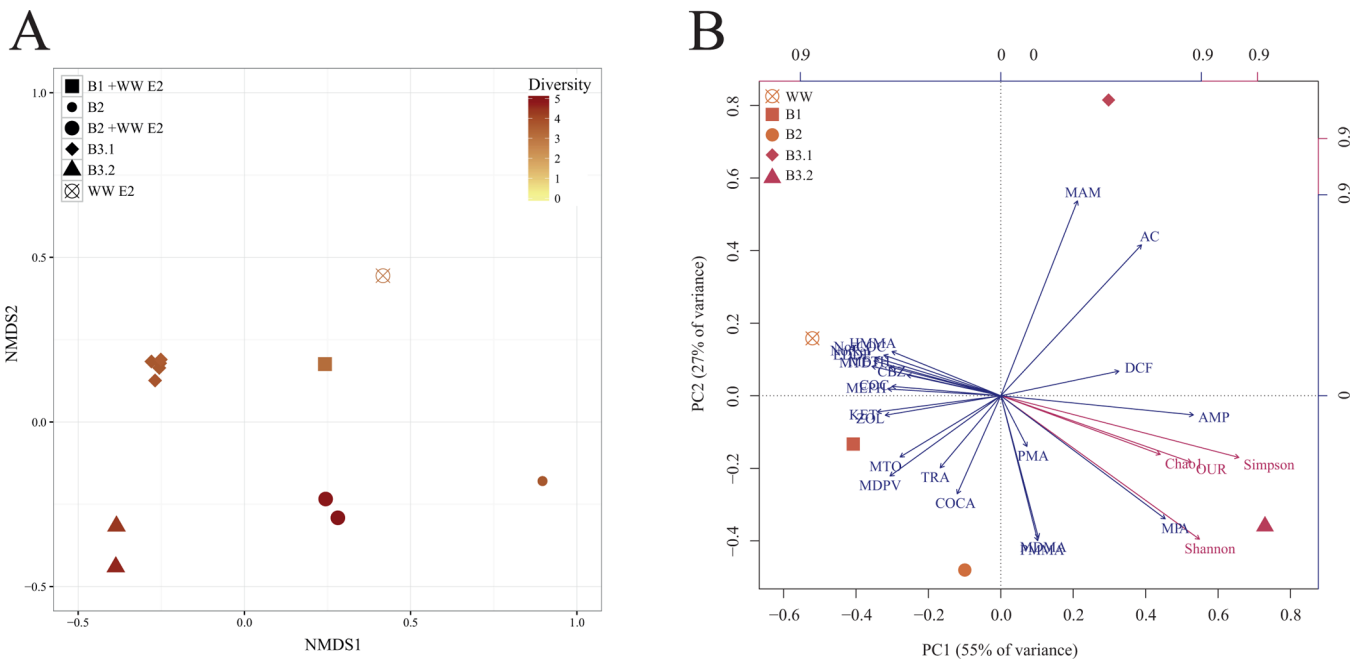


Figure 2. Nonmetric dimensional scaling analysis (NMDS) and principal component analyses (PCA). (A) the first NMDS delineates microscale differences in the microbial community compositions of the biomasses collected with or without replication at the different sampling locations. (B) the PCA biplot highlights relationships between the average microbial community compositions of each biomass and biomarker transformation rates. The NMDS (any similarity distance; stress factor of 0.047 indicating an excellent fit) and PCA (Euclidean distances only) highlight the fine-scale dissimilarities in the microbial community composition measured by v6−v8 16S rRNA gene-based amplicon sequencing from the biofilms collected at the different sampling locations and from the wastewater used for the transformation experiments: the closer the dots, the closer the microbial community compositions. These ordination analyses basically inform that the microbial community compositions of each biofilm and wastewater ecosystem were unique and that biological replicates were highly similar. Each symbol is accompanied by a color scale proportional to the diversity index of the microbial community fingerprints computed according to Shannon—lowest: gold = 2.97 to highest: purple = 4.40. In the PCA biplot, the average microbial community composition per sampling location was taken into account to allow for comparison with mean biotransformation rates. The objective was to assess whether differences in biomarker degradation patterns may link to differences in microbial community compositions and diversities and overall aerobic microbial activity. The red vectors represents 3 diversity indices, namely Chao1, Shannon, and Simpson indices, as well as the oxygen uptake rate (OUR). The blue vectors represent the biomarker biotransformation rates.

are involved in drug transformation in the sewer, using intact or suspended biofilm can result in a similar response.

To assess the biomarker transformation potential of different sewer biofilms, suspension of intact biofilm was necessary which may have influenced the process conditions. To quantify the influence of mass transport limitations on the substrate flux, we calculated the efficiency factor ε (ratio of flux into biofilm with diffusion limitations to flux with suspended biofilm without diffusion limitation).[43] Suspended biofilm overestimated the conversion rates of AMP, AC, and MAM by a factor of 1.1−1.7 depending on the thickness of active biomass in the intact biofilm, while transport limitations were negligible for all other biomarkers (see S2.6 in SI for detailed calculations). Comparing the conversion rates of the intact and suspended biofilm showed that transformation rates were lower overall than in experiments with real sewer biofilms (Figure 1F). We assume that the microbial community composition of the laboratory biofilm (LB) differed from the investigated real sewer biofilms, which influenced its transformation potential. Therefore, the diversity in bacterial community structures by means of microbial and numerical ecology methods was investigated (see S2.7 in SI).

Qualitative Relationships of Biofilm Microbial Composition and Biomarker Transformations. Amplicon sequencing analyses revealed significant differences in the microbial community compositions and diversities of the biological samples collected at each sewer location (Figure 2A and Figure S12 in SI). Multivariate numerical analyses performed by ordination via PCA and by computation of correlation patterns indicated trends in the relationships between average microbial community compositions and average biomarker transformations (Figure 2B).

The PCA biplot in Figure 2B first rapidly informed that the higher transformation rates of biomarkers measured with B3.1 and B3.2 biofilms correlate with higher microbial diversities of these biomasses. It also highlighted close transformation responses for the AMP and MPA as well as MAM and AC biomarkers in relationship with biomass and community features. For these compounds that displayed significant transformations conducted with biofilms B3.1 and B3.2, the heatmap of Pearson's linear correlations (Figure S14 in the SI) provided some hints on putative phylotypes that may display interesting metabolic features for biodegradation of the biomarkers that we investigated. Overall, the biotransformation patterns of the eight biomarkers MAM, AC, AMP, MPA, PMA, DCF, PMMA, and MDMA distinguish from others in their correlative patterns with the microbial traits of the biological samples, such as sustained by both the PCA biplot and the correlation heatmap.

Special Case: Cocaine−Benzoylecgonine Relationship. In general, one would expect an increase of BE formed from COC. The presence of BE in urine indicates prior metabolism of COC.[44] Interestingly, in reactors with biofilm, BE concentrations remained stable despite decreasing COC levels.

DOI: 10.1021/acs.est.6b04200
Environ. Sci. Technol. 2016, 50, 13351−13360

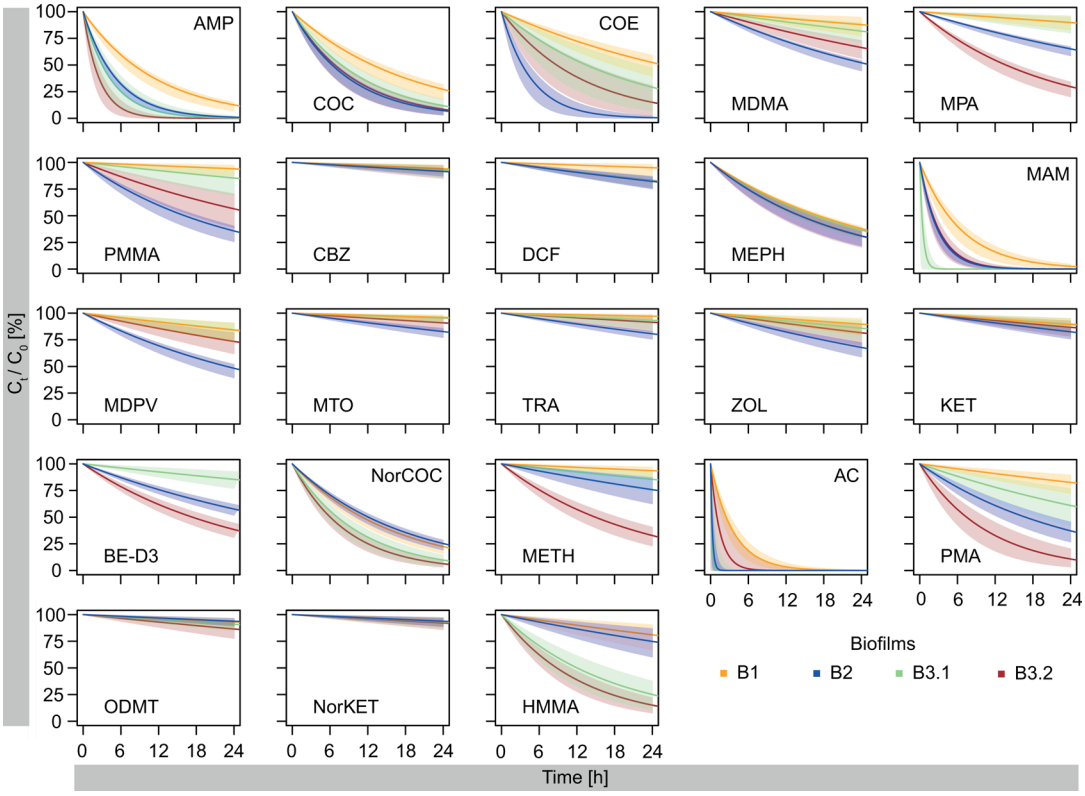


Figure 3. Lines represent the results of the transformation model (eq 1) fitted to the experimental data with A/V 33 m^2 m^{-3}, and k_{WW}, k_a, and $k_{biofilm}$ from the experiments with four different sewer biofilms. Shaded areas are the 90% credible intervals (without model structure error term). Biomarkers that were formed during batch studies as a metabolite of any spiked biomarker were not evaluated.

This observation suggests the formation of other transformation products or, alternatively, that BE was produced and transformed at equivalent rates. To investigate this, we tested the stability of BE by spiking the deuterium-labeled substance BE-D3. BE-D3 was biodegraded in reactors with biofilm B2 and B3.2, while it was stable in reactors with B1 and B3.1. In the past, BE has been assumed to be stable under different sewer conditions. Thai et al. (2014) tested the stability of illicit drugs in an aerobic gravity pilot sewer and an anaerobic pressure sewer (A/V 71 m^2 m^{-3}) and found BE-D3 to be stable over 12 h.[12,45] Therefore, we assumed that gravity sewer biofilms B2 and B3.2 might have a unique microbial composition allowing other transformation pathways to manifest.

Overall, we clearly identified four different microbial biofilm communities for the biofilms collected at the four different sampling locations. Further, the multivariate approach indicated possible links between the transformation rates of biomarkers and compositions and diversities of microbial communities in the investigated biofilms. Additionally, our *qualitative* screening—meaning that we identified the presence of these phylotypes but not their activity for the degradation of biomarkers—serves as starting point for further thorough research on the ecophysiology and functional metabolic potential and activity of these populations for the biotransformation of drug residues.

Kinetic Parameter Estimation. The parameters of the transformation model were estimated in two steps using Bayesian inference: first, k_a was estimated based on data from the abiotic acWW control experiments. Then, the resulting posterior distribution of k_a provided prior information for the second step, in which all parameters in the transformation model were estimated simultaneously (see the SI for further information).

The transformation model (eq 1) fitted to the experimental data with A/V 33 m^2 m^{-3}, and k_{WW}, k_a, and $k_{biofilm}$ for the four different sewer biofilms demonstrated a good quality of fit based on the 90% credible intervals (including the error term representing parametric, conceptual, and measurement error) (see Figure S9 in the SI).

Applying the transformation model to all time series for each biomarker resulted in variable stabilities (Figure 3). Estimated rates for each parameter of the sewer transformation model are shown in Table 1. In summary, the investigated biomarkers can be grouped in the following categories, based on their stability over 12 h:

(a) Biomarkers were stable (<20% loss) under all tested conditions. No transformation occurred for KET, NorKET, MTO, ODMT, DCF, ZOL (aerobic), and CBZ.

(b) Abiotic chemical processes dominated the transformation —with and without biofilm or suspended particulate matter — present for COC, COE, EME, NorCOC, and MEPH. Chemical hydrolysis was dependent on pH; nonetheless, all rates were within 30%.

(c) Wastewater-driven biological transformations (>20% loss) affected AMP, MAM, AC, and ZOL (anaerobic).

(d) Biological transformations of AMP, MAM, and AC were amplified by the presence of increasing amounts of biofilm. Biofilm-specific transformation rates in sewers with an A/V of >10 m^2 m^{-3} can further affect concentrations of HMMA, PMA, BED3, MDMA, MDPV, MPA, PMMA, TRA, and METH, and losses can be above 20% over 12 h. Transformation rates for

DOI: 10.1021/acs.est.6b04200
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Table 1. Transformation Rates as Estimated with Sewer Transformation Model at 22 °C and under Aerobic Conditions

	abiotic transformation k_a (h^{-1})			biofilm induced transformation $k_{biofilm}$ ($m\ h^{-1}$)												activ. sludge transformation (h^{-1})		
	autoclaved WW (acWW)			B1			B2			B3.1			B3.2			AS		
		quantiles			quantiles			quantiles			quantiles			quantiles			quantiles	
biomarker	mean	10%	90%	mean	10%	90%	mean	10%	90%	mean	10%	90%	mean	10%	90%	mean	10%	90%
AMP	0.0046	0.0023	0.0072	0.0024	0.0021	0.0027	0.0060	0.0054	0.0067	0.0073	0.0047	0.0102	0.0125	0.0093	0.0161	0.0115	0.0101	0.0130
COC	0.0352	0.0294	0.0408	0.0004	0.0001	0.0006	0.0022	0.0018	0.0026	0.0009	0.0002	0.0017	0.0013	0.0006	0.0021	0.0129	0.0107	0.0152
COE	0.0200	0.0149	0.0249	0.0001	0.0000	0.0003	0.0063	0.0045	0.0083	0.0011	0.0003	0.0020	0.0019	0.0009	0.0031	0.0121	0.0089	0.0156
MDMA	0.0007	0.0003	0.0011	0.0001	0.0001	0.0003	0.0009	0.0008	0.0010	0.0002	0.0001	0.0003	0.0005	0.0004	0.0007	0.0003	0.0002	0.0005
MPA	0.0005	0.0002	0.0009	0.0001	0.0001	0.0002	0.0006	0.0005	0.0006	0.0001	0.0000	0.0002	0.0016	0.0014	0.0019	0.0001	0.0000	0.0002
PMMA	0.0008	0.0003	0.0014	0.0000	0.0000	0.0001	0.0014	0.0012	0.0016	0.0002	0.0000	0.0003	0.0007	0.0005	0.0010	0.0002	0.0000	0.0003
CBZ	0.0004	0.0002	0.0007	0.0001	0.0000	0.0001	0.0001	0.0001	0.0001	0.0001	0.0000	0.0001	0.0001	0.0000	0.0002	0.0000	0.0000	0.0001
DCF	0.0005	0.0002	0.0008	0.0001	0.0000	0.0001	0.0002	0.0002	0.0003	0.0002	0.0002	0.0003	0.0002	0.0002	0.0003	0.0001	0.0000	0.0001
MDPV	0.0026	0.0014	0.0038	0.0001	0.0000	0.0002	0.0009	0.0008	0.0010	0.0001	0.0000	0.0002	0.0003	0.0001	0.0004	0.0002	0.0001	0.0004
MEPH	0.0383	0.0348	0.0419	0.0001	0.0000	0.0002	0.0004	0.0001	0.0006	0.0001	0.0000	0.0002	0.0003	0.0001	0.0006	0.0002	0.0000	0.0004
MTO	0.0008	0.0004	0.0013	0.0000	0.0000	0.0001	0.0002	0.0002	0.0003	0.0000	0.0000	0.0001	0.0001	0.0000	0.0002	0.0000	0.0000	0.0001
MAM	0.0097	0.0067	0.0129	0.0044	0.0035	0.0054	0.0109	0.0094	0.0124	0.1243	0.0398	0.1976	0.0104	0.0080	0.0132	0.0146	0.0118	0.0177
TRA	0.0005	0.0002	0.0009	0.0000	0.0000	0.0000	0.0003	0.0002	0.0003	0.0001	0.0000	0.0001	0.0001	0.0000	0.0002	0.0001	0.0000	0.0001
ZOL	0.0014	0.0006	0.0023	0.0001	0.0000	0.0002	0.0005	0.0004	0.0006	0.0001	0.0000	0.0003	0.0002	0.0001	0.0003	0.0001	0.0000	0.0003
KET	0.0010	0.0005	0.0016	0.0001	0.0000	0.0002	0.0002	0.0002	0.0003	0.0001	0.0000	0.0002	0.0001	0.0001	0.0002	0.0002	0.0001	0.0003
BED3	0.0006	0.0002	0.0010				0.0007	0.0006	0.0008	0.0002	0.0001	0.0003	0.0012	0.0011	0.0014			
NorCOC	0.0495	0.0424	0.0561	0.0003	0.0001	0.0006	0.0002	0.0000	0.0003	0.0010	0.0004	0.0017	0.0016	0.0010	0.0021	0.0066	0.0055	0.0078
METH	0.0009	0.0004	0.0016	0.0000	0.0000	0.0001	0.0003	0.0001	0.0005	0.0001	0.0000	0.0003	0.0014	0.0011	0.0017	0.0005	0.0003	0.0006
PMA	0.0042	0.0022	0.0064	0.0000	0.0000	0.0001	0.0011	0.0009	0.0013	0.0004	0.0001	0.0007	0.0028	0.0019	0.0037	0.0016	0.0011	0.0022
AC	0.0082	0.0045	0.0122	0.0081	0.0063	0.0101	0.1363	0.0652	0.2463	0.7639	0.0445	1.3127	0.2379	0.0094	0.5133	0.9204	0.4838	1.4041
ODMT	0.0015	0.0008	0.0022	0.0000	0.0000	0.0001	0.0000	0.0000	0.0001	0.0001	0.0000	0.0001	0.0001	0.0000	0.0002	0.0001	0.0000	0.0002
NorKET	0.0006	0.0002	0.0011	0.0001	0.0000	0.0001	0.0000	0.0000	0.0001	0.0001	0.0000	0.0001	0.0001	0.0000	0.0001	0.0001	0.0001	0.0002
HMMA	0.0018	0.0009	0.0028	0.0002	0.0000	0.0003	0.0003	0.0001	0.0005	0.0018	0.0012	0.0024	0.0024	0.0019	0.0029	0.0017	0.0012	0.0022

DOI: 10.1021/acs.est.6b04200
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biofilm B1, originating from a small, residential sewer, were overall lower than for the other three biofilms taken from larger sewers, indicating that the microbial community may influence transformation potentials.

Implication for Back-Calculation and Outlook. Our results show that sewer experiments conducted without biofilm can underestimate the transformation of certain biomarkers during in-sewer transport. Clearly, for stable biomarkers, no correction of the back calculation is required. For unstable biomarkers, further research is needed (i) investigating more biofilms (following a protocol like the one suggested in this study) to compile more kinetic rate constants in a database for objective comparison (including, for example, different biofilms from pressurized sewers) and (ii) to characterize conditions in sewer systems that influence the extent of rates. Including a correction for in-sewer biomarker stability will improve the estimate and overall uncertainty quantification of WBE back-calculations, resulting in an increasingly beneficial tool in drug epidemiology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04200.

Additional information about the investigated sewer catchments, applied analytical, statistical and experimental methods, and supplementary results are presented in Tables S1−S11, equations S1−S23, Figures S1−S17, and references (PDF)

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Funding

This project has received funding from the European Union's Seventh Framework Programme for research, technological development, and demonstration under grant agreement no. 317205 (ITN SEWPROF).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Anna-Maria Forss, Michael Plötzsch and Sandra Stäheli (chemical analyses IRM, UZH). For fruitful discussion and support we are thankful to Tobias Doppler, Adriano Joss, Claudia Bänninger-Werffeli, Karin Rottermann, Brian Sinnet, Jacqueline Traber, Kathrin Fenner, Rebekka Gulde and Heinz Singer (Eawag) and Peter Liechti, Roger Küenzi, Beat Rohrbach and Hans Lamp (ERZ, Urban Drainage Department Zurich). Many thanks to INSA Lyon for providing the Couette-Taylor reactor. Peter Vanrolleghem holds the Canada Research Chair on Water Quality Modelling.

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DOI: 10.1021/acs.est.6b04200
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