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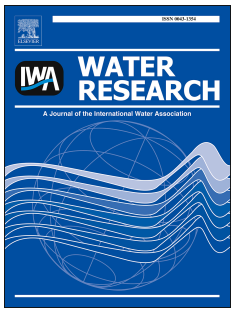

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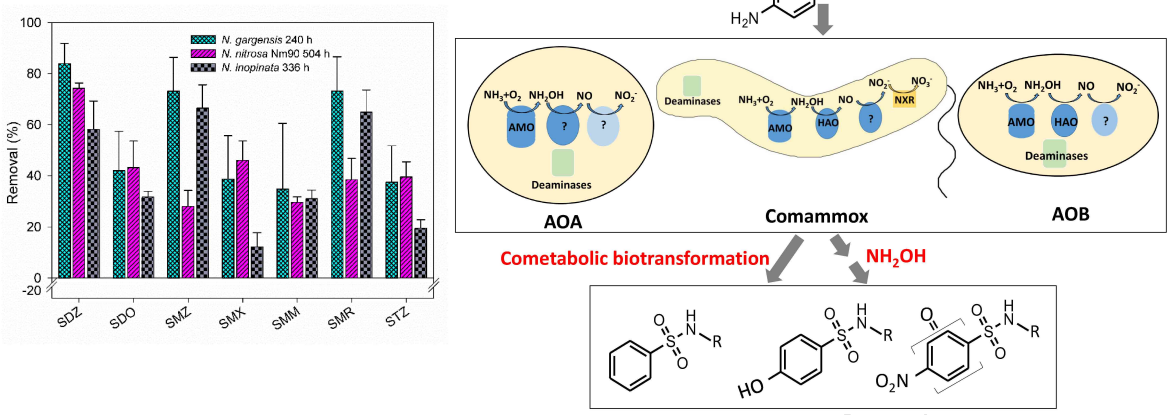

Cometabolic biotransformation and microbial-mediated abiotic transformation of sulfonamides by three ammonia oxidizers

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Abstract

The abilities of three phylogenetically distant ammonia oxidizers, *Nitrososphaera gargensis*, an ammonia-oxidizing archaeon (AOA); *Nitrosomomas nitrosa* Nm90, an ammonia-oxidizing bacterium (AOB); and *Nitrospira inopinata*, the only complete ammonia oxidizer (comammox) available as a pure culture, to biotransform seven sulfonamides (SAs) were investigated. The removals and protein-normalized biotransformation rate constants indicated that the AOA strain *N. gargensis* exhibited the highest SA biotransformation rates, followed by *N. inopinata* and *N. nitrosa* Nm90. The transformation products (TPs) of sulfadiazine (SDZ), sulfamethazine (SMZ) and sulfamethoxazole (SMX) and the biotransformation mechanisms were evaluated. Based on the analysis of the TP formulas and approximate structures, it was found that during biotransformation, i) the AOA strain carried out SA deamination, hydroxylation, and nitration; ii) the AOB strain mainly performed SA deamination; and iii) the comammox isolate participated only in deamination reactions. It is proposed that deamination was catalyzed by deaminases while hydroxylation and nitration were mediated by nonspecific activities of the ammonia monooxygenase (AMO). Additionally, it was demonstrated that among the three ammonia oxidizers, only AOB contributed to the formation of pterin-SA conjugates. The biotransformation of SDZ, SMZ and SMX occurred only when ammonia oxidation was active, suggesting a cometabolic transformation mechanism. Interestingly, SAs could also be transformed by hydroxylamine, an intermediate of ammonia oxidation, suggesting that in addition to enzymatic conversions, a microbially induced abiotic mechanism contributes to SA transformation during ammonia oxidation. Overall, using experiments with pure cultures, this study provides important insights into the roles played by ammonia oxidizers in SA biotransformation.

Keywords: biotransformation, sulfonamides, ammonia oxidizers, cometabolism, abiotic transformation.

1. Introduction

Sulfonamide antibiotics are frequently used for the treatment of bacterial diseases of humans and animals (Sukul and Spiteller, 2006). In recent years, increasing concerns have arisen about SAs in aquatic systems due to their potential adverse effects on ecosystems and public health (Baran et al., 2011; Kummerer, 2009). SAs are ubiquitous in terrestrial and aquatic ecosystems due to their widespread application, incomplete removal by wastewater treatment, and stability under typical environmental conditions (Chen and Xie, 2018). The concentrations of SAs in aquatic environments range from a few nanograms to several hundred micrograms per liter in groundwater, surface water, and wastewater (Gao et al., 2012; Zhou et al., 2016; Zhou et al., 2013a; Zhou et al., 2013b). Feedlots and municipal wastewater treatment plants (WWTPs) are the major sinks and main points of discharge of SAs into the environment, because sewage processing at such facilities was originally designed to lower the oxygen demand and to remove N and P, but not to decrease the concentrations of antibiotics (Zhou et al., 2013a; Zhou et al., 2013b). These antibiotic residues in the environment can affect the growth of aquatic organisms (Garcia-Galan et al., 2009; Park and Choi, 2008), alter the structure and function of microbial communities (Proia et al., 2013; Roose-Amsaleg et al., 2013; Underwood et al., 2011; Yan et al., 2013), and promote the development of antibiotic resistance (Martinez, 2009; Zhu et al., 2017). A systematic and mechanistic understanding of the fate of SAs during wastewater treatment and in the receiving environments is needed to help assess the environmental persistence and

ecotoxicity of SAs and their TPs, which can guide the establishment of appropriate remediation strategies or environmental regulations.

Biotransformation plays a major role in the fate of SAs in WWTPs, feedlot wastewater, and soil (Alvarino et al., 2016; Chen and Xie, 2018; Muller et al., 2013; Xing et al., 2018; Zhou et al., 2013b). A number of studies have demonstrated significant associations between SA biotransformation and nitrification (Kassotaki et al., 2016; Men et al., 2016; Xu et al., 2016). Enhanced biotransformation of SAs was observed as greater ammonia oxidation activities were reached in nitrifying activated sludge (NAS) (Xu et al., 2016). Consistently, the biotransformation of SMX in NAS was completely suppressed when nitrification was inhibited by addition of allylthiourea (ATU) (Kassotaki et al., 2016). In the nitrification process, ammonia oxidizers oxidizing ammonia to nitrite, and nitrite-oxidizing bacteria (NOB) converting nitrite to nitrate, are indispensable players. A previous study revealed that a NOB strain of the genus *Nitrobacter* was unable to biotransform a number of emerging organic contaminants (Yu et al., 2018), including asulam and the SA sulfathiazole (STZ). Therefore, it is less likely that NOB can biotransform SAs, although other nitrite oxidizers than *Nitrobacter* dominate most wastewater treatment plants (Gruber-Dorninger et al., 2015; Lücker et al., 2015). It was further demonstrated that ammonia monooxygenase (AMO) of ammonia-oxidizing microorganisms (AOMs) cometabolically biotransformed asulam and two pharmaceuticals (Men et al., 2016; Xu et al., 2016). However, most SA biotransformation studies have focused on enriched cultures of bacterial ammonia oxidizers or nitrifying sludge from WWTPs (Peng et al., 2017), and the mechanisms and pathways of SA biotransformation by different groups of AOMs in isolation have rarely been investigated.

SAs have an aromatic amine group, which is an antimicrobially active moiety. In a previous study, 4-nitro-SMX has been detected in WWTP effluents and surface waters at levels one order of magnitude lower than those of their parent compounds (Osorio et al., 2016), suggesting the oxidation of aromatic amines. Given that all aerobic ammonia oxidizers rely on a substrate promiscuous AMO, which can oxidize not only ammonia, but also some other organic compounds (Hooper et al., 1997; Roh et al., 2009), it was hypothesized that AMO might contribute to the oxidation of aromatic amines in SAs.

In municipal WWTPs, AOMs are dominated by AOB (Mussmann et al., 2011), but *amoA*-encoding archaea (AEA) have also been reported to occur (Li et al., 2016; Pornkulwat et al., 2018; Sauder et al., 2017; Zhang et al., 2009). Furthermore, complete ammonia oxidizers (comammox; (Daims et al., 2015)) encoding the enzymatic repertoire to completely oxidize ammonia to nitrate have been detected in WWTPs using molecular tools (Chao et al., 2016; Daims et al., 2015; Pjevac et al., 2017). AOA, AOB and comammox all possess AMOs (albeit from different evolutionary enzyme lineages), which converts ammonia into hydroxylamine by adding O_2 (Daims et al., 2015; Hooper et al., 1997; Prosser and Nicol, 2012). On the other hand these ammonia oxidizers differ in important physiological traits (He et al., 2012; Kits et al., 2017; Martens-Habbena et al., 2009). For example, some but not all members of the AOA as well as the comammox microbe *N. inopinata* have very high substrate affinities, while AOB generally have much higher mean apparent half-saturation constant values for ammonia ($K_{m(app)}$) (Kits et al., 2017). Given the relatively low concentrations of SAs and other micropollutants in most systems, these differences may be important for the biotransformation kinetics of SAs by AOB, AOA and comammox. Furthermore, these ammonia oxidizer groups differ in their enzymatic repertoire – while AOB and comammox encode a homologous hydroxylamine

dehydrogenase converting hydroxylamine to NO (Caranto and Lancaster, 2017; Daims et al., 2015), the hydroxylamine converting enzyme in AOA has not yet been recognized (Vajrala et al., 2013). In addition, comammox species possess the enzyme nitrite oxidoreductase and can thus in addition to AOA and AOB oxidize nitrite to nitrate (Daims et al., 2015). These differences in their biochemistry may also result in different abilities in terms of biotransforming SAs.

The goals of this study were: (i) using a pure culture representative of each group to investigate the abilities of AOA, AOB and comammox to biotransform seven SAs and (ii) to at least partly elucidate the transformation mechanisms and pathways by TP analysis. For this purpose, the biotransformation rates of seven SAs by the three selected pure cultures were determined and compared. Furthermore, the abiotic transformation of SAs by the ammonia oxidation intermediates hydroxylamine and NO were investigated.

2. Material and methods

2.1. Micropollutant selection. The seven SAs sulfadiazine (SDZ), sulfamethazine (SMZ), SMX, sulfadoxine (SDO), sulfamerazine (SMR), sulfamonomethoxine (SMM), and STZ were selected based on their high detection frequencies in natural environments and WWTPs (Gao et al., 2012; Zhou et al., 2016; Zhou et al., 2013a; Zhou et al., 2013b). The selected SAs were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and Toronto Research Chemicals (Toronto, Canada). Stock solutions of each reference compound were prepared in methanol (1 g/L), and stored at -20 °C until use. A mixture of standards (each SA at 100 mg/L) was prepared via

appropriate dilution of the stock solutions in methanol. Detailed information on the target compounds is presented in Table S1 in the supplementary material.

2.2. Cultivation of Comammox and AOMs. One representative strain from each AOM group was included: *Nitrososphaera gargensis*, an AOA, isolated from an outflow of a hot spring (Hatzenpichler et al., 2008); *Nitrosomonas nitrosa* Nm90, an AOB strain, isolated from WWTPs (Koops et al., 1991); and *Nitrospira inopinata* (the only available comammox pure culture), isolated from a pipe under the flow of hot water (56 °C, pH 7.5) raised from a 1,200 m deep oil exploration well (Daims et al., 2015). The comammox strain, *N. inopinata*, was cultivated in a modified basal medium (Daims et al., 2015) with 4 g/L $CaCO_3$ to buffer the pH at ~ 8.0. The culture was incubated at 42 °C in the dark without shaking, and 2 mM NH_4Cl were added as growth substrate every 12 days. The AOA strain, *N. gargensis*, was cultivated in the same modified basal medium (Daims et al., 2015) with 4 g/L $CaCO_3$ to buffer the pH at ~ 8.0. The culture was incubated at 46 °C in the dark without shaking, and 2 mM NH_4Cl were added as growth substrate every 6 days. The AOB strain, *N. nitrosa* Nm90, was obtained from the AOB strain collection of the University of Hamburg (Germany) and incubated at 37 °C in the dark with shaking at 80 rpm using the same basal medium with 4 g/L $CaCO_3$ amended with 2 mM NH_4Cl every week. The purity of the three AOMs was checked at the beginning of the experiments. The purity of the AOA was confirmed by a negative PCR result obtained using universal bacterial 16S rRNA gene primers (Men et al., 2016). The purity of the AOB strain and comammox were confirmed by the lack of detection of contaminating 16S rRNA gene sequences in 16S rRNA gene amplicon sequencing experiments (Men et al., 2016).

2.3. Biotransformation by Comammox and AOMs. The target pollutant biotransformation capabilities of comammox and AOMs were investigated using batch cultures. Pre-grown

158 biomass was harvested by centrifugation at 8,000 × *g* at 10 °C for 30 min and resuspended in
159 fresh medium to remove residual nitrite and to concentrate the biomass by approximately 2-3
160 times, resulting in an ammonia turnover rate of the concentrated biomass of ~ 1 mM/d for AOA
161 and AOB and ~ 0.3 mM/d for comammox. To avoid potential inhibitory effects of methanol in
162 the mixed target pollutant sock solutions (100 mg/L for each compound) on the cultures, 6 µL of
163 mixed SAs stock solution (for a starting concentration of 20 µg/L for each pollutant) was first
164 added into empty sterile bottles. After the organic solvents were evaporated, 30 mL of
165 thoroughly mixed concentrated culture containing 2 mM NH_4Cl was inoculated into 100 mL
166 glass bottles wrapped with tinfoil to maintain a dark environment for all tested AOM. The bottles
167 were loosely capped and shaken at 80 rpm for ~ 20 min to re-dissolve the target compounds.
168 NH_4Cl was added to bring the concentration to 2 mM when it decreased below 1 mM.
169 These bottles were incubated at the optimal growth temperatures for the tested comammox
170 (42 °C), AOA (46 °C) and AOB (37 °C). A first set of samples (~ 0.7 mL) was taken after
171 biomass addition. Then the samples (~ 0.7 mL) were centrifuged at 13,000 rpm at 4 °C for 10
172 min. Approximately 0.3 mL of each supernatant was transferred into 2 mL amber glass vials and
173 stored at 4 °C in the dark until liquid chromatography tandem mass spectrometry (LC-MS/MS)
174 analysis of the target compounds and their TPs. The remaining supernatant was transferred into a
175 1.5 mL microcentrifuge tube, and these tubes were stored at 4 °C for ammonia and nitrite
176 measurements. The cell pellets were stored at -20 °C for total protein measurement. Subsequent
177 samples were taken in the same way at 16, 24, 48, 72, 96, and 144 h, and 7, 10, and 14 d.
178 Abiotic control experiments to examine the hydrolysis of SAs, and the sorption potential of
179 the target pollutants to the medium matrix containing $CaCO_3$ precipitates were set up with fresh
180 medium containing either 8 or 0 g/L $CaCO_3$. Samples were taken at 0, 1 and 21 d (beginning,

middle and end-time points of the experiments). In addition, control experiments with heat-inactivated biomass were set up in the same way as the biotransformation reactors. For this purpose, the biomass was autoclaved twice at 121 °C and 103 kPa for 20 min. Next, 2 mM ammonium and 6 mM nitrite were added into the abiotic and heat-inactivated control samples to mimic the same N levels in the biological samples, and to investigate the possible abiotic SAs transformation in the presence of ammonium and nitrite. Samples were taken at the same time points during the same incubation period as for the biotransformation reactors. Furthermore, for all pure cultures positive controls demonstrating active ammonia oxidation without added target pollutants were also set up. All experiments were performed in triplicate.

2.4. Metabolic or Cometabolic Biotransformation. Whether the target SAs were metabolically utilized by the comammox and AOMs (*N. gargensis* and *N. nitrosa* Nm90) was tested separately using the same setup, but with a minimal NH_4-N concentration (0.2 mM, without reamendment) as the nitrogen source only (denoted "Lo_NH_4-N") and 100 µg/L SAs. Batch cultures amended with high NH_4-N (2 mM, with reamendment) and 100 µg/L sulfonamide compound (each) were used as positive controls (denoted "Hi_NH_4-N"). Samples were taken over a time course of 14 d.

2.5. Analytical Methods for SAs. SAs were analyzed by liquid chromatography coupled to a high-resolution quadrupole orbitrap mass spectrometry (LC-HRMS/MS) (Q Exactive, Thermo Fisher Scientific). For LC analysis, 50 µL of sample was loaded onto a C_{18} Atlantis-T_3 column (3 µm particle size, 3.0 × 150 mm, Waters) and eluted at a flow rate of 350 µL/min with nanopure water (A) and acetonitrile (B) (both amended with 0.1% formic acid) in the following gradient: 5% B: 0 - 1 min, 5% - 100% B: 1 - 8min, 100% B: 8 - 20 min, and 5% B: 20 - 26 min. The compounds were measured in full scan mode on HRMS at a resolution of 70,000 at m/z 200 and a scan range of m/z 50 - 750 in a positive/negative switching mode.

2.6. Estimation of Kinetic Parameters for the SA Biotransformation. Given that the growth substrate, ammonium, was unlimited, the cometabolic reductant and competition models of biotransformation could theoretically be simplified to a first-order model (Men et al., 2016). To quantitatively compare the biotransformation activities among the biological samples, the observed biotransformation rate constants normalized to the total protein were corrected for sorption and abiotic processes with the help of the control experiments. The median value calculated from the fitting procedure was used as the estimated k_{bio}, with the 5% and 95% percentile values representing the estimation uncertainty. Given the lag phase for AOB during the start of the incubation period and the removal plateau for the AOA and the comammox, the time points during those periods were neglected, and only the time points that showed 1st-order kinetics pattern according to the biotransformation curves were included (Figures S1-S3).

$$\frac{dS_c}{dt} = -f_{aq}(k_{bio}X + k_a) \times S_c \quad (1)$$

$$f_{aq} = \frac{S_c}{S_{ct}} \quad (2)$$

$$K_d = \frac{1-f_{aq}}{f_{aq}X} \quad (3)$$

where S_c is the aqueous concentration of the compound, f_{aq} is the dissolved compound fraction, k_{bio} is the total protein concentration-normalized biotransformation rate constant, X is the total protein concentration, k_a is the abiotic transformation rate, S_{ct} is the total concentration of the compound, and K_d is the sorption coefficient.

2.7. Cell Extraction for Measurement of the Intracellular SA Concentration. The cell extraction procedure was adopted from a previous study (Yu et al., 2018) with slight modification. Briefly, internal standards were spiked into cell pellets collected from 10 mL of culture (at a final concentration of 4 μg/L for each standard), followed by the addition of 2 mL of

lysis solvent containing methanol (0.5% formic acid): nanopure water (0.1% w/w EDTA), 50: 50 (v/v). The cells were disrupted by ultrasonication at 50 °C for 15 min, and centrifuged at 10,000 × *g* for 10 min. The supernatant was collected in a glass vial. This procedure was repeated twice for a better recovery. Finally, ~ 6 mL supernatant was evaporated to dryness under a gentle steam of dinitrogen gas at 40 °C. The analytes were redissolved in 0.5 mL of filter-sterilized fresh medium without $CaCO_3$, and these solutions were then centrifuged at 10,000 × *g* at 4 °C for 10 min. The supernatant was collected for LC-HRMS measurement of SAs and their TPs.

2.9. TP Identification. Suspect screening was used to identify potential TPs (TPs) formed during micropollutant biotransformation by the comammox and AOMs. The screening was performed by TraceFinder 4.1 EFS software (Thermo Scientific). Suspect lists of potential SA TPs were compiled using a self-written, automated metabolite mass prediction script, which took into account a variety of known redox and hydrolysis reactions, as well as conjugation reactions (Men et al., 2016). Additionally, previously reported TPs and mass shifts of typical biotransformation reactions were considered, including pterin-SAs (SDZ, SMZ and SMX), PtO-SAs (SDZ, SMZ and SMX), N4-formyl-SAs (SDZ, SMZ and SMX), N4-acetyl-SAs (SDZ, SMZ and SMX), 3-amino-5-methylisoxazole, 2-amino-4,6-dimethylpyrimidine, AcOH-SAs (SDZ, SMZ and SMX), 7,8-dihydropterin-SA (SDZ, SMZ and SMX), SA+O, pterin-O-SAs (SDZ, SMZ and SMX), pterin+H_2O-SA (SDZ, SMZ and SMX), and dihydropterin-SAs (SDZ, SMZ and SMX) (Achermann et al., 2018). Those compounds with isotope patterns that matched the predicted isotopic patterns at >70% and with a concentration that increased over the time course were subjected to further elucidation (Men et al., 2016).

Nontarget screening was further carried out to find possible TPs. TP candidates were selected based on the following criteria: (1) intensity above a set threshold with reasonable peak shape; (2) presence in the target pollutant-added samples and absence in target pollutant-added samples and heat-inactivated controls; (3) TP-like time-series pattern (i.e., trend of increasing or of increasing and then decreasing over the time course of the experiment); and (4) a reasonable chemical formula derived from the exact mass of [M+H] and isotopic pattern. Parent compounds and TPs might have different ionization efficiencies on LC-HRMS, but according to other studies such difference could be no larger than 3:1 (Gulde et al., 2016). As the reference compounds for SA TP candidates were not commercially available, to perform a relative comparison, it is reasonable to assume that the TPs and the parent compound had the same ionization efficiency, so that TPs compounds were semi-quantified using calibration curves of the corresponding parent compounds (Men et al., 2016).

2.10. Ammonium, Nitrite and Nitrate Measurements. Ammonium (NH_4^++NH_3) was measured by the colorimetric method (Kandeler and Gerber, 1988). Standards were prepared in the medium and ranged from 100 to 2000 μM NH_4Cl. Nitrite was measured by photometry with the sulfanilamide N-(1-naphthyl)ethylenediamine dihydrochloride (NED) reagent method, and nitrate was reduced to nitrite by vanadium chloride and measured as NO_x by the Griess assay (Miranda et al., 2001). Standards were prepared in the medium and ranged from 100 to 2000 μM NO_x and from 100 to 1000 μM nitrite.

2.11. Total Protein Measurement. Cell growth was measured on the basis of total protein determinations. Total protein was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Regensburg, Germany) according to the manufacturer's instructions.

3. Results and Discussion

3.1. Micropollutant biotransformation by the AOA, AOB and comammox strains. First, sorption of the target SAs to $CaCO_3$ precipitates and dead cells, as well as hydrolysis of the SAs in autoclaved medium was investigated. No significant sorption (< 10% removal) was observed. Depending on the compound, 0-35% of the added SAs was abiotically removed during an incubation time of 504 h (Figures S1-S3).

Next, the SA biotransformation capabilities of the tested AOA, AOB and comammox strains were examined (Figure 1 and Figures S1-S3). Compared to the control with heat-inactivated biomass, significant removal from 37.4% to 83.9% (end time point, two-tailed *t* test, $p<0.05$) of all SAs except SMM was recorded for the AOA *N. gargensis* (after an incubation for 240 h). For the AOB strain *N. nitrosa* Nm90, biotransformation was observed for all SAs with removals ranging from 27.9% to 74.2% (after an incubation for 504 h). For *N. inopinata*, significant removals from 31% to 66.5% (end time point, two tailed *t* test, $p<0.05$) of SDZ, SDO, SMZ, SMM and SMR (after an incubation for 336 h) were observed, while removals of SMX and STZ were less than 20%. The protein-normalized biotransformation rates of SAs by the three ammonia oxidizers are presented in Figure 2 and Table S2. The AOA *N. gargensis* exhibited high protein-normalized biotransformation rate constants (k_{bio}) greater than 0.01 L/(mg total protein • d) for three SAs (SDZ, SMZ and SMR), and the comammox strains showed $k_{bio} > 0.01$ L/(mg total protein • d) for SMZ and SMR; however, for the AOB strain, only k_{bio} of SDZ was above 0.01 L/(mg total protein • d) (Figure 2 and Table S2). The removals and protein-normalized biotransformation rate constants indicate that the AOA *N. gargensis* exhibited the best SA biotransformation performance, followed by the comammox strain and the AOB strain.

In addition, the SA removals were compared at the time when the same amount of ammonia was oxidized by the respective pure cultures (i.e., 144 h for the AOA and AOB strains and 336 h for the comammox strain to oxidize ~ 5-6 mM ammonia) (Figure 1A). After having oxidized the same amount of ammonia, the comammox strain *N. inopinata* (336 h) showed similar removals as the AOA strain (114 h) for all actively biotransformed SAs, except for SMX and STZ (Figure 1A and Figures S1-S3). In contrast, no removal was observed for these SAs by the AOB strain at 144 h. However, after an extended incubation time of up to 504 h, the removal of the SAs by the AOB strain gradually increased (Figure 1B and Figure S2). These results indicate a lag period in SA biotransformation by the AOB strain. In contrast, the biotransformation of SAs by the AOA and comammox strains were relatively higher at the beginning of the experiments, and became slower later during the incubation (Figure S1). The different k_{bio} and biotransformation processes of SAs by AOA, AOB and comammox might be due to their physiological characteristics, such as differences in substrate affinity of the AMO. The AMOs of AOA and comammox strains are reported to have a greater affinity for NH_3 than AOB (He et al., 2012; Kits et al., 2017; Martens-Habbena et al., 2009), which might result in a greater affinity for SAs and higher biotransformation rates at low SA concentrations if SA biotransformation is actually carried out by the AMOs. The AOA *N. gargensis* has greater substrate affinity than some AOB (e.g., *Nitrosomonas europaea*) but less substrate affinity than the comammox *N. inopinata* (Kits et al., 2017). Although comammox has greater affinity for ammonia than the AOA *N. gargensis* (Kits et al., 2017), the comammox treatments had low NO_2^-+NO_3^- formation rates (Table S2), which suggested low cell growth rate and low protein abundance and might have resulted in lower SA biotransformation rates than AOA.

3.2. TP identification and possible biotransformation mechanisms. To identify TPs of three biotransformed SAs (i.e., SDZ, SMZ and SMX), suspect screening was first carried out (Figure 3). For SDZ, one TP candidate was formed by all three AOMs, with an exact mass of [M+H] at 236.0482 (designated "TP236"). It has a formula of $C_{10}H_9O_2S$ (-NH from SDZ), likely a deamination product. Although the k_{bio} of SDZ by the AOB strain was smaller than that by the AOA and comammox strains, the abundance of TP236 in the AOB biotransformation samples was five times higher than that in the AOA and comammox biotransformation samples. This suggests that some other TPs might be present in the AOA and the comammox biotransformation samples, which were not included in the suspect list and thus not detected.

For SMZ, four TP candidates were detected during SMZ biotransformation by both the AOA and AOB. A TP candidate found in all the incubations has an exact mass of [M+H] at 264.0801 (designated "TP264") and a formula of $C_{12}H_{13}N_3O_2S$ (-NH from SMZ). Two other possible candidates were found at low abundance: one with an exact mass of [M+H] at 280.0750 (designated "TP280") and a formula of $C_{12}H_{13}N_3O_3S$ (-NH, +O from SMZ), and one with an exact mass of [M+H] at 325.0601 (designated "TP325") and a formula of $C_{12}H_{12}N_4O_5S$ (-2H, +3O from SMZ). It is proposed that TP264 is a deamination product, TP280 is likely formed by the oxidation of the amine group of SAs to a hydroxyl group (-OH), and TP325 likely originated from the hydroxylation of the aromatic ring plus the oxidation of the amine group into a nitro group. However, the structure of TP262 (exact mass of [M+H] at 262.0645) that was observed at a considerable abundance in all AOM-incubations remained unclear.

Three SMX TP candidates (TP237, TP239 and TP300) were detected in the AOA and AOB biotransformation treatments, with exact masses of [M+H] at 237.0328, 239.0476 and 300.0271, respectively. TP239 has a formula of $C_{10}H_9O_2S$ (-NH from SMX), likely a deamination product.

338 TP300 has a formula of $C_{10}H_9N_3O_6S$ (- 2H, + 2O, + O from SMX), and likely contains a nitro
339 group from oxidation of the amine group, as well as a -OH group added on the aromatic ring.
340 However, it is difficult to hypothesize approximate structures for TP237. SMX cannot be
341 biotransformed by the tested comammox strain, thus, no TPs of this compound were detected.

342 According to the TP formulas and tentative structures, it was hypothesized that the SDZ
343 biotransformation pathway involves deamination, and that the SMZ and SMX biotransformation
344 pathways involve deamination, hydroxylation, and nitration. The abundance of TP236 and
345 TP264 in the samples of the AOA and comammox biotransformation of SDZ and SMZ,
346 respectively, were one order of magnitude lower than that in the respective AOB
347 biotransformation samples (Figure 3). Furthermore, the abundance of SDZ and SMZ decreased
348 with increasing TP abundance in the AOB biotransformation samples (Figure 3). In the AOA
349 biotransformation treatment of SDZ and SMZ, the abundance of SDZ and SMZ initially
350 decreased, but the abundance of TP236 and TP264 did not increase. This suggests that in
351 addition to deamination, other biotranformation pathways might be involved in the AOA and
352 comammox catalyzed biotransformation of SDZ and SMZ.

353 A previous study showed pterin-SAs conjugates and related secondary products as the
354 major products during SA biotransformation by a nitrifying activated sludge community in
355 laboratory batch experiments (Achermann et al., 2018). SAs inhibit the proliferation of bacteria
356 by acting as competitive inhibitors of *p*-aminobenzoic acid in the folic acid metabolism cycle
357 (Sukul and Spiteller, 2006) by forming pterin-SA with a pteridine. However, in the present study,
358 pterin-SAs were detected only in the incubation experiments with the AOB strain and with low
359 abundance (0.1%-0.6% of the removed parent compound abundance). Despite the rather low
360 ionization efficiencies of pterin-SAs (10% relative to that of the parent SAs) (Achermann et al.,

2018), less than 0.6% of the removed parent compound abundance represents a very small portion of all identified TPs. N4-formyl-SMX was also detected in the AOB treatment in even lower abundance than pterin-SMX. N4-formyl-SMX can be formed from pterin-conjugates by a series of hydrolysis, oxidation, and decarboxylation reactions (Achermann et al., 2018). N4-acetyl-SMX was also detected in the AOB treatment at low abundance. N4-acetyl-SMX, the pig/human metabolite of SMX, is formed by N-acetylation (Mengelers et al., 1997).

Intracellular SAs and potential TPs were also analyzed based on the same analysis criteria, but none were detected, suggesting no active uptake of SAs or TPs by the living cells. The sum of peak areas of all the identified TPs was much lower than that of SA removed. The incomplete mass balance is probably due to: (1) the uncertainty inherent in the semiquantitative approach using peak areas given the difference in ionization efficiency for the parent compound and the TPs, and (2) the presence of potential TPs undetectable by the current LC-HRMS method.

SAs are biotransformed mainly by N-glucuronidation, N-acetylation, N-deamination, and hydroxylation in humans and other mammals (Sukul and Spiteller, 2006). In the present study, the three ammonia oxidizer strains formed mainly desamino-SAs, hydroxyl-SAs and nitro-SAs as TPs. The TPs 4-nitro-SMX, desamino-SMX and N-acetyl-SMX formed during SMX degradation in an enriched culture of AOB represented up to 32% of the parent compound, with 4-nitro-SMX as the most abundant TP (Kassotaki et al., 2016). 4-Nitro SMX and desamino-SMX were detected in WWTP effluents and surface waters at levels one order of magnitude lower than those of their parent compounds (Osorio et al., 2016). Deamination might be catalyzed by deaminases, which are encoded in the genomes of the AOA *N. gargensis*, the AOB *N. nitrosa* Nm90 and the comammox *N. inopinata*. In addition, AMO might play a key role in the formation of hydroxyl-SA, and nitro-SA via the possible intermediate NHOH-SA. AMOs in

ammonia oxidizers are responsible for ammonia oxidation to hydroxylamine (Daims et al., 2015; Hooper et al., 1997; Kozlowski et al., 2016), which might be involved in a further hydroxylation reaction catalyzed by AMOs after the deamination. It is also proposed that, under the action of AMOs, the amine of SAs underwent hydroxylation by reacting with the intermediate hydroxylamine; and then, nitration might occur under the action of Cu "P460" or other hydroxylamine converting enzymes in AOA and hydroxylamine dehydrogenase (HAO) in AOB and comammox strains, which can convert hydroxylamine into NO (Caranto and Lancaster, 2017; Carini et al., 2018; Daims et al., 2015; Kozlowski et al., 2016). Clearly, further research is needed to confirm the contribution of deaminases and AMOs to the biotransformation of SAs by ammonia oxidizers.

3.3. Cometabolic Biotransformation of SAs. To determine whether SA biotransformation by the three different ammonia oxidizers was metabolic or cometabolic, SA biotransformation by the AOA *N. gargensis*, AOB *N. nitrosa* Nm90, and comammox *N. inopinata* grown with minimal ammonia (Lo_NH_4-N) were compared to that grown with unlimited ammonia by re-adding ammonium back to 2 mM once it was below 1 mM (Hi_NH_4-N) (Figure 4). Three biotransformed SAs (i.e., SMZ, SDZ and SMX) were added individually to each strain. In the Hi_NH_4-N controls of *N. gargensis*, *N. nitrosa* Nm90, and *N. inopinata*, SAs were continuously biotransformed as sufficient ammonium was supplied. Again AOA showed the best SA biotransformation abilities among the tested ammonia oxidizers followed by comammox and the AOB (Figure 4 A, C & E). SMZ was more efficiently removed than SDZ, while SMX was the most recalcitrant of the three compounds (Figure 4 A, C & E). In the SA-added Lo_NH_4-N cultures, no significant SA removal was observed (Figure 4 B, D & F), except for the *N.*

gargensis culture, where the biotransformation of SDZ and SMZ ceased after 24 h when the remaining low level ammonia was depleted and no more nitrite was formed. This is probably caused by the relatively lower biotransformation capabilities of AOB and comammox strains than AOA. These results suggest that SA biotransformation was dependent on active ammonia oxidation, and these SAs were biotransformed via cometabolism (Figure 5). Based on TPs analysis and cometabolic experiments, potential transformation mechanisms and pathways of SAs by the three ammonia oxidizers were proposed (Figure 5). SAs can be biotransformed by ammonia oxidizers via cometabolism; deamination might be catalyzed by deaminases while hydroxylation and nitration are proposed to be mediated by nonspecific activities of the ammonia monooxygenase (AMO).

3.4. Abiotic MP transformation by the ammonia oxidation intermediate NH_2OH. A previous study by us revealed that abiotic transformation by AMO-mediated formation of hydroxylamine (NH_2OH) represents another pathway for asulam transformation by an AOB strain (Yu et al., 2018). As all of the three investigated AOM strains in this study can produce the intermediates hydroxylamine (NH_2OH) and NO (Kits et al., 2019; Kozlowski et al., 2016), it was tested whether SAs can also abiotically react with the ammonia oxidation intermediates hydroxylamine and NO. Abiotic SA transformation experiments after the addition of NH_2OH at concentrations occurring in AOB batch cultures were conducted (Figure S4). It was also tested whether SAs can be transformed by NO, which is an ammonia oxidation intermediate of AOM. Seven SAs were exposed to hydroxylamine and NO, separately. All SAs were transformed abiotically by NH_2OH, whereas no SAs were transformed by NO (Figure 5).

The TPs identified from SDZ and SMZ biotransformation were not detected in NH_2OH-treated abiotic transformation, suggesting different pathways for biological and abiotic transformation of these two SAs. For SMX, two TPs (TP239 and TP300) were detected in both biological and NH_2OH abiotic transformation, although their abundance was much lower during the abiotic transformation by NH_2OH. In previous studies, it was shown that SAs can abiotically react with NO_2^- at acid conditions, and that TP patterns are dependent on the pH; for example, SDZ could be transformed into desNH_2SDZ when calves or rats consumed a diet high in nitrite (Woolley and Sigel, 1982). At pH values below the acid dissociation constant of nitrous acid (pKa ~ 3.2 3.4), the formation of desamino-SMX was observed; while TP 4-nitro-SMX was detected when the solution was neutralized (pH 7-7.4) (Noedler et al., 2012). Aromatic amines can react with NO_2^- to form a diazonium salt under acidic conditions, but they cannot react under neutral or basic conditions. Depending on the reaction conditions, the diazonium cation disintegrates via the cleavage of elementary nitrogen and substitutes its diazo-group with a NO_2-group, hydrogen or OH-group. However, NO and aromatic amines cannot react to form diazonium cations in natural environments. Hydroxylamine is not stable and easy to decompose ($2NH_2OH \rightarrow NH_3+HNO+H_2O$) (Izato et al., 2017); it is possible that hydroxylamine reacted with aromatic amines (e.g., SAs) via nitroxyl (HNO) to form diazonium cations. The medium in this study was at pH 8, where the diazonium cation disintegrates via the cleavage of elementary nitrogen and substitutes its diazo-group with a NO_2-group or OH-group. That is probably why none desNH_2-SDZ was detected in the hydroxylamine treatment.

Most of WWTPs effluents, surface waters and sediments, and soil are at neutral pH condition, and NO_2^- less likely reacts with pollutants containing aromatic amines in these environments. However, in acidic soil with a pH of 4 - 5, NO_2^- has the potential to react with

pollutants containing aromatic amines. In addition, the ammonia oxidation intermediate hydroxylamine can transform pollutants containing aromatic amines extracellularly, as well.

3.5. Environmental Relevance and Implications.

SAs have been proven to undergo microbially mediated biotransformation in nitrifying WWTPs (Muller et al., 2013; Osorio et al., 2016) and in soil (Brienza et al., 2017). AOA, AOB, and comammox are prevalent in many environments such as WWTPs, soil, and surface waters (Chao et al., 2016; Daims et al., 2015; Pjevac et al., 2017; Prosser and Nicol, 2012). In this study, the AOA *N. gargensis*, AOB *N. nitrosa* Nm90, and comammox *N. inopinata* were shown to biotransform SAs to different extents. In particular, the AOA strain showed high biotransformation rates. The main reaction between SAs and AOMs mainly occurred at the aromatic amine, suggesting that other pollutants with aromatic amines likely have the potential to be biotransformed by AOMs, as well.

The amino group of SAs is an antimicrobially active moiety that, if replaced, might change the antimicrobial effect. In this study, it was observed that the biotransformation of SAs by ammonia oxidizers mainly occurred on the amino group on the para position, and desNH_2-SAs, NO_2-SAs and OH-SAs are important TPs of ammonia oxidizers produced via biotic or abiotic reaction. In acute toxicity assays, NO_2- SMX and 4-OH-SMX were found to inhibit the growth of *Vibro fishcheri* to a greater extent than the parent compound, SMX (Majewsky et al., 2014; Osorio et al., 2016). In addition to its toxicity, NO_2-SMX has the potential to convert back to SMX in anoxic environments in the absence of nitrate as an electron acceptor (Noedler et al., 2012). Pterin-SA conjugates were reported to have appreciable antibacterial activity, but this

activity was ten times weaker than that of their corresponding parent SAs (Zhao et al., 2016). Moreover, synergistic effects were observed for a mixture of SMX and trimethoprim in algae (Yang et al., 2008). TPs might also have synergistic effects with other pharmaceuticals. So far, only two target organisms *V. fishcheri* (bacteria) and *Daphnia magna* (crustacean) were used to test the acute toxicity of the TPs of SAs. Further studies on acute and chronic toxicity of the formed TPs should be carried out to obtain a comprehensive risk assessment of SAs in the environment.

4. Conclusions

In summary, this study represents an important step toward filling in the knowledge gaps on SA biotransformation by ammonia oxidizers. Firstly, the biotransformation of SAs by pure AOA, AOB and comammox cultures revealed new biotransformation pathways for SAs. Secondly, indications for TP formation by (i) deamination, (ii) hydroxylation on the aromatic ring, (iii) oxidation of the amine group of SAs to a hydroxyl group (-OH), and (iv) oxidation of the amine group into nitro group were obtained. Thirdly, experimental data suggest that SA biotransformation by ammonia oxidizers occurred via cometabolim. Finally, the intermediate hydroxylamine has the potential to abiotically transform SAs.

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701

702 **Captions**

703 **Figure 1.** Removal of seven SAs biotransformed by *N. gargensis* (AOA), *N. nitrosa* Nm 90
704 (AOB), and *N. inopinata* (comammox) (n=3). Removal (%) = (C_0-Ct)/C_0×100%. C_t represents
705 the concentrations of SAs at time T, C_0 represents the concentrations of SAs at the starting time.
706 A) Removal at 144 h for the AOA and AOB strains and 336 h for comammox strain. B)
707 Removal at 240 h for the AOA strain, 504 h for the AOB strain, and 336 h for the comammox
708 strain. SDZ, sulfadiazine; SMZ, sulfamethazine; SMX, sulfamethoxazole; SDO, sulfadoxine;
709 SMR, sulfamerazine; SMM, sulfamonomethoxine; STZ, sulfathiazole.

710

711 **Figure 2.** First-order biotransformation rate constants (k_{bio}) of SAs by *N. gargensis*, *N. nitrosa*
712 Nm90 and *N. inopinata*. SDZ, sulfadiazine; SMZ, sulfamethazine; SMX, sulfamethoxazole;
713 SDO, sulfadoxine; SMR, sulfamerazine; SMM, sulfamonomethoxine; STZ, sulfathiazole.

714

715 **Figure 3.** Comparison of SDZ (Figure 3 A, B and C), SMZ (Figure 3 D, E, and F), and SMX
716 (Figure 3 G and H) biotransformation by *N. gargensis*, *N. nitrosa* Nm90, *and N. inopinata*,
717 respectively. The y axis plots the peak abundance of SAs and their TPs in LC- HRMS/MS, mean
718 ± SD (n=3). Note: different panels have different scales. SDZ, sulfadiazine; SMZ,
719 sulfamethazine; SMX, sulfamethoxazole. TP236, desamino-SDZ; TP264, desamino-SMZ;
720 TP280, HO-SMZ; TP325, likely from the hydroxylation of the aromatic ring plus the oxidation
721 of the amine group into nitro group; TP239, desamino-SMX; TP300, likely contains a nitro

group from oxidation of the amine group, as well as a -OH group added on the aromatic ring; the structure of TP262 and TP237 remains unclear.

Figure 4. Comparison of SDZ, SMZ, and SMX biotransformation by *N. gargensis*, *N. nitrosa* Nm90, *and N. inopinata* between Hi_NH_4-N (left) and Lo_NH_4-N (right). C_t represents the concentrations of SAs at time T, C_0 represents the concentrations of SAs at the starting time. The y axis plots the ratios of the SA concentration at time T0 and T, mean ± SD (n=3). SDZ, sulfadiazine; SMZ, sulfamethazine; SMX, sulfamethoxazole. For all experiments the SAs were added individually to each culture. All experiments were performed in triplicate.

Figure 5. The potential transformation mechanisms and pathways of sulfonamides by the three ammonia oxidizers strains. AOA, ammonia-oxidizing archaea; comammox, the complete ammonia oxidizer; AOB, ammonia-oxidizing bacteria; AMO, ammonia monooxygenase; HAO, hydroxylamine dehydrogenase; NXR, nitrite oxidoreductase. For AOA, only one of several different published metabolic models for ammonia oxidation is depicted here (Carini et al., 2018).

Figure 1.

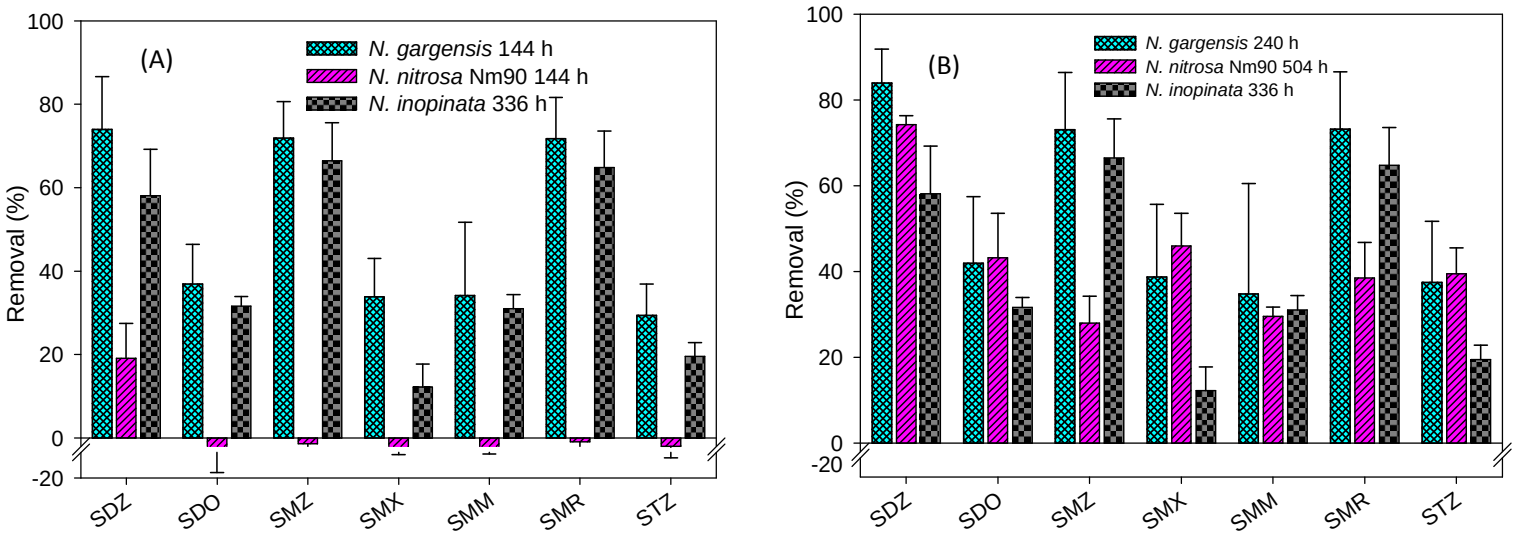


Figure 2

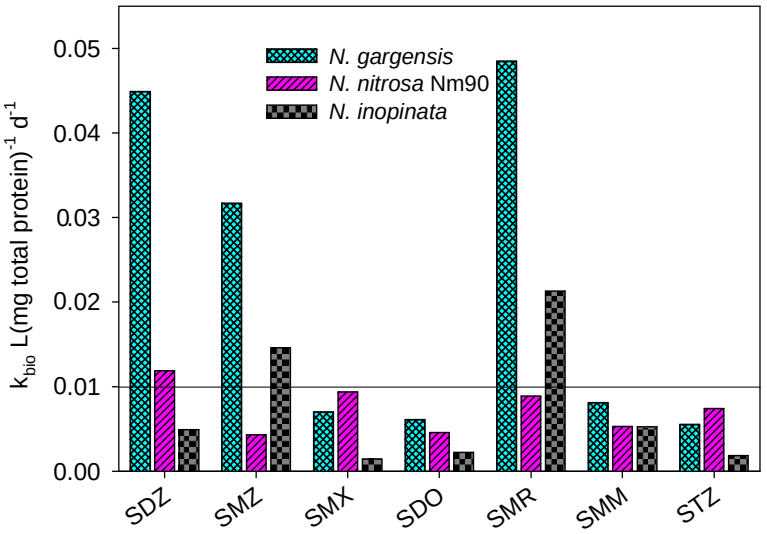
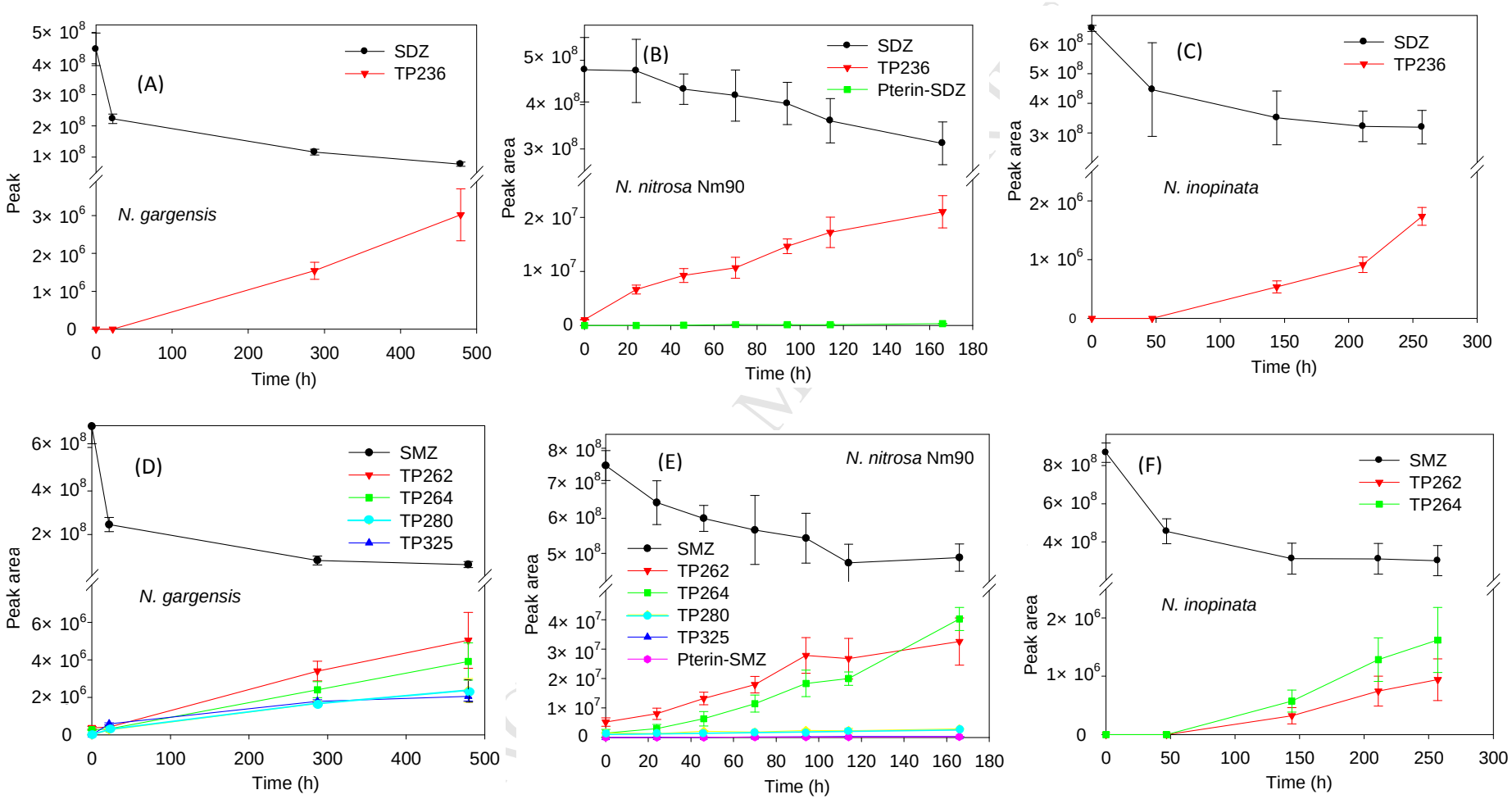
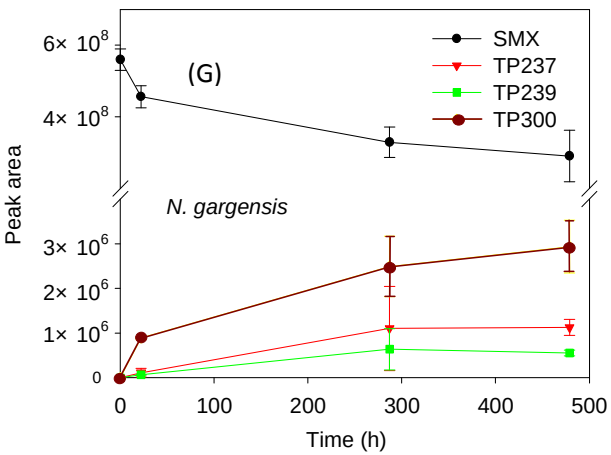


Figure 3.


(A)
SDZ
TP236
N. gargensis
Peak
Time (h)
(B)
SDZ
TP236
Pterin-SDZ
N. nitrosa Nm90
Peak area
Time (h)
(C)
SDZ
TP236
N. inopinata
Peak area
Time (h)
(D)
SMZ
TP262
TP264
TP280
TP325
N. gargensis
Peak area
Time (h)
(E)
N. nitrosa Nm90
SMZ
TP262
TP264
TP280
TP325
Pterin-SMZ
Peak area
Time (h)
(F)
SMZ
TP262
TP264
N. inopinata
Peak area
Time (h)



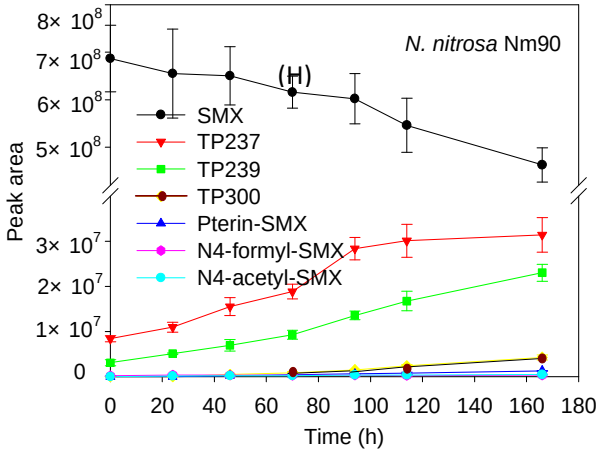


Figure 4.

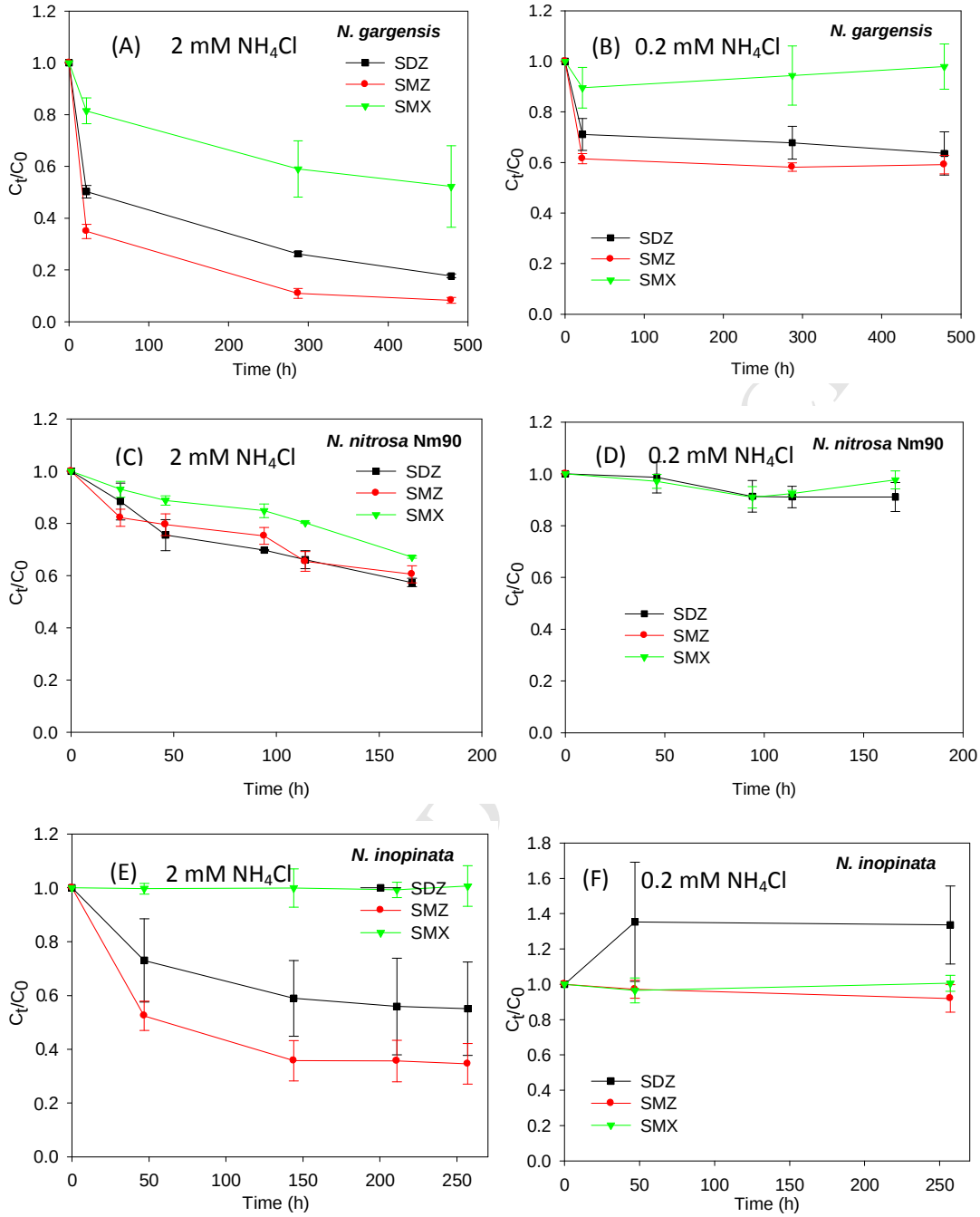
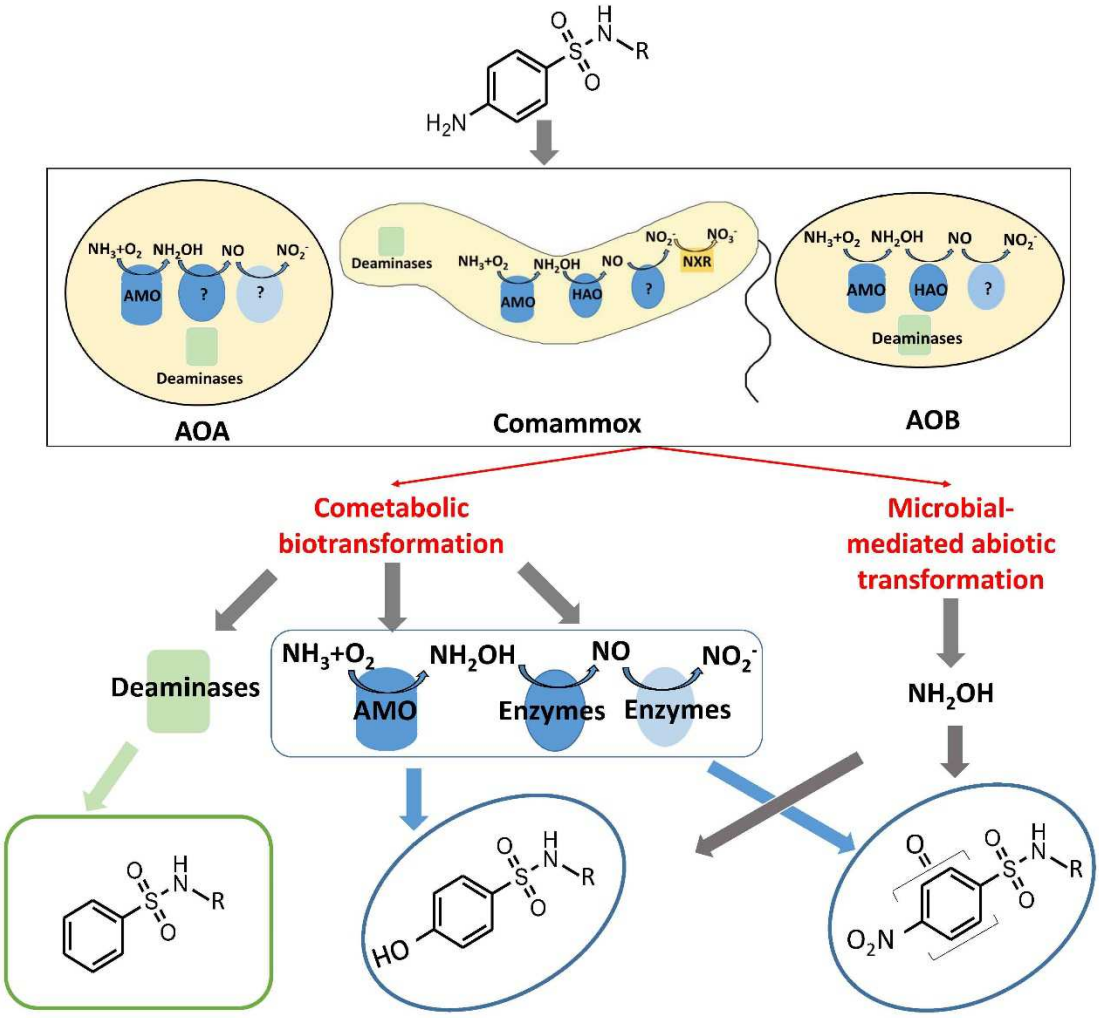

(A) 2 mM NH4Cl
N. gargensis
SDZ
SMZ
SMX
Ct/C0
Time (h)
(B) 0.2 mM NH4Cl
N. gargensis
SDZ
SMZ
SMX
Ct/C0
Time (h)
(C) 2 mM NH4Cl
N. nitrosa Nm90
SDZ
SMZ
SMX
Ct/C0
Time (h)
(D) 0.2 mM NH4Cl
N. nitrosa Nm90
SDZ
SMZ
SMX
Ct/C0
Time (h)
(E) 2 mM NH4Cl
N. inopinata
SDZ
SMZ
SMX
Ct/C0
Time (h)
(F) 0.2 mM NH4Cl
N. inopinata
SDZ
SMZ
SMX
Ct/C0
Time (h)

Figure 5.



Highlights

1. Three groups of AOMs including AOA, AOB and comammox are able to biotransform SAs.

2. The AOA strain exhibited the best SA removals, followed by the comammox and the AOB.

3. Deamination, hydroxylation and nitration are main reactions of SDZ, SMZ and SMX.

4. SAs were biotransformed by AOMs via cometabolism.

5. SAs were transformed by hydroxylamine, an intermediate of ammonia oxidation.

Conflict of interest

The authors declare no conflict of interest.