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On the occasional biodegradation of pharmaceuticals in the activated sludge process: The example of the antibiotic sulfamethoxazole

P. Drillia, S.N. Dokianakis, M.S. Fountoulakis, M. Kornaros *, K. Stamatelatou, G. Lyberatos

Department of Chemical Engineering, University of Patras, Karatheodori 1, 26500 Patras, Greece

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Abstract

Sulfamethoxazole, a common antibiotic, was found to be biodegradable under aerobic conditions. The fate of sulfamethoxazole in the activated sludge process was studied using a Sequencing Batch Reactor (SBR). Aerobic biomass was acclimated to sulfamethoxazole and a series of kinetic experiments were conducted to investigate the impact of other carbon and nitrogen sources on the degradation of the antibiotic. It was found that sulfamethoxazole serves both as carbon and nitrogen source for the enriched consortium. It was degraded whenever there was a depletion of carbon or nitrogen or both in the feeding medium, while in the presence of acetate and ammonium nitrogen (alternative carbon and nitrogen sources, respectively), sulfamethoxazole remained intact.

Keywords: Sulfamethoxazole; Antibiotic; Degradation; Kinetics; Activated sludge

1. Introduction

After administration of a pharmaceutical, a significant part of the original substance or its metabolites are excreted with urine or faeces, ending up in the municipal wastewater and municipal sewage via hospital effluents [1]. Pharmaceuticals are usually very persistent compounds to biodegradation, hardly eliminated during sewage treatment, since they have been designed to cause DNA damage to bacteria or eukaryotic cells [2,3]. The percentage removal of pharmaceuticals in municipal sewage treatment plants vary between almost zero values and almost complete removal, depending on the substance and the process taking place in the sewage treatment plant [4–13]. If not eliminated in STPs, the pharmaceuticals will be eventually released to the aquatic environment or to soil, posing potential threats to public health. For example, recent research on xenobiotic compounds such as PCBs has demonstrated, that some pharmaceuticals called endocrine disruptors have a hormone mimicking effect, even at concentrations of a few nanograms per litre [14,15].

Antibiotics are a major category of pharmaceuticals in human and veterinary medicine. They are used as growth promoters or for therapeutic purposes. Many antibiotics are hydrophilic and are therefore anticipated to be mobile ending up in the aquatic environment. They have been detected in the influent and effluent of sewage treatment plants and surface water in the range of ng/L and μg/L [16,17]. Recently published results [18] demonstrated that many veterinary antibiotics proved to be moderately persistent in surface water systems under aerobic and anaerobic conditions. During biodegradation tests performed according to OECD 301 D method, it was confirmed that certain antibiotics were active against different groups of bacteria present in wastewater [19,20]. However, other researchers have not observed any significant inhibition in the activity of anaerobic consortium [21].

Sulfamethoxazole (Fig. 1), a common antibiotic, is an antibacterial sulfonamide. It prevents the formation of dihydrofolic acid, a compound that bacteria must be able to produce in order to survive. Although it was once a very useful antibiotic, it is almost obsolete as a single agent today due to the development of bacterial resistance to its effects. Sulfamethoxazole is now used primarily in combination with

* Corresponding author.
E-mail address: kornaros@chemeng.upatras.gr (M. Kornaros).

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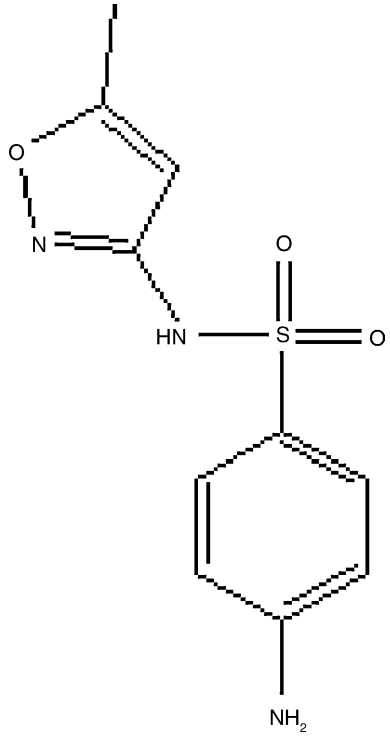

Fig. 1. The chemical structure of sulfamethoxazole.

trimethoprim a combination product known as Bactrim or Septra [22]. This antibiotic has proven to be very persistent to biodegradation according to recent researches [23,24].

In this work, the biodegradation of sulfamethoxazole was studied under aerobic conditions by an acclimated enhanced culture of aerobic microorganisms. Furthermore, the fate of sulfamethoxazole in activated sludge was investigated in the presence or absence of the degrading culture.

2. Materials and methods

2.1. Chemicals

Sulfamethoxazole (purity >97% by weight) was purchased from Sigma–Aldrich (723-46-6). Methanol of gradient grade was used for liquid chromatography (LiChrosolv, Merck) and ultra pure water was prepared in a Milli-Q purification system (Millipore). All other chemicals were obtained from Merck (Darmstadt, Germany). The solid phase extraction cartridges (SPE) were purchased from Waters (HLB® cartridges of 50 mg). Filters used for filtration were obtained from Millipore (0.22 μm) and Whatman (0.7 μm).

2.2. Analytical methods

Various parameters were measured during the experiments: total suspended solids (TSS), volatile suspended solids (VSS), total and dissolved (after filtration) chemical oxygen demand (COD) and total nitrogen concentration (persulfate digestion method) were determined according to Standard Methods [25]. The pH was measured using a portable pH meter (Hanna, HI 8224). Filtered samples were analyzed for acetate by ion chromatography on a model DX300 gradient system (Dionex, Sunnyvale, CA) using an AS11 column and a CDM-3 conductivity detector. A gradient method was used for the analysis of acetate. After the injection of a 25-μL appropriately diluted sample, using NaOH eluent at a flow rate of 2.0 mL/min, the chromatogram for acetate was obtained within 7 min. The retention time for acetate was 1.3 min.

Determination of sulfamethoxazole in the samples was performed using HPLC (X-Terra™ RP_{18} 5 μm column from Waters) combined with UV detection. Prior to the analysis in HPLC, the samples were treated using solid phase extraction techniques (Waters HLB® cartridges of 50 mg), so that the samples would be free of other organic compounds that would interfere in the analysis or inorganic ions that would decay the column. The mobile phase consisted of methanol (eluent A) and water (eluent B). The method was isocratic (50% of eluent A and 50% of eluent B). Analysis was performed at a flow rate of 1 mL/min. The wavelength used for detection was 270 nm and the retention time was 3.70 min.

2.3. Continuous removal of sulfamethoxazole in a Sequencing Batch Reactor

A Sequencing Batch Reactor (SBR) was used for studying the fate of sulfamethoxazole under aerobic operating conditions. The working volume of the reactor was 1 L. The minimum reactor volume at the end of the draw phase was one-third of the effective SBR volume. The SBR was operated at 25 °C using an 8-h cycle in the typical phase sequence: "*fill*" (30 min), "*react*" (6 h), "*settle*" (1h) and "*draw*" (30 min). The system was equipped with facilities for temperature control (electrical resistance, thermocouple and cooling water), wastewater inflow (variable speed pump), effluent discharge (variable speed pump), aeration (air pump) and mixing (magnetic stirrer). Digital timers controlled the operation of the system. The reactor was seeded with sludge from the aerobic basin of the Wastewater Treatment Plant of Patras, Greece. The *fill* phase was taking place under anoxic/anaerobic conditions, providing only mixing of the reactor content. During the "*react*" phase, air was supplied to the system ensuring dissolved oxygen (D.O.) concentration above 3 mg/L, throughout the aeration period. Aeration and agitation of the reactor ceased during the "*settle*" phase and the sludge was allowed to settle under quiescent conditions. During the "*draw*" phase, the clarified supernatant was withdrawn by pumping through a solenoid valve from a fixed port at the minimum liquid level. During one SBR cycle, samples were collected from the reactor at the beginning of the cycle, the end of "*fill*", and the end of the "*react*" phase, as well as from the effluent following the "*draw*" phase. The feed consisted of a synthetic medium (Table 1) containing sulfamethoxazole at different concentrations.

Table 1
Composition of (a) synthetic medium fed to SBR and (b) stock solution with trace elements

Component	Concentration
(a)	
KH_2PO_4	3.000 (g/L)
K_2HPO_4	5.000 (g/L)
$(NH_4)_2SO_4$	0.235 (g/L)
$MgSO_4{\cdot}7H_2O$	0.200 (g/L)
$CaCl_2{\cdot}2H_2O$	0.026 (g/L)
$CH_3COONa{\cdot}3H_2O$	0.850 (g/L)
$NaHCO_3$	0.700 (g/L)
Trace elements	0.1 (mL/feed)
(b)	
$CuSO_4{\cdot}5H_2O$	0.786 (g/L)
$FeSO_4{\cdot}7H_2O$	5.000 (g/L)
$NaMoO_4{\cdot}2H_2O$	12.609 (g/L)
$NiCl_2{\cdot}6H_2O$	4.050 (g/L)
$ZnSO_4{\cdot}7H_2O$	4.398 (g/L)
$CoCl_2{\cdot}6H_2O$	2.453 (g/L)
KI	0.750 (g/L)
H_3BO_3	3.000 (g/L)
$MnCl_2{\cdot}4H_2O$	5.000 (g/L)
EDTA	5.000 (g/L)

2.4. Acclimation of activated sludge to sulfamethoxazole

Activated-sludge mixed liquor was obtained from the Wastewater Treatment Plant of the University of Patras (Greece) and used as inoculum for the acclimation of aerobic biomass to sulfamethoxazole in a draw–fill reactor. The reactor consisted of a 250-mL Erlenmeyer flask, inoculated with activated sludge and operated in a draw and fill mode at 25 °C, while adequate aeration was provided. Every 2 days, 50 mL were withdrawn from the bioreactor and were replaced with 50 mL of feed. The feed contained: 6.93 g/L $Na_2HPO_4{\cdot}2H_2O$; 0.675 g/L KH_2PO_4; 0.2 g/L $MgSO_4{\cdot}7H_2O$; 0.02 g/L $CaCl_2{\cdot}2H_2O$; 0.3 g/L CH_3COONa; 0.25 g/L NH_4NO_3; 10 mg/L sulfamethoxazole and 1 mL/(L feed) trace elements. Trace elements solution contained: $FeSO_4{\cdot}7H_2O$, 1.0 mg/mL; $MnSO_4{\cdot}H_2O$, 1.0 mg/mL; $CuCl_2{\cdot}2H_2O$, 0.25 mg/mL; $Na_2MoO_4{\cdot}2H_2O$, 0.25 mg/mL; H_3BO_3, 0.1 mg/mL and concentrated H_2SO_4, 5.0 mL. The pH of the medium was 7.5. All nutrient media were sterilised by autoclaving at 121 °C for 20 min. Sulfamethoxazole was added after the sterilisation in order to avoid possible conversion or reaction due to the high temperature. Sulfamethoxazole sterilisation was achieved by filtration through sterilised filters (0.22 μm Millipore). The concentration of the volatile suspended solids in the bioreactor was constantly about 300 mg/L. When sulfamethoxazole degradation was observed, the consortium was gradually acclimated to increasing concentrations of sulfamethoxazole (from 10 to 50 mg/L) while, at the same time, the feed no longer contained CH_3COONa so that sulfamethoxazole was the only source of organic carbon. The addition of NH_4NO_3 (nitrogen source) was also suspended in order to prohibit the growth of autotrophic nitrifying bacteria.

A new adaptation procedure was followed afterwards: 25 mL of the acclimated culture were used as inoculum and transferred to a new batch reactor (250 mL Erlenmeyer flask) that contained fresh medium and sulfamethoxazole at a concentration of 100 mg/L. The biomass growth was very low due to the low organic carbon content of the feed. When sulfamethoxazole was fully degraded, this procedure was repeated five more times, so that an enriched mixed microbial population was finally established.

2.5. Kinetic experiments of the mixed enhanced culture

A series of kinetic experiments was conducted in order to investigate the mechanism of sulfamethoxazole degradation. For each experiment, an inoculum from the acclimated and enriched biomass was added in a batch bioreactor (Erlenmeyer flask), accounting for 10% of the working volume. The feed medium was the same as that used for biomass acclimation, but acetate and ammonium nitrogen were occasionally present, whenever the purpose of the experiment was to investigate the effect of common carbon and nitrogen sources on sulfamethoxazole biodegradation. The experiments took place in sterile conditions. Sterilised air (filtered through 0.22 μm membrane filters) was continuously provided to the bioreactor, which was placed in a shaking water bath ensuring that the liquid culture was continuously stirred at constant temperature (25 °C). During the experiments, samples were taken and the following parameters were determined: TSS and VSS concentrations, sulfamethoxazole, acetic acid and total nitrogen concentrations.

3. Results

3.1. Continuous removal of sulfamethoxazole in a Sequencing Batch Reactor

Variation of sulfamethoxazole removal during operation with different feed concentrations is depicted in Fig. 2. Initially the reactor was fed with the pharmaceutical at a concentration of 20 mg/L. In the first 4 days, no reduction of sulfamethoxazole was observed. In the 5th day, the removal efficiency of sulfamethoxazole reached nearly 100%. Eight days after start-up, the concentration of sulfamethoxazole was raised to 50 mg/L, but still no pharmaceutical was detected in the effluent. The feed concentration was further increased to 100 mg/L (54 days of operation) and 320 mg/L (70 days of operation) and the concentration of sulfamethoxazole reduced to undetectable levels. Fig. 3 shows a typical sulfamethoxazole concentration profile observed during an operating cycle, when the feed concentration was 383 mg/L. Sulfamethoxazole was removed during the reaction phase within approximately 3 h. The VSS concentration in the SBR was about 4.2 g/L during the operation.

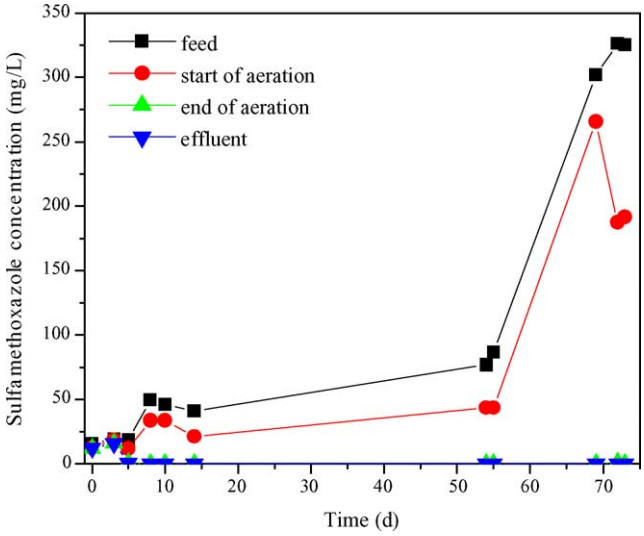

Fig. 2. Sulfamethoxazole concentration in SBR at increasing feed concentrations.

3.2. Kinetic experiments with the enhanced culture

In order to investigate the mechanism of sulfamethoxazole degradation, a series of five kinetic experiments based on the presence or not of other carbon and/or nitrogen sources (acetate and/or ammonium nitrate, respectively) were conducted in parallel. Fig. 4 shows the sulfamethoxazole degradation where the pharmaceutical was the only available carbon and nitrogen source. After a lag phase of 3 days, the concentration of sulfamethoxazole started decreasing and reached undetectable levels within 17 days. Another experiment was conducted in parallel under the same conditions, except that ammonium nitrate was added as an extra nitrogen source. Ammonium seemed to have no effect on the pharmaceutical degradation (Fig. 5).

Fig. 6 shows, the ability of the sulfamethoxazole-degrading consortium to degrade an easily biodegradable

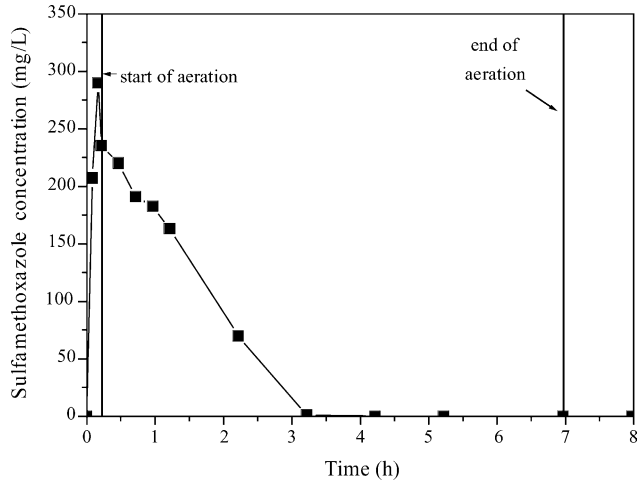

Fig. 3. Sulfamethoxazole degradation during an SBR cycle while the feed concentration was 383 mg/L.

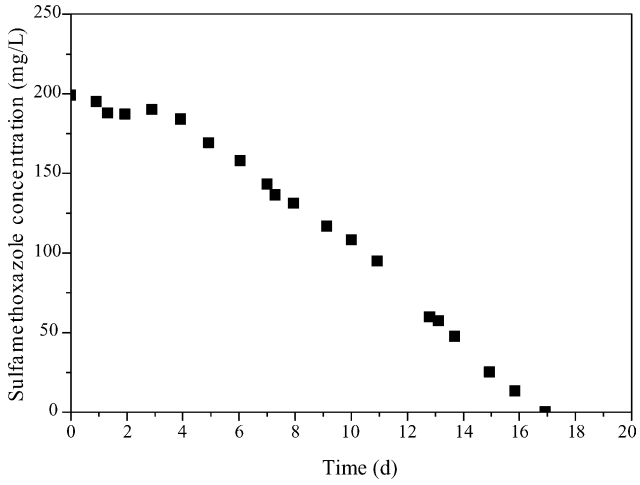

Fig. 4. Sulfamethoxazole degradation during a batch kinetic experiment where sulfamethoxazole is the only carbon and nitrogen source.

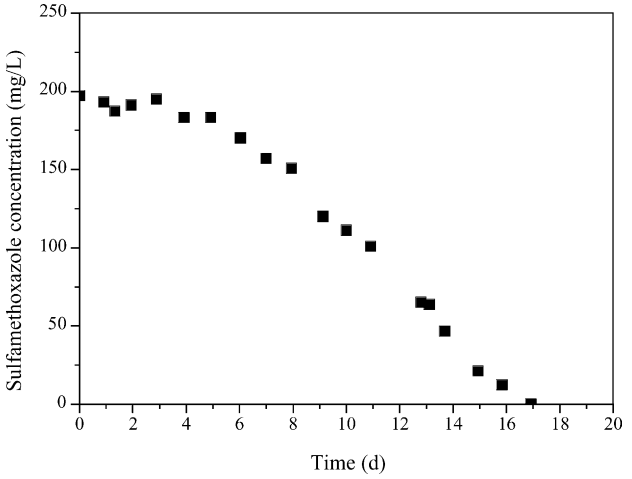

Fig. 5. Sulfamethoxazole degradation at the presence of ammonia.

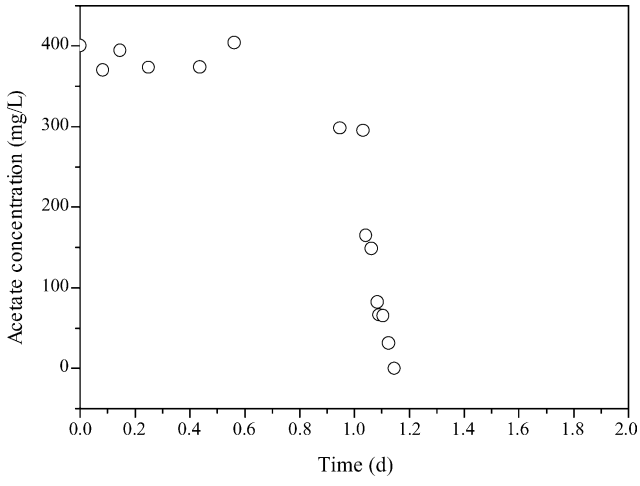

Fig. 6. Acetate consumption by the sulfamethoxazole degrading consortium at the presence of ammonia.

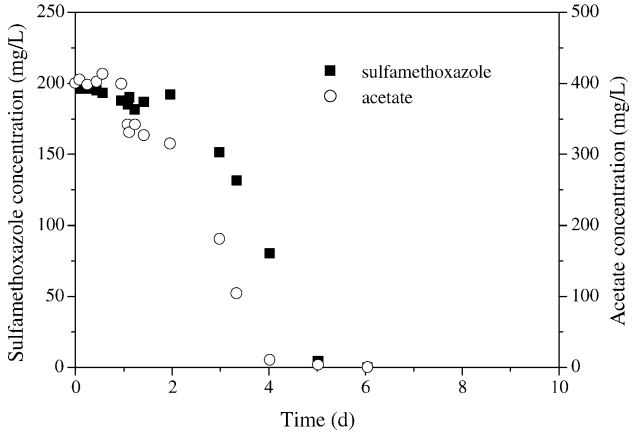

Fig. 7. Sulfamethoxazole and acetate degradation without any other nitrogen source.

organic compound such as acetate. Ammonium was also present in the culture medium, serving as nitrogen source. Acetate was completely degraded very fast. Since acetate accounts for the typical product of fermentation of the readily biodegradable fraction of wastewater organic loading in wastewater treatment plants, an experiment was conducted to study the potential co-metabolism of both substrates (sulfamethoxazole and acetate). Sulfamethoxazole and acetate were both fully degraded when ammonium was not present in the culture medium (Fig. 7). However, in the presence of ammonium, only acetate was degraded (Fig. 8), implying that in the previous experiment (Fig. 7), the pharmaceutical was utilized as a nitrogen source. A sample was taken 9 days after acetate had been completely exhausted, and it was found that sulfamethoxazole had been subsequently consumed (data not shown), indicating that the pharmaceutical had been eventually utilized in the absence of any other easily degradable organic compound. Apparently there was a significant delay so that the appropriate enzymes for sulfamethoxazole degradation were generated. Comparing acetate consumption in Figs. 7 and 8, it seems that the presence of ammonia in the medium leads to significantly faster acetate degradation.

Another series of two concurrent experiments was conducted to investigate the effect of ammonia on sulfamethoxazole and acetate degradation when it is added after sulfamethoxazole degradation has initiated. A batch bioreactor was inoculated and the feeding medium contained acetate and sulfamethoxazole without any ammonium nitrate. When sulfamethoxazole concentration started to decrease, the mixed liquor was separated into two parts and put in two different batch bioreactors (Erlenmeyer flasks). Ammonium nitrate was added only in one bioreactor while the other continued to operate under the same conditions as before the splitting. As shown in Fig. 9a, ammonium addition had no effect on sulfamethoxazole degradation rate, but increased the acetate degradation rate as shown in Fig. 9b. This verifies that once the appropriate enzymes for sulfamethoxazole degradation have been synthesized, the drug will be metabolised even in the presence of ammonia, although prolonged exposure to ammonia and acetate is expected to lead to eventual loss of the sulfamethoxazole degradation ability.

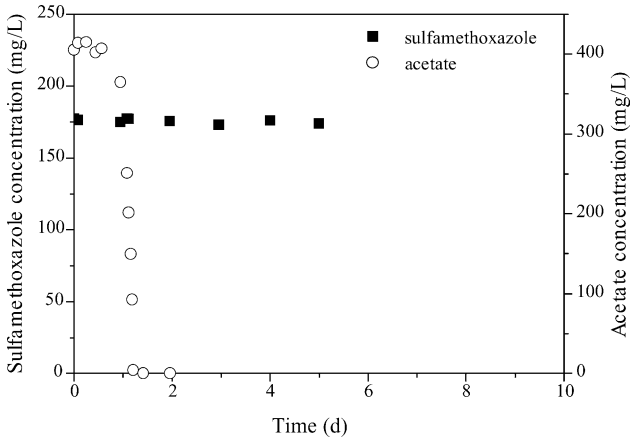

Fig. 8. Sulfamethoxazole and acetate degradation at the presence of ammonia.

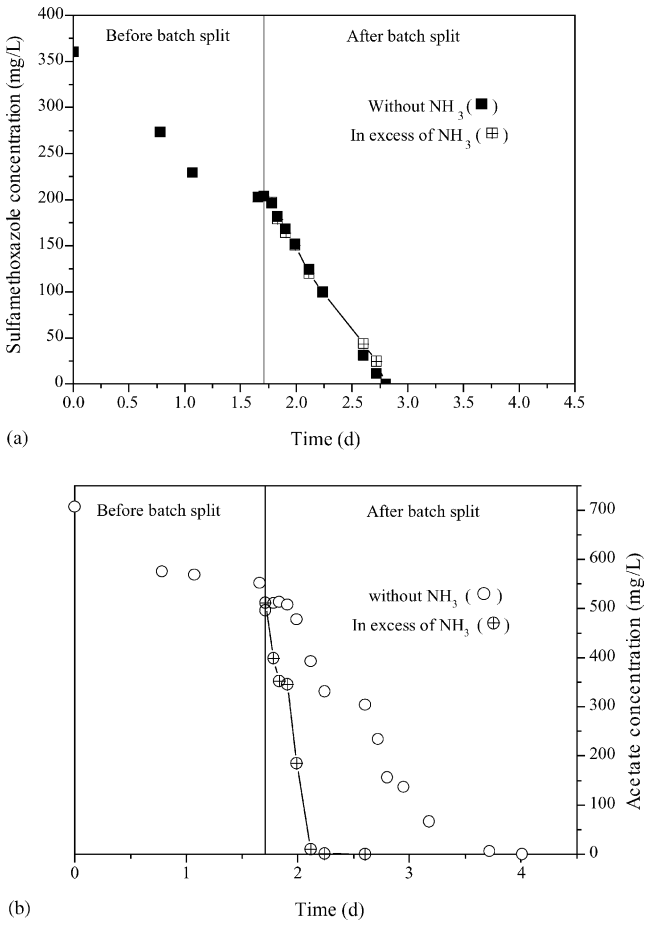

Fig. 9. Effect of ammonia on sulfamethoxazole (a) and acetate (b) degradation when it is added after sulfamethoxazole degradation has initiated.

4. Discussion

Sulfamethoxazole concentration was eliminated in an SBR even when the feed concentration was as high as 383 mg/L. During the first 4 days of operation, sulfamethoxazole was not degraded, but afterwards the removal efficiency was very high even when sulfamethoxazole concentration in the feed was increased. Since the inoculum used for the SBR start-up was not already acclimated to the pharmaceutical, the microorganisms responsible for the biodegradation of sulfamethoxazole must be common bacteria; species present in the activated sludge process. During a typical operating cycle, it took half the reaction phase (about 3 h) for the pharmaceutical to be degraded.

In order to illuminate the mechanism of sulfamethoxazole degradation, two series of kinetic experiments were conducted using an inoculum from a microorganism culture that had been adapted and enriched with sulfamethoxazole degraders. Since the pharmaceutical contains nitrogen, it seemed that this compound can serve for the microorganisms as a carbon or nitrogen source or both. The addition of ammonium nitrogen did not have an impact on the degradation rate of sulfamethoxazole (Figs. 4 and 5), meaning that once the compound was degraded to provide organic carbon, this was the rate limiting step deciding the rate of microbial growth and degradation of the pharmaceutical.

Acetate alone was rapidly biodegraded by the enhanced culture in the presence of ammonium nitrogen (Fig. 6). Comparing the acetate degradation rate as shown in Figs. 6 and 7, it seems that nitrogen becomes a limiting factor when ammonium is not added and nitrogen is then obtained from sulfamethoxazole degradation. On the other hand, the degradation rate of sulfamethoxazole is faster in the presence of acetate without any ammonia source (Figs. 3 and 7), indicating that growth of the microorganisms is enhanced by acetate (co-metabolism).

Furthermore, the presence of both acetate and ammonia in the culture medium before pharmaceutical addition prevents sulfamethoxazole degradation (Fig. 8), indicating that the microorganisms prefer acetate and ammonium nitrogen to the use of sulfamethoxazole as either carbon or nitrogen source. The lack of any degradation of the drug under such conditions implies that the microorganisms are not stressed to degrade sulfamethoxazole too, to utilize it either as a carbon or nitrogen source, since both carbon and nitrogen are made available by simpler substrates. However, this is the case only if the enzymes for sulfamethoxazole degradation have not been synthesized, something that depends on the past history of the culture. If sulfamethoxazole degradation has already initiated (e.g. in the absence of ammonium), the enzymes responsible for the biodegradation are active even when ammonium is added (Fig. 9). As anticipated, ammonium addition speeds up acetate degradation, but does not affect sulfamethoxazole degradation.

5. Conclusions

Very often certain trace compounds such as pharmaceuticals appear in the effluent of wastewater treatment plants not regularly, but erratically. Also, although some WWTPs are able to remove these compounds, others are not. The present work, through the example of the antibiotic sulfamethoxazole helps elucidate some of the possible reasons why ceratin compounds may appear in the effluent of wastewater treatment plants on occasion.

Sulfamethoxazole serves as a carbon and/or a nitrogen source for the enhanced culture of microorganisms that are able to degrade it. It seems that the enzymatic mechanism responsible for the compound degradation is not activated as long as there is a readily degradable carbon source available in excess of ammonium (which is the typical case in WWTP). However, if the conditions necessary for the activation of this mechanism prevail, then the enzymes are not inhibited in the case ammonium is afterwards present. These observations help understand the occasional presence of sulfamethoxazole in the WWTPs effluents.

In conclusion, sulfamethoxazole is expected to be detected in WWTP effluents, whenever there are easily biodegradable carbon and nitrogen sources contained in wastewaters that would prevent the initiation of the pharmaceutical degradation. In the case, there is a depletion of easily biodegradable matter (as likely may happen in extended aeration systems), sulfamethoxazole degradation is more likely to take place, so that there will be no need to maintain a bioaugmentation reactor.

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