Water Research 94 (2016) 111–119

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products

Elissavet Kassotaki [a], Gianluigi Buttiglieri [a, *], Laura Ferrando-Climent [a, b], Ignasi Rodriguez-Roda [a, c], Maite Pijuan [a]

[a] ICRA, Catalan Institute for Water Research, Scientific and Technological Park of the University of Girona, 17003, Girona, Spain
[b] IFE, Tracer Technology Department, Oil and Gas Section, Institute for Energy Technology, P.O. Box 40, NO-2027, Kjeller, Norway
[c] LEQUiA, Laboratory of Chemical and Environmental Engineering, University of Girona, Campus Montilivi, 17071, Girona, Spain

ARTICLE INFO

Article history:
Received 14 November 2015
Received in revised form
3 February 2016
Accepted 11 February 2016
Available online 15 February 2016

Keywords:
Micropollutants
Sulfamethoxazole degradation
Transformation products
Ammonia oxidizing bacteria
Nitrification rate

ABSTRACT

The occurrence of the widely-used antibiotic sulfamethoxazole (SFX) in wastewaters and surface waters has been reported in a large number of studies. However, the results obtained up-to-date have pointed out disparities in its removal. This manuscript explores the enhanced biodegradation potential of an enriched culture of Ammonia Oxidizing Bacteria (AOB) towards SFX. Several sets of batch tests were conducted to establish a link between SFX degradation and specific ammonia oxidation rate. The occurrence, degradation and generation of SFX and some of its transformation products (4-Nitro SFX, Desamino-SFX and N^4-Acetyl-SFX) was also monitored. A clear link between the degradation of SFX and the nitrification rate was found, resulting in an increased SFX removal at higher specific ammonia oxidation rates. Moreover, experiments conducted under the presence of allylthiourea (ATU) did not present any removal of SFX, suggesting a connection between the AMO enzyme and SFX degradation. Long term experiments (up to 10 weeks) were also conducted adding two different concentrations (10 and 100 μg/L) of SFX in the influent of a partial nitrification sequencing batch reactor, resulting in up to 98% removal. Finally, the formation of transformation products during SFX degradation represented up to 32%, being 4-Nitro-SFX the most abundant.

1. Introduction

In the recent years, the occurrence and fate of pharmaceutically active compounds (PhACs) has become an issue of great environmental concern due to their potential adverse effects on aquatic ecosystems and human health, and in combination with their increasing consumption. They are considered as emerging pollutants in water bodies because they still remain unregulated or are currently undergoing a regularization process, although the directives and legal frameworks are not set-up yet (Rivera-Utrilla et al., 2013).

Once administered, PhACs are metabolised to varying degrees and the excreted metabolites and/or unaltered parent compounds can also undergo further modification due to biological, chemical and physical processes in both sewage transport and treatment facilities, as well as in receiving water bodies (Jelic et al., 2015; Verlicchi et al., 2012). On the other hand, some of these substances can subsequently be transformed back to parent compounds during biological wastewater treatment (Göbel et al., 2005; Radjenović et al., 2007). Current wastewater treatment plants (WWTPs) are not specifically designed to remove these complex and persistent compounds; therefore they have been identified as a main point of discharge of PhACs into the environment (Buttiglieri and Knepper, 2008). Moreover even if parent compounds are not detected after treatment, transformation products (TPs) may still be of concern due to their potential stability or toxicity (Ternes et al., 2007).

Antibiotics, which are a major category of pharmaceuticals, have raised attention mostly because of the risk of a worldwide dispersal of concomitant resistance genes (Müller et al., 2013). Sulfamethoxazole (SFX) is a common antibiotic, which belongs to the class of sulfonamide antibiotics which were the first antimicrobial drugs utilized worldwide. SFX was among the 30 most frequently

* Corresponding author.
E-mail addresses: ekassotaki@icra.cat (E. Kassotaki), gbuttiglieri@icra.cat (G. Buttiglieri), laurafc@ife.no (L. Ferrando-Climent), irodriguezroda@icra.cat (I. Rodriguez-Roda), mpijuan@icra.cat (M. Pijuan).

http://dx.doi.org/10.1016/j.watres.2016.02.022
0043-1354/

detected organic wastewater contaminants as reported by the US Geological Survey (Kolpin et al., 2002), and also among the top 10 high priority pharmaceuticals identified in a European assessment of pharmaceutical and personal care products (De Voogt et al., 2009). In addition, it was among the most refractory pharmaceuticals tested in estuarine and coastal surface water samples with a half-life of 85 to more than 100 days, and it was suggested to be an excellent candidate as a wastewater tracer (Benotti and Brownawell, 2009).

SFX is a low adsorptive, polar sulfonamide, therefore its removal is mainly due to microbial activity (Müller et al., 2013). However, up-to-date investigations pertaining to SFX elimination through biodegradation including both laboratory and full scale studies, are marked by inconsistent results (Drillia et al., 2005; Larcher and Yargeau, 2012; Müller et al., 2013). The removal efficiency of SFX during wastewater treatment ranges from no removal in a conventional activated sludge (CAS) plant (Bendz et al., 2005) to removals greater than 98% in a CAS plant with nitrification (Levine et al., 2006). Conflicting removal efficiencies were also reported in laboratory scale studies. With CAS, Collado et al. (2013) reported SFX removals ranging from 36.5 ± 11.5% under nitrifying and denitrifying conditions. Alvarino et al. (2015) found removals of 57 ± 2% in an autotrophic nitrogen removal reactor (ELAN®) treating the supernatant of an anaerobic sludge digester. Also, Yang et al. (2012) observed SFX removals greater than 99% during batch tests lasting for 14 days and conducted with activated sludge collected from the WWTP of a food manufacturing company under aerobic conditions. Finally, Bouju et al. (2012) isolated five strains, two of these belonging to the phylum Actinobacteria while the other three to Proteobacteria, capable of mineralizing SFX. These discrepancies in reported SFX removals can be attributed to specific factors (e.g. sludge retention time, hydraulic retention time, redox conditions, pH, temperature, and micropollutant biodegradability) (Luo et al., 2014). Moreover they may be also attributed to variations in experimental conditions and especially to the use of different AS inoculum with different microbial communities (Onesios et al., 2009).

Higher removal rates of some pharmaceutical compounds have been reported in nitrifying systems (Fernandez-Fontaina et al., 2012; Tran et al., 2009). This enhanced biodegradation seems to be related to the activity of ammonia oxidizing bacteria (AOB) which could co-metabolize these compounds using one of its key enzymes, ammonia monooxygenase (AMO), responsible for ammonia oxidation (Forrez et al., 2011). It is known that AMO has a broad substrate range and it is capable of oxidizing a large variety of pollutants, simultaneously to the oxidation of ammonia, potentially being able to play a key role on several micropollutants' biodegradation (Tran et al., 2013). In the case of SFX, the results are again contradictory. Both Drillia et al. (2005) and Müller et al. (2013) found that nitrogen deficiency (no addition) enhanced SFX removal, at mg/L concentration, in lab-scale experiments that were executed under aerobic conditions with sludge withdrawn from a CAS system. These results suggest that SFX biodegradation may not be undertaken simultaneously with nitrification (process called co-metabolism) and that SFX at mg/L concentrations can serve either as organic carbon and/or nitrogen source, with autotrophic nitrifying bacteria potentially being the responsible group for SFX biodegradation in the latter case. On the other hand Fernandez-Fontaina et al. (2014) reported that SFX was slowly biodegradable (kbiol<1 L/gVSSd) at μg/L concentration with autotrophic nitrifying biomass. SFX removal was, nonetheless, enhanced at higher specific nitrification rates, this behavior being in accordance with the co-metabolic hypothesis and the fact that biotransformation of some compounds can rely on specific microbial populations. The observed SFX removal variability, combined with the extreme shortage of data on production and removal of its transformation products (TPs), which as reported by Majewsky et al. (2014) can present similar or higher ecotoxicological effects than SFX, highlights the necessity of more in depth exploration.

Up to our knowledge studies focusing on SFX removal by enriched AOB cultures while simultaneously exploring the occurrence of its TPs are currently lacking. The aim of the present work was to explore the biodegradation capacity of an enriched AOB culture (more than 80% of the microbial population belonging to the AOB group) towards SFX, under different conditions. Batch experiments were performed to evaluate i) the effect of the nitrification rate measured as specific ammonium oxidation rate (SAOR), ii) the role of AMO enzyme and iii) the effect of adding a carbon source in the nitrifying culture, on SFX degradation. In addition, the formation of two TPs of SFX: 4-Nitro-SFX and Desamino-SFX (Barbieri et al., 2012; Nödler et al., 2012) as well as of a human metabolite: N^4-Acetyl-SFX (Larcher and Yargeau, 2012), and their correlation with the parent compound were investigated. Finally, long term experiments on SFX removal, at 10 and 100 μg/L influent concentrations, were carried out in a partial nitrification sequencing batch reactor (SBR) treating synthetic reject wastewater, so as to investigate a longer hydraulic retention time (HRT) and possible acclimation factors.

2. Materials and methods

2.1. SBR operation for the enrichment of the AOB population

An 8L SBR was inoculated with activated sludge from a domestic wastewater treatment plant (WWTP) located in Girona (Spain). The reactor was operated in cycles of 6 h, consisting of feed-1 (1 min), aeration-1 (120 min), feed-2 (1 min), aeration-2 (120 min), waste (2 min), settling (103 min) and decanting (15 min). 1 L of synthetic reject wastewater (wastewater that simulates the effluent of an anaerobic digester in terms of ammonia and bicarbonate concentrations and is prepared in the laboratory) with a concentration of 1 g NH_4^+-N/L was added in each feeding period, while during the "waste" phase 2 L of the clarified supernatant were withdrawn. The HRT of the system was 24 h. The mixed liquor temperature was controlled at 30 °C using a water jacket, to mimic the common temperature conditions of reactors treating reject wastewater. Dissolved oxygen (DO) was controlled with a programmable logic controller (PLC) between 0.5 and 3 mg O_2/L and a minimum pH level was maintained at 6.7 by adding 1 M $NaHCO_3$ solution.

The synthetic wastewater composition was modified from Kuai and Verstraete (1998): 5.64 g/L of NH_4HCO_3 (1 g NH_4^+-N/L), 0.088 g/L of KH_2PO_4, 0.11 g/L K_2HPO_4 and 2 mL of trace element stock solution. The trace element stock solution was previously described elsewhere (Rodriguez-Caballero and Pijuan, 2013). Cycle studies were performed on a weekly basis to monitor the nitrification activity, where samples for the analyses of NH_4^+, NO_2^- and NO_3^- were taken along the cycle and immediately filtered through disposable Millipore filter units (0.22 μm pore size). The experiments detailed in this manuscript were conducted after more than 1 year of reactor operation, with a stable AOB population (more than 80% of the total microbial community) and with stable nitritation performance (95% of NH_4^+ converted to NO_2^- and no NO_3^- detected in the effluent).

2.2. Batch experiments

2.2.1. Batch reactor experimental setup

All batch tests were performed using a 1 L lab-scale Applikon stirred tank reactor coupled with a proportional-integral-derivative (PID) controller. Enriched AOB biomass was withdrawn from the nitritation SBR during the settling phase previously sparged with

compressed air for 5 min in order to oxidize any remaining NH_4^+. The biomass was subsequently washed with a phosphate buffer solution (PBS) in order to ensure that NO_2^- was removed completely. The initial mass of mixed liquor volatile suspended solids (MLVSS) at the beginning of all batch tests was 0.5 g. Mixed liquor samples were taken using a syringe, filtered (0.22 μm pore size Millipore filter units for ammonia, nitrite and nitrate; 0.45 μm pore size Millex® PVDF for pharmaceuticals) and immediately frozen until analysis. Aerobic conditions (>2.5 mg O_2/L) were achieved with a continuous air supply. pH was automatically controlled during the experiments at 7.8 ± 0.07 (by adding 0.6 M HCl or 1 M $NaHCO_3$ solution) and the temperature maintained at 29.7 ± 0.6 °C.

2.2.2. Set of batch experiments

Different sets of tests were performed (Table 1) with a duration of 6 h. During all tests NH_4^+ was continuously dosed for the first 4 h at a loading rate of 0.002 L/min to ensure a constant ammonia oxidizing rate. The last 2 h were monitored to investigate if and how the lack of NH_4^+ would affect the removal of SFX.

The fed ammonium in T1-T19 (Table 1, first category) was in the range 69.5–481.3 mg NH_4^+-N/L, leading to NH_4^+ loading rates from 0.12 to 0.96 mg/min and to specific ammonium oxidation rates (SAOR, which represents the amount of ammonia oxidized during the first 4 h of the test per sludge mass and time) from 0.25 to 2.1 mg NH_4^+-N/(g MLVSS min). T20 and T21 were executed in the absence of NH_4^+ and therefore with SAORs of 0 mg NH_4^+-N/(g MLVSS min). In T22–T25 (Table 1, second category) allylthiourea (ATU) was spiked (25 mg) at the beginning of the test to suppress the degradation of SFX by ammonia oxidation. ATU is a very well known inhibitor of AMO (a membrane-bound enzyme used by AOB to catalyze the oxidation of NH_3 to hydroxylamine) and has been widely used in many studies to inhibit the activity of AOB (Roh et al., 2009; Sathyamoorthy et al., 2013; Shi et al., 2004; Tran et al., 2009; Yi and Harper, 2007). The mechanism of ATU inhibition on AOB is recognized as chelation of the Cu active site in the AMO enzyme (Bédard and Knowles, 1989). Likewise, in T24–T29 100 mg of acetate (ACE) was spiked to investigate the contribution of the heterotrophic fraction of the biomass on SFX degradation. All T1-T29 tests had an initial SFX mass of 100 μg. Finally, T30–T35 experiments (Table 1, third category) were conducted adding 4-Nitro-SFX, Desamino-SFX and N^4-acetyl-SFX separately (initial spiked TP mass of 100 μg), without any addition of SFX.

Samples were taken at the beginning of the test, after half an hour, after three hours and at the end of the test (6 h) for chemical analyses of nutrients, SFX and SFX TPs. Mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) were measured at the beginning and at the end of each test. The working volume of the batch reactor increased from 0.5 L at the beginning up to 1 L at the end of the tests due to the continuous and constant NH_4^+ supply (when applicable); therefore the results presented refer to masses.

Additionally, a sorption control experiment was performed with sulfamethoxazole (SFX) and autoclaved activated sludge to assess possible losses (more details in supplementary material-SM1).

Table 1
List of conducted experiments with AOB and SFX and TPs.

Category	Name of the tests	N-NH_4^+ load (mg/min)[a]	SAOR (mg NH_4-N/g MLVSS min)	SFX (μg)[b]	ATU (mg)[b]	ACE (mg)[b]	4-Nitro-SFX (μg)[b]	N^4-Acetyl-SFX (μg)[b]	Desamino-SFX (μg)[b]
1) $NH_{4\ +\ SFX}$	T1	0.96	2.02	100	–	–	–	–	–
	T2	0.93	2.10	100	–	–	–	–	–
	T3	0.90	1.66	100	–	–	–	–	–
	T4	0.87	1.72	100	–	–	–	–	–
	T5	0.78	1.86	100	–	–	–	–	–
	T6	0.60	1.18	100	–	–	–	–	–
	T7	0.60	1.04	100	–	–	–	–	–
	T8	0.56	1.08	100	–	–	–	–	–
	T9	0.39	0.77	100	–	–	–	–	–
	T10	0.37	0.70	100	–	–	–	–	–
	T11	0.36	0.70	100	–	–	–	–	–
	T12	0.29	0.59	100	–	–	–	–	–
	T13	0.28	0.56	100	–	–	–	–	–
	T14	0.26	0.61	100	–	–	–	–	–
	T15	0.26	0.52	100	–	–	–	–	–
	T16	0.19	0.43	100	–	–	–	–	–
	T17	0.18	0.39	100	–	–	–	–	–
	T18	0.14	0.28	100	–	–	–	–	–
	T19	0.12	0.25	100	–	–	–	–	–
	T20	0	0	100	–	–	–	–	–
	T21	0	0	100	–	–	–	–	–
2) $NH_{4\ +\ SFX\ +\ ATU/ACE}$	T22	0.61	0.11	100	25	–	–	–	–
	T23	0.54	0.15	100	25	–	–	–	–
	T24	0.65	0.04	100	25	100	–	–	–
	T25	0.62	0.01	100	25	100	–	–	–
	T26	0.28	0.64	100	–	100	–	–	–
	T27	0.25	0.54	100	–	100	–	–	–
	T28	0	0	100	–	100	–	–	–
	T29	0	0	100	–	100	–	–	–
3) TP	T30	1.04	2.18	–	–	–	100	–	–
	T31	0.96	2.07	–	–	–	100	–	–
	T32	1.02	2.15	–	–	–	–	100	–
	T33	0.99	2.24	–	–	–	–	100	–
	T34	0.96	1.76	–	–	–	–	–	100
	T35	0.88	1.21	–	–	–	–	–	100

[a] NH_4^+-N load equals to ammonia oxidation rate in the batch reactor since no accumulation of ammonia was observed.
[b] The values refer to the theoretical masses added in the system.

Table 2
Mass parameters for LC-MS/MS analysis.

Compounds	Rt (min)	Precursor ion (*m/z*)	Q1	CE/DP/EP/CXP[a]	Q3	CE/DP/EP/CXP
Sulfamethoxazole	1.5	254 [M + H$^+$]	156	23/81/10/12	92	37/81/10/12
N^4-Acetyl-Sulfamethoxazole	1.6	296 [M + H$^+$]	134	35/96/10/18	65	59/96/10/10
Desamino Sulfamethoxazole	1.9	239 [M + H$^+$]	77	49/121/10/12	131	23/121/10/10
4-Nitro Sulfamethoxazole	2.1	284 [M + H$^+$]	92	77/111/10/12	189	37/111/10/8

[a] CE: collision energy, DP: declustering potential, EP: entrance potential, CXP: collision cell exit potential.

2.3. Long term experiments

The removal capability of the enriched AOB population present in the nitritation SBR was investigated under a longer HRT (1 day) and in long term conditions. The SBR was operated for 10 weeks with the presence of SFX in its influent following the same cycle previously described in section 2.1. The removal efficiency of the SBR towards SFX was investigated, at constant MLVSS (2 g/L), first at an influent concentration of 10 μg SFX/L for five weeks (days 1–35), and then of 100 μg SFX/L during the five following weeks (days 36–70).

The influent medium was sampled every day (i.e. exactly after the completion of 4 cycles) to ascertain that the concentration of SFX remained stable and to ensure that no abiotic SFX degradation was occurring (influent sample). The SBR effluent was collected in a tank and a composite 24 h sample was taken on a daily basis (effluent sample) and was correlated to the influent sample of the previous day for removal calculations. Both influent and effluent samples were collected at the same time for chemical analyses of nutrients, SFX and SFX TPs (4-Nitro-SFX, Desamino-SFX and N^4-acetyl-SFX). Long term SBR experiments were executed after all batch experiments described in the previous section were completed to avoid any inconsistency attributable to the potential biomass adaptation to SFX.

2.4. Chemical analysis

MLSS and MLVSS were analyzed according to the standard methods (APHA, 1998). NH_4^+, NO_2^- and NO_3^- were analyzed via ion chromatography (ICS5000, DIONEX.), and acetate via gas chromatography (Trace GC Ultra ThermoFisher Scientific).

Sulfamethoxazole was purchased from Sigma–Aldrich. 4-Nitro Sulfamethoxazole, N-Acetyl Sulfamethoxazole and isotopically labeled compound Sulfamethoxazole-d4 were obtained from Toronto Research Chemicals. N-(5-Methyl-3-isoxazolyl)benzenesulfonamide (Desamino Sulfamethoxazole) were provided by Dr. Tobias Licha, from the Geoscience Centre of the University of Göttingen. HPLC-grade solvents water, methanol, and acetonitrile (LiChrosolv) and 98% pure formic acid (HCOOH) were supplied by Merck (Darmstadt, Germany. Individual stock solutions were prepared on a weight basis, in methanol at 1 mg/mL and kept frozen at −20 °C. Reference standard solutions, as well as, the calibration curve were prepared by appropriate dilution in methanol-water (10:90, v/v) of the stock solution of target compounds.

Additionally, preliminary abiotic experiments were executed with SFX at different concentrations of nitrite, aiming to investigate possible chemical transformations under different sample preservation conditions (at −20 °C and at 4 °C, overnight). It was confirmed that neither chemical transformation of SFX nor chemical formation of 4-Nitro-SFX can occur in the presence of nitrite, in none of the cases (more details in supplementary material-SM2).

2.4.1. Sample preparation in short term experiments

All the samples were filtered through 0.45 μm Polyvinylidene fluoride membrane filters (PVDF, Millipore). The samples collected at time 0 and 30 min were diluted in methanol-water (10:90, v/v) to an appropriate concentration (the samples were diluted ten times in order to reassure that the results would be in the range of the calibration curve from 0.1 to 100 μg/L), while the others (time 3 and 6 h) were not diluted. 10 μL of sulfamethoxazole-d4 (internal standard) at 1 mg/L was added to 1 mL of each sample before injection in the Ultra Performance Liquid Chromatography tandem Mass Spectrometry (UPLC-MS/MS) system.

2.4.2. Sample preparation in long term experiments

The influent and effluent samples collected over time were filtered through 0.45 μm PVDF membrane filters. Influent samples were diluted in methanol-water (10:90, v/v) to an appropriate concentration and analyzed by UPLC-MS/MS. Effluent samples were preconcentrated using solid phase extraction (SPE) in order to achieve proper sensitivity for the detection of potential metabolites of sulfamethoxazole. Oasis HLB cartridges (60 mg, 3 mL) were conditioned with 5 mL of methanol followed by 5 mL of HPLC grade water at a flow rate of 2 mL/min. A suitable volume of a Na_2EDTA solution, having a concentration of 0.1 M, was added to 25 mL of effluent sample to achieve a final concentration of 0.1% (g solute/g solution) and loaded onto the cartridge at a flow rate of 1 mL/min. After sample pre-concentration, cartridges were rinsed with 6 mL of HPLC grade water, at a flow rate of 2 mL/min, and were dried with air for 5 min till total water removal. Analytes were eluted with 6 mL of pure methanol at a flow rate of 1 mL/min. Extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted with 1 mL of methanol/water (10:90, v/v). Finally, 10 μL of a 1 ng/μL of sulfamethoxazole-d4 at 1 mg/L was added to 1 mL of extract as internal standard and further analyzed by UPLC-MS/MS.

2.4.3. Ultra Performance TM-ESI-(QqLIT) MS/MS analysis

Samples were analyzed by UPLC (Waters Corp. Milford, MA, USA) coupled to a quadrupole-linear hybrid ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA,USA). Chromatographic separation was carried out using an Acquity BEH C_{18} column (50 mm × 2.1 mm i.d. 1.7 μm particle size; Waters Corp. Milford, MA, USA). The separation conditions were as follows: solvent (A) acetonitrile, solvent (B) HPLC water modified with 0.1% HCOOH at a flow rate of 0.5 mL/min. The gradient elution was: initial, 5% A; 0–3.0 min, 5–70% A; 3.0–3.5 min, 70–100% A; 3.5–5.0 min, 100 A; from 5 to 5.10 to return to the initial conditions and finally from 5.10 to 6.0 equilibration of the column. Mass spectrometer equipped with Turbo V ion spray source was operated in positive electrospray mode. Optimal source parameters were T = 650 °C, CUR = 30, CAD = Medium, GS1 = 60, GS2 = 50 and EP = 10. For quantitative purposes, two MRM transitions were monitored for each compound, the first one for quantification and the second one for confirmation of the compound (Table 2). In order to increase sensitivity and selectivity the data were recorded by using scheduled MRM™ algorithm. Target scan time was set at 0.25 s with MRM detection window of 20 s. Data were acquired and processed using Analyst 1.5.1 software.

2.5. Bacterial composition analysis

Fluorescence in situ hybridization (FISH) analysis was conducted on the biomass from the SBR at the time of the study to assess AOB abundance. FISH was performed as described in Nielsen et al. (2009) with Cy5-labeled EUBMIX probes (for general bacteria) and Cy3-labeled AOBMIX probes (for AOBs, comprising equal amounts of probes Nso1225, NEU, NmV, Cluster6a192) and Cy3-labeled Nso190. FISH preparations were visualized with a Nikon CS1 confocal laser-scanning microscope (CLSM) using Plan-Apochromat 63_oil (NA1.4) objective. Thirty images were taken from each sample for quantification. The area containing Cy3-labeled specific probe (AOBMIX + Nso190 for AOB) cells was quantified proportionally to the area of Cy5-labeled bacteria probe (EUBMIX) within each image using the daime software package (Daims et al., 2006).

FISH analysis showed that AOB bound with the FISH probe Cy3-labeled AOBMIX were dominant (more than 80% of the microbial population corresponded to AOB), as presented in the FISH micrographs (Fig. S3, supplementary material-SM3).

3. Results

3.1. Batch experiments

3.1.1. SFX degradation and TPs formation at different specific ammonium oxidation rates (SAOR)

The degradation of SFX and the formation of 4-Nitro-SFX (4-NO_2-SFX), Desamino-SFX and N^4-Acetyl-SFX were evaluated in all the experiments. A time course of the amounts of SFX, its TPs, and the ammonia oxidizing rate are shown for three representative tests (T4, T13 and T17) in Fig. 1. Also, time course data for all the experiments is provided in the Supplementary material-SM4. NH_4^+ was oxidized as soon as it entered the system and no accumulation was detected; NO_2^- was increasing throughout the tests as expected and NO_3^- was not detected in any of the experiments.

SFX experienced, at higher SAORs, a sharp decrease during the first half an hour, with a lower decrease rate in the rest of the experiment (Fig. 1a and b). At a lower SAOR, SFX removal was significantly affected and, after a first moderate decrease, no further removal was detected (Fig. 1c). A higher final SFX removal was obtained in correspondence of a higher initial SFX decrease. 4-Nitro-SFX was formed in all the tests but the ones with ATU (T22–T25), whereas the formation of Desamino-SFX and N^4-Acetyl-SFX was negligible in most of the cases. Moreover, a correlation was found between SFX degradation and 4-Nitro-SFX formation, as discussed later.

In the first category of tests (T1-T21) different NH_4^+ loading rates were applied in the system, thus different SAORs were obtained. SFX degradation was clearly related to the nitrification rate since higher removals were observed at higher specific ammonium oxidation rates (Fig. 2a). When nitrification did not occur due to the lack of ammonia, SFX degradation was still observed at around 25%. Based on the range of ammonia oxidation rates that are applied, three different levels of removal (low, moderate or high) can be expected, as it was identified in the present study: i) $0 \leq$ SAOR ≤ 0.4 mg NH_4^+-N/(g MLVSS min) (T16–T21) corresponding to a range of limited removals of 16–33% (average 25 ± 7%); ii) $0.5 \leq$ SAOR ≤ 1.2 mg NH_4^+-N/(g MLVSS min) (T6-T15) corresponding to a range of removals of 53–83% (average 67 ± 12%); and iii) $1.6 \leq$ SAOR ≤ 2.1 mg NH_4^+-N/(g MLVSS min) (T1-T5) corresponding to a range of high removals of 79–91% (average 86 ± 5%) (Fig. 2b).

Different batch tests were executed adding ATU and acetate with the intention of clarifying the contribution of the autotrophic and of the small heterotrophic fractions present in the sludge, towards SFX degradation. The effect of both compounds was investigated independently as well as in combination (Fig. 3). In the presence of ATU (T22–T23), nitrification was completely suppressed and this resulted in a null removal of SFX, providing a first indication of the role of the AOBs in the total degradation. Interestingly, when acetate was spiked in the system, in the presence (T26–T27) and absence (T28–T29) of NH_4^+, similar SFX removals of 54% and 48% were observed respectively with an average acetate consumption of 17.8 ± 8.2%. However, in the tests that were executed in the presence of both ATU and acetate (T24–T25), the removal of SFX decreased to very low levels (7.3 ± 6.3%), suggesting a connection between AMO and SFX degradation.

3.1.2. Formation of transformation products (TPs)

An efficient UPLC-QqLIT method for the determination of 4-Nitro SFX, Desamino-SFX and N^4-Acetyl-SFX in batch samples without sample pre-concentration was optimized and validated with good quality parameters. To the extent of our knowledge this is the first study on AOB enriched biomass evaluating these three TPs (more details in supplementary material-SM5).

The average formations of 4-Nitro-SFX, Desamino-SFX and N^4-Acetyl-SFX were evaluated in parallel to SFX degradation in all the batch experiments. TPs formation represented up to an average of 30% of the initial SFX mass (Fig. 4). 4-Nitro-SFX was detected in almost all tests. Moreover, it was the main detected TP since the difference between its formation and the total formation of all the

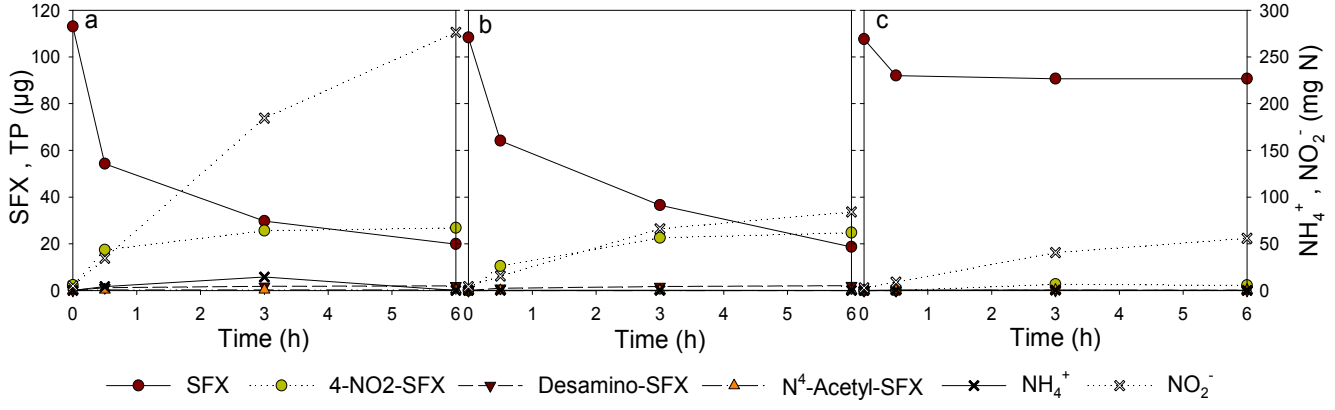

Fig. 1. Removal of SFX (µg) and TPs formation under different SAOR rates: a) 1.72 mg NH_4^+-N/(g MLVSS min); b) 0.56 mg NH_4^+-N/(g MLVSS min); c) 0.39 mg NH_4^+-N/(g MLVSS min).

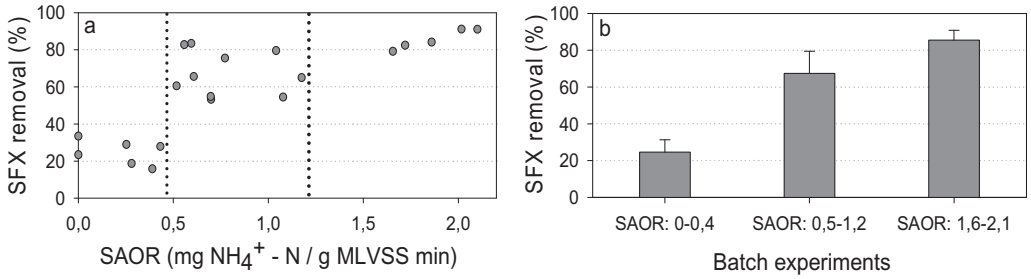

Fig. 2. a) Removal of SFX (%) as a function of the investigated SAOR rates (T1–T21) (vertical dotted lines represent the 3 different degradation zones defined); b) Average removal of SFX (%) for the three identified zones. The error bars represent the standard deviation.

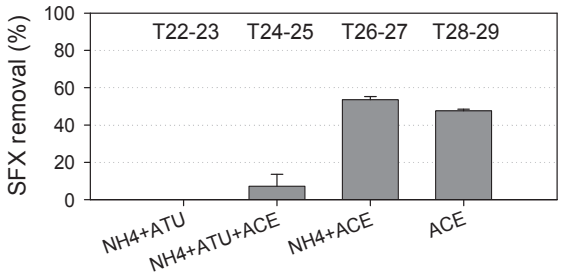

Fig. 3. Effect of ATU and acetate on SFX degradation (average removal presented as percentage). The error bars represent the standard deviation.

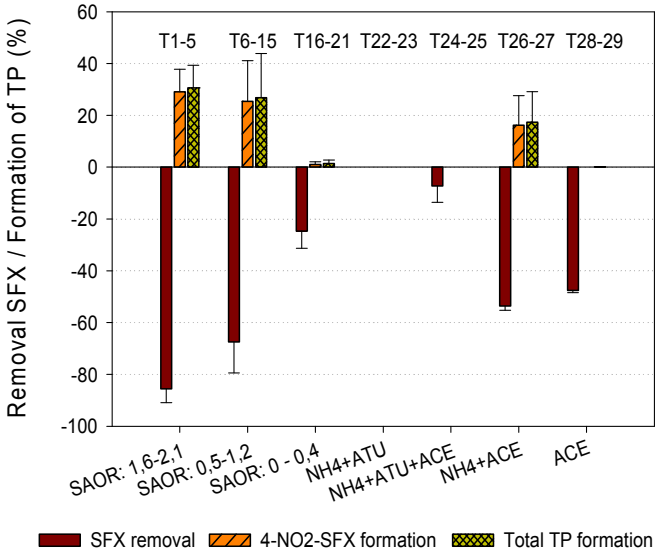

Fig. 4. Removal of SFX and TPs production for all the conducted tests. The error bars represent the standard deviation.

TPs was significantly low. The results of the first category of experiments executed with NH_4^+ and SFX (T1-T21) demonstrated that nitrification rate not only affected SFX degradation, but also the formation of the TPs. Considering the three SAOR zones previously described, higher TP formation was quantified for higher SAORs. Moreover, higher TP formation was observed for higher SFX degradation, except in the case of the tests T28–T29, where an intermediate removal of SFX led to null formation of TPs. In the case that SFX was not degraded due to the addition ATU (T22–T23), no formation of TPs was detected.

The fate of SFX TPs was also explored by means of independent experiments for each of them, and results are shown for three representative tests (Fig. 5, T31, T32 and T34). The compounds showed a relatively stable behavior during the experiments with a limited increase during the first 30 min for 4-Nitro-SFX and Desamino-SFX (Fig. 5a and b, respectively), possibly for an insufficient mixing at time zero, considering that no significant increase was observed from then onwards. N^4-Acetyl-SFX remained stable during all the experiment (Fig. 5c).

In all tests, the only detected TP was the one spiked. Moreover, it is important to stress that TPs were not cleaved back to SFX and that they could not be degraded during the batch tests.

3.2. Long term experiments

3.2.1. SFX degradation and TPs formation during the long term test in the SBR

The performance of the SBR kept stable during the 10 weeks of the long term SFX experiment. The addition of SFX in the influent of the reactor did not affect the nitritation performance (data not shown). In fact the reactor achieved more than 99% of ammonia removal. No NO_3^- was detected in the effluent.

During the first period (1–35 days), the concentration of SFX at the influent was 11.2 ± 0.8 μg/L whereas during the second period (36–70 days), the influent SFX concentration was 101.7 ± 12.2 μg/L. High SFX removals were observed at both influent concentrations with the SBR operating at a SAOR of 0.5 mg NH_4^+-N/(g MLVSS min). SFX was rapidly degraded in the system, without a previous period of acclimation of the biomass, and from the first day a removal around 98% was obtained. During the first period, at the lower influent concentration, SFX removal was 98.3 ± 3.2% (effluent concentration of 0.2 ± 0.3 μg/L, Fig. 6a). During the second period, at the higher influent concentration, the removal of SFX was 97.6 ± 3.2% (effluent concentration of 2.5 ± 3.5 μg/L, Fig. 6b).

In terms of TPs, a similar behavior was observed as in the case of the batch tests. 4-Nitro-SFX was detected in all tests and was the main TP. 4-Nitro SFX was formed up to 20.5 ± 7.8% (effluent concentration of 2.3 ± 0.9 μg/L), and up to 27.6 ± 6.1% (effluent concentration of 27.9 ± 6.4 μg/L) during the first (1–35 days) and second (36–70 days) period respectively. The total TPs formation ranged from 23.4 ± 8.7% to 32 ± 7% during the first and the second period respectively. Adversely, in this experiment Desamino-SFX and N^4-Acetyl-SFX formation remained at low levels compared to 4-Nitro-SFX, but both were detected in the system. Desamino-SFX formation ranged from 2.7 ± 1.3% (effluent concentration of 0.3 ± 0.1 μg/L) to 4.4 ± 1.1% (effluent concentration of 4.4 ± 1.0 μg/L) during the first and the second period respectively, while N^4-Acetyl-SFX ranged from 0.2 ± 0.08% (effluent concentration of 0.02 ± 0.01 μg/L) to 0.1 ± 0.02% (effluent concentration of 0.06 ± 0.02 μg/L) during the first and the second period

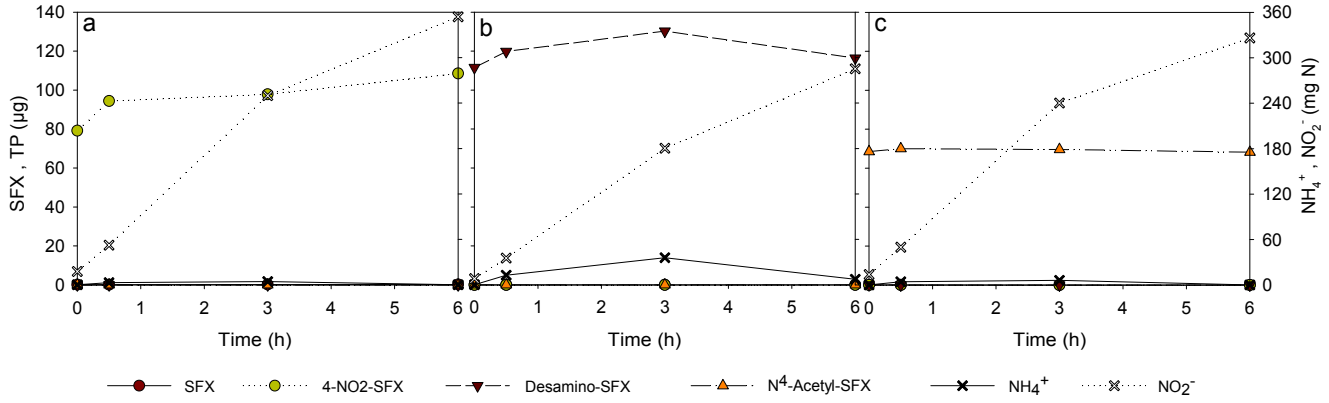

Fig. 5. Fate of TPs during the independent experiments: a) 4-Nitro-SFX (T31); b) Desamino-SFX (T34); c) N^4-Acetyl-SFX (T32).

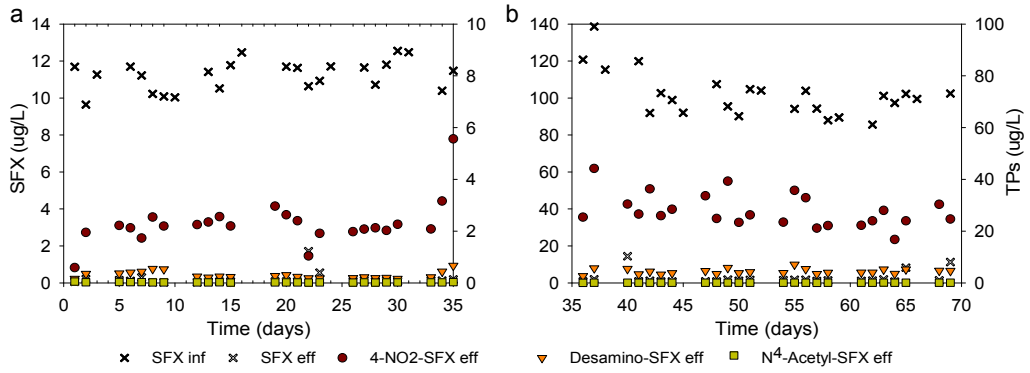

Fig. 6. Removal of SFX and TPs formation during the long term experiment executed in the SBR: a) day 1–35, influent SFX concentration of 11.2 ± 0.8 µg/L; b) day 36–70 influent SFX concentration of 101.7 ± 12.2 µg/L.

respectively.

4. Discussion

High degradation of sulfamethoxazole, up to 86%, was obtained during aerobic batch tests by the investigated enriched AOB and SFX degradation was clearly related to the nitrification rate with higher removals observed at higher SAORs. Similarly, Fernandez-Fontaina et al. (2012) observed that antibiotics and musk fragrances removal was found to be related to the nitrification performance in a nitrifying activated sludge system, having the lowest biodegradation efficiency at the lowest specific nitrification rate value of 0.12 g NH_4^+-N/(g MLVSS d). Moreover, a linear relationship was found between the nitrifying activity and the removal of ibuprofen, erythromycin and roxithromycin in an aerobic conventional activated sludge system (Alvarino et al., 2014). On the other hand, previous findings reported K_{biol} values of less than 0.5 L/(g_{ss} d) for SFX, in batch tests carried out with CAS and membrane bioreactor sludge, suggesting its poor biodegradability (Joss et al., 2006; Suarez et al., 2010).

In the present study, SFX experienced a sharp decrease during the first half an hour in most tests that could be linked to adsorption onto the biomass. However during the sorption control experiment that was executed, SFX did not show any remarkable removal during a period of four days. Similarly, Yang et al. (2011) conducted batch incubation experiments with activated sludge in order to study the sorption of sulfonamide antibiotics at an initial concentration of 100 µg/L at pH of 6.8. The study demonstrated that these compounds adsorb onto the activated sludge relatively quickly in the first 2 h, but this adsorption corresponded to a 6.5% of removal in the case of SFX. Moreover they observed that when adsorption equilibrium was established biodegradation became the dominant mechanism.

A higher removal was obtained in the long term SBR experiment with SFX being degraded up to 98% at both influent concentrations of 11.2 ± 0.8 and 101.7 ± 12.2 µg SFX/L. The SBR was operating with a SAOR of 0.5 mg NH_4^+-N/(g MLVSS min). When comparing this rate with the rates presented in Fig. 2a, a moderate removal of SFX would have been expected. However, the results demonstrated the high efficiency of a partial nitrification reactor in the removal of SFX without a previous period of adaptation, suggesting that the longer HRT applied in the reactor (compared to the batch tests) played an important role on the removal of SFX. According to the literature, the micropollutants having slow/intermediate kinetics such as some antibiotics experience less effective biodegradation at shorter HRTs (Fernandez-Fontaina et al., 2012). Moreover, another study stated that compounds with a half-life time less than WWTP HRT generally exhibit high removal efficiencies (García-Galán et al., 2011; Gros et al., 2010). Contrary to the findings of the present study, Müller et al. (2013) observed that the initial concentration of 10 mg SFX/L remained unchanged, during a period of 14 days in 2 L flask reactors operating under aerobic conditions with CAS, until adaptation of the biomass occurred. Moreover, SFX removal was found to be incomplete (36.5 ± 11.5%) in an SBR at 20 °C with CAS under nitrifying and denitrifying conditions, throughout an experimental period of two months, with no significant sign of enhancement over time, but with higher removals being obtained during the aerobic phases (Collado et al., 2013).

In the presence of ATU, nitrification was completely suppressed and this resulted in a null removal of SFX, providing an indication of the role of the AOB in SFX degradation. ATU is an inhibitor of copper-containing enzymes such as AMO, but also other monooxygenases that could possibly co-metabolize recalcitrant compounds. It was reported that any inhibition of AOB may negatively influence PhAC biodegradation in WWTPs due to the reduction in the growth rate of AOB (Sathyamoorthy et al., 2013). Likewise, the addition of ATU in an enriched nitrifier culture inhibited the nitrification completely and suppressed the removal of most selected pharmaceuticals (Tran et al., 2009). Similarly, the degradation rate constants of estrone, estradiol and ethinylestradiol were decreased significantly when ATU was added in a nitrifying activated sludge system in the presence of ammonia-oxidizing bacteria Nitrosomonas europaea (Shi et al., 2004).

Some studies have investigated the expression of the AMO enzyme under ammonia starvation. Forrez et al. (2011), demonstrated that AOB cells starving for more than 2 months contained nearly twice as much AMO as actively growing cells, although they possessed lower ammonia-oxidizing activity. In other cases, AMO was even detected in Nitrosomonas after 1 year of ammonium starvation, indicating a high resistance of AMO towards degradation (Pinck et al., 2001). These findings could explain the removals around 28.4 ± 7.1% that were observed in the present study in the absence of ammonia (T20–T21, Fig. 2a), suggesting the potential of systems where there is an abundance of AOB towards the degradation of recalcitrant pollutants (even without a previous period of acclimation).

In this study, SFX TPs were found to represent up to 30% of the initial mass of SFX during the batch tests (in case that the latter was degraded) and up to 32% of the initial SFX concentration during the long term experiment. In both types of experiments, the main TP was 4-Nitro-SFX and SFX degradation occurred simultaneously and opposite to its formation. Desamino-SFX and N^4-Acetyl-SFX were detected in several experiments, even though at lower concentration. Similar results with 4-Nitro-SFX concentration developing opposite to that of SFX, were previously observed but in different conditions (aquifer material under denitrifying conditions, Barbieri et al., 2012). Finally, the short term experiments that were executed to explore the fate of the TPs in the absence of SFX, demonstrated that TPs could not be degraded. During the long term experiment it was observed that TPs were not cleaved back to SFX, since no accumulation of the latter was detected in the system. On the contrary, in batch tests involving aquifer material and under denitrifying conditions, 4-Nitro-SFX returned to the parent compound (SFX) when the concentration of nitrite dropped (Barbieri et al., 2012). This known reversible formation of nitro-derivatives, was not observed in this study taking into account that nitrite was accumulating into the system, in both short and long term experiments. Likewise, in contrast to the findings of the present study were Desamino-SFX was not found to be degradable, formation and its subsequent degradation was observed in anoxic water/sediment batch experiments with SFX and high nitrate concentration (Nödler et al., 2012). Moreover, N^4-Acetyl-SFX, was reduced in a concentration of 80–90% presumably via biodegradation, in an advanced wastewater reclamation plant (Yang et al., 2011). Same behavior was observed in batch biodegradation studies that were performed with conventional activated sludge or with membrane bioreactor sludge, in which an exponential decrease of the concentration of N^4-Acetyl-SFX was observed over time (Joss et al., 2006). The contradictory results can be attributed to the different biomass and conditions applied.

In the present study the evaluated SFX TPs appeared to be stable, since non drop-back to the parent compound was observed, but they represent a significant percentage of the total removal. The results stress that only a comprehensive monitoring for metabolites or end products of mineralization can provide information about the real degree of biotransformation of the parent compounds. In some cases, in fact, the concentrations of parent PhACs can even increase during the treatment process (Göbel et al., 2007; Lindqvist et al., 2005; Ternes, 1998), as specifically reported for sulfonamides (Joss et al., 2006; Yang et al., 2011). Moreover, Barbieri et al. (2012) reported that TPs such as 4-Nitro-SFX, may also be formed in nitrification and denitrification processes, leading to a wrong estimation of SFX removal efficiency, as this nitro derivative can transform back into the parent compounds.

Finally, TPs can exhibit similar or higher ecotoxicological effects than the parent compound. Majewsky et al., 2014 reported that 4-Nitro-SFX was found to inhibit bacterial growth to a clearly greater extent than the parent compound, SFX, whereas N^4-Acetyl-SFX retained less than 10% of the effect of SFX on growth and luminescence inhibition. Consequently, it is urgent to consider TPs formation as well in SFX degradation studies because they can contribute to the total antibacterial and ecotoxicological effect.

5. Conclusions

- The high degradation capabilities of the enriched AOB culture towards refractory micropollutants such as SFX were demonstrated even without a previous period of adaptation.
- SFX was degraded up to 86%, and up to 98%, during the aerobic short term experiments (contact time of 6 h) and the long term experiment in the SBR (contact time of 24 h), respectively.
- SFX degradation was clearly related to the nitrification rate. Higher removals were obtained under higher specific ammonium oxidation rates (SAORs), and three zones of different SAOR corresponding to different removals were identified. Nevertheless, also with nitrification decreased or suppressed due to the lack of ammonia, SFX degradation was still observed at around 25%.
- In the presence of ATU nitrification was completely inhibited and SFX was not degraded.
- An efficient UPLC-QqLIT method for the determination of 4-Nitro SFX, Desamino-SFX and N^4-Acetyl-SFX in batch samples without sample pre-concentration has been optimized and validated with good quality parameters.
- TPs formation represented around 30% of SFX in the short and long term experiments conducted. In both cases, the main TP was 4-Nitro-SFX, whereas the formation of Desamino-SFX and N^4-Acetyl-SFX was minor. TPs could not be degraded during the batch tests.

Acknowledgments

This study has received funding from the People Program (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013, under REA agreement 289193-Project SANITAS and the European Comission FP7-PEOPLE-2011-CIG 303946 project, and from Waterfate project (CTM2012-38314-C02-01). This publication reflects only the author's views and the European Union is not liable for any use that may be made of the information contained therein. Elissavet Kassotaki acknowledges the FI fellowship (2015FI_B 00454) from the Catalan Government. Maite Pijuan acknowledges the Ramon y Cajal research fellowship (RYC-2009-04959) from the Spanish Ministry of Economy and Competitiveness. Authors acknowledge the support from the Economy and Knowledge Department of the Catalan Government through a Consolidated Research Group (2014 SGR 291) - Catalan Institute for Water Research. Thanks to Dr. Tobias Licha, from the Geoscience Centre of the University of Göttingen for providing us with the standard desamino-SFX, and to Marta Villagrasa for the

support in the analytical part.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.02.022.

References

Alvarino, T., Suarez, S., Katsou, E., Vazquez-Padin, J., Lema, J.M., Omil, F., 2015. Removal of PPCPs from the sludge supernatant in a one stage nitritation/anammox process. Water Res. 68, 701–709.

Alvarino, T., Suarez, S., Lema, J.M., Omil, F., 2014. Understanding the removal mechanisms of PPCPs and the influence of main technological parameters in anaerobic UASB and aerobic CAS reactors. J. Hazard. Mater. 278, 506–513.

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

Barbieri, M., Carrera, J., Ayora, C., Sanchez-Vila, X., Licha, T., Nödler, K., Osorio, V., Pérez, S., Köck-Schulmeyer, M., López de Alda, M., Barceló, D., 2012. Formation of diclofenac and sulfamethoxazole reversible transformation products in aquifer material under denitrifying conditions: batch experiments. Sci. Total Environ. 426, 256–263.

Bédard, C., Knowles, R., 1989. Physiology, biochemistry, and specific inhibitors of CH4, NH4+, and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev. 53, 68–84.

Bendz, D., Paxéus, N.A., Ginn, T.R., Loge, F.J., 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Höje River in Sweden. J. Hazard. Mater. 122, 195–204.

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

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, 277–279.

Buttiglieri, G., Knepper, T.P., 2008. Removal of emerging contaminants in wastewater treatment: conventional activated sludge treatment. In: Barceló, D., Petrovic, M. (Eds.), The Handbook of Environmental Chemistry, Volume: Emerging Contaminants from Industrial and Municipal Wastewaters. Springer, pp. 1–35.

Collado, N., Buttiglieri, G., Marti, E., Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., Comas, J., Rodriguez-Roda, I., 2013. Effects on activated sludge bacterial community exposed to sulfamethoxazole. Chemosphere 93, 99–106.

Daims, H., Lücker, S., Wagner, M., 2006. Daime, a novel image analysis program for microbial ecology and biofilm research. Environ. Microbiol. 8, 200–213.

De Voogt, P., Janex-Habibi, M.L., Sacher, F., Puijker, L., Mons, M., 2009. Development of a common priority list of pharmaceuticals relevant for the water cycle. Water Sci. Technol. 59, 39–46.

Drillia, P., Dokianakis, S.N., Fountoulakis, M.S., Kornaros, M., Stamatelatou, K., Lyberatos, G., 2005. On the occasional biodegradation of pharmaceuticals in the activated sludge process: the example of the antibiotic sulfamethoxazole. J. Hazard. Mater. 122, 259–265.

Fernandez-Fontaina, E., Carballa, M., Omil, F., Lema, J.M., 2014. Modelling cometabolic biotransformation of organic micropollutants in nitrifying reactors. Water Res. 65, 371–383.

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, 5434–5444.

Forrez, I., Boon, N., Verstraete, W., Carballa, M., 2011. In: Biodegradation of Micropollutants and Prospects for Water and Wastewater Biotreatment, second ed. Comprehensive Biotechnology. Elsevier.

García-Galán, M.J., Díaz-Cruz, M.S., Barceló, D., 2011. Occurrence of sulfonamide residues along the Ebro River basin: removal in wastewater treatment plants and environmental impact assessment. Environ. Int. 37, 462–473.

Göbel, A., McArdell, C.S., Joss, A., Siegrist, H., Giger, W., 2007. Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. Sci. Total Environ. 372, 361–371.

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, 3981–3989.

Gros, M., Petrović, M., Ginebreda, A., Barceló, D., 2010. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environ. Int. 36, 15–26.

Jelic, A., Rodriguez-Mozaz, S., Barceló, D., Gutierrez, O., 2015. Impact of in-sewer transformation on 43 pharmaceuticals in a pressurized sewer under anaerobic conditions. Water Res. 68, 98–108.

Joss, A., Zabczynski, S., Göbel, A., Hoffmann, B., Löffler, D., McArdell, C.S., Ternes, T. a., Thomsen, A., Siegrist, H., 2006. Biological degradation of pharmaceuticals in municipal wastewater treatment: proposing a classification scheme. Water Res. 40, 1686–1696.

Kolpin, D.W., Kolpin, D.W., Furlong, E.T., Furlong, E.T., Meyer, M.T., Meyer, M.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. Streams, 1999-2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Kuai, L., Verstraete, W., 1998. Ammonium removal by the oxygen-limited autotrophic nitrification- denitrification system. Appl. Environ. Microbiol. 64, 4500–4506.

Larcher, S., Yargeau, V., 2012. Biodegradation of sulfamethoxazole: current knowledge and perspectives. Appl. Microbiol. Biotechnol. 96, 309–318.

Levine, A.D., Meyer, M.T., Kish, G., 2006. Evaluation of the persistence of micropollutants through pure-oxygen activated sludge nitrification and denitrification. Water Environ. Res. 78, 2276–2285.

Lindqvist, N., Tuhkanen, T., Kronberg, L., 2005. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. Water Res. 39, 2219–2228.

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. 473–474, 619–641.

Majewsky, M., Wagner, D., Delay, M., Bra, S., Yargeau, V., Horn, H., 2014. Antibacterial activity of sulfamethoxazole transformation products (TPs): general relevance for sulfonamide TPs modified at the para position. Chem. Res. Toxicol. 27, 1821–1828.

Müller, E., Schüssler, W., Horn, H., Lemmer, H., 2013. Aerobic biodegradation of the sulfonamide antibiotic sulfamethoxazole by activated sludge applied as cosubstrate and sole carbon and nitrogen source. Chemosphere 92, 969–978.

Nielsen, P.H., Daims, H., Lemmer, H. (Eds.), 2009. FISH Handbook for Biological Wastewater Treatment: Identification and Quantification of Microorganisms in Activated Sludge and Biofilms by FISH. IWA Publishing, London, UK.

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, 2131–2139.

Onesios, K.M., Yu, J.T., Bouwer, E.J., 2009. Biodegradation and removal of pharmaceuticals and personal care products in treatment systems: a review. Biodegradation 20, 441–466.

Pinck, C., Coeur, C., Potier, P., Bock, E., 2001. Polyclonal antibodies recognizing the AmoB protein of ammonia oxidizers of the β-subclass of the class proteobacteria. Appl. Environ. Microbiol. 67, 118–124.

Radjenović, J., Petrović, M., Barceló, D., Petrović, M., 2007. Advanced mass spectrometric methods applied to the study of fate and removal of pharmaceuticals in wastewater treatment. TrAC – Trends Anal. Chem. 26, 1132–1144.

Rivera-Utrilla, J., Sánchez-Polo, M., Ferro-García, M.Á., Prados-Joya, G., Ocampo-Pérez, R., 2013. Pharmaceuticals as emerging contaminants and their removal from water. A review. Chemosphere 93, 1268–1287.

Rodriguez-Caballero, A., Pijuan, M., 2013. N2O and NO emissions from a partial nitrification sequencing batch reactor: exploring dynamics, sources and minimization mechanisms. Water Res. 47, 3131–3140.

Roh, H., Subramanya, N., Zhao, F., Yu, C.P., Sandt, J., Chu, K.H., 2009. Biodegradation potential of wastewater micropollutants by ammonia-oxidizing bacteria. Chemosphere 77, 1084–1089.

Sathyamoorthy, S., Chandran, K., Ramsburg, C.A., 2013. Biodegradation and cometabolic modeling of selected beta blockers during ammonia oxidation. Environ. Sci. Technol. 47, 12835–12843.

Shi, J., Fujisawa, S., Nakai, S., Hosomi, M., 2004. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium Nitrosomonas europaea. Water Res. 38, 2322–2329.

Suarez, S., Lema, J.M., Omil, F., 2010. Removal of pharmaceutical and personal care products (PPCPs) under nitrifying and denitrifying conditions. Water Res. 44, 3214–3224.

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.

Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R., 2007. Irrigation of treated wastewater in Braunschweig, Germany: an option to remove pharmaceuticals and musk fragrances. Chemosphere 66, 894–904.

Tran, N.H., Urase, T., Kusakabe, O., 2009. The characteristics of enriched nitrifier culture in the degradation of selected pharmaceutically active compounds. J. Hazard. Mater. 171, 1051–1057.

Tran, N.H., Urase, T., Ngo, H.H., Hu, J., Ong, S.L., 2013. Insight into metabolic and cometabolic activities of autotrophic and heterotrophic microorganisms in the biodegradation of emerging trace organic contaminants. Bioresour. Technol. 146, 721–731.

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment–a review. Sci. Total Environ. 429, 123–155.

Yang, S.F., Lin, C.F., Wu, C.J., Ng, K.K., Yu-Chen Lin, A., Andy Hong, P.K., 2012. Fate of sulfonamide antibiotics in contact with activated sludge - sorption and biodegradation. Water Res. 46, 1301–1308.

Yang, S.F., Lin, C.F., Yu-Chen Lin, A., Andy Hong, P.K., 2011. Sorption and biodegradation of sulfonamide antibiotics by activated sludge: experimental assessment using batch data obtained under aerobic conditions. Water Res. 45, 3389–3397.

Yang, X., Flowers, R.C., Weinberg, H.S., Singer, P.C., 2011. Occurrence and removal of pharmaceuticals and personal care products (PPCPs) in an advanced wastewater reclamation plant. Water Res. 45, 5218–5228.

Yi, T., Harper, W.F., 2007. The link between nitrification and biotransformation of 17α-Ethinylestradiol. Environ. Sci. Technol. 41, 4311–4316.