Inhibitory effect of erythromycin, tetracycline and sulfamethoxazole antibiotics on anaerobic treatment of a pharmaceutical wastewater
Sevcan Aydin, Bahar Ince and Orhan Ince

ABSTRACT
Pharmaceuticals enter ecosystems, which causes changes to microbial community structure and development of resistant genes. Anaerobic treatments can be an alternative application for treatment of pharmaceutical wastewaters, which has high organic content. This study aims to develop an understanding of the effects of sulfamethoxazole–erythromycin–tetracycline (ETS), sulfamethoxazole–tetracycline (ST), erythromycin–sulfamethoxazole (ES) and erythromycin–tetracycline (ET) combinations on the anaerobic treatment of pharmaceutical industry wastewater. The results of this investigation revealed that bacteria have a competitive advantage over archaea under all antibiotic combinations. The ET reactor showed a better performance compared to other reactors; this could be due to antagonistic effects of sulfamethoxazole. Acute inhibition in the microbial community was also strongly affected by antibiotics concentrations. This indicated that the composition of the microbial community changed in association with anaerobic sequencing batch reactor performances. The results of this research support the idea that an acute test could be used to control and improve the anaerobic treatment system.

KEY WORDS | acetoclastic methanogens, acute inhibition, anaerobic treatment, bacteria, combined effect, microbial community

INTRODUCTION
Wastewater produced from the pharmaceutical industry is characterized by high chemical oxygen demand (COD) concentration. These kinds of effluents, however, contain a wide range of recalcitrant compounds such as antibiotics which can cause serious environmental pollution and affect biological treatment processes (Alexy et al. 2004; Fountoulakis et al. 2008; Kuemmerer 2009). Owing to high organic content, anaerobic treatment is a promising technology to treat effectively pharmaceutical manufacturing wastewaters, simultaneously with the generation of bioenergy resources. For instance, Sreekanth et al. (2009) investigated COD removal efficiency of a hybrid upflow anaerobic sludge blanket reactor treating a pharmaceutical wastewater. According to the results, 65–75% and 80–94% COD and biochemical oxygen demand (BOD) removal efficiencies at an organic loading rate of 9 kg COD/(m³ d) with 60–70% of methane in biogas were achieved. Successful treatment of pharmaceutical wastewater has also been reported by Gangagni Rao et al. (2005), in which 60–70% of COD and 80–90% of BOD removal efficiencies were achieved in an anaerobic fixed film reactor. Similarly, Chelliapan et al. (2006) reported 93% COD removal efficiency in an upflow anaerobic stage reactor treating a pharmaceutical wastewater containing 0–400 mg/L tylosin. At increased tylosin concentrations of 600 and 800 mg/L, the COD removal efficiencies declined to 85% and 75%, respectively. Although research in this area is promising, the number of experimental studies investigating the combined effects of antibiotics on anaerobic treatment of pharmaceutical wastewaters are scarce. During the use of two or more antibiotics simultaneously, interactions between these components may lead to an additive (the activity of antibiotic combination is equal to the sum of their independent activity when studied separately), synergistic (the activity of antibiotic combination is greater than the sum of their independent activity when studied separately) or antagonistic (the activity of antibiotic combination is less
than the effect of the respective single antibiotics) effects. For example, Christensen et al. (2006) observed the significant synergistic effects of antibiotic mixtures including erythromycin and oxytetracycline on activated sludge samples. Aydin et al. (2015a) evaluated the acute joint inhibitory effects of sulfamethoxazole, erythromycin and tetracycline antibiotics on anaerobic microbial community. According to the results, the authors asserted that remarkable synergistic and antagonistic effects were observed in different antibiotic combination in anaerobic sequencing batch reactors (ASBRs).

The anaerobic process is complex with a number of sequential and parallel steps that are carried out by mainly four groups of micro-organisms: primary fermenting bacteria, syntrophic acetogens, homoacetogens and methanogenic archaea (Stams et al. 2002). Complex microbial interactions are critical for stable performance of anaerobic systems. Methanogens catalyze the terminal stage of the process and are normally divided into two main groups based on their substrate conversion capabilities (Amin et al. 2006). Acetoelastic methanogens are capable of converting acetate to methane and CO2, and are regarded as playing a dominant role in CH4 production since 70% of the methane produced in digesters comes from acetate. Hydrogenotrophic methanogens convert H2/CO2 to methane. These species also play a key role in the overall process by maintaining the very low partial pressures of H2 necessary for the functioning of the intermediate trophic group, the syntrophic bacteria, which are responsible for the conversion of organic acid and alcohol intermediates to direct methane precursors (Li et al. 2012). Conversely, a short contact time between high inhibitor concentration and biomass has a positive impact on acetogens, which have faster growth kinetics and better rate of adaptation than methanogens (Aydin et al. 2015a, b).

The present study was carried out to establish data for acute toxicity, which describes the adverse effects of different antibiotic combinations in a short exposure time and the adverse effects may happen within 15 days of the administration of the inhibitor substance. The term half-maximal effective concentration (EC50) refers to the inhibitor concentration that results in 50% inhibition on measured response of the microbial community. It is a common parameter used for the assessment of inhibitory impact (Ruiz et al. 2009).

The human and veterinary antibiotics sulfamethoxazole (S), erythromycin (E) and tetracycline (T) are the most commonly used pharmaceuticals. Tetracycline and erythromycin inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site by binding irreversibly to the 50S and 50S bacterial rRNA subunits, respectively (Chopra & Roberts 2001; Tenson et al. 2003). Sulfamethoxazole is the inhibitor of nucleic acid synthesis, which is a necessary component for folic acid synthesis (McDermott et al. 2003).

The goal of this research was to understand the combined effects of sulfamethoxazole–erythromycin–tetracycline (ETS), sulfamethoxazole–tetracycline (ST), erythromycin–sulfamethoxazole (ES) and erythromycin–tetracycline (ET) on the anaerobic treatment in terms of bacteria and methanogens. For this purpose, five laboratory-scale ASBRs were operated to treat synthetic pharmaceutical wastewater including ETS, ST, ES and ET combinations for 1 year. The combined effects of antibiotics were determined using specific methanogenic activity (SMA) tests. SMA tests were conducted with various antibiotic combinations and the effects on syntrophic bacteria, homoacetogens and methanogens of ASBRs were evaluated.

**MATERIALS AND METHODS**

The experimental approach

The experimental setup consisted of five ASBRs with identical dimensions and configurations, which are comprehensively explained in the studies of Aydin et al. (2014, 2015b, c). The influent antibiotic concentrations were gradually increased through successive phases, each lasting for 30 days, until metabolic collapse of the anaerobic batch reactors. The antibiotic concentrations used in each stage are given in Table 1.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Sulfamethoxazole (mg/L)</th>
<th>Erythromycin (mg/L)</th>
<th>Tetracycline (mg/L)</th>
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<tbody>
<tr>
<td>Stage 1</td>
<td>0.5</td>
<td>0.1</td>
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<tr>
<td>Stage 2</td>
<td>5</td>
<td>0.2</td>
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<tr>
<td>Stage 3</td>
<td>5</td>
<td>0.5</td>
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<tr>
<td>Stage 4</td>
<td>10</td>
<td>0.5</td>
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<td>Stage 5</td>
<td>10</td>
<td>1</td>
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<td>Stage 6</td>
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<tr>
<td>Stage 7</td>
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<td>Stage 8</td>
<td>20</td>
<td>1.5</td>
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<td>Stage 9</td>
<td>20</td>
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<td>Stage 10</td>
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<td>Stage 12</td>
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<td>3</td>
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<tr>
<td>Stage 13</td>
<td>40</td>
<td>4</td>
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</table>

Table 1 | Tested antibiotic concentrations
The performance of the reactors was observed during the operational period which was 360 days (10th) for the ST reactor, 440 days (13th) for the ET reactor, 360 days (10th) for the ES reactor and 420 days (12th) for the ETS reactor. A fifth ASBR, which was fed free of antibiotics, was operated in parallel for the entire period under identical operating conditions and serving as a control reactor (Aydin et al. 2014, 2015b, c). Acute tests were set up based on SMA test principles to evaluate bacteria and methanogens. SMA experiments were seeded with biomass collected in each stage of the ST, ES, ET and ETS reactors (Aydin et al. 2015a).

Start-up and operation of ASBRs

The five anaerobic SBRs were set up and operated as described by Aydin et al. (2014, 2015b, c). Briefly, the reactors with a 1.5 L liquid volume were inoculated using a granular sludge from an anaerobic reactor treating raki and fresh grape alcohol wastewaters and operated as ASBRs with 24-h cycles. The trace element solution was adopted from a previous study (Aydin et al. 2015a).

Acute tests

The experiments were designed to evaluate the long-term effect of the various antibiotic combinations (ETS, ST, ES and ET) using an acute test with biomass which was collected from each stage of the ASBRs in duplicate bottles. These experiments were designed as three sets: butyrate feeding to evaluate syntrophic butyrate-oxidizing bacteria, propionate feeding to evaluate syntrophic propionate-oxidizing bacteria and volatile fatty acid (VFA) mixture feeding to evaluate homoacetogens and all methanogens. Acute tests were conducted as batch studies that were based on SMA test principles (Aydin et al. 2015a). Glass serum bottles with 120 mL total volume (100 mL liquid volume and 20 mL headspace volume) were sealed with butyl rubber septa. Before the inhibition experiments, various substrate concentrations (500, 1,000, 1,500, and 2,000 mg/L) were tested in order to determine the optimum volatile suspended solids (VSS) concentrations as given in the studies of Aydin et al. (2015a, d). Batch reactors were seeded with biomass from ASBRs with 1,000 mg/L VSS. Among those tests, 1,500 mg/L was found to be the optimal substrate concentration. The test medium and the trace element solution were prepared according to the OECD Test No. 311 (OECD 2006) protocol under strict anaerobic conditions. The SMA tests were run for 18 days. The SMA bottles were stored at 35 ± 2 ºC, shaken daily by hand and headspace pressure was measured daily by a hand-held pressure transducer (Lutron PM-9107, USA).

Analytical methods

Gas compositions and VFA concentrations were determined using gas chromatographs with a flame ionization detector (Perichrom, France and Agilent Technologies 6890N, USA, respectively). The column used was Elite FFAP (30 m × 0.32 mm). The set point of the oven and maximum temperature of inlet were 100 ºC and 240 ºC, respectively. Helium gas was used as a carrier gas at a rate of 0.8 mL/min. Headspace pressure was measured daily by a hand-held pressure transducer (Lutron PM-9107, USA).

Statistical analysis

Statistical analyses were conducted in MINITAB. One-way analysis of variance and the Tukey test were used to determine whether significant differences existed for acute tests. In addition to calculating the global significance values (p-values), the data were analyzed pairwise using the Tukey test to deliver detailed significant differences between total methane production and antibiotic concentrations. Significant differences were determined at the p < 0.05 level of significance.

RESULTS AND DISCUSSION

Performance of ASBR

The composition of the VFAs in the effluents was analyzed for providing additional information about the inhibitory and toxic effects of antibiotics on the performance of studied reactors. During the entire operation period, VFAs could not be detected in the effluent of the control reactor.VFAs were also not detected in the ST and ES reactors’ effluent until the 110th day (stage 4). On this day, VFA accumulation started at 47 mg/L and 59 mg/L in the ST and ES reactors, respectively. At stage 10, VFAs were detected at 1,601 and 1,691 mg/L in the ST and ES reactors. The results indicated that the ST and ES antibiotic combination affected the VFA utilization pathways after 15 + 1.5 mg/L for ST and 1.5 + 15 mg/L for ES concentrations (Aydin et al. 2014, 2015b, c).
The first VFA in the effluent of the ETS and ET reactor was measured on the 180th and 190th day, the first day of stage 7, at 50 and 70 mg/L of acetate. On the 240th day, the VFA concentration increased to 1,210 and 1,320 mg/L in the ETS and ET reactors, respectively. The VFA increased day to day and reached 1,700 and 1,830 mg/L, respectively, until the reactor operation was terminated (Aydin et al. 2014, 2015b, c).

A clear effect of the ETS, ST, ES and ET on microbial community was observed in the VFA results. Also, the results obtained from this study support previous works in which inhibition of VFA uptake was observed in the presence of antibiotic (Sanz et al. 1996; Shimada et al. 2008; Aydin et al. 2014, 2015b, c).

The effect on the propionate and butyrate consumption by the sludge

To determine long-term effect of antibiotic combinations on syntrophic bacteria, the SMA test was performed with sludges taken from different operation stages of the ETS, ST, ES and ET reactors. Butyrate and propionate were selected as a sole carbon and energy source to examine the inhibition effects of the antibiotic combinations of ASBRs on syntrophic butyrate-oxidizing and propionate-oxidizing bacteria, respectively. Inhibition effects of all antibiotic combinations on the total methane production and VFA accumulation are shown in Figure 1(a) and 1(b) and Figure 2(a) and 2(b).

![Figure 1](https://iwaponline.com/wst/article-pdf/71/11/1620/468571/wst071111620.pdf)
Acute tests revealed that a significant ($p < 0.05$) decrease on the total methane production and VFA measurement in the presence of $15 + 1.5 + 1.5$ mg/L of ETS, $1.5 + 1.5$ mg/L of ET, and $10 + 1$ mg/L of ST and of ES combinations for butyrate substrates. As depicted in Figure 1, the highest inhibitory effect on the total methane production and butyrate accumulation was determined at the last stage of each antibiotic combination.

Figure 2 shows that a significant inhibitory effect ($p < 0.05$) on propionate uptake and total methane production was observed when $20 + 1.5 + 1.5$ mg/L of ETS, $1.5 + 1.5$ mg/L of ET, and $10 + 1$ mg/L of ST and of ES were added to the ASBRs. This was similar to the butyrate test result except for the ETS and ES reactors. Our results indicated that ST and ES combinations have a more dramatic effect on Gram-positive bacteria than Gram-negative bacteria and also these reactors were in metabolic collapse before the ETS and ET reactors. One possible explanation is that Gram-positive bacteria are important for degradation of antibiotics in anaerobic treatment.

Gartiser et al. (2007) performed a study on the individual inhibitory effects of antibiotics on methanogenic activity. The authors indicated that erythromycin and sulfamethoxazole had no inhibition effect on biogas production. Fountoulakis et al. (2008) also found that sulfamethoxazole had not affected methanogenesis even at high concentrations (400 mg/L). Similarly, Sanz et al. (1996) established 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 erythromycin had no effect on anaerobic digestion. However, in the present study, all antibiotic combinations had higher inhibitory effects on anaerobic process than did individual components. Christensen et al. (2006) also observed the significant synergistic effects of antibiotic mixtures including erythromycin 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, erythromycin induces inhibition on ammonification, nitrification and nitratation significantly. The impacts of antibiotic combinations on biological treatment systems may be different than on pure cultures, as each group of bacteria may have different kinds of response to the different antibiotics. With the addition of antibiotic combinations which have clear effects on each group of micro-organisms, bacterial groups might be inhibited; as a result, the mixture toxicity will appear to be synergistic (Backhaus et al. 2004).

**Acute effects on methanogenic activity**

The SMA test was performed to determine the VFA utilization capacity of the anaerobic sludge, taken from different stages of the ST, ES, ET and ETS reactors, on methanogens. The total methane production was 68 mL which accounted for approximately 87% of biogas produced in control reactors. One hundred percent inhibition of methane production was not observed in all test bottles. The total methane production profiles of the SMA tests are given in Figure 3.

In the ASBRs fed with ST and ES combinations, a significant inhibitory effect ($p < 0.05$) on methane production was observed at stage 3. Methane generation decreased with increasing antibiotic concentration gradually until stage 8. The inhibition rate of methane yield reached 41% and 44% at stage 8 when compared to the control bottle in the ST and ES reactors, respectively. Furthermore, the total methane production dropped to 14 and 11 mL dramatically at stage 10, which dropped 20 and 16% compared to the control reactor, for the ST and ES reactors.

ETS and ET dosage of stages 1–6 generated a methane production profile quite close to the control reactor with total biogas volumes close to 90 mL and methane volume above 67 mL. SMA batch tests showed a significant ($p < 0.05$) decrease on the total methane production in the presence of $15 + 1 + 1$ mg/L (stage 6) of ETS and $1 + 1$ mg/L (stage 6) of ET combinations. The highest inhibitory effect on the total methane production, which dropped 14 and 5% compared with control, was determined at $40 + 3 + 3$ mg/L (stage 12) ETS and $4 + 4$ mg/L (at stage 12) ET combinations. However, Amin et al. (2006) mentioned that the removal of acetic acid in the SMA test was not effected even in the presence of high erythromycin concentration such as 200–500 mg/L.

The presence and composition of the VFA concentration in all stages of the ASBRs are shown in Figure 4(a)–4(d). A significant inhibition effect ($p < 0.05$) on acetate utilization was observed at stage 3 for ST and ES combinations and stage 5 for ETS and ET combinations. Statistical analysis also showed that VFA accumulation was negatively correlated with total methane production ($p < 0.05$) and positively correlated with antibiotic concentration.

SMA results indicated that antibiotic combinations have a more dramatic effect on archaea than bacteria. These results are consistent with those of other studies and suggest that archaea are more sensitive to changes in antibiotic combinations than are bacteria present in ASBRs, and effective control is a necessity for successful operation of anaerobic

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**Figure 3** | Inhibition effect of antibiotic mixtures on total methane production in effluents of VFA mixture set.
systems (Yu et al. 2014). The most likely explanation is that bacteria are capable of faster growth kinetics and a better rate of adaptation to inhibitory components than are archaea. The Gibbs free energy change in all archaea is always very low compared to bacteria. Therefore, archaea always have slow growth rates (Ma et al. 2015).

In this study, EC$_{20}$ and EC$_{50}$ values were calculated as 11.2 mg/L and 40.2 mg/L for ETS, 11.1 mg/L and 41.2 mg/L for ET, 2.1 mg/L and 21.3 mg/L for ST, and 2.7 mg/L and 21.0 mg/L for ES, respectively. The lowest EC$_{50}$ was for the ST and ES reactors. It was determined that ET, ST, ES and ETS antibiotic mixtures demonstrated a higher inhibition than antibiotics alone, and could have a considerable inhibition effect, even if all component mixtures were present in low concentrations.

Figures 3 and 4 show that in the combinations of ET and ST, although T is the same, higher inhibitory effects of ST are observed in all stages. This would cause each antibiotic combination to affect a unique population of micro-organisms. ST combination had a greatest inhibitory effect on isobutyric acid degradation; this could be due to an effect on the Gram-positive bacteria. Conversely, propionic acid is most often utilized by Gram-negative bacteria and ES combination may be expected to inhibit sensitive strains of this microbial group. These results support Aydin et al. (2014, 2015a) who suggest that Gram-negative bacteria are important for antibiotic degradation in the anaerobic process, and this microbial group could be controlled for treat effectively pharmaceutical manufacturing wastewaters.
CONCLUSIONS

This study has indicated that anaerobic treatment tolerated lower antibiotic combinations in long-term operation and also the effects of the antibiotic mixtures are different from each other. The ET reactor showed a better performance compared to ETS, ST and ES reactors; this could be due to antagonistic effects by sulfamethoxazole.

In view of the findings of this study, SMA analysis revealed that antibiotic combinations significantly affected microbial interactions between bacteria, archaea and methanogenic archaea. Our results also indicated that exposure to antibiotic mixtures affected specific propionate, acetate and butyrate degradation pathways and also this effect was changed by ETS, ST, ES and ET combinations. The composition of the microbial community changed in association with ASBR performances. For that reason, SMA analysis could be useful to monitor anaerobic treatment systems and to assess the condition of the reactors for control and improvement of such systems.

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