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Anaerobic sulfamethoxazole degradation is driven by homoacetogenesis coupled with hydrogenotrophic methanogenesis

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More than 40 mg/L of SMX inhibits acetoclastic methanogens.

High SMX exposure puts forward homoacetogenesis with hydrogenotrophic methanogenesis.

Long-term exposure on anaerobic microorganisms

Sulfamethoxazole

Cloning and sequencing

DGGE and PCA

Q-PCR-RT-QPCR

Acetoclastic methanogens

Homoacetogenesis with hydrogenotrophic methanogenesis
ANAEROBIC SULFAMETHOXAZOLE DEGRADATION IS DRIVEN BY HOMOACETOGENESIS COUPLED WITH HYDROGENOTROPHIC METHANOMENOSIS

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Abstract

In this study, microbial community dynamics were assessed in two lab-scale anaerobic sequencing batch reactors (ASBRs). One of the reactors was fed by synthetic pharmaceutical industry wastewater with sulfamethoxazole (SMX) as the test reactor and the other without sulfamethoxazole as the control reactor. DNA based DGGE results indicated that Clostridium sp. became dominant in the SMX reactor while the inoculum was dominated with Firmicutes (61%) and Methanomicrobiales (28%). However, their abundances in the active community decreased through the last phase. Also, the abundance of hydrogenotrophs was high in each phase, while acetoclastic methanogens disappeared in the last phase. Q-PCR analysis revealed that there is a significant reduction in the bacterial community.
approximately 84%, while methanogens increased to 97% through the operation. Additionally an increase in the expression level of bacterial and methanogenic 16S rRNA (60% and 20%, respectively) was detected. Significant correlation between microbial community and the reactor operation data was found. The study demonstrated that the microbial community maintains the system stability under high antibiotic concentration and long-term operation by homoacetogenesis coupled with hydrogenotrophic methanogenesis.

**Keywords:** 16S rRNA; anaerobic sequencing batch reactor; DGGE; microbial community; PCA; sulfamethoxazole

1. **Introduction**

Antibiotics serve as the main ingredients of health and protection against infectious environment in the modern society; they are also considered among major pollutants, as they are widely used and remain persistent to biodegradation by virtue of their lethal impact on microbial metabolism (Kümmerer et al., 2004). They tend to accumulate in the environment to alarming levels, because they mostly by-pass treatment systems (Müller et al., 2013).

Sulfamethoxazole (SMX) is a synthetic antibiotic within the sulfonamide (sulfa drugs) family; a group widely used both in veterinary and human medicine, mainly to treat urinary tract infections. SMX inhibits the multiplication of bacteria, since they are competitive inhibitors of p-amino benzoic acid in the folic acid metabolism cycle (Sweetman, 2009). It is frequently detected in various ecosystems, at concentrations of 70-150 ng/L in surface waters and 200-2000 ng/L in secondary wastewater effluents (Miao et al., 2004). It should be noted that the reported concentrations
indicate largely diluted levels in wastewaters. A better approach for antibiotic abatement would be to detect and remove these chemicals at primary sources. In a comprehensive review, Larsson (2015) reports a wide array of 11 – 1065 mg/l for different antibiotics in plant effluents, although SMX was not specifically mentioned (Larsson, 2014). In another study, tylosin concentration in the effluent of one pharmaceutical plant in the UK was measured between 20 and 200 mg/L (Chelliapan et al., 2006). Similarly, fluoroquinone concentration in the influent of a joint treatment plant serving about 90 bulk drug manufacturers was determined as 28 – 31 mg/L (Larsson et al., 2007). Since these waste streams also contain high levels of different organics, they are specifically suitable for anaerobic treatment (Chelliapan et al., 2006).

The presence of antibiotics in the engineered or natural ecosystems is the major driving force for resistance selection and spreading of antibiotic resistance genes (Baquero et al., 2008; Martinez, 2009). However, there is still limited knowledge about how antibiotic residues disturb the microbial communities and promote antibiotic resistance selection. Novo et al. (2013) argued that microbial communities are affected by various environmental factors during the wastewater treatment, so it is difficult to dissociate the effect of antibiotic residues from other environmental variables. Other approaches about antibiotic effects on microbial communities are more restrictive: Antibiotics may exert two types of effect on microbial communities, which have role on wastewater treatment; (1) the selection of antibiotic resistant bacteria, (2) the failure of physiological functions important for the treatment process (Alighardashi et al., 2009; Louvet et al., 2010).

SMX has been subject to many studies investigating its fate, biodegradation by engineered microbial communities such as biological wastewater treatment systems.
and its inhibitory and toxic effects on microbial communities. Under aerobic conditions, some studies reported that SMX was biodegradable (Müller et al., 2013) while in others, it was observed to be persistent to biodegradation (Drillia et al., 2005; Kümmerer et al., 2004), exerting significant inhibitory and toxic effects depending on the nature of the substrate (Cetecioglu et al., 2012; Pala-Ozkok et al., 2013, 2011).

Investigations on the fate and effect of SMX under anaerobic conditions are limited to only a few studies (Cetecioglu et al., 2015, 2013a; Gartiser et al., 2007). Cetecioglu et al. (2013a) showed that SMX was biodegradable under both anoxic and anaerobic conditions and its biodegradability characteristic are similar under nitrate reducing, sulfate reducing and methanogenic conditions. Otherwise, Gartiser et al. (2007) revealed that SMX was almost non-biodegradable at the short-term operated batch tests (ISO standards 13641). In a recent study, it was shown that SMX was biodegradable at long-term operation of anaerobic reactor however 40 mg/L is a threshold concentration for SMX inhibition (Cetecioglu et al., 2015).

The impact of SMX was interpreted under different conditions in terms of changes in the rate of substrate utilization and/or product generation. It is always useful to know to what extent these rates change, but the fundamental issue is to clarify why they change. In this context, understanding the interactions between SMX and microbial cultures, i.e. effect of SMX on microbial dynamics, remains to be the major challenge. Changes in the composition of the microbial community may be assessed by molecular techniques. While, different methodologies have been developed in order to determine the microbial community structure of various biological systems such as clone library, next generation sequencing, fingerprinting techniques, real-time PCR, etc. (Kampmann et al., 2012; Talbot et al., 2008), only a few studies have investigated the impact of antibiotics on the microbial community dynamics under aerobic conditions (Collado et al., 2013; Deng et al., 2012; Novo et al., 2013; Pala-Ozkok et al., 2013).
The main objective of the study was to monitor the changes induced on the composition of the microbial community subject to chronic/continuous exposure to various doses of SMX under anaerobic conditions. It was also intended to correlate the microbial dynamics with specific indications related to impairment of biochemical reactions such as biogas generation, relative magnitude of remaining volatile fatty acid fractions, in the presence of gradually increasing SMX concentrations in the range of 1 to 45 mg/L in the influent stream to mimic the pharmaceutical wastewater. For this purpose, the system performance (Cetecioglu et al., 2015) and the structure of the microbial community were analysed using a lab-scale sequencing batch reactor sustained under anaerobic conditions. Microbial diversity and dynamics were determined by cloning and denaturing gradient gel electrophoresis (DGGE), respectively. Also quantitative real-time PCR (Q-PCR) and reverse transcription Q-PCR (RT-qPCR) were applied to determine the number of active microbial communities during the reactor operation.

2. Materials and Methods

2.1. The Experimental Approach

The experiments were designed to reveal shifts on microbial community and metabolic pathways during the anaerobic degradation of SMX. For this purpose, two anaerobic sequencing batch reactors (ASBRs) as control (without SMX) and SMX reactors were set-up. The operation of the ASBRs included a start-up period of around 170 days for acclimation and establishment of steady state conditions. Their performance was observed under steady state conditions, to make sure that these conditions prevailed before semi-continuous exposure to SMX dosing. A sequence of eight different phases were included in the experimental observation for SMX.
reactor: During the first phase, phase a, (Day 0-77) SMX reactor was operated with feeding of just the synthetic substrate without SMX addition whereas during the following seven phases it was operated with semi-continuous feeding of the substrate/SMX mixture: In phase b (days 78-82), the daily SMX dose was maintained at 1 mg/L; the antibiotic dose was gradually increased to 10 mg/L in phase c (days 82-92), to 25 mg/L in phase d (days 93-101), to 30 mg/L in phase e (days 102-110), to 35 mg/L in phase f (days 110-115), to 40 mg/L in phase g (days 116-127), and finally to 45 mg/L in phase h (days 127-168). The dosages of SMX were mimicked as SMX concentrations in pharmaceutical industry wastewater. The sequence of different phases was primarily designed to observe the tolerance and possible failure of the microbial community under semi-continuous exposure to SMX; this was the reason why SMX concentration was gradually increased once the expected microbial response was observed. This approach enabled to observe different responses of the system at selected/gradually increased SMX doses, which constituted the basis of the evaluation.

2.2. Reactor Operation

Two Anaerobic Sequencing Batch Reactors (ASBRs) with 1 L active volume were set-up and operated at 35 °C under dark conditions to prevent phototrophic degradation. The reactors were operated in a 24-hour cycle: filling (10 min), reaction (23 h), settling (45 min) and withdrawing (5 min). The systems was mixed continuously at 90 rpm. The systems were inoculated by an anaerobic sludge collected from the stock reactor treating model wastewater as mg COD/L (starch, 2090; glucose, 1350; NaAcetate, 240; NaButyrate, 330; NaPropionate, 490). The ASBRs were fed by 2250 mg/L COD equivalent substrate mixture and the mixed liquor volatile suspended solids (MLVSS) concentration of the reactors was 4500 mg/L. The pH of the reactors at the start of each cycle was observed to vary from 6.8 to 7.2, mainly due to the alkalinity level of approximately 1000 mg/L CaCO3. The
other start-up and operational details of the ASBRs were described in Cetecioglu et al. (2015).

2.3. Analytical procedures

Methane and volatile fatty acid (VFA) concentrations were measured using gas chromatograph (Perichrom, France and Agilent Technologies 6890N, USA, respectively). Suspended solids (SS), volatile suspended solids (VSS), total suspended solids (TS), total volatile suspended solids (TVS) and soluble chemical oxygen demand (COD) were determined according to Standard Methods (APHA, 2005).

Preparation step of wastewater and sludge samples for antibiotic measurement was given in Cetecioglu et al. (2015). SMX concentration in the wastewater and sludge samples were measured by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry following the method developed by Gros et al. (2012). The recovery of the sludge sample was 107.8±25.7% as mentioned by Cetecioglu et al. (2015).

During the operation of the reactors, biomass was sampled periodically for microbiological analysis and samples taken from the control reactor was accepted as a reference sample.

2.3. Genomic DNA (GDNA) Extraction

Genomic DNA was extracted from 1-mL sludge samples using FastDNA Spin Kit for Soil (Qbiogene Inc., U.K.) following the manufacturer's instructions. Extracted GDNA concentrations were determined using Qubit 2.0 Fluorometer (Invitrogen, UK) and
diluted to 25 ng/µl by DNase free water. The extracted GDNAs were stored at -20 °C until further analysis.

2.4. Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from 1 mL sludge sampled using Charge Switch RNA extraction kit (Invitrogen, UK) according to recommended procedure. Concentration of isolated RNAs was measured using Quant-It RiboGreen RNA Assay Kit with Qubit 2.0 Fluorimeter (Invitrogen, UK). cDNAs were synthesized from isolated RNAs immediately. The rest of the isolated RNAs were stored at -80°C.

cDNAs were synthesized using Superscript Vilo cDNA synthesis kit (Invitrogen, UK) by reverse Transcription Polymerase Chain Reaction (RT-PCR) using hexamer primers. cDNA synthesis reaction was run for 10 minutes at 25 °C, one hour at 42 °C and 5 minutes at 85 °C. The cDNA samples were stored at –20 °C until further analysis.

2.5. Polymerase Chain Reaction (PCR), cloning, sequencing and phylogenetic analysis

PCR amplification was done as described in Cetecioglu et al. (2009). Primers used in the cloning and sequencing analyses and annealing temperature are given in Table 1. Amplification was occurred in a 50 µl reaction volume including 50 ng of DNA, 10 pmol of each primer, 10 mM of each deoxynucleoside triphosphate, 1.5 mM MgCl2, 5 µl of 10X Taq buffer and 4 U of Taq DNA polymerase (iTaq, U.K.). Nested PCR approach was applied to enhance specificity in archaeal 16S rDNA amplification. For the second-round nested amplification 1 µl of the first-round product was used as template, with reaction composition being the same as previously. PCR amplification was performed in a Techne TC-5000 thermal cycler (Barloworld Scientific Ltd., U.K.)
with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at
94°C for 1 min, annealing for 1 min and extension at 72°C for 2 min and a final
extension at 72°C for 10 min. PCR products were visualized by electrophoresis
(Thermo-Scientific Ltd., U.K.) on a 1% (w/v) agarose gel in TBE buffer (89 mM Tris,
89 mM boric acid, 2 mM EDTA, pH 8.3) and gel images were recorded using a
Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after staining
with ethidium bromide.

The bacterial and archaeal PCR products of the seed sludge were cloned by TOPO
TA cloning kit (Invitrogen Ltd., UK) according to manufacturer’s instructions. Cloning
procedure and analysis of partial 16S rRNA gene sequences were done according to
Kolukirik et al. (2011).

The nucleotide sequences obtained from this study have been deposited to EBI
Gene database under accession numbers FR836435 to FR836459 for the bacterial
16S rDNA and FR836460 to FR836475 for the archaeal 16S rDNA.

2.6. Denaturing gradient gel electrophoresis (DGGE)

DNA and RNA based community profiles of Archaea and Bacteria within the reactors
were obtained using DGGE analysis of PCR amplification products from primers
Arch344f_GC-Univ522r and Bact341f_GC-Bact534r as described by Muyzer et al.
(1993). Products (10 µL) were mixed in equal volumes with loading buffer and run on
a 10% polyacrylamide gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM
EDTA; pH 8.0). A chemical denaturing gradient of urea and formamide were adjusted
to 30–60% denaturant (100% denaturant is 7 M urea and 40% (v/v) formamide). To
aid the conversion and normalization of gels, a marker consisting of 16S rDNA mix
from archaeal and bacterial clone libraries was run alongside the samples.
Electrophoresis was performed using the D-Code system (Bio-Rad Laboratories, Ltd.,
UK) at 200 V constant current at 60°C, for 4.5 h. Gel images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after staining with SybrGold (1:10000 diluted; Molecular Probes Inc., UK) according to the supplier's instructions. Images were converted, normalized and analyzed by using the Bionumerics 5.0 software (Applied Maths, Kortrijk, Belgium).

2.7. Quantitative Real Time PCR (Q-PCR)

Three primer sets targeting the bacteria, archaea and methanogens were used to quantify existing and active microbial communities by using the template extracted GDNA s and synthesized cDNA, respectively. The primers using Q-PCR analysis were given in Table 1.

The procedure recommended by Roche was followed and a Light Cycler Master Kit (Roche, Applied Science, Switzerland) was used to set up the reaction (2.0 µl master mix, 1.6 µl MgCl₂ 1.0 µl Primer F and R, 13.4 µl H₂O, 1 µl sample). Absolute quantification analysis of the GDNA was carried out with a LightCycler 480 Instrument (Roche Applied Science, Switzerland). The amplification protocol was as follows: initial denaturation for 10 min at 94 ºC followed by 45 cycles of 10 s at 94 ºC, 5 s at specific annealing temperature as given in Table 1, 16 s at 72 ºC.

Standard curves for Q-PCR constructed from clones of PCR products. PCR products were sequenced as explained previously. After the primer specificity was confirmed by the sequence analysis, these amplicons were used as standards. Dilution series of the purified PCR product were used as calibration standards for real time PCR quantification after their DNA concentrations were determined by the fluorometer (Qubit, Invitrogen, Carlsband, CA, USA). Standard curves were constructed in each PCR run and the copy numbers of the genes in each sample were interpolated using
these standard curves. For each PCR run, a melting curve analysis was performed to confirm the specificity in each reaction tube by the absence of primer dimers and other nonspecific products. Reactions for all samples were shown to have only one melting peak, which indicated a specific amplification making it suitable for accurate quantification.

2.8. **Statistical analysis and diversity indices**

To determine the statistical significance of COD removal efficiencies of the ASBR, ONE WAY ANOVA test was used. This was followed by a Post-hoc Dunnett's test and student's T-test. Graphpad Prism 4 software (Graphpad Software, USA) was used for all statistical analysis.

DGGE gel images were analyzed and cluster analyses were done by Bionumerics 5.0 software (Applied Maths, Kortrijk, Belgium). Similarities between tracks were calculated by using the band-independent, whole-densitometric-curve-based Pearson product-moment correlation coefficients (r) and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering. Minitab Release 14 was used for PCA and correlation analysis. Correlation analysis was used to identify the relationship between operational conditions and microbial diversity through operation of the reactors.

Shannon-Weaver diversity indices, Margalef’s species richness measure, and evenness measure estimates were determined with PAST version 1.77. Shannon-Weaver diversity indices were calculated according to Pielou (1966) and Shannon and Weaver (1949). The operational taxonomic unit (OTU) richness was estimated using Margalef’s Species Richness (SR) index, and was calculated (Margalef, 1958). A species evenness (J’) index was calculated (Pielou, 1966).
3. Results and Discussion

3.1. Performance of Anaerobic Sequencing Batch Reactor

The COD removal efficiency and the biogas production during 168 days of operation is given in Figure 1. COD removal efficiency of the SMX reactor was 97.8±2.5% at the steady state condition (Phase a). While COD removal efficiency in the control reactor was 96.8±2.6% through the operation, it started to decrease in a stepwise manner as 97.3±1.5%, 96.6±2.2%, 93.5±1.8%, 93.8±1.2%, 93.3±2.4% and 92.9±1.3% at Phase b, c, d, e, f, and g; respectively, at SMX reactor after SMX addition. The COD data showed that the reactor collapsed at the last phase (Phase h: 45 mg/L of SMX): COD removal efficiency was 86.1±0.9% in the first days of Phase h and decreased to 25.0±1.1% at the end of the operation.

Biogas production was also parallel to effluent soluble COD concentration and it was also dramatically dropped through the last phase. While biogas production in the SMX reactor was 1004±129 mL/d in the Phase b, this value decreased until Phase g as 980±19 mL/d. However the biogas production was measured as 920 mL/d and 96 mL/d in the first and last day of the Phase h, respectively. During the entire operation of the reactor, methane content of the biogas produced was stable (64.2%±4.7). VSS concentration and pH were 4500 mg/L and 7.0±0.2 during the operational period.

The VFAs were first detected in the beginning of phase h at the SMX reactor. At the end of the operation, propionic acid and acetic acid concentrations in the effluent were 438 mg/L and 342 mg/L, respectively. The details of performance of the reactor were presented in Cetecioglu et al. (2015).
SMX concentration was measured in wastewater and sludge in each phase and the detailed results were represented in a previous study by the authors (Cetecioglu et al., 2015). The results showed that the main mechanism to remove the SMX is driven by microorganisms. The biodegradation efficiency of SMX was 99.9% until Phase c, including Phase c. It decreased to 59% at the Phase g, while 40 mg/L of SMX was feeding to the system. It should be noted that although the removal efficiency of SMX decreased with time, mass of SMX removed (in terms of mg/d) increased. A sharp decrease in the biodegradation down to 6%, was observed at the last phase (45 mg/L of SMX).

3.2. Cloning, Sequencing and DGGE

3.2.1. Bacterial and Archaeal Clone Libraries

In this study, 260 16S rRNA gene clones were screened; and 22 bacterial and 25 archaeal operational taxonomic units (OTUs) were identified. Bacterial and archaeal coverages of the clone libraries were calculated as 91.6% and 97.1%, respectively. Identified OTUs in the clone libraries, their classification, clone frequencies and similarities between the OTUs and their cultured and uncultured closest relatives, and their respective nucleotide accession numbers are given as Table 1 and 2, respectively.

57% of Firmicutes members, which are the majority of sequences obtained from the bacterial clone library (61%), belonged to Clostridium sp., which are mostly sulfide-reducing bacteria and found in different environments. The second most abundant phylum was Bacteroidetes (21%). Members of Verrucomicrobia, unclassified bacteria, Chloroflexi, Actinobacter and Proteobacteria were distributed as 6%, 6%, 4%, 1% and 1%, respectively. Phylogenetic tree for bacterial 16S rDNA gene sequences is given in Figure 2a.
Methanogens constituted 75.36% of OTUs obtained from archaeal clone library. Phylogenetic tree for archaeal 16S rDNA gene sequences of the seed sludge is given in Figure 2b. The most abundant taxa obtained in the archaeal clone library were the *Methanomicrobiales* (28%) which have members utilizing $\text{H}_2+\text{CO}_2$, alcohols and formate, *Methanosarcinales* (24%) which mostly acetoclastic methanogens belong to genus, and *Methanobacteriales* (21%) which has members utilizing $\text{H}_2+\text{CO}_2$, formate and methanol+$\text{H}_2$ as seen in Figure 2b. The members of *unclassified euryarchaeota*, *unclassified archaea* and *Methanococcales* which has members utilizing $\text{H}_2+\text{CO}_2$, pyruvate+$\text{CO}_2$ and formate were distributes as 18%, 6% and 3%, respectively, in the seed sludge.

### 3.2.2. 16S rDNA Based Evaluation of Predominant Bands and Phylogenetic Relationship in the DGGE Fingerprinting Patterns

To assign the composition of the active predominant community and its shift during the operation time, 16S rDNA and rRNA DGGE profiles of the samples were compared to bacterial and archaeal clone libraries of the seed sludge. The comparison of the bacterial and archaeal 16S rDNA/16S rRNA DGGE band patterns obtained from SMX and control reactors was given in Supp. Table 1-8.

13 of 22 bacterial OTUs were detected in the 16S rDNA DGGE profiles. According to these results, *Clostridium species* (AB114241, AM158323, and AY648564) were always detected during the operation of both the control and the SMX reactors and their intensities did not noticeably change with respect to the SMX concentration and the operation time (Supp. Table 1 and 5). *Clostridium species*, which belong to the *Firmicutes* phylum, may frequently be observed in the anaerobic systems and they are responsible of fermentation and some *Firmicutes* species are known to produce lactic acid, ethanol and volatile fatty acids. Kampmann et al. (2012) showed that *Firmicutes* shift and contribute to a stable reactor performance under continuous
starch feeding conditions. Clostridia are already known to play a key role in the biogas-producing process, especially on the starch degradation by exo-enzymes (Hungate, 1982; Liu et al., 2009; Payton and Haddock, 1986). So, the results are not surprising for our system fed by a synthetic wastewater including starch. 13 of the bacterial OTUs were detected in the 16S rRNA DGGE profiles in the SMX reactor. According to 16S rRNA DGGE results, the uncultured _Clostridium_ species, which were detected in 16S rDNA results (AM158323 and AY 648564), also existed in every phase while some of them disappeared in the control reactor. However the abundances of _Clostridium sp._ decreased with respect to the SMX concentration and the operation time. This result also confirms the data associated with the performance of the SMX reactor; the COD removal efficiency decreased through the operation, methanogenic activity increased (Ceteciglu et al., 2015). However studies focusing on the antibiotic exposure under both aerobic and anaerobic conditions revealed that bacterial species belonging to _Firmicutes_ phylum were negatively affected in terms of abundance (Deng et al., 2012; Novo et al., 2013).

Additionally, while _Acinetobacter sp._ 1B3 (EU337120) was detected in the seed sludge, it did not be matched in DGGE band patterns of the samples taken from the reactor. However, the same species was observed to adapt to high concentration of antibiotics, due to its ability of developing resistance genes to antibiotics (Guardabassi et al., 1998). Other predominant bacterial clones were mostly uncultured species in both 16S rDNA and 16S rRNA DGGE results. These uncultured bacterial clones may probably be responsible for fermentation and homoacetogenesis, especially syntrophic VFA-oxidation (Madigan et al., 2009; Speece, 1996).
Seven bacterial OTUs disappeared during the operation. The main reason for this might be the acclimation to the synthetic wastewater or the inhibition effect of SMX (Haack et al., 2012; Wunder et al., 2013).

The analysis of the bacterial community structure in this study could also serve to identify bacterial species that would potentially trigger the enhancement or spread the antibiotic resistant genes in anaerobic systems. The data related to the occurrence and abundance of microbial populations and environmental variables measured in the same time and site, may bring a holistic perspective to the evolution of antibiotic resistance in the microbial environment (Novo et al., 2013).

Subsequently 14 archaeal OTUs were detected in the 16S rDNA DGGE profiles (Supp. Table 3, 4, 7 and 8). According to the results, acetoclastic methanogenic species (U89773, FR836463, FR836464, and FR836468) disappeared through the last phase in the SMX reactor, while they were detected in the control reactor in every phase. The abundance of hydrogenotrophic methanogens, especially Methanobacterium species, and methanogenic archeons (AY350742, AY552778, HM630570, FR836462, and FR836467) were higher than acetoclastic methanogens and they seemed almost in every operational phase of the SMX reactor. It should be noted that acetoclastic methanogens were observed as the dominant group in the control reactor operated without SMX feeding. This correlation revealed that even though bacterial species were negatively affected by higher SMX concentrations; the surviving species continued the degradation of substrate and production of VFAs from valeric acid to acetic acid.

The comparison results to determine the active species and the shifts in their abundances in the reactors with respect to the SMX concentration and the operation time were given in Supp. Table 4 and 8 from the SMX and the control reactors,
respectively. According to the results obtained from the SMX reactor, only *Methanosarcina siciliae* (U89773) was detected. This species was seen at phase h_2 (145\textsuperscript{th} day). Presumably quite a few acetoclastic methanogens were in the SMX reactor during the operation however their amount was under the detection limit. Detection in the Phase h_2 could be related to sampling. In this phase, *hydrogenotrophic methanogens* were dominant and more active through the operation of the SMX reactor. While *Methanobacterium beijingense* (AY350742) were highly dominant, abundance of other *hydrogenotrophic methanogens* decreased in the last phase (phase h). Other detected species mostly consisted of uncultured archaeal clones (Supp. Table 4). As opposed to biomass exposed to SMX, *acetoclastic methanogenic species* were more dominant and active (AB077214, X51423, GU475184, U89773) in the control reactor.

In anaerobic digestion, degradation of organic compounds to CO\textsubscript{2} and CH\textsubscript{4} relies greatly on methanogenic archaea (Madigan et al., 2009). So, the results were expected apart from the fact that *acetoclastic methanogens* were not detected. Generally, 70\% of CH\textsubscript{4} is produced from acetate in the anaerobic reactors (Whiticar et al., 1986). However Batstone et al. (2002) suggested that low hydrogen partial pressure should be provided to syntrophic consortia to utilize different intermediates and Shin et al. (2010) explained the increase of *Methanomicrobiales* in their study by this argument.

The VFA results indicated that SMX affected the propionic acid degradation and acetic acid utilization pathways in the higher concentrations of SMX. Sponza and Demirden (2007) also found similar results in an anaerobic system fed with sulfamerazine, another antibiotic from sulfonamid group. Additionally, a parallel study carried out with a different antibiotic, tetracycline, showed that acetoclastic methanogens are affected under long-term antimicrobial addition (Cetecioglu et al.,
2013b). Hydrogenotrophic methanogens have also been detected in similar studies which were focused on anaerobic treatment of pharmaceutical industry wastewater and manure digestion which includes antibiotics (Gómez-Silván et al., 2010; Stone et al., 2010). The main factors determining the major pathway and/or shift in the processes could be the existence of high levels of inhibitors, which have more negative effects on acetoclastic methanogens (Shin et al., 2010).

So the results obtained from this study propose another pathway for all phases excluding early stage of Phase h; **homoacetogenesis with hydrogenotrophic methanogenesis.** Acetate could be converted to H\(_2\) + CO\(_2\) by homoacetogens; and H\(_2\) might be used to produce methane in also early phase of the operation.

### 3.2.3. Cluster analysis of DGGE banding pattern

Shifts in the microbial diversity in the SMX and the control reactors during the operation, were estimated using the DGGE patterns. All sludge samples obtained from different antibiotic feeding regimes were compared with the corresponding samples of the control reactor. Bacterial and archaeal diversities and species richness in the seed sludge and sludges taken from the control and SMX reactors were measured by the Shannon-Weaver diversity index and Margalef’s species richness, respectively (Table 2 and 3). While 33 different bacterial species were detected in the seed sludge, this number decreased to seven at the end of the SMX reactor operation while the number of species remained at 25 in the control reactor at the end of the operation. The phylogenetic results confirmed the shift in the bacterial community in the case of SMX exposure. In another study, the authors found that bacterial community structure changed even when 50 µg/L of SMX was added to the activated sludge system (Collado et al., 2013).
While archaeal species number was nine in the control reactor (without SMX), at the end of the operation, this number in the SMX reactor changed during the operation: Firstly it increased to 21 from 13 at the end of Phase b (1 mg/L of SMX) but finally the archaeal species number dramatically decreased to ten in the last period (Phase h; 45 mg/L of SMX). Bacterial and archaeal species were generally stable between the phase c (10 mg/L of SMX) and g (40 mg/L of SMX) in the SMX reactor. Comparing microbiological observations of the SMX reactor with those of the control reactor (without SMX), it was noted that SMX addition affected bacterial community during the Phase h (45 mg/L of SMX addition). It is probably because the SMX concentration in the reactor reached to the inhibitory threshold level. While microbial diversity remained the same in previous phases, a sharp decrease in bacterial OTUs was observed at the beginning of the phase h. The diversity was recovered for a short period, but the decreasing trend in bacterial OTUs continued again until the end of the operation. This result confirmed that the biodiversity was positively related to ecosystem stability (Miura et al. 2007). While OTUs number decreased, two different methanogenic groups survived in the SMX and the control reactors as hydrogenotrophic methanogens and acetoclastic methanogens, respectively.

The bacterial and archaeal 16S rDNA phylogenetic trees obtained from the SMX and the control reactors were given in Supp. Figure 1. The analysis of DGGE band patterns revealed that the samples differentiated not only by the SMX addition but also by the operation time. The changes in bacterial community during the operation reflect the clusters obtained from DGGE band patterns, which were divided into two clusters (Supp. Figure 2a). The clusters were defined by approx. 70% pattern similarity. The pattern similarity of the samples did not change very distinctly.
Similarity of the archaeal pattern between samples changed more distinctly than the bacterial dendogram. Clustering of 16S rDNA gene DGGE banding pattern revealed two main clusters defined by less than 60% similarity for Archaea (Supp. Figure 1b).

DGGE analysis was also performed to find out the active community changes during the operation of the SMX reactor. The bacterial and archaeal phylogenetic dendograms obtained from DGGE band patterns were represented in Supp. Figure 2. The bacterial band patterns obtained from 16S rRNA gave the similar result to 16S rDNA band patterns. Phase h_3 sample differentiated from other samples obviously, in which the reactor collapsed, and biodegradation activity stopped.

The dendogram structures and similarities of the 16S rRNA archaeal phylogenetic tree were different from the dendogram of archaeal 16S rDNA. As seen in Supp. Figure 2b, the distribution of the active archaeal community reflected the clusters, which were divided into two.

No significant changes in 16S rDNA and 16S rRNA profiles of bacterial and archaeal communities were detected in the cluster analyses, while a microbial shift was found according to comparing DGGE profiles with sequencing data. The stable wastewater treatment efficiency is suggested to be linked to the stable microbial community (LaPara et al., 2002) and the shifts in our study might be explained by adaptation to antibiotic and gaining antibiotic resistance genes of some species.

3.2.4. Principle Components Analyses and Correlation

To reveal SMX effect on anaerobic microbial community, we combined the physico-chemical and analytical data with culture-independent microbial approaches and statistical analyses.
PCA of both 16S rDNA/16S rRNA bacterial and archaeal DGGE profiles from different operating phases of the SMX reactor revealed that 66-79% of the variability was explained by the first three components (Table 4).

The PCA plots of the first and second components obtained from 16S rDNA/16S rRNA bacterial and archaeal DGGE profiles are given in Supp. Figure 4. As seen in Supp. Figure 3a and 3b, the bacterial components were best explained by the data and showed clear association between cluster analyses. However the archaeal components did not give parallel results with cluster analyses (Supp. Figure 3c and 3d).

A significant correlation was found between microbial community profile and the reactor operation data as COD removal efficiency, biogas production, VFAs composition, antibiotic biodegradation and sorption.

A negative correlation was found between SMX concentration and the reactor efficiency; while SMX concentration in the effluent increased during the operation, the biodegradation efficiency decreased. So the bacterial profile was positively correlated with SMX biodegradation efficiency. Also the sorption of SMX was the highest at the end of the operation and it had a negative correlation with bacterial composition.

Biogas production and COD removal efficiency were positively correlated with both bacterial and archaeal compositions. While VFAs concentrations in the reactor were negatively correlated with bacterial composition, the positive correlation was detected with most of the archaeal components. This correlation revealed that even though bacterial species were negatively affected by higher SMX concentrations; the survival species continue the degradation of substrate and production of VFAs from
valeric acid to acetic acid. Also the correlation between acetic acid and PC1 of bacterial 16S rDNA was found the highest as -0.906; hydrogenotrophic species was found as dominant during the operation even in the last phase. According to this, negative impact of SMX on homoacetogenic pathway does not affect the reactor performance; an alternative metabolic pathway becomes a part of the activity.

3.3. Quantitative Real-Time PCR (Q-PCR)

Q-PCR analyses were accomplished for the SMX and the control reactors to detect the changes in microbial community and active population through the operation. While bacterial, archaeal and methanogenic populations increased in the control reactor during operation, the number of each population decreased in each phase of the SMX reactor, wherein the SMX concentration increased in a stepwise manner (Figure 3a). A sharp decrease was detected for archaeal and methanogenic communities in the beginning of Phase h_1 (45 mg/L of SMX), correspondingly the reactor efficiency in terms of COD removal efficiency and biogas generation significantly decreased as seen in Figure 1. The bacterial, archaeal and methanogenic communities reached to 3.75X 10^{10}, 3.01 X 10^8, and 2.58 X 10^8, respectively, at the end of the operation of the control reactor. These values decreased to 6.51X 10^5, 3.59 X 10^5, and 1.9 X 10^3, respectively, at Phase h of the SMX reactor. The highest methanogenic population was determined at Phase g (40 mg/L of SMX) as 2.22X 10^7, (Figure 3a).

The reason of the increase in the methanogenic community in Phase g could be the adaptation and shift of methanogens. Even though the acetate was accumulated in the last Phase (Phase h: 45 mg/L of SMX) (Cetecioglu et al., 2015), the main reason, that the amount of total methanogenic population was not affected, could be explained by a shift to hydrogenotrophic methanogens. A similar study by Hori et al. (2006) showed that VFA accumulation may also account for the appearance in the
hydrogenotrophic methanogen population, less sensitive than acetoclastic methanogens to increases in VFAs concentration. Similarly, Merlino et al. (2013) speculated that the partial accumulation of VFAs, particularly acetate and propionate, could be an explanation of the one order magnitude decrease of methanogens and the acetotrophic methanogen proportion.

Besides quantification of 16S rDNA concentration, 16S rRNAs (expression level of 16S rDNA) were quantified to determine the effects of SMX on the specific microbial groups. Quantification results of bacteria, archaea and methanogens were also given in Figure 3b. While the reactor operation data and specific methanogenic activity (Cetecioglu et al., 2015) results changed during the operation time, 16S rRNA expression level in the SMX reactor was not changed noticeably. Similarly Merlino et al. (2013) found that quantitative data of the functional groups in an anaerobic digester indicated rather constant abundance at the steady state despite some variations in biogas production during the period.

3.4. Conclusion

The study showed that qualitative and quantitative data on microbial community dynamics provided valuable information on the functionality of anaerobic processes during treatment of pharmaceutical wastewater including antibiotic. In particular, the results enabled evaluating the changes in the structure of microbial communities in terms of functional mechanisms of corresponding metabolic processes, establishing reasonable relationships between different microbial groups with observed functionality. Microbiological investigation provided a useful tool to get a better insight into inhibitory effects of the pharmaceuticals on the performance of anaerobic reactors. According to obtained results:
Up to 40 mg/L of SMX, biodegradation is driven mechanism to remove the SMX from the system.

45 mg/L of antibiotic is lethal dose for the anaerobic system and this causes the inactivation of acetoclastic methanogens.

Clostridium sp and hydrogenotrophic methanogens became dominant through the operation.

It can be argued that SMX exposure at high concentrations puts forward an alternative pathway; homoacetogenesis with hydrogenotrophic methanogenesis.

Acknowledgement

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References


Pharmaceutical Press, London, UK.


Figure Captions

Figure 1. COD removal efficiency and biogas generation in the ASBRs (Cetecioglu et al., 2015)

Figure 2. Neighbor-joining full-length 16S rDNA trees and distributions of phylogenetic phylums from clone sequences of a) bacterial 16S rDNA clone library, b) archaeal 16S rDNA clone library (The bar scale represents 2 nucleotide substitutions per 100 nucleotides)

Figure 3. Quantitative changes in microbial community a) 16S rDNA concentrations b) 16S rRNA concentrations at different phases of the SMX and the control reactors
Supporting Information: Figure Captions

Supp. Figure 1. Cluster analysis and similarity matrix of 16S rDNA gene DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA (a: bacterial 16S rDNA, b: archaeal 16S rDNA)

Supp. Figure 2. Cluster analysis and similarity matrix of bacterial 16S rRNA DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA (a: bacterial, b: archaeal)

Supp. Figure 3. The loadings on the first two components from principal component analysis of the SMX reactor for a) bacterial 16S rDNA, b) bacterial 16S rRNA c) archaeal 16S rDNA, d) archaeal 16S rRNA
Table 1. Primers using in PCR amplification and their annealing temperatures

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<th>Reference</th>
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<td>Bact8f</td>
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<td>Edwards et al., 1988</td>
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¹ *Escherichia coli* numbering.
² 5’-GC clamp on Arch344f and Bact341f (GCCGCGCCCGCGCGCGCGCGCGCGCGCGCGCGCGACGGGGGGACGGGG).
Table 2. Shannon-Weaver diversity indices, Margalef's species richness measure, and evenness measure estimates for DGGE profiles of sludges taken from different phases of the SMX reactor

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<th>Phase b: 82nd day (1 mg/L SMX)</th>
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<th>Phase g: 128th day (40 mg/L SMX)</th>
<th>Phase h: 136th day (45 mg/L SMX)</th>
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<td>2.568</td>
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Table 3. Shannon-Weaver diversity indices, Margalef's species richness measure, and eveness measure estimates for DGGE profiles of sludges taken from different phases of the control reactor

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Table 4. Correlation coefficients and their significance between the first three principal components from DGGE banding analysis of Bacteria and Archaea 16S rRNA gene fragments of sludges taken from different phases of the SMX reactor

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<th>Archaeal 16S rDNA</th>
<th>Archaeal 16S rRNA</th>
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<td>PC2 20.6%</td>
<td>PC3 13.2%</td>
<td>PC1 54.8%</td>
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<td>PC1 40.3%</td>
<td>PC2 23%</td>
<td>PC3 12.5%</td>
<td>PC2 13.3%</td>
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<td>-0.199</td>
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</tbody>
</table>

*, **, ***: significance at P = 0.05, 0.01 and 0.001, respectively; significant coefficients are printed in bold.
Highlights

- Sulfamethoxazole has an inhibitory effect on acetoclastic methanogenic pathway.
- Clostridium sp. and hydrogenotrophic methanogens survived under high SMX exposure.
- *Homoacetogenesis with hydrogenotrophic methanogenesis* is prominent during SMX degradation.