Performance of anaerobic sequencing batch reactor in the treatment of pharmaceutical wastewater containing erythromycin and sulfamethoxazole mixture

S. Aydin, B. Ince, Z. Cetecioglu, E. G. Ozbayram, A. Shahi, O. Okay, O. Arikan and O. Ince

ABSTRACT

This study evaluates the joint effects of erythromycin–sulfamethoxazole (ES) combinations on anaerobic treatment efficiency and the potential for antibiotic degradation during anaerobic sequencing batch reactor operation. The experiments involved two identical anaerobic sequencing batch reactors. One reactor, as control unit, was fed with synthetic wastewater while the other reactor (ES) was fed with a synthetic substrate mixture including ES antibiotic combinations. The influence of ES antibiotic mixtures on chemical oxygen demand (COD) removal, volatile fatty acid production, antibiotic degradation, biogas production, and composition were investigated. The influent antibiotic concentration was gradually increased over 10 stages, until the metabolic collapse of the reactors, which occurred at 360 days for the ES reactor. The results suggest that substrate/COD utilization and biogas/methane generation affect performance of the anaerobic reactors at higher concentration. In addition, an average of 40% erythromycin and 37% sulfamethoxazole reduction was achieved in the ES reactor. These results indicated that these antibiotics were partly biodegradable in the anaerobic reactor system.

Key words | anaerobic reactor, antibiotics, biodegradation, inhibition, pharmaceutical wastewater

S. Aydin (corresponding author)
Z. Cetecioglu
E. G. Ozbayram
A. Shahi
O. Arikan
O. Ince
Environmental Engineering Department,
Istanbul Technical University,
Maslak, Istanbul,
Turkey
E-mail: *sevcan_aydn@hotmail.com*

B. Ince
Institutes of Environmental Sciences,
Bogazici University,
Bebek, Istanbul,
Turkey

O. Okay
Naval Architecture and Ocean Engineering Department,
Istanbul Technical University,
Maslak, Istanbul,
Turkey

INTRODUCTION

Pharmaceutical companies produce a large variety of products that are used in both human and veterinary medicines. The manufacturing processes that prominent pharmaceutical companies use incorporate five main processes: fermentation, extraction, chemical synthesis, formulation, and packaging. Although all of these processes produce some wastewater, it is the fermentation and synthesis operations that generate the largest amount of waste product; also, the wastewaters from these processes contain the highest amount of organic load (Sarantopoulos *et al.* 1995; Larsson *et al.* 2007). These active compounds also cannot be completely metabolized by the human body and cannot be removed completely in sewage treatment systems; they can be found in wastewater (Kümmerer 2009; Santos *et al.* 2010). Although the concentration of these antibiotics is relatively low in raw domestic wastewater, it can be significantly higher in hospital and pharmaceutical production facilities' effluents, reaching around the 100–500 mg/L level (Kümmerer 2001; Amin *et al.* 2006). This accumulation can adversely affect and change the microbial community that is present in biological wastewater treatment processes (Selvam *et al.* 2012; Resende *et al.* 2014).

Two different biological treatment processes have been used to treat and control the wastewater that is produced as a byproduct of pharmaceutical manufacturing techniques: aerobic and anaerobic (Amin *et al.* 2006; Fountoulakis *et al.* 2008; Cetecioglu *et al.* 2013; Dorival-García *et al.* 2013). While activated sludge treatment has been used to treat wastewater, this process is unsuitable in situations where the chemical oxygen demand (COD) levels of the water exceed 1,500 mg/L (Chelliapan *et al.* 2006; Oktem *et al.* 2008). In recent years, research indicates that the high COD concentration of pharmaceutical industry wastewater makes anaerobic technology a favorable treatment (Shimada *et al.* 2008; Cetecioglu *et al.* 2012). This

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process can be applied to high strength wastewater, it uses less energy, has a lower sludge yield and nutrient requirements, is cheaper to implement, uses less space, and offers improved biogas recovery. Although research in this area is promising, there are a limited number of experimental studies investigating the effects of antibiotics on the treatment of pharmaceutical wastewater using an anaerobic process (Lallai *et al.* 2002).

Antibiotics are not present as single compound substances in the environment but they typically occur within some form of mixture (Cleuvers 2004; Pomati *et al.* 2008; Backhaus *et al.* 2004). The effects of mixtures will be different from the single compounds which can be antagonistic or synergistic. The combination effect is generally higher than the effects of its individual components even if all components are present only in low concentrations that do not provoke significant toxic effects (Cleuvers 2004; Backhaus *et al.* 2004). This study assesses the chronic impact of sulfamethoxazole (SMX) and erythromycin (ERY) on the anaerobic process. Each is a common component of pharmaceuticals that are produced for human and veterinary consumption (Chopra & Roberts 2001; Tenson *et al.* 2003; Turkdogan & Yetilmezsoy 2009; Baran *et al.* 2011).

Through examining the biodegradation of organic substrate under anaerobic conditions, the research aim to provide valid insights into the substrate treatment efficiency, biogas/methane production, and antibiotic reduction in a laboratory-scale anaerobic sequencing batch reactor (ASBR), fed synthetic wastewater amended with antibiotics at concentrations representative of the waste streams typically produced by a pharmaceutical plant throughout a year's operation. This research was also used to determine the extent to which anaerobic processes can treat pharmaceutical wastewater which contains erythromycin–sulfamethoxazole (ES) antibiotic mixtures.

MATERIALS AND METHODS

The experimental approach

The purpose of this experiment was to study how anaerobic treatment performance is impacted by the presence of ERY and SMX at concentrations relevant to pharmaceutical wastewater. Two identical 1.75 L ASBR systems were constructed for the present study. An ASBR was run in a daily 'fill and draw' mode using a synthetic substrate mixture including volatile fatty acids (VFAs), glucose, and starch that resembled the wastewater from a pharmaceutical facility. The operation of the ASBR included a start-up period of approximately 90 days for acclimation and the establishment of steady-state conditions. Its performance was observed during the next 94 days under steady-state conditions to make sure these conditions prevailed before semi-continuous exposure to ES mixtures. The influent antibiotics concentration was gradually increased through successive phases for 30 days until the metabolic collapse of the reactors, which lasted for 10 stages for the ES reactors. Antibiotic concentrations in each stage are shown in Table 1. The concentrations of antibiotics were selected based on levels of antibiotics inhibition data by Gartiser *et al.* (2007) and Cetecioglu *et al.* (2013). The antibiotics dosing stopped after total collapse of the reactor in order to observe a possible recovery of the reactor's performance during the next 30 days. A second ASBR was operated in parallel for the entire period under identical conditions, but without antibiotic dosing, serving as a control reactor.

Evaluation of the ASBR performance was predominantly based on daily measurements of soluble COD and VFA concentrations determined both in the influent and effluent streams. They were accompanied with parallel daily measurements of biogas production and composition, assessing main fractions such as CH_4, CO_2, and H_2.

Operation of ASBR systems

Two ASBRs with a liquid volume of 1.5 L were inoculated using granular sludge from an anaerobic contact reactor treating raki and fresh grape alcohol wastewater, and operated as ASBRs with 24-h cycles (10 min feeding, 23 h 40 min reaction, 1 min settling, and 9 min liquid withdrawal). The three anaerobic reactors were initially operated

Table 1 | Tested antibiotic concentrations

	Sulfamethoxazole (mg/L)	Erythromycin (mg/L)
Stage 1	0.5	0.1
Stage 2	5	0.2
Stage 3	5	0.5
Stage 4	10	0.5
Stage 5	10	1
Stage 6	15	1
Stage 7	15	1.5
Stage 8	20	1.5
Stage 9	20	2
Stage 10	25	2.5

to reach a steady state at an organic loading rate of 2.5 kg COD/m^3·d at which point daily antibiotic additions were started. Throughout the operation, hydraulic retention time of 2.5 days and a solids retention time of 30 days were used. Reactor temperatures of $35 \pm 2\,^{\circ}C$ and continuous mixing at 90 rpm were maintained. Stable operation was reached on the 90th day of reactor operation. The amount of mixed liquor volatile suspended solids was fixed at 5,000 mg/L. The composition of synthetic wastewater constituted 1,160 mg COD/L starch, 750 mg COD/L glucose, 135 mg/L COD sodium acetate, 183 mg/L COD sodium butyrate, and 272 mg/L COD sodium propionate. The trace element solution was adopted from a previous study (Cetecioglu *et al*. 2013) and contained (mg/L) $FeCl_2{\cdot}4H_2O$, 2; $CoCl_2{\cdot}6H_2O$, 2; $MnCl_2$, 0.32; $CuCl_2$, 0.024; $ZnCl_2$, 0.05; H_3BO_3, 0.05; $(NH_4)Mo_7O_{24}{\cdot}4H_2O$, 0.09; Na_2SeO_3, 0.068; $NiCl_2{\cdot}6H_2O$, 0.05; EDTA, 1 and resazurin, 0.5, HCl (36%) 0.001 mL and vitamins (mg/L) 4-aminobenzoic acid, 0.04; D(+)-biotin, 0.01; nicotinic acid, 0.1; calcium D(+)-pantothenate 0.05; pyroxidinedihydrochloride, 0.15; thiamine, 0.1 in NaP buffer (10 mM, pH 7.1) and 0.05 mg/L B12, and was added to the wastewater. The pH of the A SBRs were daily adjusted to 6.8–7.2 by addition of 1,000 mg/L $CaCO_3$ alkalinity for sustaining operational stability.

Analytical methods

Sample analysis included soluble COD, alkalinity, suspended solids, volatile suspended solids, and total volatile suspended solids, all according to *Standard Methods* (APHA 2005). Methane content in the biogas and VFA concentrations were measured using gas chromatography (Perichrom, France and Agilent Technologies 6890N, USA, respectively).

ERY and SMX assay

The ERY and SMX assay was performed using a Shimadzu high-performance liquid chromatography instrument (Shimadzu LC-10 AD) equipped with a UV light detector (UV-Vis Detector, SPD 10-A) by injecting sample solutions onto a C18 analytical column. ERY's gradient elution was applied using (A) 32 mM potassium phosphate buffer by dissolving 5.57 g dipotassium hydrogen phosphate in 1,000 mL water adjusted with concentrated phosphoric acid to pH 8.0 and a mixture (B) of acetonitrile/methanol (75/25). The gradient was run with 33% B from 0 to 28 min and 33–45% B from 28 to 60 min, post run with 33% B for 10 min. ERY was detected at 215 nm. The flow rate was 1.0 mL/min (Deubel & Holzgrabe 2007). Sulfamethoxazole was eluted with 0.1% formic acid in acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). The mobile phase composition was changed as follows: A:B = 5:95 at the beginning and then rising to 30:70 from 0 to 7 min. Equilibration was then performed from 7 to 8.5 min at 30:70 and then returned to 5:95 from 8.5 to 10 min. The flow rate was 0.7 mL/min. Detection was carried out at 270 nm (Karcı & Balcıoğlu 2009).

Statistical analysis

To determine the statistical significance of antibiotic mixtures' inhibition (ES), COD removal efficiencies of the ASBRs were compared using a one-way analysis of variance (ANOVA) test, followed by running Student's *t*-test. Significant differences were determined at the $p < 0.05$ level of significance.

RESULTS AND DISCUSSION

COD removal

Efficient COD removal was observed during stage 1 in the ES reactor: soluble COD in the effluent was reduced from an initial COD concentration of 2,500 mg/L at the beginning of each cycle to 93 ± 5 mg/L, corresponding to an efficiency higher than 95% (Figure 1). Similar COD removal could be maintained in the control reactor for the entire monitoring period. Soluble COD removal efficiency was not affected significantly ($p < 0.05$) by the addition of ES mixtures from stage 2 to stage 3 in the ES reactor influent (days 90–150). The first remarkable effect of ES mixtures on the reactor performance was detected in stage 4 on day 155. A substantial increase in the effluent soluble COD concentration to 480 ± 51 mg/L in the ES reactor was observed at stage 4, whereas the effluent soluble COD concentration of the control reactor was 64.2 ± 38.5 mg/L ($p < 0.05$). Consequently reactor performance decreased substantially after stage 9 of ES mixture (30 mg/L) addition between the 330th and 360th days. In contrast to this result, Sponza & Demirden (2007) found that the COD removal efficiency decreased from 87 to 68% when sulfamerazine (sulfonamide group antibiotics) concentration was increased from 10 to 90 mg/L. This contradiction could originate from different anaerobic reactor types, operation conditions, or antibiotic combinations used in the studies. At the end of stage 10 in the ES reactor, antibiotics dosing was stopped in order to observe any possible recovery in the reactor performance. However, the metabolic activity of the biomass could not be re-activated to induce noticeable

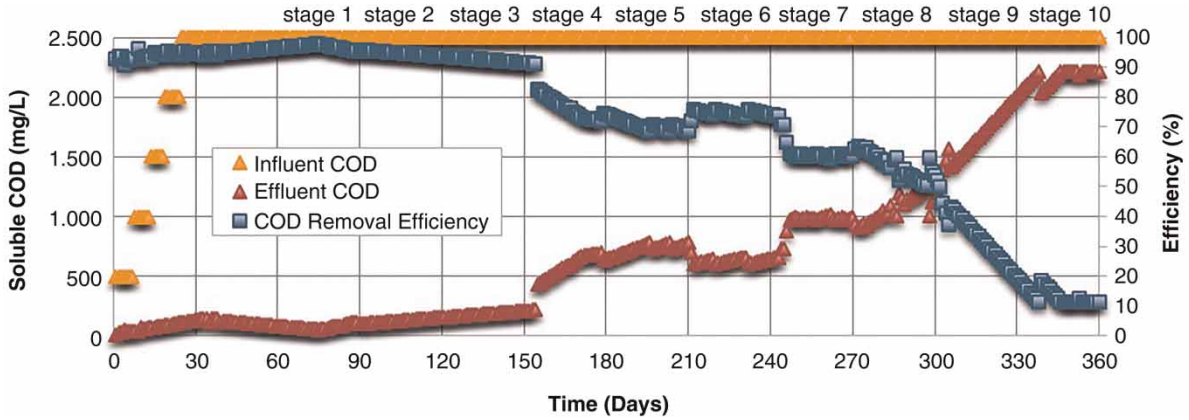

Figure 1 | COD removal efficiency in the ES reactor.

substrate utilization and the reactor operation was terminated on day 390 in the ES reactor.

Biogas production

Biogas production was monitored in all stages throughout the operation of the reactor, particularly for the assessment of methanogenic activity. Figure 2 illustrates the biogas produced in all stages of the ES reactor. Biogas production was parallel to COD removal efficiency in the ES reactors. Biogas generation showed a significant ($p < 0.05$) reduction in stage 4 for the ES reactor ($70 \pm 2\%$). Meanwhile, the amount of biogas production in the control reactor was almost constant through the operational time ($1{,}240 \pm 3$ mL/d).

Methane yield (L methane produced per gram COD removed) can be a useful parameter to assess the performance of an anaerobic reactor. Figure 3 shows that the methane yield dropped dramatically in stage 4 when the ES dosing changed from 21.5 to 20 mg/L. It dropped from 44 to 30% between days 210 and 270. Consequently methane yield decreased substantially after stage 10, with the addition of ES mixture (27.5 mg/L) on day 150. Methane production in all reactors showed a strong correlation with biogas production during the operational time, showing that the inhibitory effects of antibiotics on methanogen activity was somehow the same as that on the other anaerobic microorganisms. The average amount of methane production in the control reactor was 879 ± 4 mL/d. Therefore, the methane percentage of biogas production in the control reactor can be calculated at about 66%, indicating a methane production yield, Y_{CH4}, of 0.32 L/g COD removed. This level conforms with the default value which had been reported by Tchobanoglous *et al.* (2003).

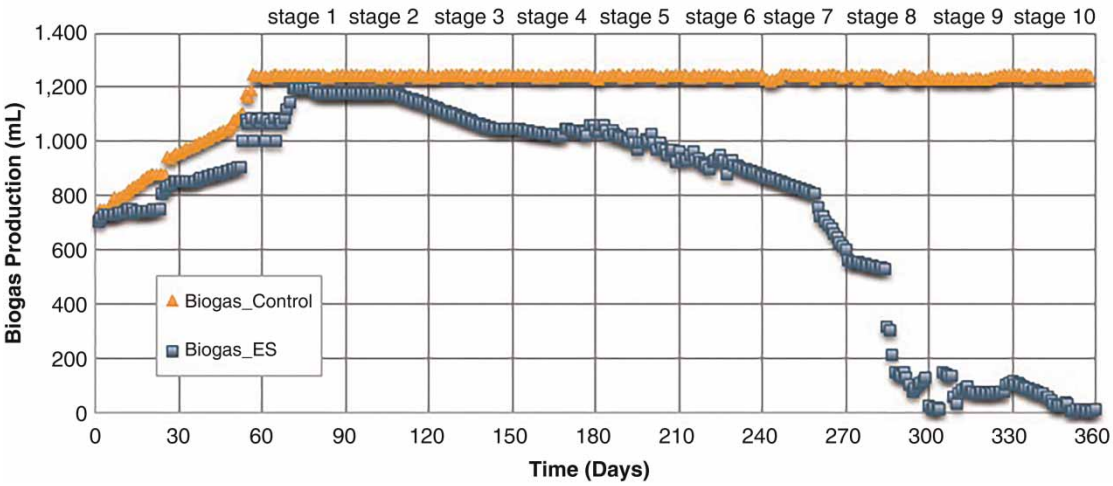

Figure 2 | Biogas production in the control and ES reactor.

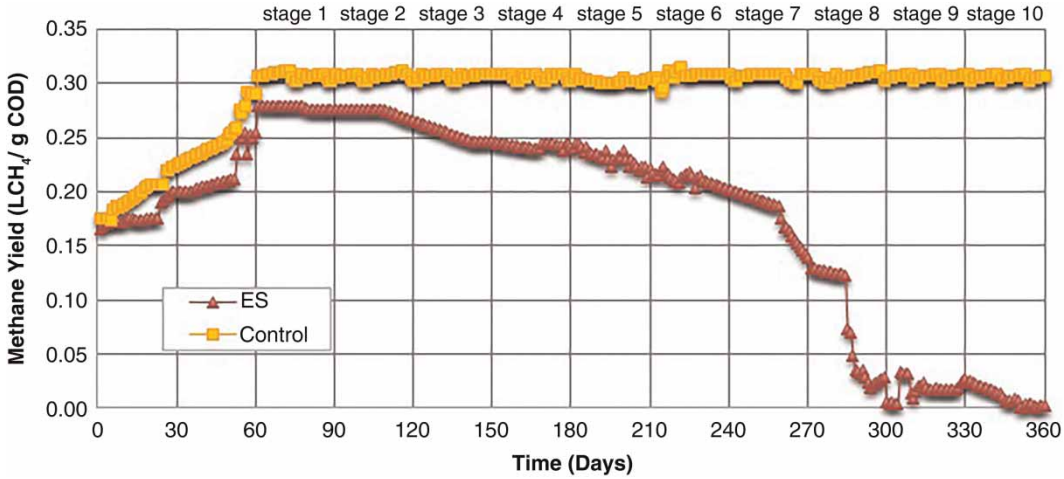

Figure 3 | Methane yield in the control and ES reactor.

Gartiser *et al.* (2007) indicated that ERY and SMX had no inhibition effect on biogas production in which yeast extract was used as substrate. Fountoulakis *et al.* (2004) also stated that SMX had not affected methanogenesis even at high concentrations (400 mg/L). Similarly, Sanz *et al.* (1996) tested inhibitory effects of antibiotics on an anaerobic system using a volatile acid mixture of acetate, butyrate, and propionate as carbon source. The authors stated that ERY had no effects on anaerobic digestion. On the other hand, a limited study about the combined effect of antibiotic on biogas production was found in the literature. Christensen *et al.* (2006) observed significant synergistic effects of antibiotic mixtures including ERY and oxytetracycline on activated sludge sample. The results pointed out that antibiotics revealed synergistic effects on activated sludge bacteria. Also, Alighardashi *et al.* (2009) pointed out that at concentrations higher than 20 mg/L ERY significantly induces inhibition on ammonification, nitritation, and nitratation. The impacts of antibiotic mixtures on a mixed culture may be different than on pure cultures because each group of bacteria may have a different kind of response to the different group of antibiotics. With the addition of antibiotic mixtures which have clear effects on each group of microorganisms, all bacterial groups are inhibited; as a result, the mixture toxicity will appear to be synergistic.

Effluent VFA composition

The presence and concentration of the VFAs in all stages of the ES reactor are shown in Figure 4. During the entire operation period, VFAs could not be detected in the effluent of the control reactor. Furthermore, VFAs were not detected in the ES reactor's effluent until the 110th day (stage 4). On this day, acetic acid and propionic acid accumulation started at 47 and 59 mg/L, respectively. Propionic acid

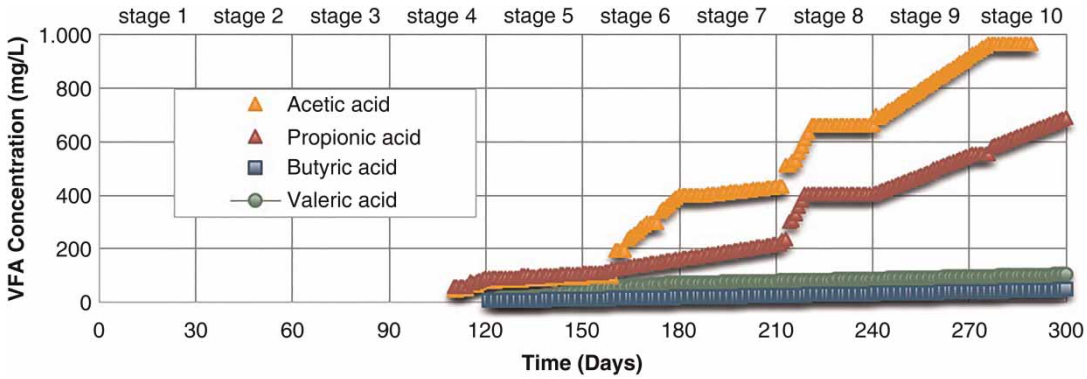

Figure 4 | VFA profile in the ES reactor.

concentration increased day to day and reached 691 mg/L at the end of operation. Acetic acid accumulation displayed a similar trend; however, the concentration was higher than butyric acid. At stage 10, acetic acid, propionic acid, butyric acid, and valeric acid were detected at 1,005, 691, 49, and 105 mg/L, respectively.

The results indicate that ES combinations have a more dramatic effect on Gram-negative bacteria than Gram-positive bacteria. The degradation of propionate is most often utilized by Gram-negative bacteria (e.g., *Syntrophobacter* spp., *Pelotomaculum* spp.), called SPOB (syntrophic propionate-oxidizing bacteria), and combinations of ES antibiotics would be expected to inhibit sensitive strains of this microbial group (Stams *et al*. 2012). Also, a clear effect of the ES reactor on acetoclastic methanogens, which utilize acetate to produce methane, was observed in the VFA results (Cetecioglu *et al*. 2012).

ERY and SMX degradation

The efficiency of ERY and SMX reduction in different stages of the ES reactor operation, using the expression defined above, is illustrated in Figures 5(a) and 5(b). SMX reduction patterns that started at 42%, increased to 44% in stage 3, and sustained at around 20% at the end of stage 10; ERY reduction started at 40% then increased to 60% at stages 2–4 and continued to 12% at the end of stage 10, where substrate/COD utilization and biogas production was practically stopped. Also, a comparison of SMX and ERY removal behavior in the ES reactor demonstrated that ERY had a higher removal efficiency than SMX.

Results obtained from the ES reactor showed that the increase of antibiotic combination dosages caused a decrease in antibiotic removal efficiency, but when repeating a constant dosage in the next phase the reactor showed a non-significant removal efficiency because of the microorganisms' developing resistance to antibiotics. Antibiotic resistance genes can be transferred between bacteria that are found in the environment through plasmids, integrons, and transposons (Pruden *et al*. 2006). It is largely accepted that an aqueous environment provides the ideal conditions through which resistant genes can be transferred between bacteria (Baquero *et al*. 2008). Fan & He (2011) established that the existence of ERY at concentrations as low as those found in the natural environment can significantly increase antibiotic resistance. Furthermore, Gao *et al*. (2012) found that there is a positive relationship between antibiotics and the numbers of antibiotic resistance genes and bacteria found in conventional wastewater treatment plants.

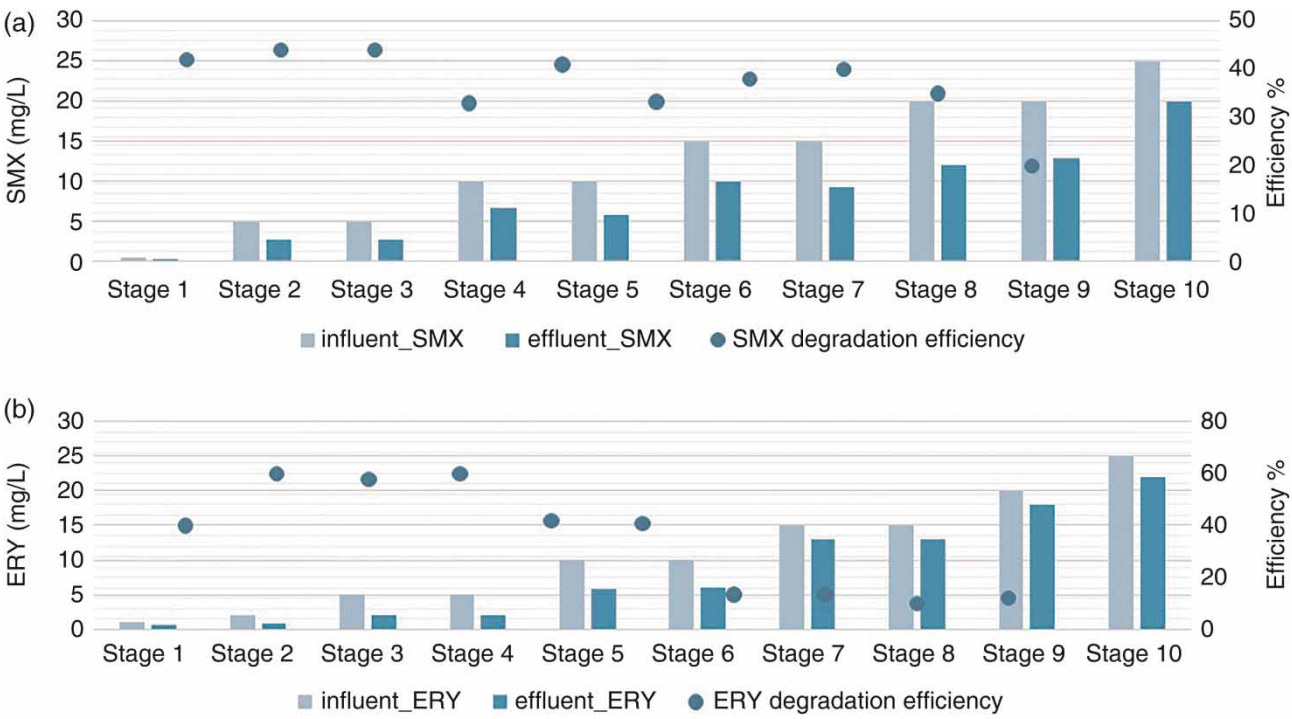

Figure 5 | Sulfamethoxazole (a) and ERY (b) measurement results of the ES reactor.

CONCLUSIONS

The results suggested that substrate/COD utilization and biogas/methane generation for the ES reactor decreased with the increase of antibiotic concentrations in influent, probably due to the inhibitory effects on certain enzymatic steps in related metabolic reactions. The fourth stage was the critical phase for the ES reactor. After this stage, the performance of the reactor decreased rapidly. For the selected conditions of the study the terminal dose for the ES reactor was 2.5 mg/L ERY and 25 mg/L SMX. According to VFA results, a clear effect of the ES reactor on acetoclastic methanogens, which utilize acetate to produce methane, was observed in the VFA results. Also, ES combinations have a more dramatic effect on Gram-negative bacteria than Gram-positive bacteria. Results obtained from the ES reactor showed that the increase of antibiotic combination dosages caused a decrease in antibiotic removal efficiency, but, when repeating a constant dosage in the next phase, the reactor showed non-significant removal efficiency because of the microorganisms' development of resistance to antibiotics.

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