Aerobic biodegradation of the sulfonamide antibiotic sulfamethoxazole by activated sludge applied as co-substrate and sole carbon and nitrogen source

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 Elimination of sulfamethoxazole (SMX) turned out to be based on biodegradation.
 Activated sludge communities utilize SMX as carbon and/or nitrogen source for growth.
 SMX biodegradation is enhanced in the presence of a C source and N deficiency.
 With SMX as co-substrate 3-amino-5-methyl-isoxazole represents the main stable metabolite.
 SMX as sole C and N source maybe yields hydroxyl-N-(5-methyl-1,2-oxazole-3-yl)benzene-1-sulfonamide as further metabolite.

Potential aerobic biodegradation mechanisms of the widely used polar, low-adsorptive sulfonamide antibiotic sulfamethoxazole (SMX) were investigated in activated sludge at bench scale. The study focused on (i) SMX co-metabolism with acetate and ammonium nitrate and (ii) SMX utilization when present as the sole carbon and nitrogen source. With SMX adsorption being negligible, elimination was primarily based on biodegradation. Activated sludge was able to utilize SMX both as a carbon and/or nitrogen source. SMX biodegradation was enhanced when a readily degradable energy supply (acetate) was provided which fostered metabolic activity. Moreover, it was raised under nitrogen deficiency conditions. The mass balance for dissolved organic carbon showed an incomplete SMX mineralization with two scenarios: (i) with SMX as a co-substrate, 3-amino-5-methyl-isoxazole represented the main stable metabolite and (ii) SMX as sole carbon and nitrogen source possibly yielded hydroxyl-N-(5-methyl-1,2-oxazole-3-yl)benzene-1-sulfonamide as a further metabolite.

1. Introduction

The discharge of antibiotics into the environment has become a major concern as this group of pharmaceuticals is not only prone to influencing microbial communities by its mode of action (Fent et al., 2006), but also because of the risk of a worldwide dispersal of concomitant resistance genes (Witte, 2004; Agersø and Petersen, 2007; Szczepanowski et al., 2009). Recent monitoring programs have revealed the presence of antibiotics in various environmental compartments such as water bodies, sediments, and soils (Heberer, 2002; Thiele-Bruhn and Beck, 2005; Ternes and Joss, 2007). Besides diffuse agricultural input (Jemba, 2002) and landfill discharge (Heberer, 2002), antibiotics mainly enter the environment via wastewater (WW) and wastewater treatment plant (WWTP) effluents. Poorly adsorptive polar substances such as sulfonamides are readily found in water bodies such as surface waters (Hirsch et al., 1999), bank filtrates (Heberer et al., 2008), and groundwater (Sacher et al., 2001; Underwood et al., 2011). The concentration levels (see review, Kümmerer, 2009) range from 370 to 2000 ng L⁻¹ in WWTP effluents, from 40 to 1900 ng L⁻¹ in surface water and from 20 to 470 ng L⁻¹ in groundwater/bank filtrate up to mg L⁻¹ in highly contaminated groundwater (Holm et al., 1995).

Considering a 50% minimum inhibitory concentration (MIC₅₀) for e.g. sulfonamide antibiotic sulfamethoxazole (SMX), ranging from 0.002 to 256 mg L⁻¹ depending on the bacteria species (Al-Ahmad et al., 1999), then the environmental concentrations found might influence microbial community activity.
SMX (Fig. 1) is a low adsorptive, polar sulfonamide antibiotic known as contaminant in drinking water resources. Hence its fate in aqueous environments is of high interest. SMX mainly enter wastewater via human excretion as unmodified SMX (13–5%) or as its human transformation products N-acetyl-SMX (43–55%) or N-SMX-glucuronide (10–13%) (van der Ven et al., 1995). Both human transformation products are readily cleaved back to the SMX molecule while passing through the sewer system or during WW treatment (Göbel et al., 2005). Previous investigations of activated sludge have shown elimination of SMX under different WW treatment conditions. Because of its low adsorptivity, it is postulated that SMX reduction is mainly due to microbial activity. However, proper balancing studies are rare (Golet et al., 2003; Carballa et al., 2007), presumably because of missing information regarding its metabolic fate and produced metabolites. In general, the literature pertaining to SMX elimination in WWTPs and natural aqueous environments is marked by inconsistent results. In WW treatment for example, low hydraulic residence times and sludge ages at high loadings were shown to be unfavorable for SMX elimination (Kreuzinger et al., 2004). On the other hand, Pérrez et al. (2005) describe a high-load sludge which readily eliminated sulfonamides. However, low-load nitrification conditions with a high sludge age were proven to be disadvantageous as a highly active community is necessary for good elimination rates (Göbel et al., 2007). Additionally, nutrient deficiencies (i.e. carbon and/or nitrogen) were pointed out as possibly being advantageous for microbial SMX reduction (Drillia et al., 2005). Studies on SMX elimination in natural systems, such as bank filtrates, also provided contradictory findings (Baumgarten et al., 2011). This is supposedly because elimination depends on various environmental factors such as in situ redox potential, available nutrients, soil characteristics, seasonal temperature, and light variations. Besides biological processes, literature indicates that abiotic SMX elimination (chemical oxidation (Sharma et al., 2006) or photochemical degradation (Andreozzi et al., 2003; Boreen et al., 2004; Trovó et al., 2009) is also possible. It has been shown that in natural environments different redox potentials due to the presence of nitrate, sulfate, humic acids, or choline soil fractions can improve xenobiotic biodegradation (Field et al., 2000; Cervantes et al., 2008).

Unfortunately, actual literature on SMX reduction (Kreuzinger et al., 2004; Göbel et al., 2005, 2007; Pérez et al., 2005) mostly refers to ‘elimination’ rather than effective ‘biodegradation’ with defined metabolites. Earlier and recent lab scale test using activated sludge as inoculum showed a high variation in aerobic biodegradation removal capacity after 48 h from <20% (Joss et al., 2006) up to 39% (Li and Zhang, 2010). In batch soil tests detected SMX half-life ranged from 1 to 11 d (Baumgarten et al., 2011; Lin and Gan, 2011). Only rarely is the literature substantiated by biological action of defined microorganisms (Gauthier et al., 2010; Larcher and Yargeau, 2011; Bouju et al., 2012). Knowledge gaps with respect to produced metabolites hinder proper balancing studies. However, appropriate experiments, which are based on realistic test conditions such as continuous food supply and sufficient adaptation periods, should facilitate assessment of SMX biotransformation in the natural environment.

The aim of the present work was to elucidate the fate of the low-adsorptive polar model antibiotic sulfamethoxazole (SMX), during aerobic WW treatment, i.e. the fate of SMX with oxygen as the terminal electron acceptor. This redox condition is described
to provoke the formation of particularly polar metabolites (Richardson and Ternes, 2011) which are then discharged with the effluent to the receiving aqueous systems. We studied aerobic utilization of SMX by activated sludge microbial communities to provide a first insight into conducive elimination conditions. The chemical structure of SMX indicates that it can possibly serve as a carbon and nitrogen source for microbial communities (Fig. 1). Therefore, our study investigated SMX biodegradation under two different conditions: (i) co-metabolism with acetate and nitrogen supplementation and (ii) where SMX acted as the sole source of carbon and nitrogen. This approach aimed to account for WW treatment under conditions of readily degradable carbonaceous substrate as well as nitrifying conditions without an external carbon supply. A major issue therein is a proper mass balance for C and N as a base for the identification of potential metabolites. As there is little information available on potential biodegradation mechanisms, possible SMX biodegradation pathways and postulated metabolites were identified with the biocatalysis/biodegradation database of the University of Minnesota (UM-BBD, http://umbbd.msi.umn.edu), for detailed information see Gao et al. (2010), Fig. 1A) and literature data (Gauthier et al., 2010, Fig. 1B).

2. Materials and methods

2.1. Chemicals

Sulfamethoxazole and sodium acetate applied as carbon sources during cultivation were obtained from Sigma–Aldrich (Munich, Germany); all other chemicals used for the synthetic medium and buffer solution were from Merck (Darmstadt, Germany). For chemical analysis, 3-amino-5-methyl-isoxazole, 3-amino-isoxazole, isoxazole, pyridine, and 2-aminoypyridine (Sigma–Aldrich) were used as standards. Solvents (methanol, water, and formic acid) were of LC–MS grade (Sigma–Aldrich). Acetonitrile gradient grade was purchased from Merck. Ultrapure water was prepared with a Milli-Q purification system (Millipore, Schwabach, Germany). Filtration was performed with 0.45 μm filters (Millipore).

2.2. Reactor setups, cultivation, dosage regime, and sampling

Bench scale tests were performed in a semi-continuous ‘fill and draw’ mode with a 2 L flask reactor containing 1.5 L synthetic media and a biomass concentration of 0.2–0.3 g mixed liquor suspended solids (MLSSs) L−1 (see Fig. 2, left). An SMX-free control (R1) and three SMX-fed setups (R2, R3-I, and R3-II) with different SMX concentrations were run in duplicate (A and B) and inoculated with activated sludge from the high-load stage of a two-stage municipal WWTP treating 1 million population equivalents.

Cultivation experiments focused on SMX biodegradation by co-metabolism with acetate and nitrogen supplementation (R2 and R3-I) as well as on SMX biodegradation where SMX was the sole source of carbon and nitrogen (R3-II). A synthetic medium (85 mg L−1 KH2PO4, 217.5 mg L−1 K2HPO4, 334 mg L−1 Na2HPO4·2H2O, 22.5 mg L−1 MgSO4·7H2O, 36.4 mg L−1 CaCl2·2H2O, and 0.25 mg L−1 FeCl3·6H2O) according to DIN EN ISO 9888 (DEV, 2000) was amended with 0.1 mL L−1 Hoagland trace element solution. Dosage regimes and final concentrations of the carbon and nitrogen sources as well as SMX are listed in Table 1.

The reactors were placed in an incubator shaker at 150 rpm and 22 °C in the dark to prevent abiotic SMX degradation by photolysis. Aerobic conditions of 7–8 mg L−1 O2 were achieved with a continuous air-supply (Fig. 2, left) and monitored once a week. Sampling and subsequent dosage occurred twice a week (Table 1 and Fig. 3). For chemical analyses of dissolved organic carbon (DOC), SMX, SMX metabolites, and nitrogen species, 200 mL of supernatant were sampled after evaporation correction and 30 min of sludge sedimentation (without aeration, Fig. 2, right). Subsequently, 200 mL of the nutrient solution amended with acetate, SMX, and ammonium nitrate (see Table 1) were supplied and 1 mL of supernatant was sampled for SMX analysis immediately after dosage (Fig. 2, right).

After 6 weeks experimental time (day 0–42 of cultivation) the SMX-free and SMX-adapted sludges were washed to remove DOC and N residues for a restart. This washing step was carried out as follows. All sludge supernatant was removed after 30 min of sedimentation. The remaining sludge was washed twice with phosphate buffer saline (10 mM Na2HPO4/NaH2PO4, 120 mM NaNCl, pH 7.2) and 1.5 L new synthetic media was added (see Table 1). The reactors were subsequently run for another 4 weeks (day 42–71).

2.3. Sample preparation

The collected supernatants were centrifuged at 15000g for 5 min to remove suspended solids before determining DOC, SMX, and its postulated metabolites 3-amino-5-methyl-isoxazole, 3-amino-isoxazole, isoxazole, as well as the nitrogen species total nitrogen TN, ammonia, and nitrate. Grab samples of activated sludge were collected at the end of the experiment on day 71 (R2 and R3-II). For a liquid/liquid extraction, the sludges were centrifuged at 15 000g for 10 min. The obtained pellets were lyophilized, supplemented with acetonitrile, and treated with ultrasonication for 20 min. Subsequently, the

![Fig. 2. Reactor setups ((A) continuous air supply, (B) stirrer, and (C) incubation chamber), and sampling and dosage routine.](image-url)
sludges were centrifuged at 15 000 g for 10 min, the supernatants filtered (pore size 0.45 μm), and SMX was analyzed directly with LC–MS/MS and reported as mass per MLSS.

2.4. Analysis of dissolved organic carbon (DOC), nitrogen species, and mixed liquor suspended solids (MLSS)

DOC was determined according to EN 1484 DEV H3, total nitrogen according to DIN 38409-H27, ammonium according to DIN EN ISO 11732-HA1, E23, nitrate according to DIN EN ISO 10304-1/-2, gen according to DIN 38409-H27, ammonium according to DIN EN ISO 11465 (DEV, 2000).

2.5. Analysis of SMX and its postulated metabolites

SMX analysis without previous enrichment (detection 50–1000 ngL⁻¹) was performed by liquid chromatography using a low pressure Jasco X-LC system (Tokyo, Japan) connected to an API 4000 Q-Trap hybrid triple-quadrupole mass spectrometer with a Turbo Ion Spray source at 450 °C. Isotopically labeled compound, used as internal standard 10 mg L⁻¹-sulfamethoxazole, was the sole carbon and nitrogen source (R3-II).

During cultivation a stable biomass concentration of approximately 0.2 mg L⁻¹ was maintained in all reactors (R1, R2, and R3). At day 42, after 6 weeks of cultivation, the control reactor and SMX-fed reactors were restarted with SMX-free and SMX-adapted sludge communities, respectively. This was done to (i) observe the DOC and N-species development in the control reactor and the reactor fed SMX as a co-substrate (R2) without any storage of DOC or N in the sludge system and (ii) operate a reactor where SMX was the sole carbon and nitrogen source (R3-II).

3. Results

3.1. Elimination of SMX

In both SMX-fed reactors R2 and R3-I (co-metabolism = Co-Met, Fig. 3A and B, respectively), the initial SMX concentration of 10 mg L⁻¹ at first remained unchanged. This indicates that the community had not yet utilized it as a co-substrate and dosage compensated for sampling losses. Adaption occurred between day 0 and 14 after which the initial SMX concentration of 10 mg L⁻¹ was completely removed from the water phase. During

### Table 1

<table>
<thead>
<tr>
<th>Dosage intervals (d)</th>
<th>SMX-free control R1</th>
<th>SMX R2 Co-Met</th>
<th>SMX R3-I Co-Met</th>
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<tr>
<td></td>
<td>Acetate-C (mgL⁻¹)</td>
<td>NH₄NO₃-N (mgL⁻¹)</td>
<td>SMX (mgL⁻¹)</td>
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<td>1.0</td>
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<td>10</td>
<td>0</td>
<td>1.0</td>
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<td>10</td>
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<tr>
<td>58, 62, 65, 69</td>
<td>10</td>
<td>0</td>
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SMX R3-II SMX as sole C/N source

Carbont loaded with pyridine and 2-aminopyridine were used as internal standards. The LOQ was 0.1 mg L⁻¹.
further cultivation with 1.3 mg L\(^{-1}\), SMX was completely eliminated within a few days. Even with an increased dosage of 12 mg L\(^{-1}\) from day 17 to 22 in R3-I, SMX was reduced to an undetectable level within a few days.

After restarting R2 with washed SMX-adapted activated sludge at day 42, it was observed that the feed containing 10 mg L\(^{-1}\) SMX as a co-substrate was removed within 3 d (Fig. 3A, right). This is similar to the co-metabolism study in R3-I until day 38 (Fig. 3B, left).

After restarting R3-II where SMX was the sole carbon and nitrogen source (Fig. 3B, right), the SMX dosage of 50 mg L\(^{-1}\) was no longer detected after 3 d. The subsequent dosage of 19 mg L\(^{-1}\) was eliminated within a few days. A second SMX dosage of 50 mg L\(^{-1}\) twice a week from day 58 on resulted in only a partial SMX reduction followed by an accumulation of SMX in the system.

Very low SMX concentrations of 100 \(\mu\)g g\(^{-1}\) SS (R2) and 400 \(\mu\)g g\(^{-1}\) SS (R3-II) were detected in the activated sludge at the end of the experiment. This proved that SMX elimination by sorption processes was negligible and verified that the SMX removal was primarily biological.

3.2. Elimination of dissolved organic carbon (DOC) during SMX biodegradation

DOC concentrations in the supernatant were monitored to determine whether SMX was totally mineralized or if any metabolites were produced (Fig. 4). The start DOC concentrations after acetate and SMX supply ranged from 87 to 88 mg L\(^{-1}\) in the SMX-free reactor R1 and from 93 to 94 mg L\(^{-1}\) in the SMX-fed Co-Met reactors (R2 and R3-I) where the higher DOC values are related to the SMX-C dosage of 4.7 mg L\(^{-1}\). In the SMX-fed reactor R3-II with SMX as sole carbon source the measured DOC start values of 24 mg L\(^{-1}\) represented the SMX-C start dosage (see Table 1). These results demonstrate that the DOC mainly consists of the added acetate-C and/or SMX-C, and the contribution of dead biomass and residual sludge fraction represents a negligible fraction of 2–4 mg DOC L\(^{-1}\) shown in the control R1 (see Fig. 5). In all three reactors, a DOC elimination of 50% was observed after only 4 h with values up to 90% in SMX-free R1 and 85% in SMX-fed R2 and R3-I after day 3. This observation suggests a rapid utilization of the supplied acetate.

R1 generally achieved very high levels of DOC elimination. From day 7 to 38 values varied between 70% and 90% and, after the restart, were greater than 95%. In contrast thereto, a lower DOC elimination was detected in the reactors fed SMX as a co-substrate. In R2, elimination ranged between 70% and 85% from day 3 to 71 and was measured 60% in R3-I from day 3 to 38. Even lower DOC elimination was achieved in R3-II after the restart when SMX was fed as the sole carbon and nitrogen source with 36.5% at day 45 decreasing down to 10% at day 55. Presumably the R3-II activated sludge was able to maintain a greater amount of metabolic activity directly after the restart due to the acetate supply from day 0 to 38. The effect of supplying SMX as the sole carbon and nitrogen source was a decline in microbial metabolic activity from day 45 to 55. Adaption of the biomass to SMX as the sole carbon and nitrogen source might have increased DOC elimination up to
33.6% at day 62. The high fluctuations in DOC elimination (large standard deviations) from day 65 to the end are assumed to be the result of varying SMX accumulation in R3-II duplicates (see Fig. 3B).

3.3. SMX metabolites

The analysis of the postulated metabolites such as 3-amino-5-methyl-isoxazole, 3-amino-isoxazole, and isoxazole (see Fig. 1A) demonstrated that only 3-amino-5-methyl-isoxazole was present in the SMX-fed reactors. At day 14 in R2 and R3-I, where SMX was supplied as a co-substrate, 3-amino-5-methyl-isoxazole was detected at 2 mg L\(^{-1}\) and SMX was no longer present. Results of the co-metabolism experiments between day 14 and 38 indicated that 3-amino-5-methyl-isoxazole was enriched up to 8 mg L\(^{-1}\) in R2 and 30 mg L\(^{-1}\) in R3-I (see Fig. 6B and C, right, shown as 3-amino-5-methyl-isoxazole-N values). After the restart wash step, 3 and 14 mg L\(^{-1}\) of newly produced 3-amino-5-methyl-isoxazole were detected in R2 and R3-II, respectively. Within 3 d, concentrations up to 15 mg L\(^{-1}\) were measured in R2. In R3-II where SMX
was the sole carbon and nitrogen source, an accumulation of 3-amino-5-methyl-isoxazole up to 45 mg L\(^{-1}\) was determined at day 62 (see Fig. 6B and C, left, shown as 3-amino-5-methyl-isoxazole-N values).

To elucidate whether 3-amino-5-methyl-isoxazole represented the main and, moreover, a stable SMX metabolite, its measured concentrations given as DOC units were correlated with (i) DOC values (see Fig. 5A and C) and (ii) calculated metabolite concentrations (see Fig. 5B and D) during stable SMX elimination.

In co-metabolism studies in R2 and R3-I from day 14 to 38, the linear trend line of DOC and 3-amino-5-methyl-isoxazole-C (Fig. 5A) is characterized by a high coefficient of determination \(R^2 > 0.9\). The individual data points indicate a good correlation in R3-I and a less distinct correlation in R2, probably due to the low concentration (1–4 mg L\(^{-1}\)) of 3-amino-5-methyl-isoxazole-C being achieved during low SMX dosage. The trend line crosses the x-axis at 3 mg DOC L\(^{-1}\), which represents the residual DOC sludge fraction in R1. It is hypothesized that this DOC fraction accounts for the higher DOC values in comparison to the 3-amino-5-methyl-isoxazole-C concentration in the SMX-fed reactors. This fraction was lower after the restart, presumably because the sludge washing removed any residual DOC (Fig. 5C). During co-metabolism, the concentrations of 3-amino-5-methyl-isoxazole analyzed corresponded with the metabolite concentrations calculated from the amount of SMX supplied, i.e. 40% of SMX total carbon content assuming a quantitative transformation to 3-amino-5-methyl-isoxazole (Fig. 5B).

Different results were obtained in R3-II where SMX was the sole carbon and nitrogen source. When compared with DOC, a distinctly lower 3-amino-5-methyl-isoxazole-C concentration was detected and the difference was observed to increase when more SMX was supplied (Fig. 5C). Calculated metabolite concentrations were distinctly higher than those measured (Fig. 5D, compare solid trend line and dashed line as above). This indicates that further metabolites may exist.

3.4. Different nitrogen species

Ammonification presumably from sludge detritus and mostly autotrophic nitrification processes both in the control R1 and the SMX co-metabolism reactors R2 and R3-I led to increased nitrate-N concentrations from day 0 to 17 (Fig. 6A-C). Any nitrate produced could not be denitrified because of the high oxygen and low DOC supplies. A stable nitrate-N accumulation of 20 to
24 mg L\(^{-1}\) was detected in R1 with slightly higher concentrations of 23 to 27 mg L\(^{-1}\) found in SMX-fed R2 and R3-I. Higher nitrate concentrations in the SMX-fed reactors may have resulted from utilization of the amine functional group on the aromatic SMX ring which was not completely assimilated. In contrast, ammonium was only found at the very start. Total nitrogen was found to quantitatively consist of nitrate-N and 3-amino-5-methyl-isoxazole-N.

After restart on day 42, R1 only showed ammonium and nitrate directly after dosage (Fig. 6A). Thereafter, neither ammonium, nitrate, nor total nitrogen were detected, demonstrating (i) complete assimilation of the N supply and (ii) that the system did not retain any excess nitrogen for ammonification. In R2 with SMX as a co-substrate (Fig. 6B right), neither ammonium nor nitrate were detected apart from the analysis directly after the restart. Besides very low amounts of SMX-N, total nitrogen primarily consisted of 3-amino-5-methyl-isoxazole-N, accumulating up to 4.7 mg L\(^{-1}\) at day 62. This observation demonstrates that both supplied ammonium nitrate and nitrogen originating from the SMX amino group were almost completely assimilated. In R3-II where SMX was the sole C and N source (Fig. 6C right), ammonium-N was determined to be 0.7 mg L\(^{-1}\) at day 45. It disappeared during further cultivation. This nitrogen species originated from the amino functional group on the aromatic ring of SMX as no other nitrogen source was available. It was (i) utilized by heterotrophic bacteria for assimilative metabolism and/or (ii) oxidized to nitrate by auto-trophic nitrifiers. The latter was confirmed by nitrate-N being detected in very small amounts from day 45 on and increasing to 6 mg L\(^{-1}\) at day 62. Total nitrogen comprised nitrate-N, SMX-N (from day 50 to 62, presumably due to SMX dosage overload), and 3-amino-5-methyl-isoxazole-N accumulating from day 45 on, up to a concentration of 21 mg L\(^{-1}\).

4. Discussion

The lower DOC elimination during SMX feeding when compared to the control, might have been caused by the antibiotic effect of SMX. It may have potentially inhibited the metabolic activity of the sludge communities, resulting in a decrease of carbon utilization of both supplied acetate and residual DOC sludge in the sludge fraction. However, Al-Ahmed et al. (1999) showed that an SMX concentration of 0.002–256 mg L\(^{-1}\) inhibited the growth of most bacteria only by 50% (MIC\(_{50}\)). 100% growth inhibition was mostly not achieved before applying 1000 mg SMX L\(^{-1}\) (Enne et al., 2002). Therefore, the supplied SMX concentrations of 10 up to maximal 50 mg L\(^{-1}\) in this study most likely did not affect the activity of the bacterial sludge community. The decrease of DOC elimination in the SMX-fed reactors probably indicates that SMX was not completely mineralized, but rather accumulated in the form of stable metabolites.

4.1. SMX metabolites

Potential metabolic biodegradation pathways for SMX (Fig. 1A) were selected by means of the biocatalysis/biodegradation database of the University of Minnesota (UM-BBD, http://umbbd.msi.umn.edu). It predicts the likelihood of microbial catabolic reactions using biotransformation rules derived from reactions found in the UM-BBD (Gao et al., 2010). As a first catabolic reaction, it proposed the cleavage of SMX into 3-amino-5-methyl-isoxazole and 4-aminobenzene sulfonate which is performed by specific microbial communities. The ability to mineralize the metabolite 4-aminobenzene sulfonate has been shown to exist in many microorganisms. Most likely, the methyl group from the 3-amino-5-methyl-isoxazole is split. However, no metabolic rule in the database describes the further transformation of 3-amino-isoxazole into isoxazole. Thus, primarily 3-amino-5-methyl-isoxazole and 3-amino-isoxazole are to be discussed as possible metabolites.

The present study showed that 3-amino-5-methyl-isoxazole was the main stable SMX biodegradation product for activated sludge being fed SMX as a co-substrate as further possible metabolites were never detected. According to the DOC elimination rates, a total mineralization of SMX seems to be unlikely. Furthermore, a quantitative transformation to 3-amino-5-methyl-isoxazole was proven by the good correlations of (i) DOC and 3-amino-5-methyl-isoxazole-C concentrations and (ii) measured and calculated 3-amino-5-methyl-isoxazole values. This finding is confirmed by previous work (Hartig, 2000; Eibes et al., 2011). However, for the present work activated sludge fed SMX as the sole carbon and nitrogen source, there is evidence for further metabolites in addition to 3-amino-5-methyl-isoxazole. The higher DOC amount in comparison to the detected 3-amino-5-methyl-isoxazole-C fraction indicates that SMX was not quantitatively cleaved into 3-amino-5-methyl-isoxazole and 4-aminobenzene sulfonate. This is because the latter component is known to be readily degradable and its enrichment thus unlikely. Furthermore, the increase of nitrate in the system is likely due to the cleavage of the functional amino group on the aromatic ring of SMX ending up in the potential metabolite hydroxyl-N-(5-methyl-1,2-oxazole-3-yl)benzene-1-sulfonamide (Fig. 1B). This SMX metabolite is also proposed by Gauthier et al. (2010) from studies of SMX biodegradation by pure cultures of actinomycete Rhodococcus rhodochrous strains (see postulated SMX biodegradation pathway Fig. 1B).

Presumably the metabolic pathways will differ depending on nutrient and environmental conditions. In the presence of readily degradable carbon sources, the microorganisms might be more active causing a more quantitative SMX biotransformation. This would result in probably only one main stable metabolite as was shown in the SMX-adapted activated sludge where acetate was supplied for energy and carbon. On the other hand, microorganisms dependent on SMX as their sole energy, carbon, and/or nitrogen source might be less active and end up with a metabolite being situated ‘higher up the biodegradation chain’.

4.2. Potential SMX biodegradation in wastewater treatment and the environment

After a sufficient adaption time, SMX proved to be readily utilized by municipal activated sludge communities under aerobic mesophilic conditions (i) as a co-substrate in the presence of acetate and ammonium nitrate and (ii) as the sole carbon and nitrogen source. The first observation is consistent with sequencing batch data from Drilila and co-workers (2005). However, their further specific kinetic experiments with SMX-adapted activated sludges turned out to be somewhat conflicting. They demonstrated SMX biodegradation with SMX as the sole carbon and nitrogen source. On the other hand, however, their addition of ammonium did not affect SMX biodegradation. The most effective SMX reduction occurred in the presence of acetate without any other nitrogen source. In the Drilila study the presence of both acetate and ammonium led to biodegradation of acetate only with SMX remaining untouched. This implies that (i) during nitrogen deficiency only SMX is utilized as a nitrogen source and (ii) the presence of both acetate (500 mg L\(^{-1}\)) and ammonium-N (50 mg L\(^{-1}\)) impedes SMX biodegradation (Drilila et al., 2005). These observations suggest that SMX biodegradation may not be undertaken via co-metabolism.

In the present work there is evidence as well that carbon and nitrogen deficiency appear to enhance SMX biodegradation. The low initial nitrogen concentration of 2.3 mg L\(^{-1}\) and the very low nitrogen dosage concentration of 0.35 mg L\(^{-1}\) supplied in the
present experiments likely resulted in a nitrogen deficiency, especially after the restart where no additional nitrogen source was present in the system. As a result of the rapid consumption of acetate present in the system at the start (see DOC elimination) and the subsequent low acetate dosage (12 and 19 mg L⁻¹ acetate-C) throughout, it can be concluded that acetate was the limiting element. This indicates that SMX was utilized as a carbon source. This is corroborated by the kinetic enhanced culture studies by Drillia and co-workers (2005), which showed that SMX was consumed only after acetate had been completely utilized. Therefore the SMX biodegradation mechanisms represent a metabolic reaction which might rather be attributed to diauxic mechanisms than to co-metabolism. If this is the case microorganisms metabolized the easily degradable carbon source, e.g. acetate first and only after the first substrate had been exhausted the microbial populations switched to SMX. However, the utilization of acetate as first substrate provided enough energy for (i) biomass growth and/or (ii) metabolic activity which is required for persistent substrate biodegradation. This was confirmed by the approaches where SMX was supplied as the sole carbon and nitrogen source causing a lower SMX biodegradation potential.

With the knowledge gained in this study about SMX biodegradation conditions, it is assumed that structurally similar sulfonamides could be degraded under these conditions. In the literature it has been discussed that different sulfonamide antibiotics, characterized by similar chemical structures, display uniform biodegradation patterns in WWTPs (Ingerslev and Halling-Sørensen, 2000; Perez et al., 2005). The enzymes required for primary biodegradation are suggested to be class-specific rather than compound-specific, enabling adapted microbial communities to biodegrade a set of structurally similar sulfonamides.

At a large scale, membrane bioreactors (MBRs) can obviously provide conditions more conducive to sulfonamide biodegradation than conventional activated sludge systems as was shown by García-Galán et al. (2012). The authors postulated that an SMX removal efficiency of 51.8 and 54.6% in MBRs as compared to 46.4% in conventional activated sludge (CAS) might be caused by the long sludge retention time often applied in MBRs which permits (i) sufficient adaptation for heterotrophs to degrade persistent pollutants and (ii) growth of slow growers such as nitrifiers (García-Galán et al., 2012). Moreover, the mixed liquor is assumed by García-Galán and coworkers to be more active presumably due to exoenzyme and soluble oxidants being retained in MBRs, whereas being eliminated in CAS. This might lead to a somewhat higher SMX biodegradation activity in MBRs as was also observed by Göbel et al. (2007) who found an elimination of about 80% in MBRs as compared to 60% in CAS and fixed bed reactors. Interestingly, they did not find any dependency on SRT as long as it is above 1d. They claim antibiotics elimination to rely rather on nutrient removal efficiency than on long SRTs and HRTs. In an effective nitrifying/denitrifying (N/DN) zone, SMX biodegradation could occur due to nitrogen deficiency conditions therein. Autotrophic nitrifiers might then find better growth conditions with ammonium being supplied through cleavage of the SMX amino group, making them the driving force for SMX biodegradation under nitrifying conditions. For other micropolutants such as estrogen, the biodegradation capacity of nitrifying sludges and ammonium-oxidizing bacteria is well investigated and described by different research groups (Shi et al., 2004; Dytczak et al., 2008). However, the concurrent low F/M ratio with a corresponding low biomass activity in a nitrifying zone might require longer adaptation and thus, slow down SMX biodegradation. This would then result in the need for a higher sludge age (Perez et al., 2005). An anaerobic downstream denitrification step with additional energy feed could in turn provide energy for sulfonamide degraders. Moreover, denitrifying anaerobic organic soil systems characterized by N-deficiency and sufficient energy sources represent a unique environment for effective SMX biodegradation (Heberer et al., 2008). Mineral soils on the contrary, would be less effective because an energy source is missing. This is corroborated by Accinelli et al. (2007) who describe SMX to dissipate more rapidly from silt loam soils as compared to sandy soils. Moreover, they confirm better SMX dissipation in the presence of an energy source, in their case 5% (v/w) of liquid swine slurry. However, in over-fertilized soils with a high N-content, SMX biodegradation might be jeopardized by a lack of N-deficiency conditions.

5. Conclusions

Elimination of SMX in an activated sludge bench scale system was primarily based on biodegradation as SMX adsorption was negligible.

SMX biodegradation under aerobic and mesophilic conditions in the dark with semi-continuous SMX dosage as either a co-substrate or as the sole carbon and nitrogen source, proved that activated sludge communities readily utilized SMX as energy, carbon and/or nitrogen source for growth. Two metabolic bacteria groups which might be responsible for SMX biodegradation are (i) heterotrophic bacteria assimilating SMX-C and/or SMX-N and (ii) autotrophic nitrifying bacteria oxidizing the functional amino group on the aromatic ring of SMX.

With SMX as a co-substrate, 3-amino-5-methyl-isoxazole was the main stable metabolite. When SMX represented the sole carbon and nitrogen source, a higher DOC in comparison to the detected 3-amino-5-methyl-isoxazole-C fraction pointed to a further metabolite, possibly hydroxyl-N-(5-methyl-1,2-oxazole-3-yI)benzene-1-sulfonamide.

Biodegradation of SMX under aerobic and mesophilic conditions was enhanced when readily degradable carbon sources were added as they provided energy for heterotrophic biomass growth and metabolic activity. Moreover, elimination efficiency also improved under nitrogen deficiency conditions.

Acknowledgement

We gratefully acknowledge financial support by the Bavarian State Ministry of the Environment and Public Health.

References


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