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Biotransformation of Sulfonamide Antibiotics in Activated Sludge: The Formation of Pterin-Conjugates Leads to Sustained Risk

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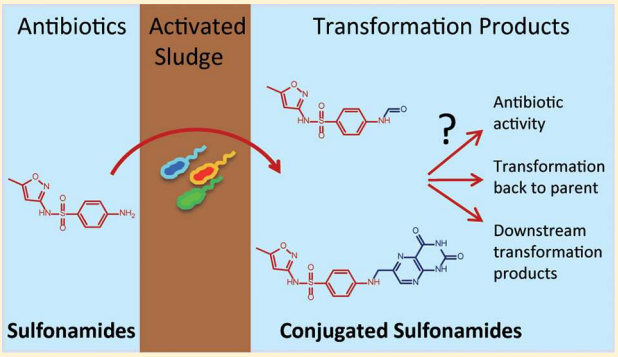
Supporting Information

ABSTRACT: The presence of antibiotics in treated wastewater and consequently in surface and groundwater resources raises concerns about the formation and spread of antibiotic resistance. Improving the removal of antibiotics during wastewater treatment therefore is a prime objective of environmental engineering. Here we obtained a detailed picture of the fate of sulfonamide antibiotics during activated sludge treatment using a combination of analytical methods. We show that pterin-sulfonamide conjugates, which are formed when sulfonamides interact with their target enzyme to inhibit folic acid synthesis, represent a major biotransformation route for sulfonamides in laboratory batch experiments with activated sludge. The same major conjugates were also present in the effluents of nine Swiss wastewater treatment plants. The demonstration of this biotransformation route, which is related to bacterial growth, helps explain seemingly contradictory views on optimal conditions for sulfonamide removal. More importantly, since pterin-sulfonamide conjugates show retained antibiotic activity, our findings suggest that risk from exposure to sulfonamide antibiotics may be less reduced during wastewater treatment than previously assumed. Our results thus further emphasize the inadequacy of focusing on parent compound removal and the importance of investigating biotransformation pathways and removal of bioactivity to properly assess contaminant removal in both engineered and natural systems.



INTRODUCTION

The widespread presence of antibiotics in the environment raises significant concern because their concentrations are high enough to potentially affect sensitive aquatic ecosystems, and their ubiquity may support the propagation of antibiotic resistant genes.[1−5] Sulfonamides, one of the oldest families of antibiotics, are still widely used as human and veterinary drugs.[6−8] They have been detected in various environmental matrices, including soil, wastewater, surface water, and groundwater.[6,9−11] Particularly for sulfamethoxazole, the sulfonamide antibiotic with the highest reported human consumption, concentrations in the upper ng/L to lower μg/L range are frequently detected in municipal wastewater.[12−17] Biological wastewater treatment plays an important role in reducing the load of chemicals collected by the sewer system prior to discharge to surface waters.[18] Several studies have investigated the sulfonamide removal capacity of biological wastewater treatment systems,[12−16] reporting variable degrees of removal[19] and contradictory results regarding optimal removal conditions.[20−25]

Although removal alone has been repeatedly demonstrated to be insufficient in assessing environmental risk,[26,27] only limited research has been focused on obtaining a clear picture of the fate of sulfonamides during wastewater treatment. Previous investigations have demonstrated that sorption to activated sludge solids and abiotic processes play a minor role, and that the majority of the observed removal in activated sludge is linked to biotransformation.[21,23] However, sulfonamide biotransformation pathways and related transformation product (TP) formation have only been studied little so far. A number of studies focus on the sulfonamide biotransformation

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capabilities of isolated microbial strains. Several of these studies report the formation of the SMX cleavage product, 3A5MI,[28,29] or analogous cleavage products for other sulfonamides.[30] One pure culture study performed with ^{14}C-labeled SMX demonstrated partial mineralization to $^{14}CO_2$.[31] Another reported biotransformation pathway is related to the original mode of action of sulfonamide antibiotics. The bacteriostatic effect of sulfonamides is based on the competitive inhibition of dihydropteroate synthase (DHPS), a key enzyme involved in intracellular folic acid synthesis. In studies with *Escherichia coli* and, more recently, in different algal species, sulfonamides were shown to act as alternate substrates for the DHPS enzyme, leading to the formation of pterin-sulfonamide conjugates.[32−35] Whereas these studies show that certain microorganisms are able to transform sulfonamides or even use them as a carbon source, pure culture results generally cannot be extrapolated to mixed communities.[36]

Limited research has been focused on the elucidation of sulfonamide biotransformation in complex activated sludge communities. In a recent study with ^{14}C-labeled SMX, the parent compound was degraded under aerobic as well as anaerobic conditions, but mineralization rates were below 5% under all measured conditions, suggesting that the majority of the spiked micropollutants was still present in the form of unidentified transformation products.[21] In a different study, a number of SMX metabolites were detected, including products potentially formed via acetylation, hydroxylation, nitration, deamination, or formylation.[37] However, no attempts were made to prioritize the detected TPs, and their relative importance remains unclear. Yet, such knowledge is highly desirable to properly assess risk, given the fact that sulfonamide metabolites with modifications at the *para*-amino position, including those that are formed when sulfonamides interact with their target enzyme to inhibit folic acid synthesis,[32−34] have been shown to still exhibit antibiotic activity.[38,39]

The objective of this study therefore was to gain a comprehensive view of the transformation pathways and products of sulfonamide antibiotics during wastewater treatment. To this end, we used a combination of two complementary methods, i.e., mass balance analyses with ^{14}C-labeled sulfonamides and in-depth transformation product screening with high-resolution mass spectrometry of samples from laboratory batch experiments with activated sludge and field study samples from wastewater treatment plants. Additional experiments were performed with five nonradiolabeled sulfonamides in parallel (i.e., sulfamethoxazole, sulfadiazine, sulfamethazine, sulfapyridine, and sulfathiazole) to evaluate the generalizability of our findings to the entire family of sulfonamide antibiotics.

METHODS

Batch Experiments. For the experiments with radiolabeled sulfonamides, ^{14}C-SMX (aniline[^{14}C]-sulfamethoxazole, Hartmann Analytic) and ^{14}C-SDZ (2-pyrimidyl[^{14}C]-sulfadiazine, Bayer HealthCare) were used. Further details on chemical reference materials are provided in Section S1 of the Supporting Information (SI). For all of the experiments, amber glass bottles (100 mL, Schott) were used as batch reactors. To maintain aerobic conditions, caps with two holes were used as previously described.[40] After the sulfonamide addition, the reactors were placed on a circulating shaker table (160 rpm) for the duration of the experiment, and samples were collected at different time points as specified below. Batch reactor experiments with SMX and SDZ; with SMZ, SPY, and STZ; and with pterin-STZ, respectively, were conducted in three separate campaigns. Therefore, fresh activated sludge was collected three times (March 21, 2017 (AS1); June 12, 2017 (AS2); and July 18, 2017 (AS3), less than 3 h before the experiments were started) from the same aerated nitrifying treatment basin of the same WWTP (ARA Neugut, Dübendorf, Switzerland, details in Section S2). During all three experimental campaigns, pH values remained in the range between 7.87 and 8.28 at all measured time points. Results from the measurements of the total suspended solids (TSS), pH, and nitrogen species (NH_4^+, NO_2^-, and NO_3^-) and a comprehensive table of all of the batch experiments, including the added chemicals, sampling time points, and sample volumes, are provided in the SI (Section S2).

Experiment with Radiolabeled and Nonlabeled SMX and SDZ. Triplicate biotransformation batch reactors with radiolabeled and nonlabeled substances were established in parallel, both for SMX and SDZ. Additionally, abiotic controls with autoclaved (30 min at 125 °C) activated sludge and radiolabeled sulfonamides were established in triplicate, and three reactors with only activated sludge added served as nonspiked controls. Biotransformation batch reactors with ^{14}C-labeled compounds were spiked (40 μL each, 1.53 kBq/μL (SMX), and 1.02 kBq/μL (SDZ) in methanol), resulting in final initial activities of 61.2 kBq (SMX) and 40.8 kBq (SDZ), respectively, corresponding to initial concentrations of 405 μg/L (SMX) and 115 μg/L (SDZ). Reactors with nonlabeled sulfonamides were spiked (40 μL of each, 250 mg/L, in methanol/water 1:3) to achieve initial concentrations of 250 μg/L (SMX and SDZ). To ensure equal starting conditions in terms of carbon availability, the reactors with nonlabeled sulfonamides and the nonspiked controls were amended with 30 and 40 μL of methanol, respectively.

Samples were collected over 72 h. The samples were centrifuged at 21500 × *g* for 10 min at room temperature, and the supernatant was transferred into amber HPLC vials. For the nonradioactive samples, internal standard solution was added (final sample concentration: 24 μg/L, details in Section S3). Samples were stored at 4 °C and then analyzed by high-performance liquid chromatography (HPLC) coupled to a high-resolution mass spectrometer (see below for details) within 7 days. For the reactors spiked with radiolabeled sulfonamides, 30 μL of supernatant was transferred into a 6 mL polypropylene vial and mixed with 5 mL of scintillation cocktail (IrgaSafe Plus, PerkinElmer) for liquid scintillation counting (LSC). In parallel, 750 μL of supernatant was pipetted into amber HPLC vials, stored at 4 °C, and then analyzed by HPLC coupled to a diode array detector (DAD) and an LSC detector within 3 days.

To analyze the radioactivity adsorbed to or incorporated into the biomass, the compressed solid material resulting from centrifugation was obtained after removal of the remaining supernatant. The cell pellet was washed with NaOH (1 mL, 1 M in water), and the washing liquid was collected after another centrifugation run (15 min at 21500 rpm). The entire washing liquid (1 mL) was mixed with scintillation cocktail (5 mL, Hionic Fluor, PerkinElmer) and analyzed using LSC. The washed cell pellet was stored at −20 °C until analysis in the sample oxidizer, as described below. The washing solution contained radioactivity both from ^{14}C-SA weakly adsorbed to the microbial cells and from the remaining aqueous supernatant that was not removed after centrifugation. Since a major

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fraction of the radioactivity originated from the remaining aqueous fraction and, as we could show for SMX, the fraction attributed to sorption was not exceeding 4% at all of the investigated time points, the measured radioactivity of the washing solution was not considered in the mass balance analysis (details in Section S4).

Experiment with Nonlabeled SPY, SMZ, STZ, and N4-Acetyl-SMX. Biotransformation experiments with the SAs SMZ, STZ, and SPY and the TP N4-acetyl SMX were run in duplicate. To estimate the degree of sorption and abiotic degradation, SAs were added to duplicate control reactors with autoclaved (121 °C and 103 kPa for 20 min) activated sludge and activated sludge filtrate, respectively. Nonspiked reactors (duplicate) were run to serve as controls in suspect screening. Samples were collected over 72 h and processed similarly to the methods described above. For the biotransformation reactors, 25 μL of each sulfonamide or N4-acetyl-sulfamethoxazole solution (100 mg/L, in methanol/water 1:9) was spiked into the according batch reactors, resulting in a starting concentration in the reactors of 50 μg/L. Samples were centrifuged (5 min at 21130 × *g*), and the supernatant (750 μL) was transferred to amber HPLC vials. After the addition of the internal standard solution, samples were stored for 3−7 days at 4 °C until HPLC-MS/MS analysis.

Experiment with Pterin-STZ. A reference standard for pterin-STZ was custom synthesized by SynphaBase, Switzerland.[34] Six batch reactors were filled with activated sludge, of which three were spiked with pterin-STZ (25 μL, 100 mg/L of pterin-STZ, in methanol/water 1:9) and three served as nonspiked controls. Samples were collected over time and centrifuged (10 min at 21130 × *g*), and the supernatant (500 μL) was transferred to amber HPLC vials and stored for 3−5 days at 4 °C until LC-MS/MS analysis.

LC-HRMS/MS Analysis. All nonradioactive samples were analyzed by reversed-phase HPLC coupled to a high-resolution tandem mass spectrometer (HRMS/MS) (Q Exactive or Q Exactive Plus, Thermo Fisher Scientific). The separation of the analytes was achieved using a C18 silica-based column (Atlantis-T3, particle size 3 μm, 3.0 × 150 mm, Waters) at 30 °C. Samples from the experiments with nonlabeled SPY, SMZ, and STZ were measured using an additional guard cartridge (particle size 3 μm, 3.9 × 20 mm, Waters), leading to higher retention times (RTs). For all of the samples, 100 μL of sample was injected onto the column at a flow rate of 300 μL/min. Further analytical details including chromatographic separation and mass spectrometric analysis are provided in Section S5. For quantification, calibration curves were prepared in nanopure water (Barnstead Nanopure, Thermo Scientific) ranging from 0.2−300 μg/L (SMX and SDZ) and 0.2−75 μg/L (SPY, SMZ, ST,Z and TPs). To account for compound losses and interferences during LC-HRMS measurements, internal standards were added to all of the samples, including the calibration samples (details in Sections S3 and S5). Quantification was performed using Tracefinder EFS 3.2 (Thermo Scientific) for all parent sulfonamides and for transformation products for which reference standards were available. The lowest calibration standards with a meaningful, detectable peak (reasonable peak shape, a minimum of 4 scans per peak, and a minimum intensity of 1E04 in Xcalibur Qualbrowser 3.0 (Thermo Scientific)) were regarded as limits of quantification (Table S5.2).

Analysis of Radioactive Residues. Total radioactivity of the samples was assessed using a liquid scintillation counter (Tri-Carb 2800TR, PerkinElmer). Sample aliquots were mixed with scintillation cocktail prior to the measurements, as described above. For analysis of the radioactivity accumulated in the solids, the biomass fractions of the samples were combusted for 1.5 min in a 307 PerkinElmer Sample Oxidizer. The resulting $^{14}CO_2$ was absorbed by Carbo-Sorb E (PerkinElmer), and LSC cocktail (Permafluor E+, PerkinElmer) was added. The radioactivity was then assessed by liquid scintillation counting. Radioactive supernatant samples were analyzed using a HPLC 1200 Series (Agilent Technologies), including a diode array detector (DAD) coupled to a subsequent liquid scintillation counter (Ramona Star, Raytest). The DAD signal was recorded at 285 nm (4 nm bandwidth), which is close to the maximum of an absorption peak between 250 and 300 nm in the absorption spectra of both SMX and SDZ.[41,42] An identical column and gradient program and identical eluent mixtures and injection volumes for chromatographic separation as described for the LC-HRMS/MS measurements above were used.

Suspect Transformation Product Screening. For the identification of TPs, a suspect screening was performed using Compound Discoverer 2.1 (Thermo Scientific) and Xcalibur Qualbrowser 3.0 (Thermo Scientific). Mass lists for the suspect screening were compiled using in silico prediction with the EAWAG pathway prediction system (EAWAG-PPS, http://eawag-bbd.ethz.ch/predict/). Additionally, previously reported transformation products and mass shifts of typical biotransformation reactions were considered. Details on the compilation of the suspect TP lists and Compound Discoverer settings are further described in Section S6.

A suspect TP screening was performed for SMX, SDZ, SMZ, STZ, SPY, N4-acetyl-SMX, and pterin-STZ. For the N4-acetyl-SMX and the pterin-STZ spike experiments, the suspect TP lists of SMX and STZ, respectively, were used. Measurements from replicate batch reactors were grouped in Compound Discoverer. Further analysis and presentation of results were performed based on the mean values from the replicate sample values. Detected features matching suspected TP masses were further assessed. Clear differences in time trends between sample and nonspiked controls were ensured, and isotope patterns were compared with calculated isotope patterns of corresponding suspected molecular formulas. For TPs for which reference standards were available, retention times of the suspected TP and the reference could be compared. For TPs for which no reference standards were available, MS^2 spectra were compared with library spectra or interpreted manually. In doing so, the MS^2 spectra of the TPs were compared with the spectra of the parents or related TPs and with fragments predicted using Mass Frontier 7.0 (HighChem). On the basis of this procedure, confidence levels were assigned to each of the detected TPs, as proposed by Schymanski et al., ranging from level 5 "exact mass", level 4 "unequivocal molecular formula", level 3 "tentative candidate(s)", level 2 "probable structure", to level 1 "confirmed structure".[43] Details and MS^2 spectra are provided in Section S7.

Wastewater Treatment Plant Samples. From each of nine Swiss WWTPs, three influent and three effluent samples of the biological treatment step were obtained (1-L aliquots of three consecutive 24-h composite samples and flow-proportional sampling, except for WWTP3, in which no SMX was detected in influent or effluent, see the Results section) and combined to 72-h composite samples (3 L) in our laboratory. The samples were collected during May and August 2013 and

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stored at −20 °C until sample preparation. The samples were enriched by solid phase extraction (SPE) and analyzed by HPLC-MS/MS using a modified protocol based on Moschet et al.[44] (further details in Section S8). A suspect TP screening was performed for the five SMX TPs N4-acetyl-SMX, PtO-SMX, pterin-SMX, Ac-OH-SMX, and N4-formyl-SMX, using Trace-finder 4.1 (ThermoFisher Scientific), and confidence levels were assigned according to Schymanski et al.[43] Reasonable peak shapes were ensured by visual inspection, and isotope patterns were compared with predicted isotope patterns to confirm molecular formulas. Confidence levels assigned in batch experiments were adopted if for at least two fragments (found in batch experiments or library spectra) their extracted ion chromatograms matched the retention times and peak shapes of the corresponding MS^1 extracted chromatograms. To compensate for matrix effects and possible analyte losses during sample preparation and analysis, the detected peak areas were normalized by the peak areas of the internal standard (isotope-labeled SMX). Influent SMX concentrations were quantified using the internal standard method described for the laboratory experiments.

RESULTS

Biotransformation as Main Removal Mechanism. Analysis of concentration−time series from batch experiments revealed mean removals of 99.3 (sulfathiazole, STZ), 88.7 (sulfapyridine, SPY), 63.8 (sulfadiazine, SDZ), 63.5 (sulfamethazine, SMZ), and 58.9% (sulfamethoxazole, SMX) after 72 h (from initial concentrations of 405 (SMX), 115 (SDZ), and 50 μg/L (SMZ, SPY, and STZ)). These removals are comparable to previously reported removals from laboratory studies.[24,45] The calculation of TSS-normalized pseudo-first-order rate constants resulted in values of 0.083 ± 0.006 (SMX), 0.093 ± 0.009 (SDZ), 0.11 ± 0.01 (SMZ), 0.45 ± 0.01 (STZ), and 0.20 ± 0.01 (SPY) $L/(g_{TSS} \times d)$ and coefficients of determination (r^2) of at least 0.95 (see details in Section S2). In the experiments with ^{14}C-labeled SMX and SDZ, the sum of the recovered radioactivity in the aqueous and solids fraction accounted for 97.3−105.3% (SMX) and 97.2−99.0% (SDZ) of the initially added radioactivity at all of the time points (Figure 1, Section S9). This indicated that the loss of radioactivity as $^{14}CO_2$, and therefore, the degree of mineralization must be low, which was consistent with the low fractions of $^{14}CO_2$ recovered from CO_2 traps in preliminary experiments, and the fact that we did not find any evidence of significant amounts of dissolved $^{14}CO_2$ accumulated in the batch reactors (details in Section S10).

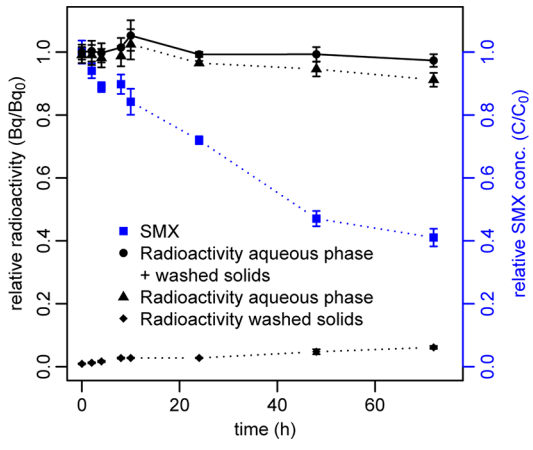

Figure 1. Biotransformation of ^{14}C-SMX. Radioactivity measured in washed solids, aqueous phase, and the sum thereof as fractions of the total radioactivity measured after spiking. Relative concentration of SMX over time is shown in blue. Error bars represent the standard deviation from triplicate reactors.

The fraction of radioactivity recovered from the washed solids (see the Methods section for details on solids washing step) was generally small but showed an increase over time (72 h) with final maximum values accounting for 6% (SMX) and 3% (SDZ) of the initially spiked radioactivity. Experiments with autoclaved activated sludge further confirmed that only small fractions of the spiked sulfonamides (SAs) were sorbed to the activated sludge, and that abiotic transformation was negligible (Section S11). Therefore, the observed declines in parent sulfonamide concentrations can be confidently attributed to biotransformation. Together with the observation that the major part of the radioactivity remained in the aqueous fraction, this indicates that dissolved transformation products must be increasingly present in the supernatant toward later time points.

Identification of Biotransformation Products. To first ensure that all of the analytes present in the supernatant samples of the ^{14}C-labeled SMX biotransformation experiment (i.e., SMX and transformation products) could be fully recovered from chromatographic separation, separate radio-scintillating counting of the HPLC effluent was performed and revealed a mean recovery of 100.3 ± 6.4% for the 0, 24, 28, and 72 h time points (relative to the total injected radioactivity of a single replicate). The HPLC-UV-DAD and the HPLC-LSC chromatogram both displayed a single dominant SMX peak in the first supernatant sample collected from the biotransformation experiment (Figure 2). In the UV-DAD chromatogram, the intensity of the SMX peak decreased with time, whereas a new peak emerged at a slightly higher retention time (+0.72 min). These observations are consistent with broadening and flattening of the SMX peak in the less highly resolved HPLC-LSC chromatogram. The overall peak area in the HPLC-LSC chromatogram (within the retention time window of 10−16 min as shown in Figure 2) showed only a slight decrease (−12.4 ± 7.9%), suggesting that the major transformation products containing the ^{14}C-labeled aniline moiety elute at similar retention times as the parent compound.

Suspect transformation product screening of samples from the batch experiments with nonlabeled SMX led to the detection of 11 transformation products (TPs) (Table 1). In Table 1, the TPs are ordered according to their time-integrated intensities, which were calculated as the sum of measured peak areas at the different sampling time points. Interestingly, with PtO-SMX (a conjugate of 2,4(1H,3H)-pteridinedione, RT shift: +0.79 min), pterin-SMX (RT shift: 0.77 min), and Ac-OH-SMX (RT shift: +0.72 min), three of the five TPs with the highest time-integrated intensities show similar retention time shifts relative to the parent as the emerging TP peak observed in the UV-DAD-chromatogram (Figure 2, Section S12). This finding suggests that the emerging TP peak in the UV-DAD-chromatogram is at least partially caused by the emergence of these three TPs. Moreover, with the exception of 3-amino-5-methylisoxazole (3A5MI), all of the detected TPs from SMX show similar RTs as SMX (RT shifts between −0.32 and +1.5 min, Table 1). Since all of these TPs still contain the radiolabeled aniline moiety, their emergence is consistent with the observation that the overall peak area in the HPLC-LSC chromatogram, despite some broadening and flattening, is

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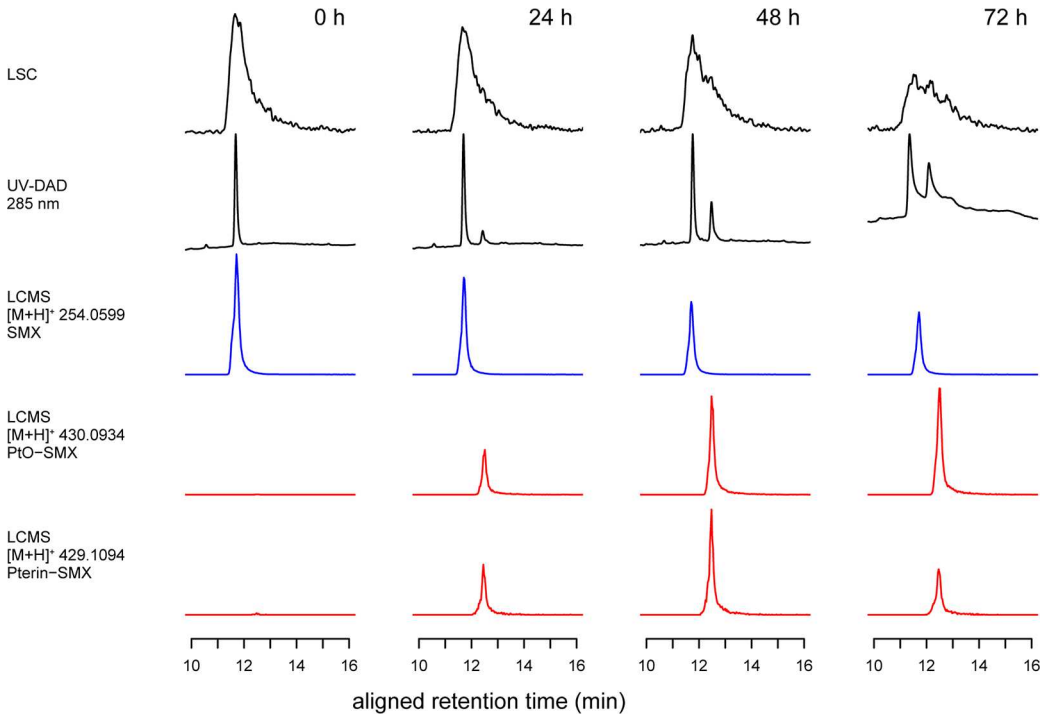

Figure 2. Chromatograms obtained from different detectors at 0, 24, 48, and 72 h after the start of the SMX biotransformation experiment. LC-MS and LSC signals were aligned with the UV-DAD signal using the peaks corresponding to SMX in the first sample (0 h). Signal intensities were normalized by maximal intensities over all four time points with the exception of the UV-DAD signals, for which normalization was performed for each sampling point separately (matrix interferences complicated comparisons of peak intensities and areas). Full chromatograms are shown in Section S12.

only slightly reduced. This suggests that the majority of SMX might indeed be transformed to the TPs given in Table 1 (for further discussion of mass balance aspects, see the section on Biotransformation Pathways).

HPLC-DAD-LSC chromatograms and recovered radioactive fractions from suspended solids and aqueous fractions of the SDZ biotransformation experiments confirmed the findings for SMX described above (Section S9). Similar to SMX, we observed a broadening and flattening of the sole LSC-peak present, and collection of the HPLC effluent and separate radio-scintillation counting again confirmed that all radio-activity could be recovered from the column (mean recovery for one replicate and time points 0, 24, 48, 72 h: 104.3 ± 3.9%). Also, a decrease of the parent SDZ peak was observed in the UV-DAD chromatogram. All eight SDZ-TPs detected in the suspect screening (Table 1) corresponded to changes in molecular structure that were already observed for SMX. Furthermore, also for SMZ, SPY, and STZ, similar product spectra as for SMX and SDZ were observed (Table 1). In particular, the TPs PtO-SA, Ac-OH-SA, and formyl-SA were consistently observed to rank among the five TPs with the highest time-integrated intensities for all five sulfonamides studied.

Across all five sulfonamides, PtO-SA was the intensity-wise most dominant TP (i.e., it ranked first for three out of the five sulfonamides (SMX, SDZ, and STZ) and had the lowest overall rank sum across all five sulfonamides). PtO-sulfonamides represent a modified pterin-sulfonamide and can presumably be formed by hydrolysis of the latter at position 2 of the pterin condensed ring structure (see discussion below). In our experiments, pterin-SAs were detected for all five investigated sulfonamides. The formation of pterin-SA via dihydropterin-SA has been described previously[32,34,35] and is related to the actual mode of action of sulfonamides as antibacterial agents. Sulfonamides not only act as competitive inhibitors of dihydropteroate synthase but can also act as alternative substrates leading to the formation of pterin-sulfonamide conjugates, for which retained antibacterial activity has recently been reported.[38] Whereas this process has been described for pure cultures of bacteria and phytoplankton species, its potential significance in activated sludge has not been recognized so far.

Biotransformation Pathways. To obtain more information about the actual biotransformation pathways, we analyzed area-time trends of the detected TPs as presented in Figure 3 for SMX and in Section S13 for SDZ, SPY, STZ, and SMZ. For SMX, we observed that TPs PtO-SMX, pterin-SMX, and SMX +O, which we tentatively identified as N4-hydroxy-SMX (confidence level 3 according to Schymanksi et al.,[43] see Table 1 and details in Section S7), show a strong immediate increase upon incubation, whereas N4-formyl-SMX was formed in larger amounts only later in the experiment. Similarly, the N4-formyl TPs of the other sulfonamides also showed a pattern of delayed or slower increase (see Section S13), potentially indicating an indirect formation via other TPs.

To investigate the pterin-related biotransformation pathway further, we performed an experiment in which we directly spiked pterin-STZ into activated sludge. Compared to the TP formation in the STZ spike experiments, the observed peak areas of PtO-STZ, Ac-OH-STZ, and formyl-STZ were clearly higher (in the case of PtO-STZ by more than 1 order of magnitude), which could not be explained by the minor amounts (approximately 5%) of the STZ parent compound present as an impurity in the pterin-STZ standard (Figure

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Table 1. Summary of Detected Transformation Products Formed from the Five Investigated Sulfonamides

TP	SMX			SDZ			SMZ			SPY			STZ		
	rank[a]	RT shift (min)[b]	confidence[c]	rank[a]	RT shift (min)[b]	confidence[c]	rank[a]	RT shift (min)[b]	confidence[c]	rank[a]	RT shift (min)[b]	confidence[c]	rank[a]	RT shift (min)[b]	confidence[c]
PtO-SA	1	0.8	2b	1	1.8	2b	2	1.1	2b	4	1.5	3	1	1.5	3
pterin-SA	2	0.8	2b	6	1.8	2b	5	1.0	2b	7	1.2	3	4	1.4	1
N4-formyl-SA	3	1.0	2b	3	0.9	2b	4	0.1	2b	2	0.6	2b	3	1.1	2b
Ac-OH-SA	5	0.8	3	2	1.3	3	1	1.1	3	1	1.3	3	2	1.2	3
SA+O[d]	4	−0.3	3	4	−0.8	4	6	−0.5	4	3	−3.1	4			
pterin-SA+H_2O	7	0.4	3	8	1.5	3									
N4-acetyl-SA	8	1.5	1	5	1.8	1	3	0.9	1	5	1.3	1			
dihydropterin-SA	9	0.6	2b												
pterin-SA + O	10	1.1	4	7	2.2	4									
SA+$C_3H_2O_3$	11	0.4	4							6	0.2	4			
3A5MI	6	−4.1	1												
2A46DP[e]							7	−5.1	2b						

[a]For each parent SA, TPs are ranked according to time-integrated intensities across all of the samples. [b]RT shift indicates the difference in retention time between the transformation product and parent SA. [c]Confidence levels according to Schymanski et al.[43] are assigned to all of the TPs (details in the Methods section and Section S7). [d]For SMX+O, the molecular structure of N4-hydroxy-SMX was proposed (details in Section S7). [e]2-Amino-4,6-dimethyl-pyrimidine.

Figure 3. Time trends of transformation products of SMX. Peak areas of selected TPs are shown as the percentage of the SMX peak area in the first sample after spiking. Area-time trends for the remaining TPs detected for SMX and TPs formed from the other investigated SAs are shown in Section S13.

S13.6). This experiment revealed that not only PtO-STZ was formed from pterin-STZ, but also Ac-OH-STZ and formyl-STZ. On the basis of these results, we developed a tentative, generalized pathway for the formation of PtO-SA, Ac-OH-SA, and formyl-SA from pterin-SA, as shown in Figure 4. The biotransformation of pterins to 2,4(1H,3H)-pteridinediones (here called PtOs) has earlier been observed in the context of folic acid biodegradation and can be catalyzed by the enzyme pterin deaminase.[46,47] Although N4-formyl-SA and Ac-OH-SA TPs could theoretically also be formed directly from the parent sulfonamides, our results suggest that they are rather formed as part of the pterin-conjugate pathway, involving a series of hydrolysis, oxidation, and decarboxylation reactions acting on either the pterin-SA or the PtO-SA, as depicted in Figure 4. For SMX and SPY, a TP with the mass of the possible intermediate SA+$C_3H_2O_3$ was additionally detected, and for SMX and SDZ, a TP with the mass corresponding to pterin-SA+H_2O was found, both lending additional support to the suggested pterin-conjugate pathway. Additionally, other potentially pterin-related TP peaks were detected corresponding to molecular formulas of pterin-SA+O. Detailed structural analyses of these TPs were complicated by the detected low peak areas and, hence, not further pursued. In addition to the pterin-conjugate pathway-related TPs, we also observed the previously described formation of N4-acetyl-SAs.[37] Separate biotransformation experiments with a direct spike of acetyl-SMX confirmed rapid back-transformation to SMX,[12] suggesting that SMX and acetyl-SMX are reversibly converted into each other (details in Section S14). Additionally, for SMX and SMZ, products resulting from the cleavage of the sulfonamide bridge were observed, namely 3A5MI and 2-amino-4,6-dimethyl-pyrimidine.

We could quantify the concentrations of the N4-acetyl metabolites, the cleavage product 3A5MI, and pterin-STZ based on the mass spectrometric data. However, together they only accounted for less than five percent of the removed SMX at all time points. Lacking authentic standards, no accurate quantification was possible for the other TPs. To obtain an approximate assessment of the mass balance over the course of the experiment, we estimated relative ionization efficiencies for the remaining TPs (Section S15) based on structural similarity

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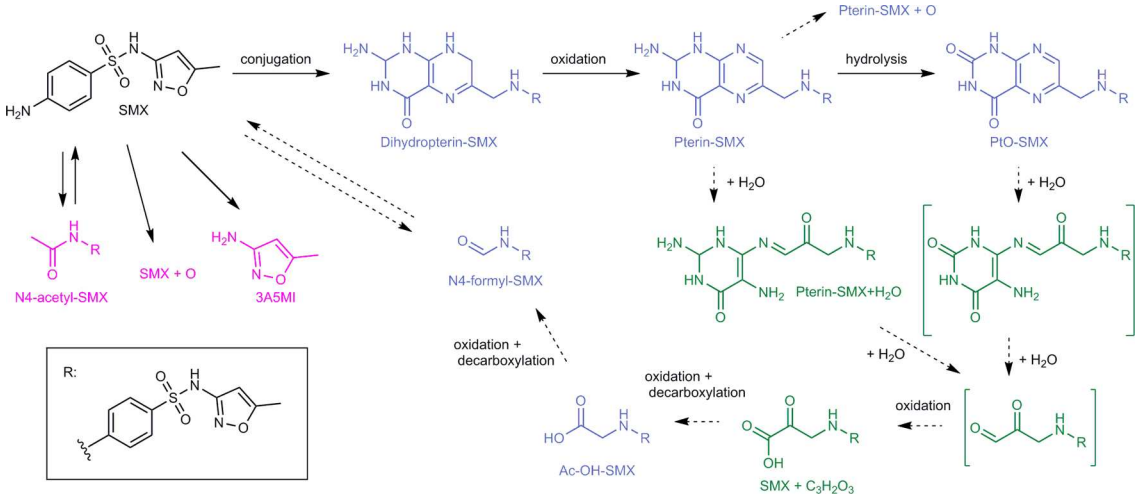

Figure 4. Proposed biotransformation pathway exemplified for SMX. As confirmed for STZ, PtO-SMX, Ac-OH-SMX, and N4-formyl-SMX are suggested to form via pterin-SMX and the detected intermediate dihydropterin-SMX (blue). TPs corresponding to masses of pterin-SMX+H_2O and SMX+$C_3H_2O_3$ were detected but low peak areas did not allow for structure elucidation, and the exact pathway to Ac-OH-SMX and N4-formyl-SMX remains speculative (green). The structures in brackets represent suggested intermediates. N4-acetyl-SMX, 3A5MI, and a TP with the mass SMX+O may form directly from SMX (pink).

Table 2. Transformation Products from SMX Detected in Wastewater Treatment Plant Effluents[a]

		WWTP1		WWTP2		WWTP3		WWTP4		WWTP5		WWTP6		WWTP7		WWTP8		WWTP9	
	lvl[b]	inf[c]	eff[d]	inf	eff	inf	eff	inf	eff	inf	eff	inf	eff	inf	eff	inf	eff	inf	eff
SMX	1	100	54	100	38	nd	nd	100	65	100	166	100	38	100	66	100	47	100	59
N4-acetyl-SMX	1	85	3	59	11	nd	nd	88	nd	559	131	43	nd	112	nd	111	2	165	24
pterin-SMX	2b	nd	10	nd	1	nd	nd	nd	1	nd	4	nd	nd	nd	nd	nd	nd	nd	7
PtO-SMX	2b	7	23	5	10	nd	nd	nd	5	nd	25	7	7	nd	7	nd	nd	nd	19
Ac-OH-SMX	4	5	3	nd	18	nd	nd	nd	19	nd	92	nd	12	nd	12	nd	3	nd	5
N4-formyl-SMX	2b	nd	1	nd	5	nd	nd	nd	3	nd	10	nd	8	nd	4	nd	24	nd	4

[a]TP and effluent SMX peak areas are presented as fractions of the peak areas of influent SMX in the respective WWTPs. In WWTP3, no SMX or SMX TPs were detected in influent or effluent. nd: not detected. [b]Level indicates the confidence level of detected TP structure according to Schymanski et al.[43] [c]Indicates influent. [d]Indicates effluent.

to those TPs for which authentic standards were available. On the basis of the authentic standards, the ionization efficiency of pterin-STZ was found to be 1 order of magnitude lower than that of STZ (i.e., ratio of instrument response factors of pterin-STZ over STZ: 0.1). Assuming that PtO-SAs exhibit similar ionization efficiencies as the structurally similar pterin-SAs, PtO-SA emerged as the TP that contributed the most to the overall mass balance for all sulfonamides. Whereas the mass balance for SMX itself was closed when accounting for all observed TPs (Figure S15.1), some of the other sulfonamides, particularly SPY and STZ, revealed a lack of explainable mass loss, notably at later time points (Figures S15.2–5). This loss suggests that we might still lack a complete representation of later generation TPs formed out of the initial pterin-SA adducts. Although extrapolation of ionization efficiencies involves large uncertainties,[48,49] and the calculated mass balances should therefore be interpreted with caution, they led to strikingly similar conclusions as the results from the ^{14}C-SMX experiments. In the UV-DAD chromatogram, one dominant TP peak was emerging with a similar retention time shift relative to SMX as the pterin-related TPs PtO-SMX, Ac−OH-SMX, and pterin-SMX detected by LC-HRMS. Furthermore, the majority of the radioactivity eluted close to SMX and the above-mentioned TPs. DAD-UV chromatograms and the LSC-chromatograms thus support the dominance of the pterin-conjugate pathway in the biotransformation of sulfonamides.

Detection of Pterin-Sulfonamides in WWTPs Effluents. By applying a suspect TP screening to effluent samples collected from nine Swiss WWTPs, the presence of pterin-conjugate pathway-related sulfonamide TPs could be confirmed. In Table 2, peak areas of TPs and effluent SMX are displayed as fractions of the influent SMX peak area (all peak areas were normalized using internal standards, quantified SMX influent concentrations were between 70 and 870 ng/L, except for one WWTP where no detectable SMX concentrations were found). For seven out of the nine WWTPs, SMX peak areas showed a decline between 34 and 62% from influent to effluent. In one WWTP (WWTP5), an increase in SMX of 66% between influent and effluent was observed. This can be explained by the large relative peak area of influent N4-acetyl-SMX, which is known to be transformed back to SMX in WWTPs.[12] In the effluent samples, peaks corresponding to pterin-SMX or PtO-SMX were found for seven WWTPs, and in eight WWTP effluents, peaks corresponding to Ac-OH-SMX and N4-formyl-SMX, were detected. Only in two cases (Ac-OH-SMX in WWTP1 and PtO-SMX in WWTP6), higher or similar TP peak areas were found in influent samples compared to effluent samples. Reassuringly, we found no TP peaks in the effluent of the only WWTP in which no SMX or N4-acetyl-SMX was detected in the influent (WWTP3). Effluent TP peaks

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displayed peak areas that were roughly 1 to 2 orders of magnitude lower than the influent SMX peak areas (e.g., $PtO_{effluent}/SMX_{influent}$: 13.7 ± 8.5%). However, because of the previously discussed rather low ionization efficiencies, relatively small pterin-SA peak areas can represent significant amounts of pterin-SAs. Therefore, the results presented in Table 2 do not only provide evidence for the formation of pterin-SAs during activated sludge treatment in full-scale WWTPs but also show that the released pterin-conjugate pathway-related TPs potentially represent major fractions of the biotransformed SMX. Finally, the diversity of the operational and design parameters of the nine WWTPs (see Section S16) suggests that the formation of pterin-SAs is of general relevance and not limited to the activated sludge used in our biotransformation experiments.

IMPLICATIONS

We demonstrate the significance of the biotransformation of sulfonamides through the pterin-conjugate pathway and the formation of a suite of derivative transformation products in both batch biotransformation studies with activated sludge and in municipal wastewater treatment plants. Although sulfonamides have been previously reported to act as alternate substrates for DHPS and form pterin-SA conjugates,[32] this is the first report of the dominant contribution of this transformation pathway during wastewater treatment.

The relevance of these findings is highlighted by a number of studies that have demonstrated the potential of diyhdropterin- and pterin-SAs to occupy the active site of the DHPS enzyme,[32,38,50] and to exhibit antibacterial activity by inhibition of the DHPS enzyme.[38] In a recent study, dihydropterin-STZ was even used as a lead structure to develop novel antibacterial agents based on the replacement of dihydropterin with a quinoxaline moiety.[51] In addition to sulfonamide conjugates, other transformation products of SMX modified at the N4-group, including N4-nitro-SMX and N4-hydroxy-SMX (here tentatively detected and denoted as SMX+O), have been shown to exhibit similar or even higher antibacterial activity than SMX.[39] Although no potency information is currently available for the major transformation product PtO-SA, it is a pterin-related conjugate and may demonstrate similar antibacterial activities. Additionally, according to our results, only small fractions of sulfonamides were cleaved at the sulfonamide bridge, resulting in transformation products with an undisputable loss of antibiotic activity.[39] The fact that N4-acetyl-SMX was demonstrated here and by others[12,52,53] to be readily back-transformed to parent SMX in different environments thus further raises the question of the potential of other pterin-conjugate pathway products, such as PtO-SAs, N4-formyl-SA, or Ac-OH-SA, to be transformed back to the parent sulfonamide in the environment. Taken together, our results suggest that although activated sludge treatment in WWTPs reduces the load of parent sulfonamides to the environment, it may well lead to the formation and environmental release of sulfonamide transformation products with similar potential to exert antibiotic activity as the parent compounds.

More generally, our results emphasize the claim that the quantification of the removal of antibiotics alone is insufficient, and that transformation products and pathways must be elucidated thoroughly to understand and evaluate the risks related with the usage and subsequent release of antibiotics to the environment. The same point has previously been underscored for other water treatment processes (e.g., aqueous ozonation[54]) and for other classes of biologically active micropollutants (e.g., steroid hormones, for which a variety of transformation reactions has been shown to lead to only minor structural modifications and hence transformation products with retained or even strongly enhanced endocrine-disrupting activities,[26,55] or pesticide active ingredients[27]). Unfortunately, this point is still often ignored in practice. Bioassays that allow for measuring relevant end points, such as antibiotic activity in complex matrices, and hence enable an effect-driven approach in transformation product analysis may support the consideration of the transformation products in future studies.[56,57]

Finally, our findings are also highly relevant in that they provide a potential explanation of seemingly contradictory findings on optimal conditions for sulfonamide removal in WWTPs. In a number of studies, an association of sulfonamide removal with the addition of readily available carbon sources or measures of heterotrophic activity was found.[20−24,58] Yet, others provide evidence suggesting an involvement of ammonia oxidizing microorganisms,[25,59] such as the correlation of SMX removal with nitrifying activity in an enriched culture of ammonia oxidizing bacteria.[25] In turn, this latter finding stands in contradiction with the fact that sulfonamide degradation was mostly insensitive to the inhibition of the nitrifiers in batch experiments with activated sludge.[45] Our results offer a new view on these discussions in that they demonstrate that sulfonamide biotransformation in activated sludge communities is apparently, to a large extent, related to their interference with folic acid synthesis. Because this pathway is integral to cellular production and maintenance, one can expect sulfonamide biotransformation to pterin-SAs to correlate with bacterial growth. This in turn explains why both nitrifier enrichment cultures and pure heterotrophic cultures both fed with their respective growth substrates, i.e., ammonium or different carbon sources, respectively, show enhanced sulfonamide removal. Sulfonamide biotransformation has also been observed to occur readily under a number of conditions differing from those prevailing in aerated bioreactor experiments with activated sludge. For instance, sulfonamide removal has also been observed under anaerobic[21,58,60] and anoxic[21,61] conditions, in microbial communities from river sediments[52] and river biofilms,[8] and by different algal species.[35] Based on our results, the observed ubiquitous trait of sulfonamide biotransformation in microbial communities becomes a logical consequence of the transformation of sulfonamides through the pterin-conjugate pathway.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b06716.

Information on chemical reference compounds, details on biotransformation experiments; analytical details, listing of parameters used for the suspect transformation product screening, structure elucidation of transformation products by analysis of MS^2 fragmentation spectra; mass spectra, additional results of biotransformation and control experiments and analysis of WWTP samples, and estimation of mass balances (PDF)

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Notes

The authors declare no competing financial interest.

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