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Carbon stable isotope fractionation of sulfamethoxazole during biodegradation by *Microbacterium* sp. strain BR1 and upon direct photolysis

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ABSTRACT

Carbon isotope fractionation of sulfamethoxazole (SMX) during biodegradation by *Microbacterium* sp. strain BR1 (*ipso*-hydroxylation) and upon direct photolysis was investigated. Carbon isotope signatures ($\delta^{13}C$) of SMX were measured by LC-IRMS (liquid chromatography coupled to isotope ratio mass spectrometry). A new LC-IRMS method for the SMX metabolite, 3-amino-5-methylisoxazole (3A5MI), was established. Carbon isotope enrichment factors for SMX (ε_C) were -0.6 ± 0.1‰ for biodegradation and -2.0 ± 0.2‰ and -3.0 ± 0.2‰ for direct photolysis, at pH 7.4 and pH 5, respectively. The corresponding apparent kinetic isotope effects (AKIE) for *ipso*-hydroxylation were 1.006 ± 0.001; these fall in the same range as AKIE in previously studied hydroxylation reactions. The differences in SMX and 3A5MI fractionation upon biotic and abiotic degradation suggest that compound specific stable isotope analysis (CSIA) is a suitable method to distinguish SMX reaction pathways. In addition, the study revealed that the extent of isotope fractionation during SMX photolytic cleavage is pH-dependent.

INTRODUCTION

The contamination of aquatic systems with pharmaceuticals has become an increasing environmental problem in recent years. Pharmaceuticals are usually found in low concentrations (ng L^{-1} to µg L^{-1}), but their presence may lead to unpredictable and irreversible ecotoxicological effects.[1] Antibiotics raise particular concerns, due to potential spread of antibiotic resistance.[2, 3] Sulfamethoxazole (SMX) is a representative of sulfonamide antibiotics (SAs), widely used in veterinary and human medicine.[4-6] SMX is incompletely metabolized and enters the environment with wastewater discharge, animal manure and aquaculture. Conventional wastewater treatment technologies cannot cope with removal of SAs, and SMX is frequently detected in wastewater treatment plants' (WWTP) effluents, surface water and ground water.[6-8] Once released into the environment, it may undergo different degradation processes, such as biodegradation, chemical reactions and photodegradation.

Biodegradation is among the key routes of SMX removal from the environment and has been observed in soils, sediments[9, 10] and in WWTPs.[6, 11-13] Multiple sulfonamide degrading strains have been described, using SMX as the sole carbon and energy source, including *Microbacterium*[9, 14-16], *Pseudomonas*[15, 17], *Achromobacter*[18], *Brevundimonas*[15] and *Variovorax*[15] strains. The biodegradation pathways for most of these organisms are not known. *Microbacterium* sp. strain BR1 is a strain for which the most detailed research on the pathway was carried out[19] and is thus a good model organism for further studies. In this strain, SMX degradation is initiated by hydroxylation of the carbon atom attached to the sulfonyl group (*ipso*-hydroxylation), which leads to the release of sulfite, 3-amino-5-methylisoxazole (3A5MI), and benzoquinone-imine. The latter is concomitantly transformed to 4-aminophenol.[19] The accumulation of 3A5MI was also identified during SMX degradation by *Achromobacter denitrificans* PR1[18] and by *Pseudomonas psychrofila* HA-4.[17] However, the

co-accumulation of aniline, 4-aminophenol and sulfanilamide by strain HA-4 may suggest the occurrence of different pathways.[17]

Photodegradation is another important process contributing to the elimination of light-sensitive compounds such as SAs from the environment, especially from sunlit surface waters.[20-22] It also potentially contributes to limiting the release of pharmaceuticals from WWTPs to the environment. UV-based wastewater treatment technologies have been proposed and are currently being tested for the removal of micropollutants, including sulfonamides, from the WWTP effluents.[23-25] Previous works have found SMX to be degraded predominantly by direct photolysis.[20, 22, 26] A few studies have characterized SMX photolysis products, the most commonly detected being sulfanilic acid, 3A5MI, and SMX tautomers (structural isomers).[20-22, 26-28] Photodegradation of SMX may occur through cleavage at various positions, but the γ- and δ- cleavage as well as the photoisomerization by rearrangement of the isoxazole ring seem to represent the main pathways (Fig. 1 A).[20-22, 27, 28]

A thorough understanding of SA's removal routes is crucial for an assessment of their environmental impact and for the evaluation of novel wastewater treatment technologies targeted at these pollutants but the knowledge available so far is not comprehensive. Few metabolites of biodegradation and photodegradation have been identified, as described above.[17-22, 26, 28] Recent reports on phylogenetic variability of microbial strains utilizing sulfonamides as growth substrates and the first data on their metabolites, may imply some heterogeneity of metabolic pathways.[15, 17-19] Understanding sulfonamide degradation processes is currently a pressing research question and distinguishing between bio- and photodegradation may be particularly important in the context of wastewater treatment.

However, the analytical methods at hand are rather limited. Monitoring of sulfonamides in the environment and in WWTPs mainly relies on concentration analysis often carried out by liquid chromatography mass spectrometry (LC–MS).[29, 30] Yet it is very difficult to obtain information on the involved reactions and degradation pathways based on LC-MS analysis,

because the metabolites are often unknown or can be further transformed. Additionally, in the case of SMX, the same metabolite, 3A5MI, is released upon both bio- and photodegradation.[17-22, 26, 28]

Compound specific stable isotope analysis (CSIA) is another tool that has the potential to provide additional information on the organic contaminants' transformation processes in complex environments.[31, 32] In CSIA, the changes in isotope composition of the parent compound are monitored during (bio)transformation processes and the isotope enrichment of the contaminant investigated provides evidence for its (bio)degradation without the need for metabolite analysis. This happens because the chemical bonds in the molecules formed by light isotopes react with different kinetics than the bonds with heavier isotopes (kinetic isotope effects, KIE).[31] The extent of isotope fractionation depends on the type of transformation reaction and the isotope fractionation investigations might be used as an indicator for distinct (bio)transformation pathways.[32] However, so far the application of CSIA has mostly been restricted to environmental contaminants amenable for gas chromatography separation technique, such as monoaromatic hydrocarbons (BTEX), fuel additives (MTBE) and chlorinated solvents.[33] An interface coupling liquid chromatography (LC) with isotope ratio mass spectrometry (IRMS) has been available since 2004 and some LC-IRMS methods have emerged for applications in archeology, food authentication and environmental sciences.[34, 35] However, to our knowledge, there is no study available on the investigation of pharmaceuticals' (bio)degradation by LC-IRMS.

Here for the first time we evaluated the applicability of CSIA for the assessment of SMX transformation pathways. The isotope fractionation of SMX during biotic degradation by *Microbacterium* sp. strain BR1 and abiotic transformation via direct photolysis was determined. CSIA was based on LC-IRMS measurements. The method for SMX was adapted after Kujawinski et al.[36], and a new method for the SMX metabolite, 3A5MI, was established.

MATERIALS AND METHODS

Information about all chemicals used in this study can be found in Supporting Information, hereafter SI.

Biodegradation experiment with *Microbacterium* strain BR1. Biodegradation of SMX was studied in batch culture experiments under growth conditions described before.[19] Briefly, cultures were prepared in 250-mL Erlenmeyer flasks filled with 100 mL of minimal mineral medium (MMO)[37] amended with yeast extract (0.5 mg L^{-1}) and vitamins (0.5 mL L^{-1}).[38] SMX (0.5 mM) was added as an electron donor and carbon source. A culture (OD 1) grown on 25% (v/v) Standard I medium containing 0.5 mM SMX[19] served as inoculum (0.05% v/v). The inoculum was washed twice and resuspended in MMO beforehand.[19] Cultures were incubated at 28°C in a rotary shaking incubator (130 rpm). Abiotic controls without inoculum were incubated under identical conditions. Experimental treatments and controls were set up in triplicate.

Photodegradation experiment. A Suntest XLS+ (Atlas Materials Testing Solutions GmbH, Linsengericht-Altenhaßlau, Germany) system equipped with xenon lamp and temperature sensor was used as the source of artificial sunlight in the wavelength range of 300-800 nm. During the experiments, the radiation intensity was maintained at 765 W m^{-2} and the reaction temperature was kept at 35°C. Photodegradation of SMX was studied in two solutions, MMO containing phosphate buffer and in double distilled water (dd H_2O). The pH values of 1 mM SMX solutions in MMO and dd H_2O at the beginning of the experiments were 7.4 and 5, respectively. The SMX solutions were prepared in 100 mL non-stained glass bottles (SIMAX GL 45 acc. to DIN; Kavalierglass, Sázava, Czech Republic) and incubated in the Suntest XLS+ system until SMX was completely degraded. Control (dark) experiments were conducted by protecting the reaction vessels with aluminum foil under identical conditions. Experimental treatments and controls were set up in triplicate.

Sampling and sample preparation. Duplicate samples (one mL) were taken from each treatment (biodegradation and photodegradation experiments and all controls) at the beginning of the experiment and at subsequent degradation points. The biotic samples were centrifuged for 10 min at 16,100 × *g* (4°C) and filtered with Titan3 PVDF 0.45 µm filters (Schmidlin Labor, Switzerland), in order to remove the cells from the supernatant. All samples were stored in glass vials at -20°C. One replicate was used for concentration analysis by HPLC, the other for carbon stable isotope analysis by LC-IRMS. The latter was additionally filtered (0.2 µm filter, Wicom, Heppenheim, Germany) and acidified with 85% (15.2 M) *ortho*-phosphoric acid to adjust to pH 2 prior to analysis.

Analytical methods.

HPLC. A high performance liquid chromatography (HPLC) system series 1200 (Agilent Technologies, Germany) equipped with Intersil ODS-3 column (GL Sciences. Inc., Böckten, Switzerland) and an EC 4/3 Universal RP (Macherey-Nagel, Düren, Germany) guard column was used for SMX and 3A5MI concentration analysis, as described further in SI.

LC-IRMS. High pressure liquid chromatography coupled via LC-IsoLink Interface to a Finnigan MAT 253 isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) (HPLC-IRMS) was used to determine the carbon stable isotope ratios of SMX and 3A5MI. The HPLC system was equipped with HTC PAL autosampler from CTC Analytics (Zwingen, Switzerland), a Surveyor MS Pump Plus (Thermo Fisher Scientific, Bremen, Germany) and a Jetstream 2 Plus column thermostat from Sigma-Aldrich (Steinhagen, Germany). SMX and 3A5MI were separated on an YMC Triart column (75 mm × 2 mm, 3 µm) (YMC, Kyoto, Japan), equipped with a 10 mm × 2.1 mm pre-column (Waters, Eschborn, Germany) and an Atlantis T3 column (150 mm × 3 mm, 3 µm) (Waters, Eschborn, Germany), respectively. Five mM sodium phosphate buffer (pH 3) was used as the eluent for SMX, as described before[36], and Milli-Q water was used for 3A5MI analysis. Flow rate was from 300 to 500 µL min^{-1} for both methods. Solutions were prevented from regassing by a continuous

flow of helium gas. The oven temperature was kept isocratic at 65 and 80°C for SMX analysis and 25 and 30°C for 3A5MI, respectively. Variation of flow rates and oven temperatures were due to increased pressure in the LC-IsoLink Interface caused by replacements of oxidation reactor and CO_2 separation unit (details are described in SI). *Ortho*-phosphoric acid (1.5 M) and sodium persulfate (200 g L^{-1}) were pumped at flow rate of 100 µL min^{-1}. All samples were injected in the range of concentrations from method detection limits to 1.20 µg and 0.72 µg of carbon on column for SMX and 3A5MI, respectively (details are described in SI). All samples were measured in triplicate and the typical uncertainty of analysis was <0.5‰.

Calculation of reaction kinetics. As the total SMX undergoes photochemical degradation with first-order kinetics, its time trend could be described by the following equation:

$$C = C_o \exp(-k_{SMX}\, t) \qquad (1)$$

where C is the SMX concentration value at the time t, C_o the initial concentration, and k_{SMX} the first-order degradation rate constant.

Quantification of isotope fractionation. The isotope fractionation during SMX degradation was calculated by applying the logarithmic form of Rayleigh-equation (Eq. 1):

$$\ln\left(\frac{\delta_t + 1000}{\delta_0 + 1000}\right) = \frac{\varepsilon}{1000} \ln\left(\frac{C_t}{C_0}\right). \qquad (2)$$

Changes in concentrations $\ln(C_t/C_0)$ were plotted versus changes in isotope ratios $\ln[(\delta_t+1000)/(\delta_0+1000)]$, thereby obtaining ε from the slope of the linear regression. δ_t, C_t, and δ_0, C_0 are the stable isotope ratios in delta notation (see SI) and the concentrations of a compound, at a given point in time and at the beginning of a transformation reaction, respectively.

For a general mechanistic interpretation of the isotope discrimination, enrichment factors have to be converted to apparent kinetic isotope effect (AKIE). AKIE for SMX was calculated according to the following equation (Eq. 2):

$$AKIE = \frac{1}{1 + z \cdot \varepsilon / 1000} \quad (3)$$

where z is the number of atoms of an element in identical reactive positions.[31] The determination of slopes and 95% confidence intervals were done via linear regression (not forced through zero) using Excel Analysis Toolpak (Microsoft). The derivation of AKIE is given in detail in SI.

Photodegradation of molecules containing different C isomers. The isotope effect in direct photolysis is accounted for by the fact that ^{13}C-containing SMX undergoes degradation with different kinetics than ^{12}C-SMX. To quantify the difference, the isotope ratios were first transformed to obtain the percentage of ^{13}C in SMX (~1.07%). Because SMX contains ten C atoms, on average for every 1000 SMX molecules, 893 would contain only ^{12}C atoms (^{12}C-SMX) and 107 would contain nine ^{12}C and one ^{13}C (^{13}C-SMX). The two kinds of molecules would undergo degradation with different kinetics (first-order rate constants k_{12} and k_{13}, respectively) and their concentration ratio would change over time due to the differential degradation. The consequence is a change in the isotope ratio of the remaining SMX, as long as photodegradation proceeds. By considering that k_{12} and k_{13} would not be very different from the experimentally observed k_{SMX} (in particular, it was assumed $k_{12} \approx k_{SMX}$ because ^{12}C-SMX molecules have the largest statistical weight), one can derive $k_{12}\ k_{13}^{-1}$ from the experimental values of the isotope ratio as a function of the irradiation time. The $k_{12}\ k_{13}^{-1}$ ratio allows some inference to be made about the process behind the observed isotope effect. Details of model assumption and equations, as well as the relevant fit of the experimental data are provided in SI.

RESULTS AND DISCUSSION

Carbon isotope fractionation during biodegradation

The biodegradation and corresponding carbon isotope fractionation of SMX was investigated with *Microbacterium* sp. strain BR1 which uses the compound as a carbon and energy source and releases 3A5MI as a dead-end metabolite. In the experiment presented in Fig. 2 (A, D) 500 µM SMX was fully degraded after approximately 56 h and 3A5MI was produced in stoichiometric concentration. The carbon isotope composition of SMX changed from -29.4 ± 0.1‰ at the start of the experiment to -28.0 ± 0.2‰ for 91% biodegradation after 54 h (Fig. 2 D). The degradation product, 3A5MI, could be first detected after 44h and its isotope composition at this time point was -29.2 ± 0.4‰. The 3A5MI concentration increased towards the end of experiments up to 500 µM, but its isotope composition remained constant over this time and was around -29‰ at all measured degradation points. Due to stability of concentration, no isotope analysis was performed in abiotic controls. Enrichment factor (ε) for carbon isotope fractionation was calculated using the Rayleigh equation (Table 1, Fig. 2) and was -0.6 ± 0.1‰ (R^2=0.86). The corresponding AKIE was 1.006 ± 0.001.

As proposed by Ricken et al.[19], the degradation of SMX by strain BR1 is initiated by *ipso*-hydroxylation, followed by electron rearrangement resulting in a concerted cleavage. The latter produces a 3-imino-5-methylisoxazole intermediate (further transformed to 3A5MI by accepting a proton), sulfur dioxide, which is further hydrated to sulfite, and benzoquinone-imine which is reduced to 4-aminophenol. Indeed, the AKIE (1.006 ± 0.001) upon SMX *ipso*-hydroxylation derived in this study falls in the same range as AKIE in previously studied hydroxylation reactions, for example aromatic ring hydroxylation of benzene ($AKIE_C$ = 1.005 ± 0.0006) and toluene ($AKIE_C$ = 1.006 ± 0.001).[39, 40] Hence, the isotope fractionation results obtained in this study seem to support the reaction pathway described by Ricken et al. [19]. Furthermore, Ricken et al.[19] proposed NADH-dependent monooxygenase to initiate the

hydroxylation reaction and the cleavage of C=C bond. Monooxygenation would proceed via direct addition of an oxygen atom to the ring. Our results also fit in the theoretical range for epoxidation-like C=C bond cleavage ($AKIE_C$ = 1.00 to 1.01).[31] Therefore, the isotope fractionation data may act as an indicator of a degradation pathway and reaction mechanism. However, further study with other SMX-degrading strains is recommended to verify this hypothesis.

Carbon isotope fractionation during direct photolysis

Kinetics. Direct photolysis of sulfonamides is strongly affected by pH.[20, 21, 27, 28] Therefore, photodegradation of 1000 µM SMX was investigated at two different initial pH values, 7.4 (MMO) and 5 (dd H_2O). SMX ($pK_{a,1}$ = 1.6±0.2; $pK_{a,2}$ = 5.7±0.2)[20] is present in the solutions in the more reactive neutral form at lower pH values (pH 4), while at higher pH (pH 8) the more stable anionic form prevails.[20, 21] As previously shown, SMX photolysis was slower at higher pH. The photolysis experiments under identical light conditions resulted in 89% SMX removal after 224 h and 97% SMX degradation within 53h at pH 7.4 and pH 5, respectively (Fig. 2 B, C). The photolysis rate constants were $k_{SMX,\ pH\ 7.4}$ = (9.3±0.5)·10^{-3} h^{-1} and $k_{SMX,\ pH\ 5}$ = (4.45±0.15)·10^{-2} h^{-1}. The assay at pH 7.4 in MMO contained some photoactive compounds such as NO_3^- (0.235 mg L^{-1} $Co(NO_3)_2 \cdot 6H_2O$). Nitrate could generate oxidant species such as hydroxyl radicals under irradiation and thus account for indirect photolytic effects. However, previous studies have shown that even much higher nitrate concentrations (up to 20 mg L^{-1}) did not make any added contribution to the SMX photolytic fate[22]. Indeed, direct photolysis is the main process responsible for SMX photochemical loss under a wide range of conditions.[20, 22, 41] Therefore, the indirect photolysis is not taken into consideration as a possible process contributing to SMX photodegradation in this study and it is assumed not to have any influence on further discussed isotope effects.

Isotope fractionation. During the photolysis experiment at pH 7.4 (MMO) the SMX carbon isotope ratios changed from -28.3 ± 0.2‰ to -23.8 ± 0.1‰ for 89% transformation (Fig. 2 B,

E). The metabolite, 3A5MI was first detected after 48h and its concentration increased over the course of SMX photodegradation, reaching 160 µM (16% of the initial SMX) at the end of the experiment after 224h (Fig. 2 B). The isotope composition of 3A5MI slightly shifted from -26.9 ± 0.0‰ (48 h) to -25.9 ± 0.3‰ (224 h). During photolysis at pH 5 (dd H_2O), SMX became even more enriched in ^{13}C compared to the experiment at pH 7.4 (MMO), and $\delta^{13}C$ shifted from -28.6 ± 0.04‰ to -18.0 ± 0.1‰ upon 97% removal. 3A5MI could be detected after 6 hours, with initial isotope composition of -28.2 ± 0.01‰. After 53 hours, when SMX was almost fully degraded, 3A5MI concentration increased to around 230 µM (23% of the initial SMX). The $\delta^{13}C$ shift of 3A5MI was also more pronounced than at pH 7.4 (MMO), reaching the value of -25.4 ± 0.5‰ (Fig. 2 C, F). The isotope fractionation observed during direct photolysis was significantly higher compared to biodegradation with strain BR1. The enrichment factors (ε) were -2.0 ± 0.2‰ and -3.0 ± 0.2‰ at pH 7.4 (MMO) and pH 5 (dd H_2O), respectively. The corresponding AKIE were 1.021 ± 0.002 at pH 7.4 (MMO) and 1.031 ± 0.004 at pH 5 (dd H_2O). The Rayleigh plots show good correlation between SMX concentration and isotope composition ($R^2_{pH\ 7.4}$=0.94, $R^2_{pH\ 5}$=0.95) (Table 1, Fig. 2).

In both photolysis experiments, the mass balance of substrate and product showed a non-stoichiometric conversion indicating that SMX was only partially degraded to 3A5MI and/or the latter was further transformed. This finding is in agreement with previous studies on SMX photochemical behavior, which showed that SMX simultaneously undergoes cleavage at various positions resulting in different degradation products (Fig. 1 A).[20, 21, 27, 28] The photolytic degradation of 3A5MI was also previously shown and it could be the case here, as a slight 3A5MI enrichment in ^{13}C was observed in the experiment at pH 5 (dd H_2O).[26, 42]

The calculated AKIE values (pH 7.4: 1.021±0.002; pH 5: 1.031±0.004) were within the range of typical values for nucleophilic substitution S_N2 (1.03-1.09) and S_N1 (1.00-1.03).[31]

Photolysis reaction pathways. Fig. 1 A shows potential SMX cleavage sites during direct photolysis. δ-cleavage and formation of sulfanilic acid seems to be the dominant pathway, observed in all studies on sulfonamides photodegradation.[20, 21, 27, 28] Sulfanilic acid may account even for 35% of the degraded SMX.[20] However, this cleavage does not involve carbon atoms and it is not likely to contribute to the carbon isotope fractionation of SMX. The *intrinsic* KIE can only be directly observed if the bond changes involving the element of interest (here carbon) represent the rate-determining step in the overall process. Therefore, carbon isotope fractionation will rather result from other degradation pathways.

Recently, Bonvin et al. detected a new SMX photodegradation product, (5-methylisoxazol-3-yl)sulfamate, resulting from γ-cleavage which has not been reported before. This pathway was quite significant as (5-methylisoxazol-3-yl)sulfamate accounted for 11% of degraded parent compound.[27] In the same study, the formation of aniline resulting from γ-cleavage was also observed and it confirmed a previous finding by Zhou and Moore.[27, 28] Cleavage at ε position was observed by Boreen et al. and sulfanilamide was detected, but to much lesser extent than the product of δ-cleavage.[20] Beta-cleavage with removal of the NH_2 was proposed by Bonvin et al. but the reaction product was not confirmed with an authentic standard.[27] Gamma-, ε- and β-cleavage are likely to cause carbon isotope fractionation. Furthermore, SMX and its degradation products undergo photoisomerization at isoxazole ring and thus exist in the aqueous solution in different tautomeric forms.[21, 28] Considering that photoisomerization would involve ε-cleavage of a C-N bond[21], it might contribute to the observed AKIE (*see below*). The photoisomerization process would likely proceed by formation of two radical species surrounded by a cage of water molecules. The radicals in the cage could either recombine to form the parent compound or an isomer, or escape to the solvent bulk where they could undergo additional reactions.[42b] The possible occurrence of two radical species formed by the breaking of a C-N bond may suggest that at least part of the observed isotope

effect could be a magnetic one, where the occurrence of a ^{13}C atom would favor radical-radical recombination. Magnetic effects often cause large isotope fractionation,[43] but the data treatment provided in the SI suggests that the present experimental data do not provide unequivocal evidence for such an effect (although they do not exclude it).

Recently, Perisa et al.[21] detected desulfonated photoproducts of various sulfonamides and in addition to the cleavage of S-N bond which was also observed, they proposed another key degradation mechanism. This mechanism can be described as nucleophilic attack of the nitrogen atom at the carbon atom of the phenyl ring, accompanied by extrusion of SO_2 and formation of carbon-nitrogen bond. The experiments with SMX resulted in detection of ions specific for this cleavage.[21] This pathway would likely cause carbon isotope fractionation.

pH dependent isotope fractionation. Previous studies have shown that the same degradation products were observed in the photolysis solutions at different pHs, but the rates of product formation and the reaction kinetics were strongly affected by pH.[20-22] SMX photolytic degradation proceeds via different pathways, and the proportion of SMX removed by various reaction mechanisms may differ depending on the SMX protonation state. It has also been reported that pH and buffer concentration could affect the formation kinetics of zwitterionic forms (a possible occurrence in the case of SMX as well), which undergo peculiar reactivity that may influence the isotope fractionation.[44] In the experiments performed here, when SMX was totally or almost totally degraded, a maximum of 23% of the initial SMX was transformed to 3A5MI at pH 5 (dd H_2O), while at pH 7.4 (MMO) this metabolite accounted only for 16% of the initial SMX concentration. It is quite likely that different protonation states of SMX undergo photochemical transformation processes to a different extent, with variable impact on the AKIE. For instance, a δ cleavage is not expected to cause a KIE on carbon, and conditions favoring this pathway would decrease the observed AKIE. Moreover, conditions favoring an important photoisomerization of SMX could also reduce the observed

isotope effect (*see below*). In previous studies it was also shown that the neutral SMX form transforms faster than the anionic form.[20, 21] This is in agreement with our study, and the differential photoreactivity between different protonation states could be one of the possible explanations for the differences in carbon isotope fractionation at pH 5 (dd H_2O) and pH 7.4 (MMO). Boreen et al. also showed that pH of the solution together with the protonation state alters the SMX absorbance spectrum, however the highest spectral changes are observed for wavelengths below 300 nm, which are not relevant to our study. [20] However, some pH dependent change in the magnitude of light absorption above 300 nm occurs[20] and thus may also contribute to the observed variability in fractionation.

In addition to the different properties of the ground states, excited states could undergo acid-base equilibria that are different from those of the ground states and/or have lifetimes that are affected by pH. Excited-state reactivity, in addition to bond breaking, could play a role in isotope fractionation.[44a] In some instances the excited states (e.g. the triplet ones) are much stronger acids or bases than the ground ones, which allows for protonation or deprotonation reactions after light excitation. Sometimes, as in the well-known case of 4-hydroxybenzophenone,[44b] the acid-base reactions of the excited states are connected with their very fast deactivation, which quenches the photoreactivity in the aqueous solution. This was clearly not the case of SMX, but the solution pH could still affect the excited-state lifetime. For instance, as a consequence of reactions with/quenching by H^+ or OH^-, ~neutral conditions may correspond to a minimum of the deactivation kinetics in the excited state.[44c]

Intrerestingly, radical species derived from SMX have often different pKa values compared to the parent molecule,[45] which could introduce an effect of pH in reaction pathways that follow the primary photolysis step.

Implications for the environmental applications of CSIA for SMX

In this study, we assessed for the first time the applicability of CSIA to monitor the biodegradation and photolysis of the antibiotic SMX. Application of CSIA for emerging contaminants, such as pharmaceuticals, is still in its infancy. Only recently a GC-IRMS method with prior derivatization step has been developed and tested for diclofenac.[46] However, derivatization is not applicable to many non-volatile compounds and can cause bias in isotope ratios.[34] Coupling of IRMS with LC allows the determination of carbon isotope ratios of polar, thermolabile, and high molecular weight compounds without the need for derivatization. However, very few LC-IRMS methods for pharmaceuticals have been published so far.[36, 47] LC-IRMS has scarcely been used in contaminant (bio)degradation studies[48] and has not yet been applied to pharmaceuticals' (bio)degradation. Therefore, our work expands the environmental application of CSIA.

A significant difference in isotope fractionation during biotic and abiotic SMX decomposition was observed, showing that CSIA has the potential for distinguishing these two degradation processes. Furthermore, isotope enrichment of 3A5MI was observed during photolytic SMX degradation while 3A5MI formed during biodegradation was not fractionated. This may provide an additional line of evidence for evaluating SMX degradation pathways.

Isotope fractionation during direct photolysis is variable and it depends on the experimental conditions. Most likely, in the case of SMX, pH has an influence on the reaction mechanism and it thus affects the isotope fractionation. Further research is needed on the connection between photolysis pathways and isotope fractionation and it would benefit from a multi-dimensional (C, H, N, S) approach.

For CSIA to be reliably applied to the assessment of degradation pathways and to distinguish between biotic and abiotic processes in environmental and wastewater samples, some limitations still need to be overcome. First of all, the environmental concentrations of SMX are typically much lower than those applied in this study, therefore it will be crucial to

develop pre-concentration methods and/or more sensitive analytical methods. These methods should not introduce additional isotope effects.

A systematic study on the metabolic pathways employed by several bacterial strains and on the associated isotope fractionation is needed. Indeed, isotope fractionation may vary within the same biodegradation process carried out by various microbial strains, as shown for chlorinated ethenes.[49, 50] To overcome the uncertainty associated with such variability, a multi-dimensional approach could be applied but the relevant methods are still to be developed.

In summary, this work opens a new chapter in CSIA development - the monitoring of sinks, sources and environmental behavior of water-soluble contaminants, among which pharmaceuticals are of particular concern. However, it has to be noted, that application of CSIA to larger molecules is challenging, as the more atoms of the same element are present within a compound, the smaller is observable isotope fractionation. These additional atoms "dilute" the observed fractionation effects. Therefore, there is an upper limit for the size of a molecule that can be analyzed by CSIA and thus also for application of compound specific isotope analysis to certain pharmaceuticals [51].

Associated Content

Supporting Information

Chemicals; Monitoring of the time trend of SMX and 3A5MI by HPLC; Accuracy and detection limit of LC-IRMS method; Quantification of apparent kinetic isotope effect; Mass and magnetic isotope effects upon direct photolysis.

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Notes

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Tables

Table 1

Stable isotope enrichments factors (ε_C), the R^2 and 95% confidence intervals (CI 95%) and derived AKIE for SMX biodegradation by *Microbacterium* strain BR1, direct photolysis at pH 7.4 (MMO) and at pH 5 (dd H_2O).

	ε_C	R^2	CI (95%)	AKIE	Std error
Microbacterium strain BR1	-0.6	0.86	0.1	1.006	0.001
Direct photolysis at pH 7.4 (MMO)	-2.0	0.94	0.2	1.021	0.002
Direct photolysis at pH 5 (dd H_2O)	-3.0	0.95	0.2	1.031	0.004

Figures

Figure 1

Potential direct photolysis cleavage sites (A) and different states of protonation (B) of SMX as proposed for sulfonamides by Boreen et al. [20], where SH_2^+, SH and S^- represent the cationic form, the neutral form and the anionic form, respectively.

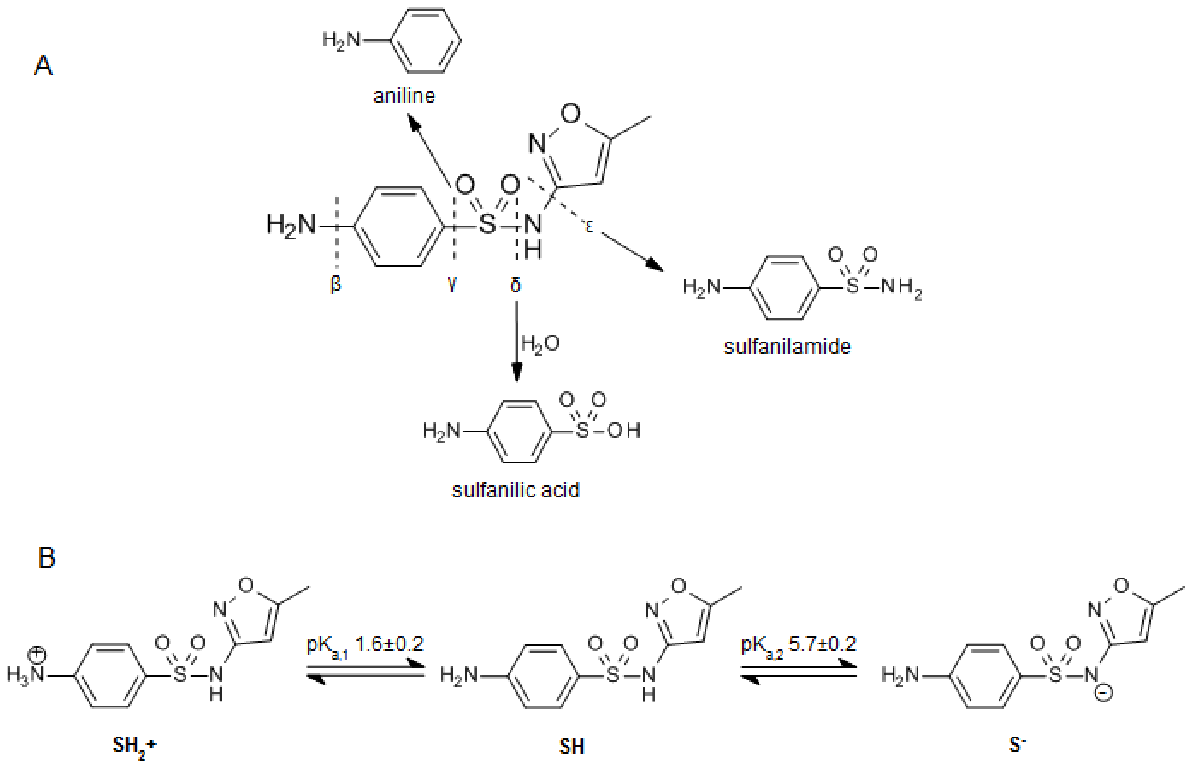

Figure 2

Change in concentration (A, B, C) and carbon isotope composition (D, E, F) of SMX (♦) and its degradation product 3A5MI ( ) during biodegradation by *Microbacterium* strain BR1 (A, D) and direct photolysis at pH 7.4 (MMO) (B, E) and at pH 5 (dd H_2O) (C, F).

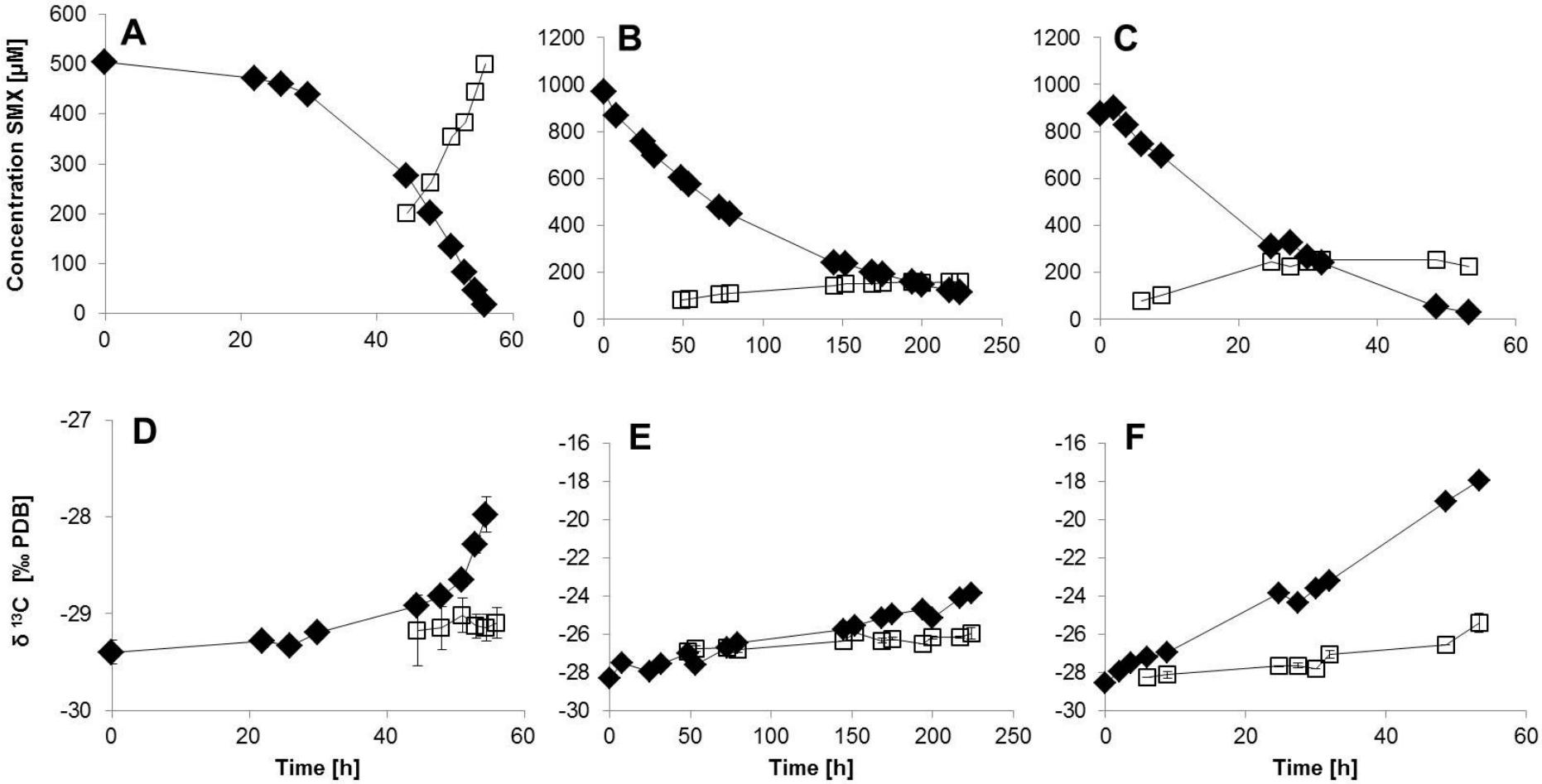

Figure 3

Carbon stable isotope fractionation of SMX during biodegradation by *Microbacterium* strain BR1 (▲) and during direct photolysis at pH 7.4 (MMO) ( ) and at pH 5 (dd H_2O) (♦).