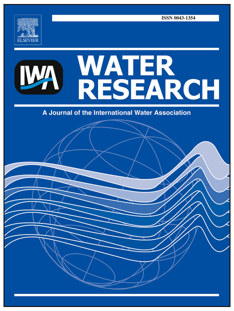

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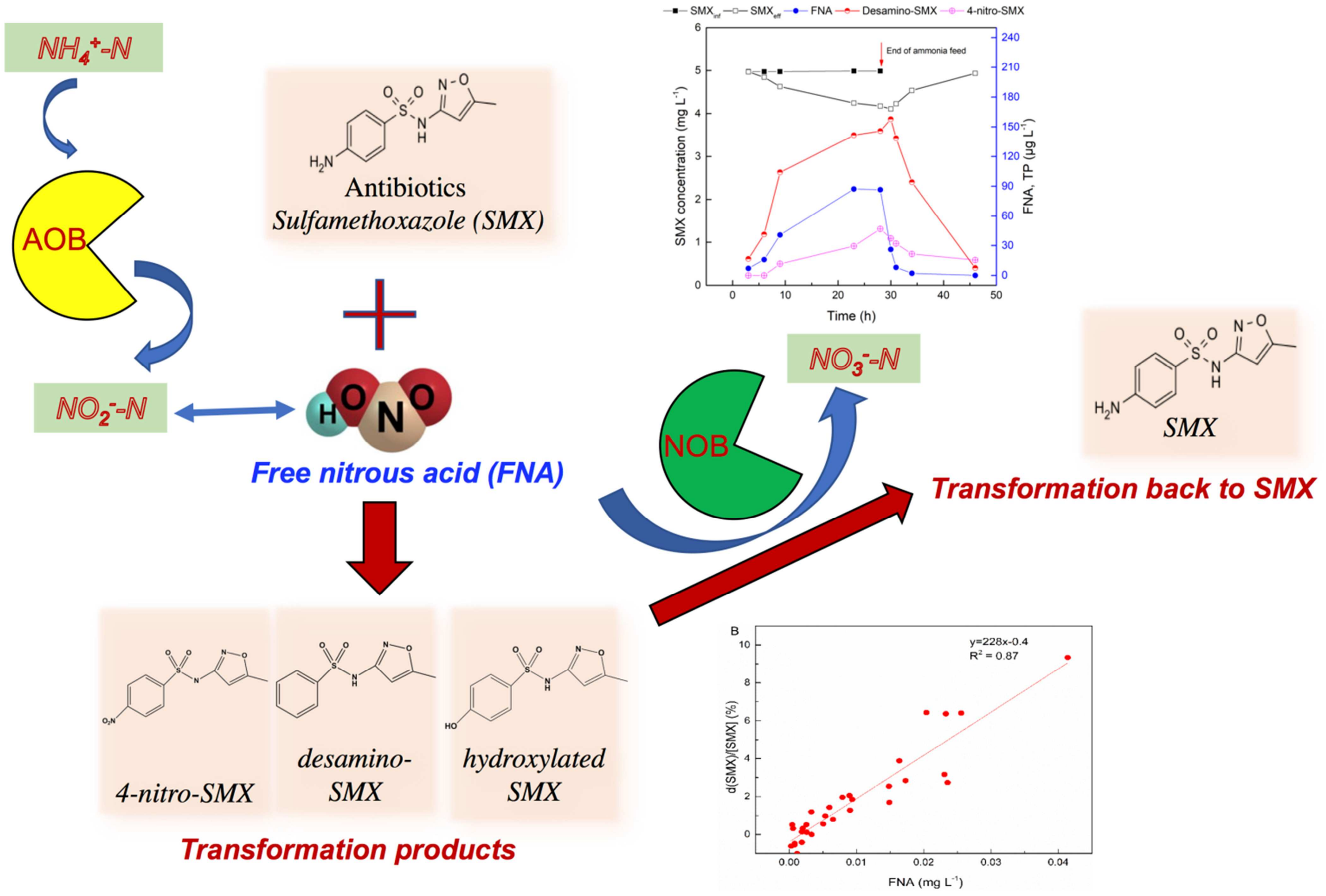
PII: S0043-1354(18)30828-5
DOI: 10.1016/j.watres.2018.10.031
Reference: WR 14142

To appear in: *Water Research*

Received Date: 19 June 2018
Revised Date: 14 September 2018
Accepted Date: 11 October 2018

Please cite this article as: Sun, F., Wu, D., Chua, F.D., Zhu, W., Zhou, Y., Free nitrous acid (FNA) induced transformation of sulfamethoxazole in the enriched nitrifying culture, *Water Research* (2018), doi: https://doi.org/10.1016/j.watres.2018.10.031.

Graphical Abstract



Free nitrous acid (FNA) induced transformation of sulfamethoxazole in the enriched nitrifying culture

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Abstract

The sulfonamide antibiotics sulfamethoxazole (SMX) has been frequently detected in the wastewater. It has been reported that part of SMX can be transformed by the co-metabolism of ammonia oxidizing bacteria (AOB) during nitrifying process. However, previous studies reported inconsistent or even contradictory results in terms of SMX degradation and/or transformation. Literature study revealed that nitrite may play certain role in SMX transformation, which has been neglected previously. In this study, the transformation behavior of SMX was investigated with and without the presence of nitrite in an enriched nitrifying culture. The results clearly show that the elimination of SMX occurred with the presence/accumulation of nitrite, and a linear regression was observed between SMX elimination efficiency and free nitrous acid (FNA) concentration, indicating that FNA was the major factor responsible for the SMX transformation. By reacting with FNA, SMX transformation products, such as 4-nitro-SMX, desamino-SMX and hydroxylated SMX, were detected. However, when FNA concentration decreased, these intermediates may be retransformed back to SMX. These findings improved our understanding on SMX transformation in a biological system and highlighted the role of nitrite/FNA in the sulfonamide antibiotics degradation.

Keywords: Sulfamethoxazole; Nitrification; Free nitrous acid; AOB; Transformation product

1. Introduction

In recent years, pharmaceutical compounds have been frequently detected worldwide in aquatic environments (Arlos et al. 2015, Blair et al. 2013, Sui et al. 2011). Even though the detected concentration levels are low, longtime exposure to pharmaceutical compounds may alter microbial diversity of the environment (Brodin et al. 2013, Demoling et al. 2009). Antibiotics are important pharmaceuticals that are prescribed in human medicine as well as for veterinary and agricultural propose. However, the broad application of antibiotics also raises some concerns on the risk of spread of antibiotic-resistance bacterial genes and the negative impacts on the living creatures in the aquatic ecosystems (Brodin et al. 2013, Marti et al. 2014, Martínez 2008, Sanderson et al. 2016). Antibiotics can reach open waters through wastewater treatment plants (WWTPs) effluent, landfill discharge, animal farming wastewater, and runoff from agricultural with livestock manure application (Padhye et al. 2014, Wei et al. 2011, Wu et al. 2015).

Sulfonamide sulfamethoxazole (SMX) is one important representative antibiotic that is extensively used to treat a wide variety of bacterial infection (such as urine, respiratory and intestinal infections) in human medicine and livestock production (Jia et al. 2017). Approximately 45-70% of SMX was excreted via urine. Its human-metabolized transformation products can also be easily retransformed back to SMX (Göbel et al. 2005, Radke et al. 2009). As a consequence, SMX is frequently detected at high concentrations (Göbel et al. 2005), mostly in WWTPs ranging from several hundred ng L^{-1} to μg L^{-1}.

There are several studies focused on the fate and biodegradation of SMX in natural and engineered ecosystems. It was found that its microbial mediated *degradation occurred preferentially under particular redox conditions such as anaerobic sulfate-reducing (Jia et al. 2017), anaerobic hydrogenotrophic methanogenic (Cetecioglu et al. 2016), denitrifying (Nödler et al. 2012) or iron-reducing conditions (Mohatt et al. 2011). In the sulfate-reducing conditions, SMX degradation was achieved by altering the isoxazole ring via biodegradation followed by adsorption, whereas for the denitrifying and iron-reducing conditions, the transformation was highly dependent on intermediates that were produced from other bioreactions. For instance, nitric oxide (NO) and nitrite produced during the denitrification (Nödler et al. 2012), and Fe(II) generated via Fe(III) reduction (Mohatt et al. 2011) could cause a rapid abiotic transformation of SMX.*

In contrast, conclusions on the SMX biodegradation are markedly inconsistent under aerobic conditions. Some studies reported that SMX was persistent to biodegradation (Benotti and Brownawell 2009). However, in other studies, it can be biodegraded via various pathways. Bouju et al. (2012) isolated five strains that were capable of SMX mineralization, of which two belonged to the phylum *Actinobacteria* and three belonged to the members of *Proteobacteria*. Hai et al. (2011) found 65% of SMX removal in a MBR treating high concentration COD and total nitrogen. Katipoglu-Yazan et al. (2016) showed that almost 100% of SMX was transformed to unknown by-products in a culture fed with ammonium and COD. Apart from the pure strains study for SMX elimination, it seems that SMX reduction is somehow related to nitrogen removal process.

Previous study demonstrated that nitrifying activated sludge had higher removal efficiency of pharmaceutical compounds compared to the system without nitrification (Fernandez-Fontaina et al. 2012, Helbling et al. 2012). Such enhanced biodegradation seems to be related to the activity of ammonia oxidizing bacteria (AOB), which could co-metabolize the compounds using one of its key enzymes - ammonia monooxygenase (AMO). It is known that AMO is capable of oxidizing a wide range of pharmaceuticals, and potentially plays a key role in their biodegradation. However, the results of SMX elimination through nitrification process were not consistent. Both insignificant removal and complete removal in different nitrification reactors were claimed (Kassotaki et al. 2016, Suarez et al. 2010). These discrepancies indicate the complexity of SMX elimination behavior during the nitrification process.

Nitrite is one of the intermediates formed in the nitrification process, and high accumulation of nitrite is common in the partial nitrification process. It is possible that SMX may undergo the similar reactions as that under the denitrification conditions. To date, there is no study to evaluate the role of nitrite in SMX transformation during the nitrification process. Therefore, the overarching objective of this study is to investigate the transformation behavior of SMX under different levels of nitrite which achieved by manipulating specific ammonium oxidation rates (SAORs) of a nitrifying culture. After the exposure of SMX to nitrite, the change of SMX and its transformation products with gradually reduced concentrations of nitrite were further investigated. The SMX transformation kinetics were investigated. The transformation metabolites were identified, and possible transformation pathways were proposed.

2. Materials and methods

2.1 Chemicals

SMX (CAS 723-46-6) was purchased from Sigma-Aldrich (USA) and of analytical grade. 4-nitro-sulfamethoxazole (4-nitro-SMX, CAS 29699-89-6) was purchased from Toronto Research Chemicals (Canada).

N-(5-methylisoxazol-3-yl)benzenesulfonamide (desamino-SMX, CAS 13053-79-7) was purchased from Specs (www.specs.net) and their purity was > 95%. All the other chemicals were purchased from Sigma-Aldrich and of analytical grade or higher.

2.2. Batch experiments set-up

2.2.1 Nitrifying sludge source

Two enriched nitrifying culture was withdrawn from two lab-scale sequencing batch reactor (SBR). The first SBR (5 L) was operated to enrich full nitrification microbes (Culture I) mostly containing AOB and nitrite oxidizing bacteria (NOB). The working cycle consisted of 480 min reaction phase including 5 consecutive intervals of 10 min feeding (aeration on) and 70 min aeration, followed by 5 min wasting, 45 min settling, 25 min decanting and 5 min idle. The synthetic wastewater for this reactor comprised 1 g $(NH_4)_2CO_3$ – N L^{-1}, 0.064 g L^{-1} each of KH_2PO_4 and K_2HPO_4, and 2 mL of trace element stock solution that was detailed previously (Chen et al. 2017). 1.5 L of synthetic wastewater was fed every cycle resulting in a hydraulic retention time (HRT) of 1.1 d. pH was recorded with programmed logic controllers (PLC) and controlled at 7.5 ± 0.1 by dosing 1 M $NaHCO_3$. The mixed liquor volatile suspended solid (MLVSS) concentration was maintained at approximately 1.5 ± 0.1 g L^{-1} with a sludge retention time (SRT) of 5 days. The reactor had stable performance for more than 1 year before the culture was used for this study. Nitrite and nitrate concentration in the effluent was around 10 mg L^{-1} and 900 mg L^{-1}, respectively.

Another SBR with a working volume of 5 L was operated at 30 ± 1 °C to enrich AOB (Culture II). Each full cycle consisted of 2 consecutive intervals of 5 min feeding and 200 min aerobic phases, as well as 20 s wasting, 60 min settling and 9 min decanting at the end of the full cycle. The synthetic wastewater comprised 33.78

g L^{-1} $(NH_4)_2CO_3$, 35 g L^{-1} $NaHCO_3$, 0.064 g L^{-1} each of KH_2PO_4 and K_2HPO_4, and trace minerals. 2 L synthetic wastewater was fed into the reactor per cycle, resulting in a HRT of 20 h. Dissolved oxygen (DO) was controlled between 0.1 and 0.4 mg L^{-1} and pH was maintained in the range of 6.9-8.0. The reactor was operated for more than 1 year before sludge was used for this study. The effluent nitrite and nitrate concentration was stabilized at 550-570 mg L^{-1} and 20-30 mg L^{-1}, respectively.

2.2.2 Batch test 1: effect of different SAORs on SMX transformation

700 mL culture I sludge was withdrawn and washed twice with PBS buffer, and resuspended with synthetic solution containing 5 mg L^{-1} SMX and 1000 mg NO_3^--N L^{-1}. Nitrite was undetectable after the washing step. The washed sludge was then transferred into a 2 L reactor. 1 L synthetic wastewater containing 5 mg L^{-1} SMX, 4.72 g L^{-1} $(NH_4)_2SO_4$ (equivalent to 1000 mg NH_4^+-N L^{-1}), 12 g L^{-1} of $NaHCO_3$ and trace elements, was continuously fed into the reactor at various flow rates to create different NH_4^+ loading rates. Aeration was provided by an air pump and DO was saturated during the aerobic phase. At the end of each loading-rate phase, mixed liquor in the reactor was washed and resuspended again with solution containing 5 mg L^{-1} SMX and 1000 mg NO_3^--N L^{-1}. Then, a new cycle was conducted. All the tests were conducted at room temperature (22 ± 1 °C). To exclude the photochemical degradation of SMX, the batch reactor was wrapped with aluminum foil during the experiment.

Different specific ammonia oxidation rates (SAORs) were achieved by introducing the feed to the reactor at 20 different flow rates (Table S1). SAOR was gradually elevated from 3.1 mg N g^{-1} VSS h^{-1}, where nitrite accumulation was not found, to 18.9 mg N g^{-1} VSS h^{-1} where nitrite was accumulated, then gradually reduced back to 3.1 mg N g^{-1} VSS h^{-1}. In the cycles where nitrite was accumulated, a prolonged aeration was applied once the feeding stopped. This was to allow accumulated nitrite to be completely converted to nitrate. Such arrangement aimed to investigate if and how the decrease of nitrite would affect SMX transformation without the activity from AOB. In each cycle, mixed liquor samples were collected at pre-designed time intervals for further analysis.

2.2.3 Batch test 2: effect of concentration of SMX and nitrite on SMX transformation

In order to have controlled nitrite accumulation level, enriched AOB culture was used in this set of batch experiment (Table 1). 500 mL mixed liquor was withdrawn from the enriched nitrifying culture II for each batch test. The mixed liquor from parent reactor was washed twice with ammonium free synthetic medium, and resuspended into synthetic medium supplemented with SMX to a final volume of 0.55 L. Batch tests I –III were carried out with SMX concentrations of 1 mg L^{-1} and IV – VI were with 5 mg L^{-1} SMX, respectively. $(NH_4)_2SO_4$ was added in one pulse to achieve an initial concentration of 200 mg NH_4^+-N L^{-1} for batch test I and IV and 400 mg NH_4^+-N L^{-1} for batch test II and V, whereas in batch test III and VI, ammonium was added in two pulses with total ammonium loading the same as that in batch II and V. pH was monitored online and DO was controlled at 1.0 ± 0.1 mg L^{-1} using a gas mixture of N_2 and air.

Abiotic reactions of SMX with nitrite were assessed in a similar way as the biological experiments described above. Briefly, tests of biomass-free abiotic reactions were performed in aqueous solutions that contained SMX with the presence of different levels of nitrite (0, 100, 200, 300, 400 mg NO_2^--N L^{-1}) under two pH (7.40 and 7.80) conditions. In each test, 100 mL aqueous solution containing 1 mg L^{-1} SMX and the designed nitrite was prepared in 1 mM PBS buffer which was used to maintain a constant pH. All reactions were carried out in duplicates.

2.3 Analytical methods

2.3.1 Ultra-fast liquid chromatography (UFLC) analysis

The concentration of SMX and nitrite was determined using the method described previously (Sun et al. 2017). In brief, samples were analyzed by UFLC with an SPD-M20A diode array detector using a Shimadzu LC system (LC-20AD, Japan). The chromatographic separation was carried out using Agilent Proshell 120 EC-C 18 column (4.1 ×100 mm, 2.7 μm) and an SPD-M20A diode array detector using a Shimadzu LC system (LC-20AD, Japan). The detector wavelength was set at 270 nm. The retention time of SMX and nitrite was 7.5 min and 4.5 min, respectively.

2.3.2 LC-MS/MS analysis

Transformation intermediates of SMX were further analyzed by an Agilent 1290 Infinity HPLC coupled with an Agilent 6460 Triple quadruple mass spectrometry, which was equipped with an electrospray ionization (ESI) source using Agilent Jet Stream Technology (Agilent, USA). Separations were performed using a 100 mm × 2.1 mm Kinetex 2.6 μm C18 column. The flow rate was set to 0.2 mL min^{-1} and the injection volume was 5 μL. The mobile phase consisted of water (A) and acetonitrile (B) modified with 0.1% formic acid. For identification of transformation products, the MS system was operated in MS2 scan mode and in both positive and negative ionization modes. The mass range between *m/z* 30 and *m/z* 600 was scanned to obtain full scan mass spectra. For quantification of the transformation products concentration, the MS system was operated in MRM mode and in negative ionization mode.

2.3.3 LC-QTOF/MS analysis

Further identification of the transformation product without reference standards was performed on an Agilent 6550 iFunnel Q-TOF LC/MS (Agilent, USA) instrument. The QTOF/MS instrument was operated with an electrospray ion source using Agilent Jet Stream Technology in positive and negative ionization mode. The column and mobile phase were the same as that of LC-MS/MS analysis. The chromatographic separation was performed with a gradient program as follows: the initial mobile phase composition (10% B) was maintained for 0.5 min, followed by a linear gradient to 90% B over 16 min, kept for 8 min and then set to 10% B again before the next run (5 min). Samples were scanned in both positive and negative mode in separate runs, with a scan range of m/z 100 to m/z 1000.

2.3.4 Chemical analysis

Samples withdrawn from the reactor were immediately filtered through 0.45 μm cellulose acetate membrane syringe filter before analysis. pH, ammonium, nitrate, MLSS and MLVSS were determined in accordance with standard methods (APHA 1998). The potential adsorption of SMX onto the sludge was evaluated using the previous study (Xiao et al. 2017). Free nitrous acid (FNA) concentration was calculated through the nitrite concentration, pH and temperature, by the equation: FNA = $[NO_2^- \text{-N}]/(Ka \times 10^{pH})$ with the Ka value determined using the formula Ka = $e^{-2300/(273+T)}$for a given temperature T (°C) (Anthonisen et al. 1976).

3. Results and discussion

3.1 SMX transformation at different SAORs

The concentration of ammonium, nitrite and nitrate at various SAORs are displayed in Fig. 1A. With the increased SAORs, nitrite was gradually accumulated, nitrate concentration decreased accordingly, while ammonium concentration was always below 5 mg L^{-1}. The result suggests the ammonium feeding rate applied in this study was lower than the maximum SAOR. The correlation between SMX transformation rate and SAORs is shown in Fig. 1B. It was found that when SAOR increased in the range of 3.1 – 18.9 mg N g^{-1} VSS h^{-1}, SMX transformation rate fluctuated at 2.3 ± 1.2 μg g^{-1} VSS h^{-1}. At this stage, ammonium in the reactor was completely oxidized to nitrate without observed nitrite accumulation, and the SMX transformation rates were similar.

With the further increase of SAOR (> 18.9 mg N g^{-1} VSS h^{-1}), nitrite accumulation was detected. Meantime, a sharp increase of SMX transformation rate was observed. For example, when nitrite concentration in the reactor reached 480 mg N L^{-1} at SAOR of 31.8 mg N g^{-1} VSS h^{-1}, the SMX transformation rate was as high as 34.3 μg g^{-1} VSS h^{-1}. Previous studies have demonstrated that adsorption of SMX to activated sludge was negligible (Achermann et al. 2018, Müller et al. 2013). At this stage, it is hypothesized that the elimination of SMX could be attributed to biological degradation and/or microbial intermediates (e.g. nitrite) interference. A possible reason for the faster reduction of SMX may be that the enriched nitrifying culture had been acclimated to SMX after certain operation period. Hence, the SAOR was again decreased to lower rates to examine the SMX transformation (Fig. 1 - Phase II).

In all batch tests, the same amount of ammonium (mass) was loaded into the reactor. As illustrated in Fig. 1B – phase II, with the decrease in SAOR, SMX transformation rate declined and a very low SMX transformation rates (1.33 µg g^{-1} VSS h^{-1}) was observed when SAOR decreased to 15.6 mg N g^{-1} VSS h^{-1} where nitrite was not accumulated. The SMX transformation rates in phase II coincided with phase I relatively well. Thus, it can be concluded that SMX transformation rate was correlated to SAOR rather than the acclimation of the culture to SMX. Interestingly, the increment extent of SMX transformation rate was much higher than that of SAORs (8.2 folds versus 1.7 folds), which indicates that other than potential biological transformation of SMX, there may be other SMX elimination pathways. It is proposed that the enhanced SMX transformation at high SAOR was probably also due to the presence of nitrite. Similar findings were reported in some denitrification processes for wastewater treatment or in soil system that enhanced SMX elimination was related to increased nitrite concentration (Brienza et al. 2017, Nödler et al. 2012). Kassotaki et al. (2016) observed higher degradation of SMX when nitrite accumulated in an enriched AOB culture. However, that study concluded co-metabolism was the major mechanism for SMX degradation.

Two SAORs, i.e. 12.5 and 22.9 mg N g^{-1} VSS h^{-1}, were chosen to illustrate the dynamic transformation of SMX in the specific feeding cycles. The choice of the two SAORs is to represent the cases of full nitrification without nitrite accumulation and partial nitrification with nitrite accumulation. With the SAOR of 12.5 mg N g^{-1} VSS h^{-1}, nitrite was not accumulated in the feeding period, and very low SMX transformation rates were observed in the 60 h feeding period (0.7 – 2.1 μg g^{-1} VSS h^{-1}) (Fig. 2A). In the batch of SAOR of 22.9 mg N g^{-1} VSS h^{-1} (Fig. 2B), it could be observed that compared to SMX transformation without nitrite accumulation, a higher transformation of SMX was obtained in the reactor underwent nitrite accumulation. During the first 10 h, when nitrite concentration gradually increased from 132 to 478 mg L^{-1}, the average SMX transformation rate increased from 1.8 to 9.0 μg g^{-1} VSS h^{-1}, correspondingly. After that, when nitrite concentration further increased to 697 mg L^{-1} at 24 h, the average SMX transformation rate jumped to 29.4 μg g^{-1} VSS h^{-1}. When the feed stopped at 29 h, nitrite concentration increased to 721 mg L^{-1}, at the same time, the average SMX transformation rate reached its highest level of 53.9 μg g^{-1} VSS h^{-1}. Following that, nitrite decreased as a result of prolonged aeration without feeding. In this case, the reformation of SMX was observed as evidenced by a reverse SMX transformation (-21.3 – -71.3 μg g^{-1} VSS h^{-1}) (Fig. 2B). Thus, the transformation of SMX was closely related to the availability of nitrite. The accumulation of nitrite most likely triggered the rapid transformation, while the decrease of nitrite led to the recovery of SMX. However, it should be noted that nitrite concentration was not strictly linear with SMX transformation rate.

3.2 Relationship between SMX transformation and nitrite

To further verify the role of nitrite, SMX transformation in response to different initial ammonium concentration in the enriched AOB culture at two SMX levels (1 mg L^{-1} and 5 mg L^{-1}) were performed (Fig. 3). As illustrated in Fig. 3, after pulse addition of 200 and 400 mg NH_4^+-N L^{-1}, nitrite concentration in the reactor increased linearly with time, and pH underwent an increase-decrease-increase trend (with and without the presence of NH_4^+-N). Under such condition, SMX transformation was very slow at the beginning, but became faster with the decrease of pH. It was also observed that SMX transformation at pulse feed of 400 mg N L^{-1} of initial ammonium was higher than that at pulse addition of 200 mg NH_4^+-N L^{-1} (0.134 vs 0.07 mg L^{-1} at initial SMX of 1 mg L^{-1}). However, in Fig. 3C and 3F where a second pulse feed was added, it is noteworthy that SMX concentration suddenly increased right after the second pulse addition of ammonium, implying the negative impact of high pH (due to high pH of new feed) on SMX transformation although the nitrite was present. Thus, at this stage, it is hypothesized that the transformation of SMX may be related to both nitrite and pH, namely, free nitrous acid (FNA).

Fig. 4 summarizes the SMX removal efficiency, nitrite and FNA concentration in all the batch tests. By conducting linear regression based on the experimental data, the results show FNA was linearly correlated to SMX removal within the predicted 87% confidence, demonstrating that SMX transformation was driven by FNA in the nitrite accumulation environment. The results also proved that FNA may play the major role in SMX transformation. In order to test this, the abiotic SMX transformation experiments with the addition of nitrite at concentrations occurring in the enriched cultures were also conducted. The abiotic batch results showed that, at the initial SMX and nitrite concentration of 1 mg L^{-1} and 400 mg N L^{-1}, 16% and 6% of SMX was transformed under pH of 7.40 and 7.80 respectively (Fig. S1), confirming that the reaction between SMX and nitrite occurred, and both nitrite and pH (FNA), were important for the transformation of SMX. Hence, FNA can act as both biological substrate and/or inhibitor (Zhou et al., 2012; Zhou et al., 2011) as well as chemical reactant in a biological system. The transformation rate of SMX can be described by the following equation:

$$(-d[SMX])/dt = k_{HNO_2,SMX}[HNO_2][SMX] \qquad (1)$$

where $k_{HNO_2,SMX}$ represents the second-order rate constant for reactor of SMX with HNO_2. $[HNO_2]$ and $[SMX]$ are the concentration of HNO_2 and SMX, respectively.

3.3 Formation of transformation products

To elucidate the transformation pathway of SMX with enriched nitrifying sludge, the samples taken from typical SAOR batch tests were examined by both LC-MRM/MS and LC-ESI/MS analysis. LC-ESI(-)/MS analysis revealed two transformation products, i.e. desamino-SMX, 4-nitro-SMX, were formed along with the elimination of SMX in the enriched nitrifying sludge (Fig. S2). Time course of the concentration of SMX, its transformation products and FNA under two SAORs are shown in Fig. 5. The trends of SMX transformation and transformation products production were similar in both SAORs. With the increase of FNA concentration, a sharpe increase of desamino-SMX was observed, together with another intermediate, 4-nitro-SMX. Desamino-SMX (Table 2) was found to be the main transformation product and generated by the substitution of the aromatic NH_2 in SMX by hydrogen. 4-nitro-SMX (Table 2) was another main product, which should be originated from the reaction with NO_2^- and/or FNA present in the reactor. It was previously proposed that high concentration of nitrite could lead to nitric oxide (NO) production due to chemical/biological nitrite reduction (Liu et al. 2017). Produced NO could react with aromatic amines in SMX to form unstable diazonium cations (Jia et al. 2017). Diazonium cations can undergo numerous consecutive reactions with available reaction partners, such as nitrite and hydrogen (Nödler et al. 2012).

Moreover, the results indicate that the desamino-SMX production rate was much higher than that of 4-nitro-SMX. For example, when SMX reduced from 4.988 mg L^{-1}

to 4.175 mg L^{-1} as a result of the elevated FNA (Fig. 5A at SAOR of 22.3 mg N g^{-1} VSS h^{-1}), the concentration of desamino-SMX and 4-nitro-SMX increased to 145.7 and 46.7 μg L^{-1}, respectively. However, it should be noted that the formation of the two products only accounted for 23% of the SMX transformed. From the LC-MS-MS chromatogram (Fig. S3), it was observed that another potential transformation product was present, and the compound - hydroxylated SMX was proposed based on the accurate mass provided by ESI. It can be generated by the substitution of the aromatic NH_2 in SMX by OH (Table 2). In addition to being transformed in the enriched nitrifying culture, the abiotic test showed that it was possible that SMX abiotically transformed with the presence of nitrite. Desamino-SMX and 4-nitro-SMX increased with the elevated nitrite concentrations, and the relative abundance of potential transformation product also increased (Fig. S4).

Due to the lack of reference standards of hydroxylated SMX, LC-MS/MS and LC-QTOF/MS were used to confirm this potential transformation product. For screening analysis, the total ion chromatogram (TIC) and extracted ion chromatogram (EIC) of the suspect ion mass of a tentative compound were obtained on both LC-MS/MS and LC-QTOF/MS. The same peak and mass spectrum obtained for hydroxylated SMX on both LC-MS/MS (Fig. S5) and LC-QTOF/MS (Fig. S6) indicated this compound was present. For further confirmation, the high-resolution mass spectrum of the tentative compound obtained from LC-QTOF was compared against the calculated value of accurate mass and isotope distribution. In the positive mode, the calculated value of accurate mass of $C_{10}H_{10}N_2O_4S$ is 255.0434, and its isotope distribution is 255.0434 (100%), 256.0462 (12.61%) and 257.0415(6.03%), while the observed value of mass of tentative compound was 255.0443 (Fig. S7A), and its isotope distribution was 255.0443 (100%), 256.0469 (12.81%) and 257.0423

(5.15%) (Fig. S7B), suggesting the tentative compound greatly matched hydroxylated SMX with formula $C_{10}H_{10}N_2O_4S$ (Table 2). In the negative mode, the tentative compound also matched hydroxylated SMX (Fig. S7C and S7D). Moreover, the ^1H NMR results also indicated that hydroxylated SMX existed in the solution (Fig. S8). Together, these results confirmed hydroxylated SMX was formed during SMX transformation. However, to elucidate the exact mechanisms of the transformation, qualification with the reference standards would be required.

This study also revealed that the transformation products of SMX were unstable. When the FNA concentration gradually decreased due to prolonged aeration, SMX could be reformed and SMX in the bulk could be replenished (Fig. 5A and B). It is likely that the transformation products were retransformed to SMX that can be evidenced by the reduction of desamino-SMX, 4-nitro-SMX and hydroxylated SMX simultaneously in the system. Interestingly, not all the transformation products decreased immediately when FNA concentration decreased, and they also showed varied reduction rates (Fig. 5). Desamino-SMX continued the increasing trend at the beginning of FNA declining stage but it was reduced at the rate of 9.67 ±1.32 $\mu g\ L^{-1}\ h^{-1}$ when FNA was no longer present (from 31 h onwards), while 4-nitro-SMX concentration decreased at 1.48 ± 0.53 $\mu g\ L^{-1}\ h^{-1}$ during the FNA depleting period.

The retransformation of 4-nitro-SMX compounds to SMX has been demonstrated in several studies under reducing conditions (Mohatt et al. 2011, Nödler et al. 2012). In this study, the similar result was also observed for 4-nitro-SMX under the aerobic conditions. It is important to note that another two products, such as desamino-SMX and hydroxylated SMX, were also retransformed to SMX. It is interesting to know, for the first time, the SMX transformation products can be retransformed when nitrite/FNA was removed from the bulk liquid. However, the underlying mechanisms are subjected to debate. According to the current data, it is assumed that the transformation of aromatic nitro groups occurs both biotically and abiotically with the major transformation induced by the presence of FNA.

3.4 Implications of this study

This study reported the transformation of SMX by enriched nitrifying sludge. The conditions of full nitrification without nitrite accumulation as well as partial nitrification with nitrite accumulation and reduction were manipulated to investigate the role of nitrite during SMX transformation. The study revealed that FNA rather than nitrite was likely the true factor that can chemically transform SMX by formation of several intermediates, such as 4-nitro-SMX, desamino-SMX and hydroxylated SMX. As aromatic NH_2 is a common feature in many sulfonamide antibiotics, similar transformation behavior may be extended to other sulfonamide antibiotics in the nitrification process.

It is well known that nitrite is an important intermediate product involved in ammonium oxidation or nitrate reduction process, and it is also widely distributed in the environment ranging from <1 ppm to thousands ppm (Sun et al. 2016). Monitoring the nitrite/FNA mediated chemical reactions and their intermediates could improve the understanding of the environmental fate and behavior of some emerging organic matters. Moreover, many nitroaromatic compounds are clearly more toxic than its corresponding aromatic amines compounds (Kovacic and Somanathan 2014). To date, limited information on the toxicological effect of SMX transformation products, such as 4-nitro-SMX, desamino-SMX, is available, evaluation of their effect on microbial activity and composition is necessary.

4. Conclusions

The transformation of SMX in two enriched nitrifying cultures was identified. It was found out that the elimination of SMX occurred with the presence of nitrite, and a linear regression was observed between SMX elimination efficiency and FNA concentration. When SAORs were in the range of 3.1 – 18.9 mg N g^{-1} VSS h^{-1} without nitrite accumulation, SMX transformation fluctuated at a low rate of 2.3 ± 1.2 μg g^{-1} VSS h^{-1}. With the further increase of SAOR to nitrite accumulation, a sharp increase of SMX transformation rates were observed, indicating that FNA was mainly responsible for the SMX transformation. Three intermediate products, namely, 4-nitro-SMX, desamino-SMX and hydroxylated SMX, were detected during SMX transformation. However, when FNA concentration decreased, these intermediates can be retransformed back to SMX.

Acknowledgements

The authors would like to thank Dr. Yuan Han from Nanyang Environment and Water Research Institute at Nanyang Technological University for her thoughtful discussion on LC-MS/MS and LC-QTOF/MS data analysis. The authors would also like to thank Dr. Claire Boulange and Wai Kin Tham from the Singapore Phenome Centre at Nanyang Technological University for their help in the structure elucidation of the compounds of interest using LC-MS/MS and ^{1}H NMR.

References

Achermann, S., Bianco, V., Mansfeldt, C.B., Vogler, B., Kolvenbach, B.A., Corvini, P.F.X., Fenner, K., 2018. Biotransformation of sulfonamide antibiotics in activated sludge: The formation of pterin-conjugates leads to sustained risk. Environ. Sci. Technol. 52 (11), 6265–6274.

Anthonisen, A., Loehr, R., Prakasam, T., Srinath, E., 1976. Inhibition of nitrification by ammonia and nitrous acid. J. Water Pollut. Con. F. 48 (5), 835-852.

APHA 1998 Standard Methods for Examination of Water and Wastewater, American Public Health Association, Washington, DC, USA.

Arlos, M.J., Bragg, L.M., Parker, W.J., Servos, M.R., 2015. Distribution of selected antiandrogens and pharmaceuticals in a highly impacted watershed. Water Res. 72, 40-50.

Benotti, M.J., Brownawell, B.J., 2009. Microbial degradation of pharmaceuticals in estuarine and coastal seawater. Environ. Pollut. 157 (3), 994-1002.

Blair, B.D., Crago, J.P., Hedman, C.J., Klaper, R.D., 2013. Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. Chemosphere 93 (9), 2116-2123.

Bouju, H., Ricken, B., Beffa, T., Corvini, P.F.-X., Kolvenbach, B.A., 2012. Isolation of bacterial strains capable of sulfamethoxazole mineralization from an acclimated membrane bioreactor. Appl. Environ. Microbiol. 78 (1), 277-279.

Brienza, M., Duwig, C., Pérez, S., Chiron, S., 2017. 4-nitroso-sulfamethoxazole generation in soil under denitrifying conditions: Field observations versus laboratory results. J. Hazard. Mater. 334, 185-192.

Brodin, T., Fick, J., Jonsson, M., Klaminder, J., 2013. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science 339 (6121), 814.

Cetecioglu, Z., Ince, B., Orhon, D., Ince, O., 2016. Anaerobic sulfamethoxazole degradation is driven by homoacetogenesis coupled with hydrogenotrophic methanogenesis. Water Res. 90, 79-89.

Chen, C., Zhu, W., Huang, X., Zhang, T.C., Wu, W., Sun, F., 2017. Effects of HRT and loading rate on performance of carriers-amended Anammox UASB reactors. Water Environ. Res. 89 (1), 43-50.

Demoling, L.A., Bååth, E., Greve, G., Wouterse, M., Schmitt, H., 2009. Effects of sulfamethoxazole on soil microbial communities after adding substrate. Soil Biol. Biochem. 41 (4), 840-848.

Fernandez-Fontaina, E., Omil, F., Lema, J.M., Carballa, M., 2012. Influence of nitrifying conditions on the biodegradation and sorption of emerging micropollutants. Water Res. 46 (16), 5434-5444.

Göbel, A., Thomsen, A., McArdell, C.S., Joss, A., Giger, W., 2005. Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. Environ. Sci. Technol. 39 (11), 3981-3989.

Hai, F.I., Li, X., Price, W.E., Nghiem, L.D., 2011. Removal of carbamazepine and sulfamethoxazole by MBR under anoxic and aerobic conditions. Bioresour. Technol. 102 (22), 10386-10390.

Helbling, D.E., Johnson, D.R., Honti, M., Fenner, K., 2012. Micropollutant biotransformation kinetics associate with WWTP process parameters and microbial community characteristics. Environ. Sci. Technol. 46 (19), 10579-10588.

Jia, Y., Khanal, S.K., Zhang, H., Chen, G.H., Lu, H., 2017. Sulfamethoxazole degradation in anaerobic sulfate-reducing bacteria sludge system. Water Res. 119, 12-20.

Kassotaki, E., Buttiglieri, G., Ferrando-Climent, L., Rodriguez-Roda, I., Pijuan, M., 2016. Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products. Water Res. 94, 111-119.

Katipoglu-Yazan, T., Merlin, C., Pons, M.N., Ubay-Cokgor, E., Orhon, D., 2016. Chronic impact of sulfamethoxazole on the metabolic activity and composition of enriched nitrifying microbial culture. Water Res. 100, 546-555.

Kovacic, P., Somanathan, R., 2014. Nitroaromatic compounds: Environmental toxicity, carcinogenicity, mutagenicity, therapy and mechanism. J. Appl. Toxicol. 34 (8), 810-824.

Liu, S., Han, P., Hink, L., Prosser, J.I., Wagner, M., Bruggemann, N., 2017. Abiotic conversion of extracellular NH_2OH contributes to N_2O emission during ammonia oxidation. Environ. Sci. Technol. 51 (22), 13122-13132.

Marti, E., Huerta, B., Rodriguez-Mozaz, S., Barcelo, D., Jofre, J., Balcazar, J.L., 2014. Characterization of ciprofloxacin-resistant isolates from a wastewater treatment plant and its receiving river. Water Res. 61, 67-76.

Martínez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321 (5887), 365.

Mohatt, J.L., Hu, L., Finneran, K.T., Strathmann, T.J., 2011. Microbially mediated abiotic transformation of the antimicrobial agent sulfamethoxazole under iron-reducing soil conditions. Environ. Sci. Technol. 45 (11), 4793-4801.

Müller, E., Schüssler, W., Horn, H., Lemmer, H., 2013. Aerobic biodegradation of the sulfonamide antibiotic sulfamethoxazole by activated sludge applied as co-substrate and sole carbon and nitrogen source. Chemosphere 92 (8), 969-978.

Nödler, K., Licha, T., Barbieri, M., Pérez, S., 2012. Evidence for the microbially mediated abiotic formation of reversible and non-reversible sulfamethoxazole transformation products during denitrification. Water Res. 46 (7), 2131-2139.

Padhye, L.P., Yao, H., Kung'u, F.T., Huang, C.H., 2014. Year-long evaluation on the occurrence and fate of pharmaceuticals, personal care products, and endocrine disrupting chemicals in an urban drinking water treatment plant. Water Res. 51, 266-276.

Radke, M., Lauwigi, C., Heinkele, G., Mürdter, T.E., Letzel, M., 2009. Fate of the antibiotic sulfamethoxazole and its two major human metabolites in a water sediment test. Environ. Sci. Technol. 43 (9), 3135-3141.

Sanderson, H., Fricker, C., Brown, R.S., Majury, A., Liss, S.N., 2016. Antibiotic resistance genes as an emerging environmental contaminant. Environ. Rev., 1-14.

Suarez, S., Lema, J.M., Omil, F., 2010. Removal of Pharmaceutical and Personal Care Products (PPCPs) under nitrifying and denitrifying conditions. Water Res. 44 (10), 3214-3224.

Sui, Q., Huang, J., Deng, S.B., Chen, W.W., Yu, G., 2011. Seasonal variation in the occurrence and removal of pharmaceuticals and personal care products in different biological wastewater treatment processes. Environ. Sci. Technol. 45 (8), 3341-3348.

Sun, F., Su, X., Kang, T., Wu, S., Yuan, M., Zhu, J., Zhang, X., Xu, F., Wu, W., 2016. Integrating landfill bioreactors, partial nitritation and anammox process for methane recovery and nitrogen removal from leachate. Sci. Rep. 6, 27744.

Sun, F., Xiao, Y., Wu, D., Zhu, W., Zhou, Y., 2017. Nitrite-driven abiotic transformation of sulfonamide micropollutants during freezing process. Chem. Eng. J. 327, 1128-1134.

Wei, R., Ge, F., Huang, S., Chen, M., Wang, R., 2011. Occurrence of veterinary antibiotics in animal wastewater and surface water around farms in Jiangsu Province, China. Chemosphere 82 (10), 1408-1414.

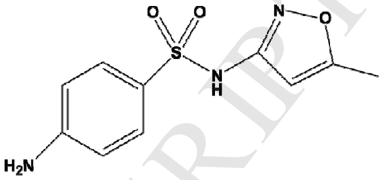
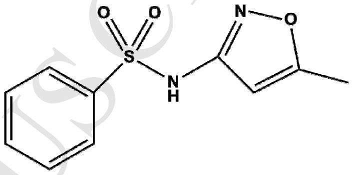
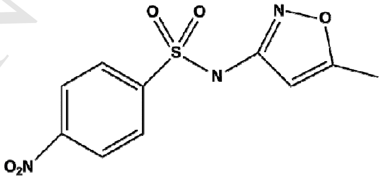
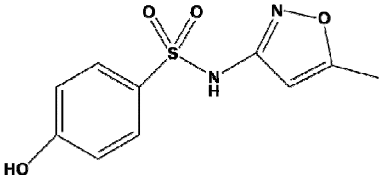
Wu, D., Huang, Z.T., Yang, K., Graham, D., Xie, B., 2015. Relationships between antibiotics and antibiotic resistance gene levels in municipal solid waste leachates in Shanghai, China. Environ. Sci. Technol. 49 (7), 4122-4128.

Xiao, Y.Y., Yaohari, H., De Araujo, C., Sze, C.C., Stuckey, D.C., 2017. Removal of selected pharmaceuticals in an anaerobic membrane bioreactor (AnMBR) with/without powdered activated carbon (PAC). Chem. Eng. J. 321, 335-345.

Table 1 Experimental conditions applied in the batch tests 2.

Test series	SMX (mg L^{-1})	NH_4^+-N (mg L^{-1})
I	1	200
II	1	400
III	1	200 + 200
IV	5	200
V	5	400
VI	5	200 + 200

Table 2 Structure, ESI-MS/MS quantifier and qualifier transitions of SMX and the transformation products

Peak	Compound	Molecular weight	Quantifier	Qualifier	Proposed structure
A	SMX	253	252 - 156.1	252 - 91.9	
B	Desamino-SMX	238	237 - 141.1	237 - 77.1	
C	4-nitro-SMX	283	282 - 138.1	282 - 186.1	
D	Hydroxylated SMX	254	-	-	

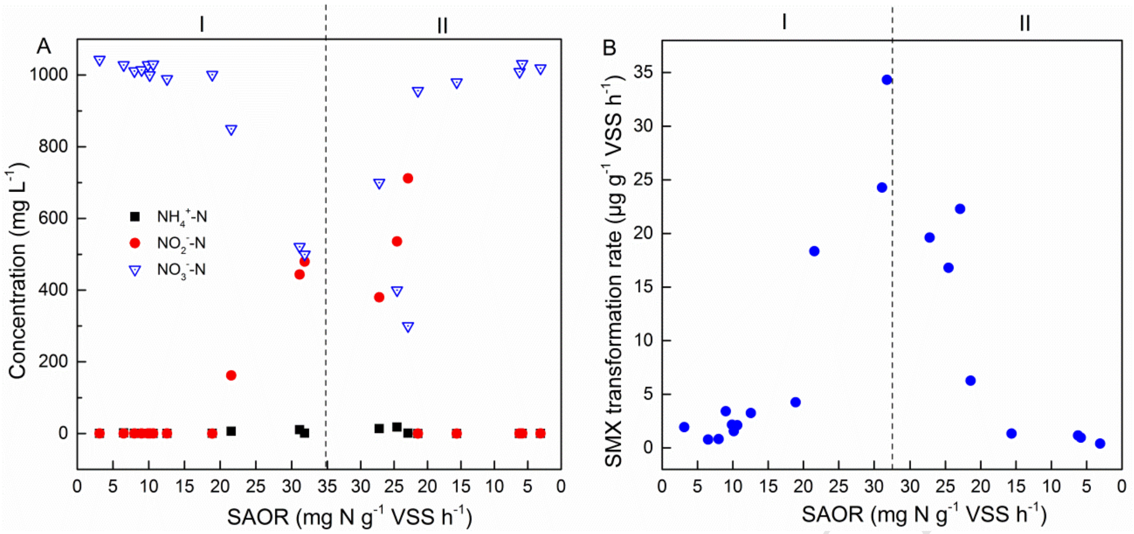


Fig. 1 Profiles of nitrogen conversion (A) and SMX transformation rates (B) under different SAORs

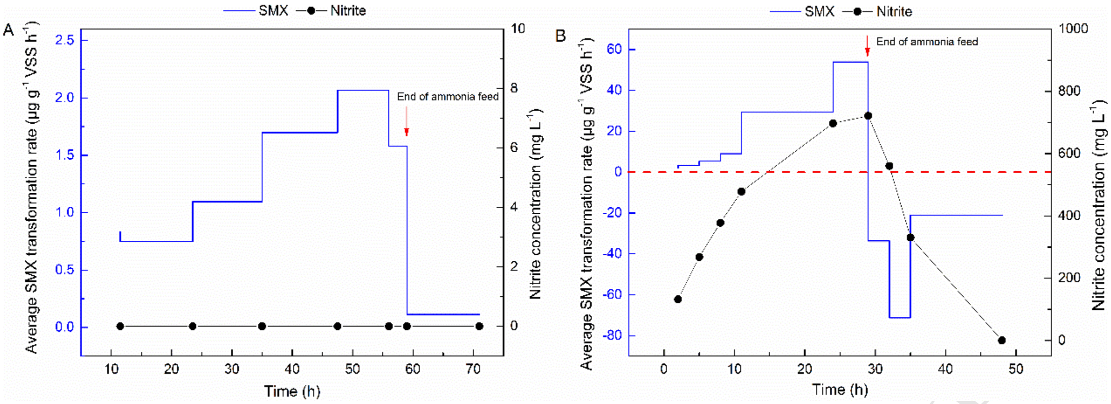


Fig. 2 The average transformation rates of SMX with time under two typical SAORs: A) SAOR = 12.5 mg N g^{-1} VSS h^{-1}; B) SAOR = 22.9 mg N g^{-1} VSS h^{-1}.

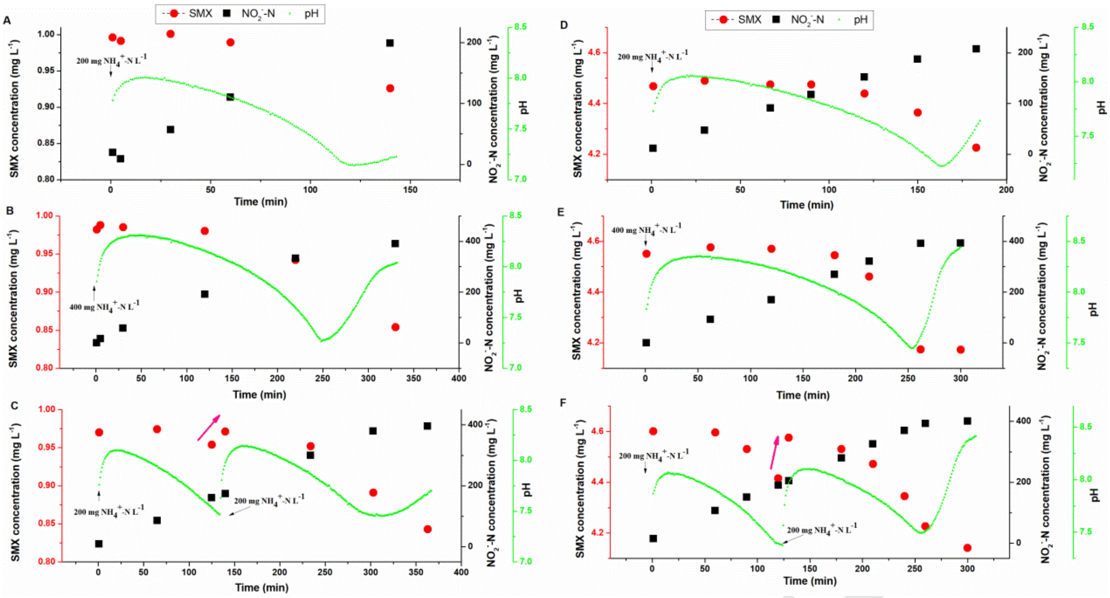


Fig. 3 The profile of SMX concentration in response to different initial ammonium concentrations at two SMX levels (1 mg L^{-1} and 5 mg L^{-1}). (A) 1 mg L^{-1} SMX with pulse addition of 200 mg NH_4^+-N L^{-1}; (B) 1 mg L^{-1} SMX with pulse addition of 400 mg NH_4^+-N L^{-1}; (C) 1 mg L^{-1} SMX with twice-pulse addition of 200 mg NH_4^+-N L^{-1}; (D) 5 mg L^{-1} SMX with pulse addition of 200 mg NH_4^+-N L^{-1}; (E) 5 mg L^{-1} SMX with pulse addition of 400 mg NH_4^+-N L^{-1}; (F) 5 mg L^{-1} SMX with twice-pulse addition of 200 mg NH_4^+-N L^{-1}.

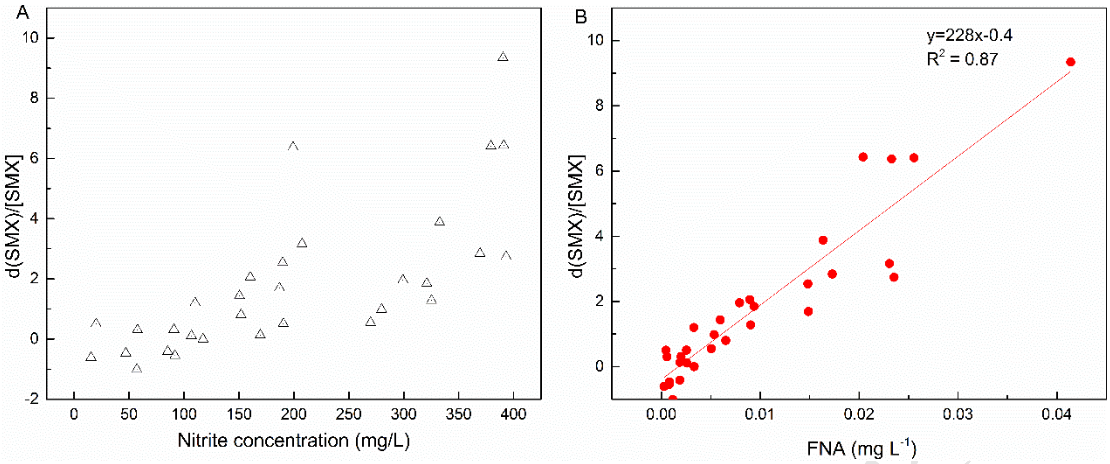


Fig. 4 Correlation of SMX transformation with nitrite (A) and FNA (B) concentration in the reactor

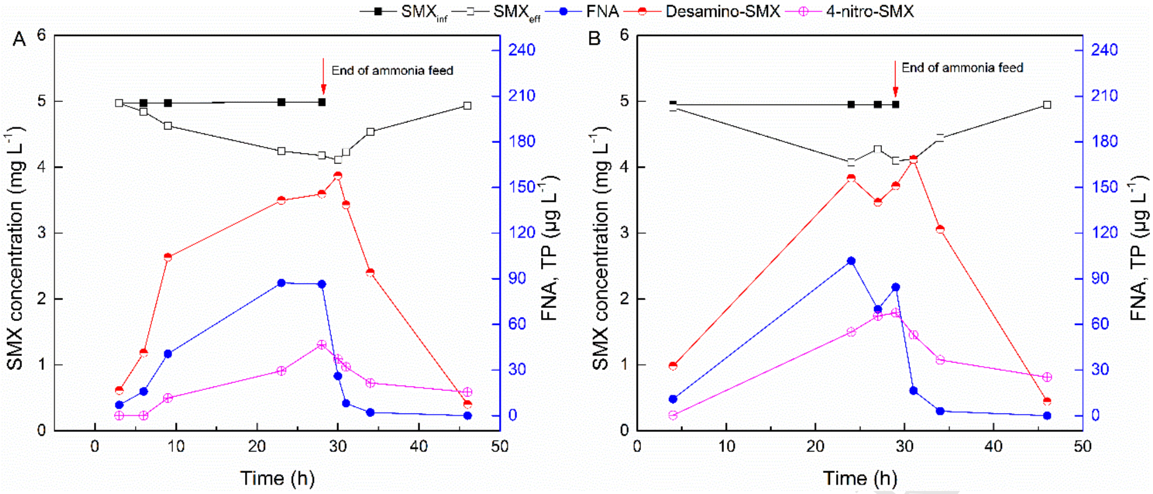


Fig. 5 Concentration of SMX, its transformation products (TPs) and FNA under two similar SAORs of 22.3 mg N g^{-1} VSS h^{-1} (A and B)

Highlights

- ✓ Transformation behavior of SMX was investigated in an enriched nitrifying culture
- ✓ The elimination of SMX mostly occurred with the presence/accumulation of nitrite
- ✓ A linear regression was observed between SMX elimination and FNA concentration
- ✓ By reacting with FNA, several SMX intermediates were detected
- ✓ When FNA concentration decreased, SMX intermediates were retransformed back to SMX