Biodegradation studies of N *4-acetylsulfapyridine and* N *4-acetylsulfamethazine in environmental water by applying mass spectrometry techniques*

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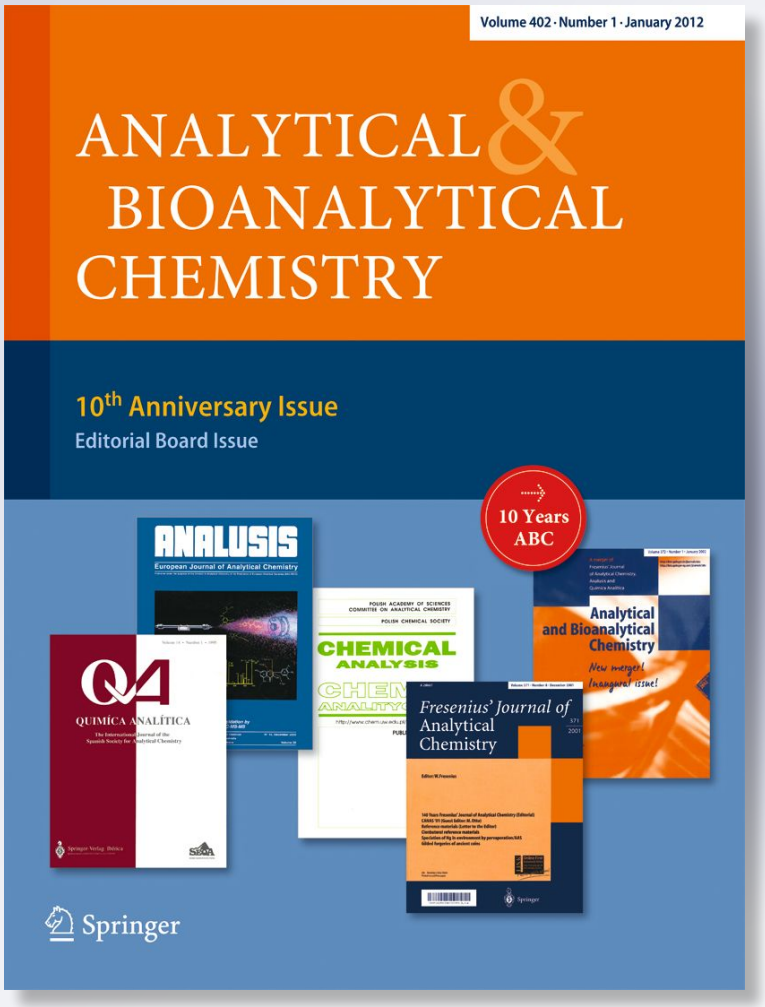

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ORIGINAL PAPER

Biodegradation studies of N^4-acetylsulfapyridine and N^4-acetylsulfamethazine in environmental water applying mass spectrometry techniques

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Abstract This work evaluates the biodegradation of N^4-acetylsulfapyridine (AcSPY) and N^4-acetylsulfamethazine (AcSMZ), metabolites of two of the most commonly used sulfonamides (SAs) in human and veterinary medicine, respectively. Aerobic transformation in effluent wastewater was simulated using aerated fixed-bed bioreactors (FBBRs). No visible changes in concentration were observed in the AcSMZ reactor after 90 days, whereas AcSPY was fully degraded after 32 days of experiment. It was also demonstrated that AcSPY was transformed back to its parent compound sulfapyridine (SPY). The environmental presence of these two metabolites in wastewater effluent had been previously investigated and confirmed, together with three more SA acetylated metabolites and their corresponding parent compounds, in 18 different wastewater treatment plants in Hesse (Germany). Sulfamethoxazole (SMX) and SPY were the two SAs detected most frequently (90% and 89% of the samples, respectively) and in the highest concentrations (682 ng L^{-1} for SMX and 532 ng L^{-1} for SPY). To conclude, hazard quotients were calculated whenever toxicity data were available. None of the SAs studied posed an environmental risk.

Keywords Sulfonamides · Metabolites · Biodegradation · HPLC-MS/MS analysis · Wastewater

Introduction

During the last decade, scientific interest has shifted from studies dealing with the environmental occurrence of pharmaceuticals to their fate and especially the presence of their transformation products (TPs) [1, 2]. Although metabolites are usually pharmacologically less active and less toxic than the parent compound, some of them may still be biologically active when entering the environment and pose a potential risk to the ecosystem [3]. Nevertheless, their environmental presence has become made evident through different studies [4, 5].

Sulfonamides (SAs) represent one of the most frequently used families of antibiotics in Europe, after tetracyclines [6]. They are mainly used in veterinary treatments but also, to a lesser extent, in human medicine, although nowadays the increasing number of resistant bacteria against SAs has made their use inappropriate in human therapies [7]. However, several SAs are still being detected in different monitoring studies in wastewater treatment plants (WWTPs) at different concentrations, indicating that some of these antibiotics are still prescribed to treat different human infections. Their high polarity and solubility in water makes them very mobile once released into the environment and have led to their detection in all kinds of environmental waters

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and also in solid matrices [8–10]. The environmental presence of their metabolites has also been published recently in different works [11–13].

Although the metabolism of SAs is species-dependent [14, 15], they are generally metabolized in the liver by phase I oxidation and phase II acetylation; glucuronide conjugation and aromatic hydroxylation can also take place yielding the N^1 and N^4 derivatives [15, 16]. The amounts of metabolites and unchanged SAs that are excreted depend on the form of the drug and the animal age and species. Livestock will usually excrete up to the 50–90% of the administered dose, usually within several days after treatment; the parent drug makes up for the 9–30% of the excreted amount, and usually acetic acid conjugates comprise 5–60% of the dose [17]. SAs used in human therapies are ultimately excreted into domestic sewage and discharged to WWTPs. Usually, removal efficiencies for SAs and the majority of other pharmaceuticals are calculated measuring the difference of concentration between the levels detected in influent and effluent wastewaters, without considering the formation of metabolites or other TPs [18–20]. The presence of acetylated metabolites such as N^4-acetylsulfamethoxazole (AcSMX) or N^4-acetylsulfamethazine (AcSMZ) in WWTP effluents [8, 11, 13, 21] reinforces the possibility that, during wastewater treatment, these and other different acetylated metabolites are totally or partially cleaved by bacteria and reverted back to the original drug. This deconjugation could explain the higher SAs concentrations in the output than in the input of the WWTPs, which were encountered in recent works [12, 19]. AcSMX was the first SA metabolite to be detected in WWTP effluents in concentrations up to 2.2 μg L^{-1} [22].

As a consequence of its recent inclusion in the scope of monitoring studies, there is a substantial lack of ecotoxicological data regarding adverse effects of these acetylated metabolites (and of SAs in general). However, the risk assessment guidelines set up by the European Medicines Agency (EMEA) for the marketing authorization of new human medicinal products have been used in different occasions to establish the environmental risk posed by drugs that are already being consumed and present in aquatic or terrestrial environments [23, 24]. Briefly, the environmental risk assessment protocol is a two-phase tiered process that begins with an approximate calculation of the predicted environmental concentration (PEC) of the drug in water. These guidelines [25] recommend that any drug with a PEC value > 10 ng L^{-1} in surface water should progress to phase II. Standard acute toxicity tests will be carried out in this stage in order to estimate predicted no-effect concentration (PNEC) or non-observed effect concentration (NOEC). Finally, the ratio of the PEC to PNEC, known as the hazard quotient (HQ), indicates whether a potential environmental impact is implicit and further testing might be needed (HQ> 1). It is also recommended that, when the total concentration of metabolites is a 10% greater than the concentration of the corresponding parent drug, the metabolites are also to be further investigated (phase II tier B) in order to determine their ecotoxicological effects. The EMEA Committee for Medicinal Products for Veterinary Use also established similar guidelines [26].

The aim of this study is to investigate the occurrence and fate of five acetylated metabolites of SAs and the respective parent drugs in 18 different WWTPs in the state of Hesse (Germany) during the year 2008. The biodegradability of sulfapyridine (SPY) and N^4-acetylsulfapyridine (AcSPY), together with AcSMZ, was further studied using a fixed-bed bioreactor (FBBR). Finally, the potential environmental risk posed by each of the studied SAs and metabolites was considered, following the EMEA guidelines.

Experimental section

Chemicals and materials

High purity standards (>99%) of the five selected SAs, namely sulfadiazine (SDZ), sulfamethazine (SMZ), AcSMZ, SMX, SPY, and sulfamerazine (SZI), were purchased from Sigma Aldrich (St Louis, MO, USA). Analytical standards for N^4-acetylsulfadiazine (AcSDZ), AcSMX, AcSPY, and N^4-acetylsulfamerazine (AcSZI) and the internal standard, d_4-sulfathiazole (99.9% purity), were purchased from Toronto Research Chemicals (ON, Canada). High-performance liquid chromatography (HPLC)-grade solvents (water, methanol (MeOH), acetone, and acetonitrile (ACN)) and formic acid (98–100% purity) were supplied by Merck (Darmstadt, Germany). Stock standard solutions for each of the analytes were prepared in MeOH at 1 mg mL^{-1} and stored at −2 °C until use. Standard solutions of the mixtures of all compounds at appropriate concentrations were prepared by the corresponding dilution of the stock solutions in MeOH.

Oasis HLB cartridges (60 mg, 3 mL) were provided by Waters (Milford, MA, USA).

Monitoring study

Sampling area and sample collection

WWTP effluent samples were taken from 18 different WWTPs located in the rural district of Darmstadt-Dieburg, in the state of Hesse (Germany). The land use in this district is mainly agriculture. The proportion of urban/industrial water is approximately 90:10. All the WWTPs followed a similar treatment scheme: pretreatment, primary treatment (phosporous elimination), and conventional activated sludge (CAS) as secondary treatment. Sampling was carried out

every 3 weeks, from 11 April to 12 June of 2007. Three weekly composited samples (volume of 1 L) were prepared collecting hourly small volumes during that period. The mixed samples were stored in amber glass bottles in the dark and frozen until analysis.

Sample preparation

WWTPs effluent samples were extracted and purified by means of a 12-fold vacuum extraction box J.T. Baker (Phillipsburg, NY, USA). The extraction method was based on that used by Buttiglieri et al. [27]. Briefly, 200 mL of WWTP effluent, previously filtered through 0.45-μm glass fiber filters, were preconcentrated on Oasis HLB cartridges; these had been previously conditioned with 2 mL of *n*-hexane, 6 mL of MeOH, and 10 mL of HPLC water. After the sample loading, the cartridges were dried under a gentle nitrogen flow for 40 min, and eluted with 3×1.5 mL acetone/ethyl acetate (1:1; *v/v*). The extracts were evaporated to dryness under a gentle nitrogen stream and finally reconstituted with 500 μL of a mixture of H_2O/MeOH (80/20; *v/v*) with 5 mM ammonium formate. d_4-sulfathiazole was used as internal standard and was added to each sample before extraction at a concentration of 500 ng L^{-1}.

Liquid chromatography-tandem mass spectrometry

Reversed-phase HPLC was performed using two Series 200 Micro Pumps (Perkin-Elmer, Norwalk, CT, USA). Separation was carried out on an Atlantis C18 (Waters) (150×2.1 mm, 3 μm) LC analytical column, preceded by a guard column with the same packing material. The mobile phase consisted of HPLC-grade water (A) and ACN (B), both with 10 mM formic acid. The initial conditions of the elution gradient programmed were A/B 75:25%. From 0 to 11 min, the eluent B was increased to 100% held for 2 min and returned to initial conditions in 3 min. Finally, the column was equilibrated for further 7 min before the next injection. The flow rate was 0.2 mL min^{-1}, and the injection volume of standards and sample extracts was set at 10 μL.

Tandem mass spectrometry (MS/MS) analysis was performed on a 3200 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray electrospray (ESI) interface in the positive ionization mode. For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction monitoring mode. For each analyte, the two most abundant product ions were monitored: The more abundant was used for quantitation and the other one for confirmation. Table 1 summarizes the MS/MS conditions for the SAs investigated. The temperature of the source was set at 700 °C, and a positive capillary voltage of 3.5 kV was applied; nitrogen was used as both nebulizing and collision gas (when performing MS/MS experiments).

Degradation experiments

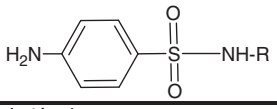
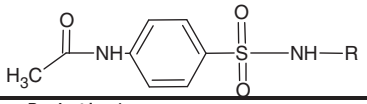
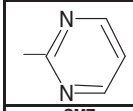
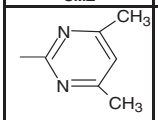
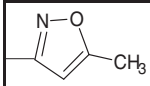
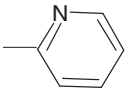
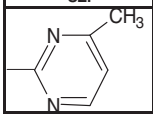
FBBRs consisted of a tank or storage bottle connected to a fixed bed, consisting of a glass column filled with glass beads (18 cm filling level, 350 mL volume); the effluent wastewater was circulated on a closed loop through this column by means of a vacuum pump, allowing the microorganisms of the water matrix to accumulate on the surface of the glass beads [28]. The flow rate was 16 mL min^{-1} on average. A second membrane pump aerated the water in the storage bottle. Samples were taken through a three-way valve at the top of the fixed bed. A scheme of the experimental design is depicted in Fig. 1.

Four different FFBRs were set up in order to assess the biodegradability of SPY, AcSPY, and AcSMZ. The fourth reactor corresponded to a blank control. Each of them were run with 5 L of WWTP effluent from the municipal WWTP Beuerbach (Hesse, Germany) and spiked with the corresponding analyte at 200 μg L^{-1}. Toxicity of the SAs investigated against the microorganisms of the reactors was considered negligible, as SAs concentrations up to 0.5 mg L^{-1} exert no negative effects on aerated reactors containing richer bacterial populations [29]. The measured pH of the wastewater was 6.9 (average value). The experiment ran for 60 days in the case of SPY, AcSPY, and the blank and for 92 days for AcSMZ, at room temperature and neutral pH. The reactors were kept in the dark to avoid photodegradation. Volume losses due to evaporation were compensated with the addition of equal volumes of the same water matrix. FBBR samples were taken periodically along the experimental period, filtered through a 0.45-μm membrane filter and stored at −20 °C until analysis.

UPLC-QqTOF-MS

Chromatographic separation followed by accurate MS analyses of each of the target analytes and their corresponding TPs was carried out quadrupole tandem time of flight (QqTOF) coupled to a Waters Acquity ultra-performance liquid chromatography (UPLC) system (Micromass, Manchester, UK). Regarding chromatography, a Waters Acquity BEH C18 column (10×2.1 mm, 1.7 μm particle size) was employed. Flow rate was set at 0.3 mL min^{-1}, with eluent A, HPLC-grade water, and eluent B, ACN, both containing 10 mM of formic acid. The elution started at 5% B for 2 min; it was then linearly increased to 60% of B in 7 min, further increased to 95% of B in the following 2 min, and then returned to initial conditions. Total run time, including the conditioning of the column and back to initial conditions, was 13 min. The injection volume of the sample was set to 5 μL.

Table 1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) parameters for the different SAs and their respective acetylated metabolites analyzed

	Sulfonamide						N^4-Acetylsulfonamide					
												
R	Precursor Ion	Product ion 1 Product ion 2	RT (min)	DP (V)	CE (V)	CXP (V)	Precursor Ion	Product ion 1 Product ion 2	RT (min)	DP (V)	CE (V)	CXP (V)
SDZ							AcSDZ					
	251	251/156 251/108	4.83	46 46	27 30	10 8	293	293/134 293/198	4.77	65 65	30 30	12 12
SMZ							AcSMZ					
	279	279/156 279/124	6.24	26 26	30 35	10 10	321	321/134 321/124	5.18	86 86	35 35	4 4
SMX							AcSMX					
	254	254/156 254/108	11.20	56 56	25 27	10 10	296	296/134 296/298	11.40	60 60	30 30	10 10
SPY							AcSPY					
	250	250/156 250/92	4.99	51 51	28 31	12 6	292	292/134 292/198	4.63	70 70	30 30	8 8
SZI							AcSZI					
	265	265/92 265/156	5.61	61 61	47 27	6 8	307	307/134 307/110	5.02	60 60	35 35	8 8

Compound-dependent parameters: *CE*, collision energy (V); *DP*, declustering potential (V); and *CXP*, collision cell exit potential (eV)

MS analyses were performed in the positive ionization mode, and the operating conditions were as follows—desolvation gas flow, 350 L h^{-1}; source and desolvation temperatures, 120 °C and 350 °C, respectively; capillary voltage, 3,000 V; sample voltage, 30 V; and extraction cone voltage, 1 V. The collision energy was held at 4 V for MS analyses, and the applied collision gas was argon. For continuous internal mass calibration, an independent reference (valine–tyrosine–valine) was used as a lock mass, with *m/z* 380.2185 and was acquired in all the measurements. MS data was recorded scanning from *m/z* 50 to *m/z* 500.

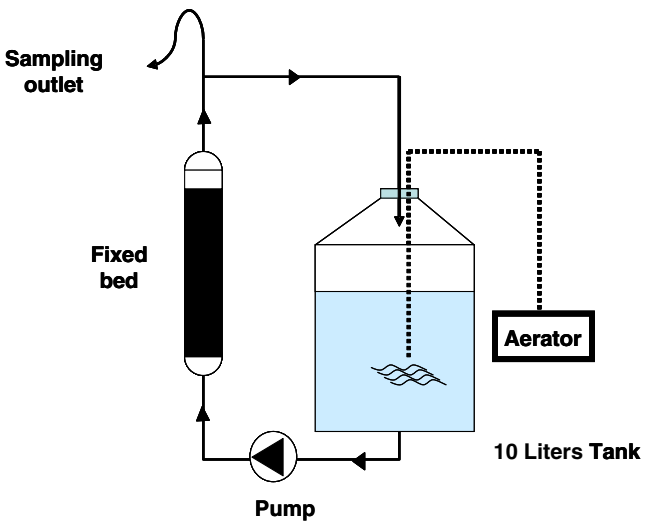


Fig. 1 Schematic setup of the FBBR

Results and discussion

Monitoring study

Method validation

Performance parameters are listed in Table 2. Calibration curves were linear for all compounds in the range 0.5–100 ng L^{-1}. Linearity is expressed as the regression coefficient (r^2) and was always equal or above 0.998. Accuracy of the method is given by the recovery values of the SAs studied and ranged from 85% to 137%. Instrumental variation is expressed as the relative standard deviation of three consecutive injections of a standard mixture at 5 ng L^{-1}. Values ranged from 2.8 (SMZ) to 16.5 (SDZ). Quantitation was performed based on the internal standard approach, adding d_4-sulfathiazole to all the samples and aqueous

Table 2 Performance parameters of the offline solid-phase extraction–LC-MS/MS method applied in the monitoring study and results from the monitoring study

Method performance						Concentration				
SAS	R% (± SD)	r^2	RSD	MLOD	MLOQ	Maximum (ng L^{-1})	Minimum (ng L^{-1})	Average (ng L^{-1})	Median	Relative frequency (%)
SDZ	85.3 (14.08)	0.9986	7.2	3.79	12.63	152	10.1[a]	54.9	42.6	72.0
AcSDZ	92.8 (14.16)	0.9996	6.5	2.16	7.19	25.3	3.66[b]	14.2	13.3	39.0
SMZ	114.9 (3.25)	0.9998	9.4	0.56	1.87	27.4	0.91[c]	8.2	1.9	13.0
AcSMZ	109.8 (6.02)	0.9976	5.5	1.20	4.00	–	–	–	–	–
SMX	137.5 (19.18)	0.9996	5.4	1.51	5.04	532	55.1	229.6	207	91.0
AcSMX	105.4 (9.00)	0.9998	11.5	0.85	2.84	190	2.8	44.3	26.3	59.0
SPY	123.3 (8.40)	0.9996	4.6	2.14	7.14	682	47	309.3	233	89.0
AcSPY	115.5 (12.46)	0.9986	8.9	2.97	9.90	415	11	134.7	119.5	74.0
SZI	125.3 (6.63)	0.9986	4.4	0.12	0.41	–	–	–	–	–
AcSZI	122.3 (11.21)	0.999	4.9	1.29[d]	4.30[d]	–	–	–	–	–

R% recovery values, R^2 coefficient of determination, *RSD* relative standard deviation (%), *MLOD* method limit of detection (average value), *MLOQ* method limit of quantification (average value)

[a] MLOQ value for this sample=4.27

[b] MLOQ value for this sample=3.10

[c] MLOQ value for this sample=0.25

[d] Values obtained from the lowest calibration point of the curve, as it was not detected in any of the samples

standards for the calibration curve at a concentration of 500 ng L^{-1} before SPE. Method limits of detection and quantification (MLODs and MLOQs, respectively) were calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively, in the different samples analyzed. MLOD values were in the range of 0.12 ng L^{-1}–3.8 ng L^{-1} (for SZI and SDZ, respectively). Values given for AcSZI correspond to the lowest point of the corresponding calibration curve, as it was not present in any of the samples.

Results

As shown in Table 2, SMX and SPY, typically used in human medicines, were the two SAs most frequently detected (90% and 89% of the samples, respectively) and in the highest concentrations (682 ng L^{-1} for SMX, 532 ng L^{-1} for SPY); these data agree with previous publications in which these two SAs were also the most commonly detected not only in WWTPs, but also in river and groundwaters [12, 30]. AcSMX and AcSPY were also the most ubiquitous metabolites and were detected in the highest concentrations. On the contrary, neither AcSMZ nor AcSZI was detected in any of the effluent samples. SZI was also not detected in any of the samples, whereas SMZ was detected in seven of them, although, in three occasions, levels were below MLOQ.

Figure 2 shows the box plots for SMX, SDZ, SPY, and their respective acetylated metabolites, using the average concentration values of the three samples taken in each of the WWTPs. Medians indicate that concentrations were generally skewed to lower values, except for AcSPY, which shows an even distribution of the concentrations. All the metabolites exhibit a narrower distribution of their concentration levels in contrast with their respective parents, which show a higher concentration range, especially at levels above the median. However, the metabolites also presented the three only values out of the box plot range: AcSMX showed two concentrations out of the interquartile range and AcSPY, one.

Degradation study

The biodegradability under aerobic conditions of SPY and AcSPY was evaluated using two FBBRs containing the parent drug and the acetylated metabolite, respectively, at 200 μg L^{-1}. The selection of SPY is justified by the fact that, as mentioned in the previous section, it is one of the most commonly detected SAs (together with SMX), whereas the latter is commonly within the scope of different research studies regarding WWTPs [32–34]; to the authors' knowledge, both SPY and AcSPY have barely been investigated. Another FBBR was set up for AcSMZ, at the same concentration. Although it was not present in any of the German WWTPs monitored in this study, this metabolite has been detected in WWTP effluents as well as in river waters

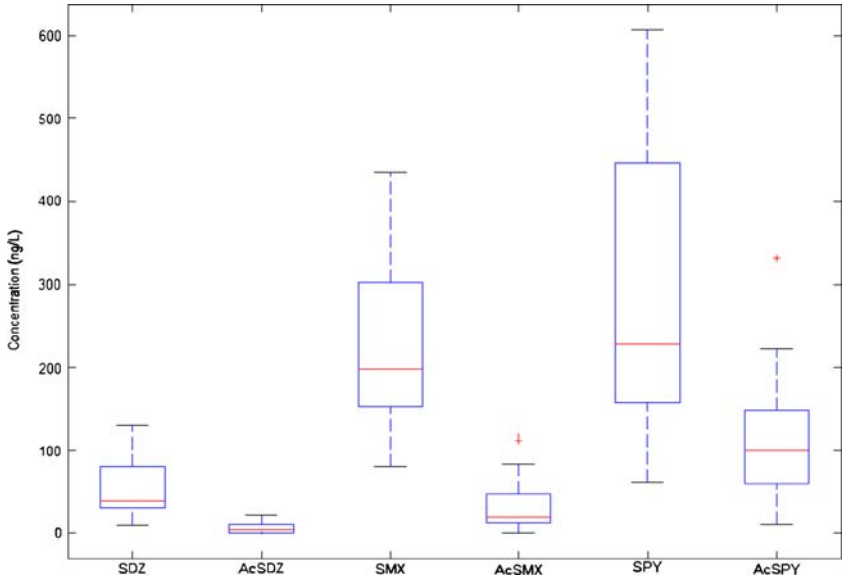


Fig. 2 Box *plots* showing the distribution of the concentrations of the corresponding SAs and their acetylated metabolites in the 18 German WWTPs studied. Concentrations are given as the average values of the three samples taken in each WWTP

and groundwaters in Spain [12, 30]. Although the spike concentrations used are not environmentally realistic, these levels were required for the identification of degradation intermediates (see section "Identification of degradation products of SPY and AcSPY"). SPY is usually detected at the highest concentrations in WWTP effluents, together with SMX, and usually in the nanogram-per-liter range.

The concentration profiles of the assayed compounds in the FBBRs are displayed in Figs. 3 and 4. No background concentrations for any of the SAs under study were detected in the blank FBBR (data not shown).

The initial concentration of SPY was unaltered till the 20th day. From then on, concentration was reduced to 50% within only 7 days and down to 1% by the end of the experiment, after 60 days. Total depletion of the initial concentration was not accomplished within the length of the experiment. It has been demonstrated that, in general, biodegradation is initially negligible when a new compound is introduced in the system till the specific degrading microbiota grow enough to make degradation relevant [29]. Then, SAs can serve as nitrogen and carbon sources for the microorganisms [35]. The decrease of SPY concentration in the FBBR could therefore be attributed mainly to biodegradation, with an initial lag phase required by the microbial community to adapt their enzymatic systems to use the antimicrobial as a nutrient and the following accelerated degradation.

As shown in Fig. 4, the degradation profile for AcSPY is quite different. T_0 corresponded to the first sample taken on the same day after the spike, and in the case of AcSPY, mixing was not fully accomplished, and the initial total concentration was detected on the following day. Concentration seemed to diminish gradually and irregularly during the first 25 days, till only the 50% of the initial concentration remained. At this point, concentration dropped quickly, and the metabolite was fully removed from the reactor in 7 days. However, dissolved organic carbon measurements were not carried out, and the full degradation of either SPY or AcSPY in the FBBRs cannot be confirmed. The slow decrease of AcSPY from the first day, without presenting a lag period for the degrading microbiota to acclimatize and/or develop, may indicate that no biodegradation was taking place during that first stage, but abiotic processes such as hydrolysis or adsorption onto biomass. The latter was not considered as biomass is present in the reactor in low amounts if compared, for instance, to CAS processes. However, the glass beads of the fixed bed could represent a significant adsorption site [36], and AcSPY, despite its high polarity, could have been gradually retained in the bed. Nevertheless, the marked decline in the profile from day 25 till day 32, similar to the decline of SPY in Fig. 3, suggests that, in fact, adaptation took place for the microorganisms during an initial lag phase (simultaneous to the slow disappearance of the metabolite). The concentration of SPY in the same reactor was detected from the first day and started to increase simultaneously to the decrease of the metabolite, reaching a peak concentration on day 39, when AcSPY was fully depleted (Fig. 4). Bacteria degrading the metabolite could break the amidic bond between the SA and the acetyl group, yielding the parent compound. But, as SPY was detected from the first day of the experiment, both abiotic and biotic processes should be regarded. It is also noticeable that the maximum concentration reached by this newly formed SPY equaled the concentration of AcSPY at which biodegradation started (half of its initial concentration); this correspondence suggests that, before day 23, the transformation of the metabolite yielded different byproducts and SPY only in small quantities, whereas, from that day on, the increase of SPY was more relevant. However, SPY remained only briefly in the FBBR and started

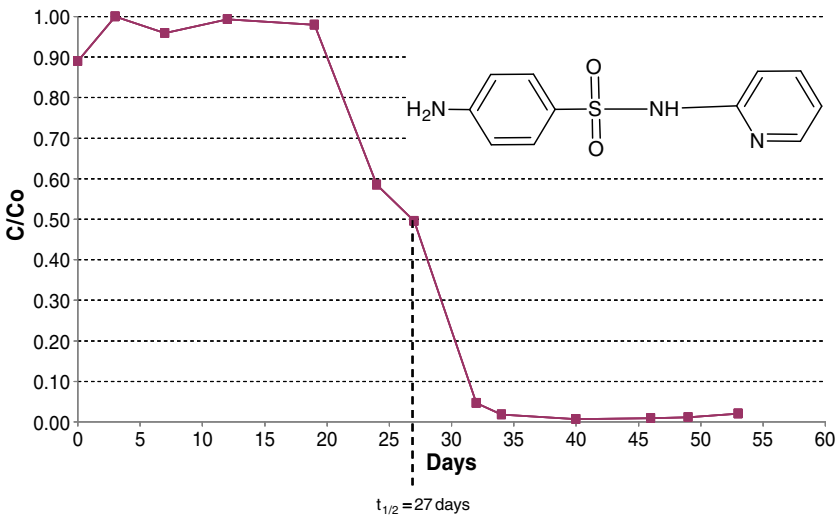


Fig. 3 Degradation profile of SPY on a FBBR containing effluent wastewater spiked at 200 μg L^{-1}

decreasing right after reaching the maximum concentration. Then, it was degraded at a similar rate as the spiked SPY in the first FBBR, and the concentration decreased to its half in 7 days. No lag phase for adaptation could be determined in this case, suggesting two possibilities:

a): The degraders involved in the depletion of the metabolite were capable of degrading not only the amidic bond of the metabolite but also different structural parts of the parent compound; Ingerslev et al. demonstrated that the specific degraders developed during exposure to four different SAs could easily degrade four other SAs in activated sludge and attributed this capacity to the hydrolysis of common structural parts [29]. The observation of the degradation of AcSPY and SPY on the same FBBR could demonstrate that SAs can be vulnerable to their metabolites degrading bacteria, as these acquire general properties needed for degradation of both metabolite and parent drug during the lag period.

b): Different types of bacteria might be devoted to the degradation of both species separately. In this case, a lag phase could be inferred from the moment the newly formed SPY was present in the reactor at enough concentration for bacteria to start their adaptation until the degradation started.

The newly formed SPY, similar to what could be observed in the FBBR for SPY, was not fully depleted by the end of the experiment.

In order to check if a different acetylated metabolite followed this behavior, a third FBBR was set up containing AcSMZ at the same initial concentration (200 μg L^{-1}). However, the metabolite concentration remained almost unaltered during the whole experiment. The length was extended to 90 days, but no changes were observed in the profile (Fig. 5). Similar results were achieved in different laboratory-scale FBBRs set up for other pharmaceuticals such as carbamazepine or clotrimazole [37, 38], which were considered as recalcitrant. In these studies, the spike concentration of the target compound was lower and the possibility of surpassing the toxicity threshold of the degrading bacteria disregarded. A plausible explanation for the outcome of the AcSMZ FBBR could be that the toxicity threshold for AcSMZ has been exceeded. The lack of data

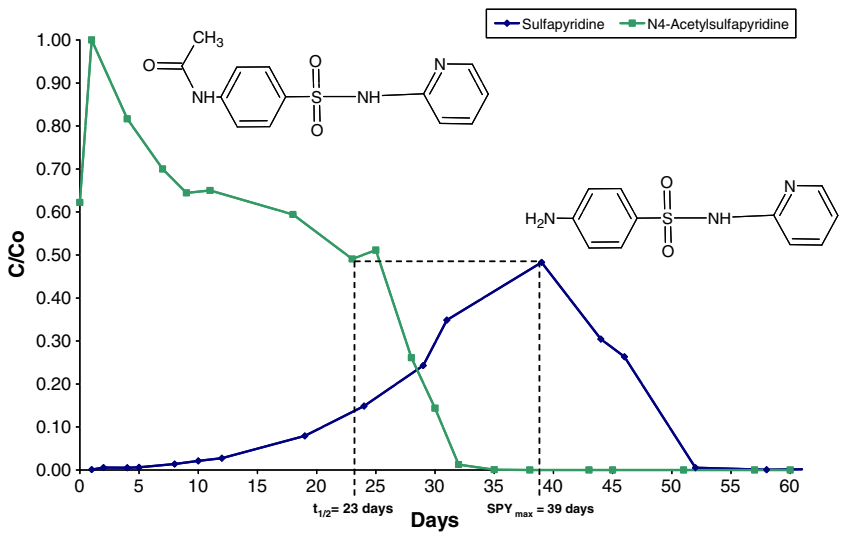


Fig. 4 Degradation profile of AcSPY on a FBBR containing effluent wastewater spiked at 200 μg L^{-1} and simultaneous appearance of newly formed SPY

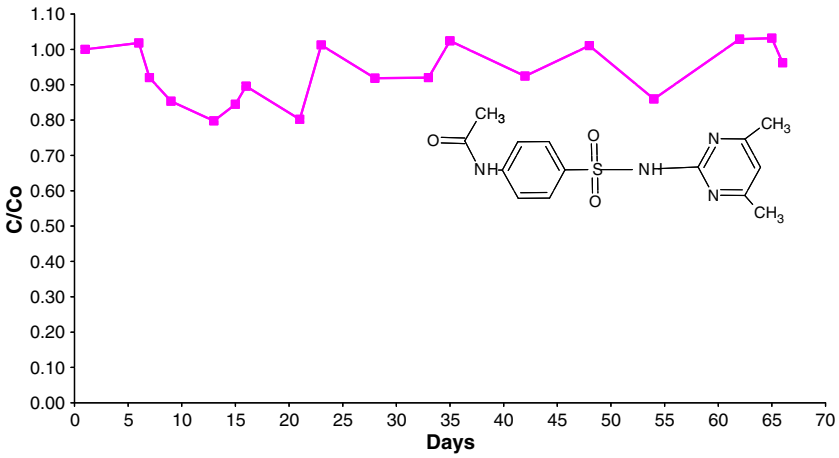
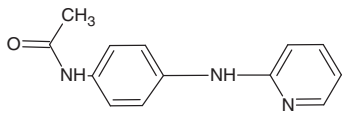
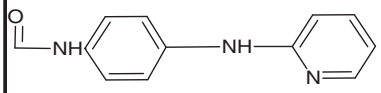
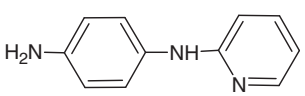


Fig. 5 Degradation profile of AcSMZ on a FBBR containing effluent wastewater spiked at 200 μg L^{-1}

regarding ecotoxicity of SAs and their metabolites hinders a clear conclusion for this result.

Despite lag phases observed, the FBBRs for SPY and AcSPY in this study are much longer than the average WWTPs hydraulic retention times (usually below 24 h [19]), the continuous exposure to different SAs and metabolites could lead to the adaptation of microbiota to these drugs. Besides, the content of biomass in CAS is much higher, and therefore the biodegradation should be accelerated. However, several studies on the presence and fate of SAs in WWTPs show rather incomplete elimination rates. In the particular case of SPY, these rates are diverse and hard to interpret. In a recent study carried out in seven different WWTPs during two consecutive years [12], the estimated elimination rates for SPY were dissimilar; in 2007, the elimination rate varied from 43% to 77% in four of the WWTPs; SPY concentration in the effluent was higher than in the influent in two of the WWTPs, and in one of them, SPY was only detected in the effluent. In 2008, the elimination rate ranged from 6% to 96%. Although AcSPY was not included in the scope of this study, these results strongly suggested its presence in the influent wastewaters and its deconjugation during the aerobic treatment. Göbel et al. reached the same conclusion when observing the SMX concentrations in the effluents, which were more than twice the inflowing load in some cases [39]. Elimination rates for AcSMX were usually above 80%, whereas those for SMX were often negative. A relevant factor to bear in mind regarding degradation rates is the temperature of the WWTP. Lower temperatures usually mean longer lag phases for the microbiota and slower degradation rates. For instance, in the study by García et al. previously mentioned [12], the variability in the elimination rates of each year could be also due to the fact that the sampling campaign in

Table 3 Accurate mass measurements of the degradation products determined by UPLC/ESI (+)-QqTOF in MS mode

Fragment (m/z)	Samples	Observed mass	Calculated mass	Elemental composition	Error (mDa)	DBE	Proposed structural composition
228	AcSPY-38 AcSPY-65	228.1142	228.1137	$C_{13}H_{14}N_3O$	2.3	8.5	
214	AcSPY-32 AcSPY-38 AcSPY-65 SPY-32 SPY-35 SPY-38	214.0984 214.0963	214.0980	$C_{12}H_{12}N_3O$	1.3 2.6	8.5	
186	AcSPY-32 AcSPY-65 SPY-32 SPY-35 SPY-38	186.1039 186.1030	186.1031	$C_{11}H_{12}N_3$	2.3 1.4	7.5	

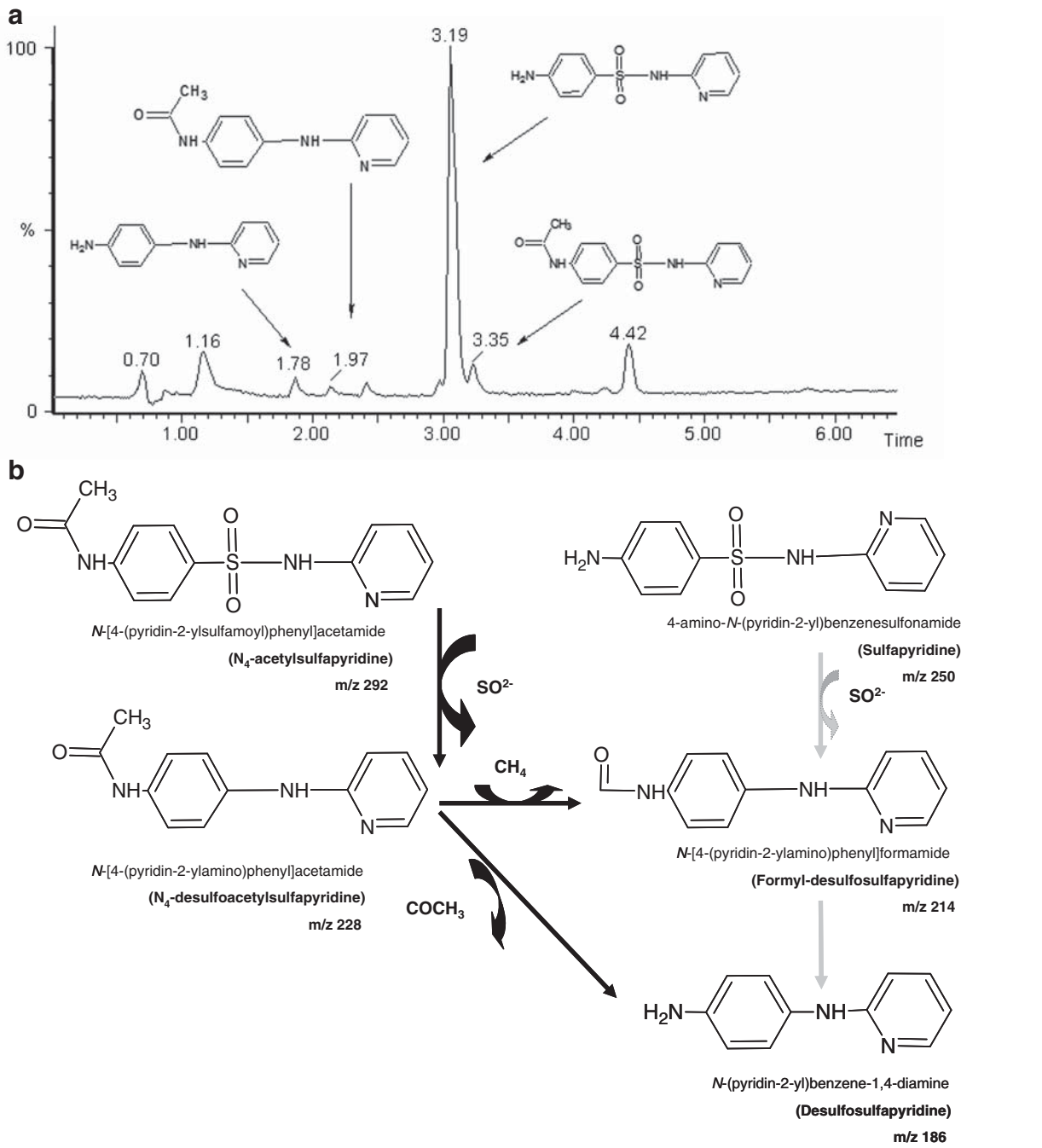


Fig. 6 Chromatogram corresponding to AcSPY degradation experiment (day 38) (**a**) and proposed transformation products of AcSPY and SPY detected during the FBBR experiment (**b**)

2007 was carried out in autumn, whereas in 2008, samples were taken in summer. Also, the amount of nutrients in the water matrix should be considered when estimating elimination rates. Drillia et al. demonstrated that the degradation of different pharmaceuticals started only when carbon or nitrogen were depleted, and then microbiota used up these molecules as alternative nutrient source [35]. Higher amounts of organic matter in the wastewater would lead to longer lag phases or an inefficient elimination.

Identification of degradation products of SPY and AcSPY

As shown in Fig. 4, the decrease of AcSPY in the FBBR partly corresponded to its back-transformation in SPY. The remaining concentration may have been fully mineralized but also transformed into different metabolites. A similar case was presented in the SPY FBBR. Samples corresponding to days 30, 32, 38, and 65 of the AcSPY degradation experiment, in which AcSPY reached its minimum, and similarly, samples

Table 4 Percentage of the acetylated metabolites concentration with respect to the concentration of their respective parents, MECs (values corresponding to the maximum concentration detected, in mg L^{-1}), PNECs (value corresponding to the EC_{50} values divided by a safety factor of 1,000), and HQs estimated for the SAs metabolites investigated

SAS	Metabolites percentage	Ec_{50}	MEC	PNEC	HQ
SDZ	–	–	1.52E-04	–	–
AcSDZ	25.9	–	2.53E-05	–	–
SMZ	–	344.7	2.74E-05	3.45E-01	0.00008
AcSMZ	–	–	–	–	–
SMX	–	78.1	5.32E-04	7.81E-02	0.00681
AcSMX	19.3	–	1.90E-04	–	–
SPY	–	27.4*	6.82E-04	2.74E-02	0.02489
AcSPY	43.5	8.2*	4.15E-04	8.20E-03	0.05061
SZI	–	–	–	–	–

EC_{50} values given are for *V. fischerii* after an exposition time of 15 min [31]

–: values not available. *: unpublished results

of days 32, 35, and 38 in the case of the SPY FBBR were individually investigated in order to identify different possible intermediate compounds. The use of the tandem quadrupole time-of-flight (QqTOF) instrument in MS full-scan mode provided the exact masses of the potential unknowns. After chromatographic separation with UPLC, two new unidentified peaks appeared in the chromatogram of both AcSPY and SPY samples. A full confirmation with product ion scans (MS/MS) could not be performed because the concentrations of the intermediate products were not high enough to reach the LODs of the QqTOF. The chemical structures proposed were confirmed with the double-bond equivalents (DBE) obtained.

AcSPY transformation products A new chromatographic peak showed after 1.98 min in sample AcSPY-38, with a base peak *m/z* 228.1182 in the corresponding mass spectra. The proposed elemental composition for this mass is $C_{13}H_{14}N_3O$ (theoretical *m/z* 228.1137; Table 3) and corresponds to the loss of the sulfonate group in AcSPY (see Fig. 6). This finding agrees with previous studies in which both degradation and photodegradation products of different SAs such as sulfamethazine or sulfadiazine were defined as SO_2 extrusion products [40–42]. This new product was also detected after 65 days in the AcSPY, when AcSPY was not longer detectable.

A second peak appeared at a chromatographic retention time of 5.7 min in both the AcSPY and SPY samples. It corresponded to the mass *m/z* 214.1006, and its elemental composition was attributed to $C_{12}H_{12}N_3O$; the molecular structure is proposed in Fig. 6 as N^4-formyl-SPY-desulfonate and could be identified in AcSPY samples of days 32, 38, and 65, and in SPY samples of days 35 and 38.

SPY transformation products Besides the peak attributed to N^4-formyl-SPY-desulfonate, a second peak was detected in the three SPY samples investigated, at a chromatographic retention time of 1.78 min; it corresponded to an *m/z* of 186.1039. In this case, the elemental composition that best fitted was $C_{11}H_{12}N_3$, and the molecular structure proposed corresponds to the desulfonated SPY (Fig. 6).

Environmental risk assessment

Following the EMEA guidelines, HQs were calculated to estimate the potential adverse effects of the SAs levels detected on non-target organisms. HQs were also estimated for the metabolites, altough their average concentrations were not a 10% greater than the levels detected for the corresponding parents (Table 4). This quotient is usually calculated as the ratio between the PEC and the PNEC or NOEC. When PNEC values are not available, an alternative PNEC can be derived by dividing the lowest EC_{50} or LC_{50} values (50% lethal concentration) by a safety factor of up to 1,000 [43, 44]. Chronic toxicity data would be more representative of the environmental conditions, but this information was lacking for SAs metabolites and acute toxicity test values (EC_{50} or LC_{50}) were used up as chronic toxicity data after being normalized by a safety factor of 1,000. Likewise, measured environmental concentrations (MECs) were used in the calculation instead of PECs [18–20]. In order to set up a worst-case scenario, MEC values used corresponded to the maximum values detected in the monitoring study whereas EC_{50}–LC_{50} values used were the lowest found in the literature. In all cases, the MECs were higher than the boundary value of 0.01 μg L^{-1} established by EMEA in tier 1. Only two publications showed ecotoxicity data for other SAs metabolites and only against the green algae *Selenastrum capricornotum* [45] and against the marine bacteria *Vibrio fischerii* (unpublished results). Table 4 summarizes the EC_{50}–LC_{50} values taken and the HQ calculated. For AcSPY, the corresponding HQ value was below the boundary of 1 established by the EMEA guidelines, and therefore the levels detected in the effluents would pose no ecological risk (Table 4). Similar results were obtained for the rest of SAs, which HQs could be estimated. In previous studies, only SMX yielded HQs higher than 1 in the same matrix [12, 19, 24] but only against green and blue–green algae, which are generally the most sensitive taxa.

Conclusions

Nowadays, SAs are one of the most common water-polluting antibiotics. The presence of their metabolites has become relevant only recently. As demonstrated in the results of the monitoring study carried out, three of the five acetylated metabolites investigated were present at detection frequencies and concentrations similar to their parent compounds. Aerobic degradation experiments, simulated in laboratory scale FBBRs, have demonstrated that AcSPY, the acetylated metabolite detected at the highest frequency and concentrations, is completely eliminated after 35 days but not fully mineralized as half of its initial concentration reverts back to the parent compound, SPY. The rest is probably transformed to different degradation products. Two of them could be identified in this work but not fully confirmed due to the low concentrations. It could be concluded that biodegradation of both SPY and AcSPY was carried out by the same adapted microorganisms, although different bacteria communities could have also degraded the different molecules separately. In this and a second FBBR, in which the fate of SPY was studied, it was not fully degraded and was still present at the end of the experiments, although at very low concentrations. Despite the low biomass content, it seems that adsorption may play a role in the initial decrease of AcSPY in the reactor, as the glass beads of the fixed bed could retain the metabolite. On the contrary, the concentration of AcSMZ was invariable after 90 days of experiment.

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