ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

COMBINED EFFECTS OF ANTIBIOTICS ON THE ANAEROBIC MICROBIAL COMMUNITY STRUCTURE, INVESTIGATION OF METABOLITES AND ANTIBIOTIC RESISTANCE GENES

Ph.D. THESIS

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Department of Environmental Engineering

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ANTİBİYOTİKLERİN ANAEROBİK MİKROBİYAL KOMUNİTE ÜZERİNDEKİ ETKİSİNİN BELİRLENİP, METABOLİT OLUŞUMU VE ANTİBYOTİK DİRENÇ GELİŞİMİNİN İNCELENMESİ

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Sevcan AYDIN, **a Ph.D.** student of ITU **Graduate School of Science, Engineering and Technology** student ID **501102802**, successfully defended the **thesis** entitled " **COMBINED EFFECTS OF ANTIBIOTICS ON THE ANAEROBIC MICROBIAL COMMUNITY STRUCTURE, INVESTIGATION OF METABOLITES AND ANTIBIOTIC RESISTANCE GENES**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my parents and my sister,

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(Molecular Biologist)

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ABBREVIATIONS

ACAS : Acetyl-coA synthetase
ARG : Antibiotic Resistance Gene
ATAD : Autothermal thermophilic aerobic digestion
cDNA : Complementary DNA
CFZ **:** Cefazolin
COD **:** Chemical Oxygen Demand
DGGE **:** Denaturing Gradient Gel Electrophoresis
ERY **:** Erythromycin
ES **:** Erythromycin and Sulfamethoxazole mixture
ET **:** Erythromycin and Tetracycline mixture
ETS **:** Erythromycin, Tetracycline, and Sulfamethoxazole mixture
FISH **:** Fluorescence *in situ* Hybridization
FTHFS **:** Formylterahydrofolate synthetase
GC **:** Gas Chromatograph
gDNA **:** Genomic DNA
HRM **:** High Resolution Melt
HRT **:** Hydraulic Retention Time
mcrA **:** Methyl-coenzyme M reductase
MLVSS **:** Mixed liquor volatile suspended solid
OLR **:** Organic loading rate
OTC **:** Oxytetracycline
PCR **:** Polymerase Chain Reaction
qPCR **:** Quantitativr real-time PCR
RT-PCR **:** Reverse Transcription Polymerase Chain Reaction
SBR **:** Sequencing Batch Reactor
SMX **:** Sulfamethoxazole
SRT **:** Solids retention time
ST **:** Sulfamethoxazole and Tetracycline mixture
TET **:** Tetracycline
TPs **:** Transformation Products
VFA **:** Volatile fatty acid
WWTPs **:** Wastewater treatment plants

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COMBINED EFFECTS OF ANTIBIOTICS ON THE ANAEROBIC MICROBIAL COMMUNITY STRUCTURE, INVESTIGATION OF METABOLITES AND ANTIBIOTIC RESISTANCE GENES

SUMMARY

Antibiotics which are one of the most commonly used pharmaceutical products have a wide range of uses in both human and veterinary medicine. These unfamiliar substances to existing enzyme systems are discharged to receiving bodies without or a little mineralization from treatment plants and accumulated in soil and sediment day by day. In this thesis, aims to develop an understanding of triple effects of sulfamethoxazole-erythromycin-tetracycline (ETS) and dual effects of sulfamethoxazole-tetracycline (ST), erythromycin-sulfamethoxazole (ES) and erythromycin-tetracycline (ET) on the microbial community structure, development of antibiotic resistance genes (ARGs), and how this relates to anaerobic sequencing batch reactors (SBRs) performance throughout a year operation. This study also evaluated understanding the link between anaerobic microbial diversity and biodegradation of antibiotic combination and the occurrence of transformation products (TPs) in anaerobic reactors by amendments of different antibiotics combination and stepwise increasing concentrations. Concentrations of the antibiotics in the influent were gradually increased until metabolic collapse of the SBRs, which corresponded to ETS (40+ 3+ 3 mg/L), ES (2.5 + 25 mg/L), ET (4+ 4 mg/L) and ST (25 + 2.5 mg/L). Although COD removal efficiency and biogas/methane generation for ETS, ES, ET and ST fed SBRs decreased with increases of antibiotic concentrations, ETS and ET batch reactor showed a better performance compared to ST and ES batch reactors; this could be due to antagonistic effects of sulfamethoxazole. Anaerobic pre-treatment of these antibiotics can be a suitable alternative for concentrations at 10 + 1 mg/L, 1 + 10 mg/L, 2 + 2 mg/L, 20 + 2 + 2 mg/L for ST, ES, ET and ETS reactor respectively which corresponds to min 70% COD removal efficiency. Antibiotic measurement and TPs formed results of this study also indicated that the anaerobic process eliminated 1 mg/L, 15 mg/L, and

0.5 mg/L for ERY, SMX and TET antibiotics respectively which corresponds to average of 80% antibiotic degradation efficiency during SBRs operation. 16S rRNA quantification and Ion Torrent result could reflect the anaerobic SBRs performance. The results of this research support the idea that molecular and metagenomic analysis could be used for providing of the control and the improvements on the anaerobic system treating pharmaceutical wastewater. Additionally, evaluation of molecular data is useful for suggesting the potential to control ultimate microbial community composition via bioagmentation to successful for antibiotic biodegradation. Overall, the results reveal a valuable information about effect of antibiotic combinations on the development of ARGs in the anaerobic SBRs, even after anaerobic treatment, contribute to the spread of these genes in aquatic environment, which may increase the risk of ARGs to public health.

ANTİBİYOTİKLERİN ANAEROBİK MİKROBİYAL KOMUNİTE ÜZERİNDEKİ ETKİSİNİN BELİRLENİP, METABOLİT OLUŞUMU VE ANTİBYOTİK DİRENÇ GELİŞİMİNİN İNCELENMESİ

ÖZET

Antibiyotikler, insan ve veterinerlik alanında en yaygın olarak kullanılan ilaçlardır. Canlılar tarafından alınan bu aktif bileşikler, canlı metabolizmasında ya hiç değişmeden ya da çok az dönüştürülmüş halde metabolizmadan atılmaktadırlar. Atılan bu aktif antibiyotik kalıntıları arıtma tesislerinden arıtılamadan deşarj oldukları için doğrudan alıcı ortama girmekte, toprak ve sudaki konsantrasyonları günden güne artmaktadır. Geleneksel olarak atıksu arıtma sistemleri, aşırı nutrient, mikrobiyal kirlilik problemleri, azot ve fosfor giderimi hedef alınarak geliştirilmiştir. Bu yüzden farmasötikler gibi doğal ve sentetik bileşiklerin birçoğu klasik arıtım sistemlerde tam olarak giderilemeyip nehirler, yeraltı suları ve içme suları gibi sucul ortamlara deşarj edilmektedir. Ekosistem üzerinde önemli etkileri olan antibiyotikler, insan sağlığı ve çevre için ileri derece risk oluşturmaktadır. Ayrıca bu maddeler, patojen bakterilerin antibiyotiğe direnç geliştirmesine neden olarak, öncelikle halk sağlığı, ardından da tarım ve hayvancılıkta önemli problemler ortaya çıkartmaktadır.

Gerçekleştirilen tezde, insanlarda sıklıkla görülen enfeksiyonel hastalıkların tedavisinde kullanılan eritromisin, sülfonamid ve tetrasiklin antibakteriyel ve antibiyotik etken maddeleri, anaerobik ardışık kesikli reaktörlerde, kolay ayrışan organik maddeler ile beslenmiş ve bu maddelerin davranışları ve diğer organik maddelerin giderimi üzerindeki kronik etkileri konvansiyonel ve spesifik parametreler ile tespit edilmiştir. Bu kapsamda ET (Eritromisin-Tetrasiklin), ST (Sülfametoksazol-Tetrasiklin), ES (Eritromisin- Sülfametoksazol), ETS (Eritromisin-Tetrasiklin-Sülfametoksazol) ve kontrol olmak üzere 5 adet anaerobik ardışık kesikli reaktör (AKR) bir yıl süresince işletilmiştir. Antibiyotiklerin üçlü ve ikili kombinasyonlarının miktarları reaktörler çökene kadar kademeli olarak arttırılmış ve anerobik AKRlerdeki mikrobiyal kominite, antibiyotik direnç gelişimi ve antibioyotik biyodegredasyon potansiyelleri ve transformasyon ürünlerinin oluşumu

13 ay boyunca gözlenmiştir. Ayrıca antibiotiklere karşı mikroorganizmaların çeşitli gen transfer yöntemleri ile kazandığı bilinilen 'Antibiyotik Direnç Genleri (ADG)'nin oluşumu ve kantifikasyonu DNA ve RNA bazında, qPCR (gerçek zamanlı kantitatif PCR) ve İllumina HiSeq 2000 yöntemleri kullanılarak gerçekleştirilmiştir. Sistemde antibakteriyel ve antibiyotiklerin giderimi ve çamurda birikimini Yüksek Performanslı Sıvı Kromatografi (HPLC) ve LC/MS/MS (QTRAP) ile ölçülmüş olup bu etken maddelerin bilinen metabolitleri de ORBİTRAP sistemi (Micromass, Manchester, UK) ile tespit edilmiştir. Son olarak, bu etken maddelerin varlığında ortamda bulunan organik maddelerin ve seçilmiş farmasötiklerin gideriminden sorumlu mikrobiyal komünite ve aktif gruplar/türler kurulacak olan 16S rDNA/rRNA klon kütüphaneleri ile tanımlanmıştır. Mikrobiyal komünitenin profilindeki değişimleri izlemek için Denaturan gradyan jel elektorfarezi (DGGE) ve Ion Torrent PGM analizleri kullanılmıştır. Seçilen antibiyotik kombinasyonlarının anaerobik proseslerde yer alan Bakteri, Arke, Metanojenik Arkelere ve homoasetojenik ve metanojenik metabolizmada yer alan enzimlere spesifik etkisi qPCR kantifikasyonu ile belirlenmiştir.

Antibiyotik içeren suların arıtımında anaerobik arıtma sistemlerindeki arıtım performansı esas olarak mikroorganizma topluluğunun birbiri ile ilişkilerine ve bu aktif maddeleri biyokimyasal döngüleri yoluyla parçalama potansiyellerinin etkilenmesine bağlıdır. Tezin gerçekleştirilmesi sonucunda görülmüştür ki her antibiyotik kombinasyonu anaerobik arıtıma farklı şeklilerde etki etmektedir. Tüm reaktörlerde antibiyotik konsantrasyonundaki artışına bağlı olarak KOİ gideriminde, biyogaz ve metan üretiminde düşüş gözlenmesine rağmen, ETS ve ET antibiyotik kombinasyonlarının ST ve ES kombinasyonlarına göre daha iyi bir arıtım performansı gözlenmiş olup, bunun da sülfametoksazol antibiyotiğinin antibiyotik kombinasyonları üzerinde gösterdiği antagonistik etkiden kaynaklandığı düşünülmektedir. Proje sonucunda tüm antibiyotik kombinasyonlarının arıtım performansları incelendiğinde, en az %70 KOI gideriminin sağlandığı 10 + 1 mg/L (ST), 1 + 10 mg/L (ES), 2 + 2 mg/L (ET), 20 + 2 + 2 mg/L (ETS) kombinasyonlarına kadar anaerobik arıtımın uygun olduğu görülmektedir.

mRNA düzeyinde kantifikayonu yapılan metil koenzim redüktaz ve asetil koA sentaz enzimi sayesinde, antibiyotik kombinasyonlarının anaerobik sistemin bu son iki aşamasından en çok asetoklastik metanojenlerin yer aldığı basamağın etkilendiğini göstermektedir. Bu sonuçlar reakörlerdeki VFA birikimi ile de doğrulanmaktadır. Tüm reaktörler incelediğinde hepsinde en çok biriken VFA'nin asetat olduğu görülmektedir. Bu da kullanan antibiyotik kombinasyonlarının ortak olarak tüm reaktörlerde en çok asetoklastik metanojenleri etkilediğini göstermektedir. 16S rRNA qPCR sonuçları tüm işletme boyunca farklı anaerobik reaktörlerin performansını birebir yansıtmaktadır. Ayrıca mikrobiyal ve aktif türlerin qPCR ve DGGE sonuçları göstermektedir ki antibiyotikler anaerobik mikrobiyal topluluğu etkileme potansiyeline sahiptir ve bu mikrobiyal popülasyonun inhibisyonu VFA sonuçlarındanda görüldüğü gibi, ciddi olarak organik madde parçalamasını etkilemektedir. Ayrıca elde edilen veriler sonucunda moleküler ve metagenomik analiz sonuçlarının antibiyotiklerin artan konsantrasyonlarının arıtımı boyunca değişen anaerobik reaktörlerin performansını yansıttığı görülmektedir. Bu da reaktör işletilmesinde ve özellikle antibiyotik gibi toksik maddelerin giderilmesi sırasında giderim verimini arttırmak için moleküler ve metagenomik anazlilerin kullanılabileceğini göstermektedir. Ayrıca anaerobik arıtım sırasında antibiyotik direnç genlerinin oluştuğu gözlenmiş olup bu direnç genlerinin miktarının da artım boyunca arttığı gözlemiştir, arıtım sonucunda oluşan bu direnç genleri insan sağlığı için bir tehdit oluşturmaktadır.

ADG ile qPCR sonuçları değerlendirildiğinde bakterilerin direnç kazanmasının sonucunda bunların sayılarının arttığı ve sistemin tekrar eden antibiyotik konsantrasyonlarında daha verimli bir arıtım gerçekleştiğini göstermektedir. Aynı zamanda antibakteriyel ve antibiyotiklere karşı mikrobiyal komünitenin kazandığı direnç genlerinin anaerobik sistemlerdeki davranışı incelenmiş olup bunların çeşidinin ve miktarlarının bu artım sürecinde ne kadar fazla artığı gözlenmiştir. Bu artış ekosisteme ve insan sağlığı üzerine gerçek bir tehdit oluşturmaktadır. Ayrıca, tespit edilen antibiyotik transformasyon ürünleri sayesinde de antibiyotiklerin arıtımda giderilmiş olarak görülmesine rağmen biyolojik olarak daha aktif yapılara dönüşerek inhibisyon etkilerini arttırdıkları gözlenmektedir.

Projenin gerçekleşmesiyle, antibiyotik içeren atıksu kompozisyonu-mühendislik parametreleri-mikrobiyal komünite-metabolik yol izleri ilişkisi detaylı olarak

incelenmiş olup bu maddelerin anaerobik proseslere etkisi mikro ve makro seviyede ortaya konmuştur. Elde edilen sonuçlar göstermektedir ki anaerobik arıtımda antibiyotikler yüksek oranda arıtılsa bile, atıksularda bu antibiyotik kalıntılarının varlığı, antibiyotiğe dayanıklı türlerin yayılmasına ve gelişmesine katkı sağlamaktadır. Ayrıca bu proje ile çevrede yüksek konsantrasyonlarda bulunan ve tüm canlıları olumsuz yönde etkileyen antibiyotik kalıntıları ve metabolitlerin, atıksularda yer alan antibiyotiklerin yetersiz arıtımından da kaynaklandığı görülmektedir. Bu aktif maddelerin etkilerinin en aza indirilmesi için antibiyotik içeren sularda sadece antibiyotik kalıntılarının değil aynı zamanda metabolitlerinin de tespit edilip, bunların deşarjı ile ilgili bir yasal düzenleme yapılması gerektiğini düşündürmektedir.

1. INTRODUCTION

Biological treatment processes offer the ideal conditions in which a high diversity of microorganisms can grow and develop. Antibiotics have also the potential to adversely affect the microbial community that is present in biological wastewater treatment processes. The antibiotics that exist in waste streams directly inhibit substrate degradation and also have an influence on the composition of the microbial community and, as such, they provide the ideal setting for the acquisition and proliferation of antibiotic resistance genes.

1.1 Purpose of Thesis

Antibiotics have the potential to adversely affect the microbial community that is present in biological wastewater treatment processes. The antibiotics that exist in waste streams directly inhibit substrate degradation and also have an influence on the composition of the microbial community (Aydin et al., 2015a). The aim of this research was to determine how the bacterial communities change in anaerobic SBRs during biodegradation of antibiotic combinations and the occurrence of transformation products (TPs) that contains sulfamethoxazole-erythromycin-tetracycline (ETS), sulfamethoxazole-tetracycline (ST), erythromycin-sulfamethoxazole (ES) and erythromycin-tetracycline (ET) throughout a year operation of anaerobic reactors. Biodegradation, sorption and TPs formed of antibiotic combination during the long-term operation of anaerobic SBRs were analyzed by on-line turbulent flow chromatography (TFC) coupled to LC-(ESI)-LTQ Orbitrap and a Waters Acquity Ultra-Performance™ liquid chromatograph system coupled to a hybrid quadrupole-linear ion trap QqLIT. Ion Torrent PGM and PCR-DGGE were also used for the microbial community composition analysis at each stage of reactors. Moreover, this research aimed to provide information about the effect that antibiotic combinations have on the development and spread of ARGs in anaerobic SBRs. Real-time PCR (qPCR) assays was used to determine the effect that different antibiotic combinations had on the total and active Bacteria, Archae and

Methanogenic Archae. Three primer sets that targeted metabolic genes encoding formylterahydrofolate synthetase, methyl-coenzyme M reductase and acetyl-coA synthetase were also used to determine the inhibition level on the mRNA expression of the homoacetogens, methanogens and specifically acetoclastic methanogens, respectively. qPCR was also used to detect the effect that different concentrations of antibiotic mixtures had on the development of ARGs. For this purpose, ten tetracycline resistance genes were selected. These included three different types of ARGs: the efflux pump genes *tetA*, *tetB*, *tetC*, *tetD*, *tetE*; the ribosomal protection genes *tetM*, *tetS*, *tetQ, tetW*; and a single enzymatic modification gene *tetX*. Three sulfamethoxazole resistance genes (*sul1*, *sul2*, *sul3*) and five erythromycin resistance genes (the efflux pump gene *msrA*; the ribosome protected genes *ermA*, *ermF*, *ermB*; and enzymatic inactivation gene (*ereA*) were also selected according to the resistance mechanism. The class 1 integron gene (intI 1) was also detected as a sensitive indicator of the potential for horizontal gene transfer. In order to verify abundances of ARGs from qPCR results, effluent and sludge samples were extracted from the ST and STE reactors at Stage 10 and Stage 12 respectively for detection all ARGs using the Illumina HiSeq 2000.

1.2 Literature Review

Pharmaceutical manufacturing wastewaters contain high concentrations of COD and antibiotics. The high COD of the wastewaters makes them a favorable alternative for anaerobic treatment (Chelliapan et al., 2006; Oktem et al., 2008). However, the anaerobic process is complicated, Bacteria and Archaea work together to convert complex polymers into methane through a number of steps that must be followed in a sequential and parallel manner. Hydrolysis is first rate-limiting step in anaerobic degradation of organic matter. The hydrolysis of macromolecules (lipids, proteins and carbohydrates) under anaerobic conditions is converted by specific extracellular enzymes, the reaction rates of which are influenced by pH, cell residence time and the waste constituents in the digester produced by hydrolytic bacteria, which is quite heterogenic (*Clostridium thermocellum*, *Clostridium bifermentas*, *Peptococcus sp.*, *Clostridium butyricum*, *Bacillus subtilis*). Following this stage, soluble oligomers and monomers are taken up by facultative and obligatory anaerobic bacteria and are converted to VFAs (acetate, probionate, butyrate, lactate) and alcohols in acidogenesis, which is mainly formed by *Propionibacterium sp.* and *Clostridium sp.*

Acidogenesis stage is followed by acetogenesis. Two different types of acetogenic mechanisms can be pronounced. First of them is acetogenic hydrogenation which involve the production of acetate as an end product, either from the fermentation of hexoses or from CO_2 and H_2. Second of them is the acetogenic dehydrogenation which refers to the anaerobic oxidation of long and short chain volatile fatty acids (Stams et al., 2012; Qiu et al., 2013). Methanogenesis is the final and may be the most important step of the anaerobic digestion process, which is a special group of Archaea called Methanogens (Aydin et al., 2015b). This population is strictly anaerobic and considered the rate-limiting step in the whole anaerobic digestion process due to the slow growth rate of the methanogens comparing to acidogens and accordingly, the performance of anaerobic digesters and the quality of the reactors depend on the activity of methanogens (Shima et al., 2008; Aydin et al., 2015a, b). This population is generally categorized in two main groups according to their substrate conversion capabilities; acetate utilizers (Acetotrophic methanogens) and hydrogen utilizers (Hydrogenotrophic methanogens). Acetotrophic methanogens are responsible for converting acetate to methane and CO_2 and, as such, they play an extremely important role in CH_4 production, as 70% of the methane that is produced as an output of the process is derived from acetate. Hydrogenotrophic methanogens are responsible for converting H_2/CO_2 to methane. The percentage of these two groups in anaerobic reactors can change due to operational parameters (temperature, pH, type of the substrate, alkalinity, HRT, SRT, reactor configuration) and instabilities or differences in these conditions such as the occurrence of antibiotics (Shima et al., 2005).

Biological treatment processes offer the ideal conditions in which a high diversity of microorganisms can grow and develop. This may create favorable conditions that aid the proliferation of antibiotic resistance genes (ARGs), which could pose health risks to humans via various pathways (Ma et al., 2011; Rodríguez-Mozaz et al., 2014; Liu et al., 2013). Nevertheless, the behaviors of ARGs during anaerobic wastewater treatment processes under high antibiotic combinations levels have been rarely stated.

Tetracycline, sulfamethoxazole and erythromycin antibiotics are most commonly used in human and veterinary medicine. For each class of antibiotics, more than one mechanism can often result in resistance. At present, 38 tetracycline resistance genes (*tet* genes) and three oxytetracycline resistance gene (*otr* genes) have been identified

in the aquatic environment (Zhang et al., 2013; Liu et al., 2014). Three specific mechanisms of tetracycline resistance have been identified: antibiotic efflux pumps, target modification with ribosomal protection protein (RPP), and inactivating enzymes as seen as Figure 1. Three sulfonamide resistance genes (*sul1*, *sul2* and *sul3*) coded for different types of dihydropteroate synthase (DHPS) in the folic acid pathway of bacteria, which allow for insensitive to sulfonamide (Selvam et al., 2012). *The sul1* gene is found on integrons and most multi-resistant gram-negative bacteria with Class 1 integrons carry the *sul1* gene. The *sul2* gene is frequently associated with the small, multi-copy, non-conjugative IncQ plasmid group. The sulfamethoxazole resistance gene *sul3* is also often found in horizontally transferable elements that are transposons and plasmids. As such, any study that is concerned with the detection of sulfonamid-resistance bacteria in wastewaters should focus on these three common sul genes (Gao et al., 2012). Three different mechanisms of erythromycin resistance have been described by efflux pump genes (*mefA/E* and *msrA/B*), modification of the target site to avoid binding of macrolides (erythromycin ribosomal methylase genes, *erm*), and destruction of the lactone ring directly (e.g., esterase genes [*ereA* and *ereB*], macrolide-2'-phosphotransferase gene, *mphA)*. Between these resistance genes, *erm* genes (A, B, C, E, F, T, V, and X), *mef* genes (A, E ,and I), *msrA*, *ereA/B*, and *mphA* genes have been identified in wastewater and biological wastewater treatment systems. Of these, the *ermB* gene is frequently the most dominant gene found in the wastewater samples (Wang et al., 2014).

ARGs can be transferred very rapidly and widely between the microorganisms that are present in the environment through mobile genetic elements such as plasmids, integrons and transposons. The process by which elements transfer these genes across different species or strains is referred to as horizontal gene transfer (HGT). The existence of the integron-integrase gene (intI) in integrons is the most important indicator of HGT in prokaryotes. The modification of functional bacteria genes may also result in antibiotic resistance. These spontaneous mutations will develop resistance through the process of the mutation of the bacterial chromosome, and can then be transferred to the next generation (vertical gene transfer). It is also largely accepted that an aqueous environment provides the ideal conditions within which resistant genes can be transferred between bacteria (Pruden et al., 2006; Zhang et al., 2013). Munir et al. (2011) investigated raw wastewater, effluent and biosolids in order to monitor tetracycline and sulfonamide resistant bacteria, tetracycline resistant

genes (*tetW* and *tetO*) and the sulfonamide resistant gene (*sul1*). They used qPCR assays and conventional heterotrophic plate count methods to evaluate the concentration of antibiotic-resistant bacteria in the samples. This study revealed that the disinfection process did not reduce ARGs. Furthermore, the ARGs were found to be in a range of 5.61×10^6 - 4.32×10^9 copies/g in biosolids. Zhang et al. (2013) used qPCR assays to determine eight tetracycline resistance genes (tetA, *tetB*, *tetC*, *tetE*, *tetM*, *tetS* and *tetX*) within a sequencing batch reactor. The results indicated the presence of widespread tetracycline resistance genes in the activated sludge under the pressure of trace tetracycline concentration. Furthermore, Birošová et al. (2014) analyzed 33 antibiotics and antibiotic-resistant microorganisms in two wastewater treatment plants (WWTPs) in Bratislava, Slovakia. Antibiotic-resistant strains were also detected by a process of bacteriological counting. They compared influent and effluent wastewater to monitor the differences in antibiotic concentrations across different seasons. They found that seasonal changes in antibiotic consumption resulted in the increased coliforms and streptococci found in sewage sludge. According to the results, the majority of coliform bacteria are resistant to ampicillin and gentamicin.

It is widely acknowledged that the presence of antibiotics in wastewaters has a direct impact on the microbial community due to the occurrence of antibiotic resistance bacteria and genes in the bioreactor (Ma et al., 2011; Munir et al., 2011; ; Wang et al., 2012; Zhang et al., 2013). This is especially the case in pharmaceutical wastewaters, which contain high non-lethal antibiotic concentrations (below the wild-type inhibitory concentration). Spontaneous mutation in the bacterium's DNA is much higher at sub-MIC concentrations, which are inhibited in terms of growth but are not usually killed, than at lethal concentrations (Amin et al., 2006; Kummerer, 2009; Larsson et al., 2007; Andersson and Hughes, 2012). It is, therefore, predicted that antibiotic resistant mutants will develop quickly and will be more stable in bacterial populations that are characterized by high, non-lethal antibiotic concentrations as opposed to lethal or lower antibiotic concentrations. Moreover, high levels of antibiotics have been shown to increase and stimulate HGT and activate mobile genetic elements among the bacterial community (Beaber et al., 2004; Ubeda et al., 2005). This means that sub-lethal high concentrations allow an increase in the genetic variations that are involved in the occurrence and dissemination of resistance. The other disadvantage of high antibiotic concentrations

is that the bacteria may develop several resistance mechanisms; this will ultimately result in multidrug resistance (Baharoglu et al., 2011).

1.3 Unique Aspect

The high amount of COD that is present in the wastewaters produced by pharmaceutical manufacturing plants makes them a favorable alternative for anaerobic processes. However, the anaerobic process is complicated and involves a number of different bacterial and archaeal species. Biodegradation of antibiotics and occurrence of TPs and ARGs may depend on shifts in microbial community structure during anaerobic treatment. Furthermore, many existing studies have evaluated the effect that individual antibiotics have on biological treatment systems. Despite the fact that antibiotics are not present as single compound substances in environmental compartment, there is very little literature available on the inhibition effects of antibiotic mixtures.

1.4 Organization of the Thesis

The thesis contains three data chapters (Chapters 2-4), preceded by an introduction (Chapter 1) and followed by thesis conclusions and recommendations (Chapter 5). Each data chapter consists of a combination of the overall research objectives, while specifically focusing on long-term effects of antibiotic combination on anaerobic treatment process and microbial community structure and how this relates to biodegradation of antibiotic combination and the occurrence of antibiotic resistance genes.

Chapter 2 presents data on combined effects of erythromycin-tetracycline-sulfamethoxazole (ETS) and sulfamethoxazole-tetracycline (ST) antibiotics on performance of anaerobic SBRs were studied. A control reactor was fed with a wastewater free of antibiotics while the other two reactors were fed with ETS and ST. Influences of ETS and ST mixtures on COD removal, VFA production, antibiotic degradation, biogas production and composition were investigated. Concentrations of the antibiotics in the influent were gradually increased until metabolic collapse of the SBRs. The effects of the ETS mixtures are different from the ST mixtures, ertyhtromycin (bacteriostatic antibiotics) can have an antagonistic effect on sulfamethoxazole and tetracycline. Anaerobic pre-treatment of these

antibiotics can be a suitable alternative to traditional chemical treatments for concentrations at 10 mg/L of S and 1 mg/L of T, 20 mg/L of S, 2 mg/L of T and 2 mg/L of E for ST and ETS reactor respectively which corresponds to min 70% COD removal efficiency.

Chapter 3 examines long-term effects of erythromycin-tetracycline-sulfamethoxazole (ETS) and sulfamethoxazole-tetracycline (ST) antibiotic combinations on the microbial community structure and how this relates to anaerobic sequencing batch reactors (SBRs) performance. Quantitative real-time PCR was used for determining the effect of antibiotic combinations on total and active bacteria, archae and methanogenic archae by using the template extracted gDNAs and synthesized cDNA. Furthermore, three primer sets targeting metabolic genes encoding formylterahydrofolate synthetase (FTHFS), methyl-coenzyme M reductase (mcrA) and acetyl-coA synthetase (ACAS) were used to determine the inhibition level on mRNA expression of the homoacetogenesis, methanogenesis and specifically acetoclastic methanogenesis, respectively. These microorganisms are vital in anaerobic degradation of organic waste, and also targeting these gene expressions offer the potential for control and the improvements on the anaerobic system. The results of this investigation are revealed that acetogens have a competitive advantage over archaea under ETS and ST combinations. Although COD removal efficiency and quantification of microbial populations on both ETS and ST reactors are decreased by the increasing of antibiotic concentrations, the ETS batch reactor has a better performance than the ST batch reactor. According to the expression of genes results, the syntrophic interaction of acetogens and methanogens is critical to the performance of the ETS and ST reactors. A strong relationship between two groups of microorganisms has been shown at stage 3 and 7 of the ST and ETS reactors, respectively. Failure to maintain the stability of these microorganisms caused a decrease in anaerobic SBRs performance and instability.

Chapter 4 evaluated how anaerobic sequencing batch reactors were affected by amendments of different antibiotics and stepwise increasing concentrations. The compositions of microbial community were determined in the seed sludge using 16S rRNA gene clone libraries and PCR-DGGE analyses were used for the detection of microbial community changes upon antibiotics additions. According to PCR-DGGE

results, the syntrophic interaction of acetogens and methanogens is critical to the performance of the ETS and ST reactors. Failure to maintain the stability of these microorganisms resulted in a decrease in the performance and stability of the anaerobic reactors. Assessment of DGGE data is also useful for suggesting the potential to control ultimate microbial community structure, especially derived from Gram-negative bacteria, through bioaugmentation to successful for antibiotic biodegradation.

Chapter 5 presents thesis conclusions and recommendations for future research.

2. COMBINED EFFECT OF ERYTHROMYCIN, TETRACYCLINE AND SULFAMETHOXAZOLE ON PERFORMANCE OF ANAEROBİC SEQUENCİNG BATCH REACTORS

This chapter has been published in Bioresource Technology (Aydin, S., Ince, B., Cetecioglu, Z., Arikan, O., Ozbayram, E.G., Shahi, A., Ince, O., 2015. Bioresour. Technol. 186, 207-214)

2.1 Introduction

Antibiotics which are one of the most commonly used pharmaceutical products have a wide range of uses in both human and veterinary medicine. These active compounds to existing enzyme systems are discharged to receiving bodies without or a little mineralization from treatment plants and accumulated in soil and sediment day by day (Kümmerer, 2009). This accumulation may causes proliferation of antibiotic resistance pathogens, which are the important threat against public health and change in the native microbial population in ecosystem (Resende et al., 2014). Although concentrations of these antibiotics are relatively low in raw domestic wastewater (100 ng/L to 6 μg/L) they can be significantly higher in hospital and pharmaceutical industry effluents, reaching around the 100-500 mg/L level (Martin et al., 2012). Additionally, antibiotics typically occur within some form of mixtures of which effects can be antagonistic or synergistic. The mixtures effect are generally stronger than effects of its individual components even if all components are only present in low concentrations that do not provoke significant toxic effects (Aydin et al., 2015).

Biological treatment processes have been reported to treat effectively some by/end products of pharmaceutical manufacturing wastewaters (Shi et al., 2014). Although activated sludge treatment processes have been used to treat these type of wastewaters, they can be unsuitable when the COD levels exceed 1500 mg/L. The high COD of the wastewaters makes them favorable alternative for anaerobic bio-technology. Anaerobic digestion also uses less energy, has a lower sludge yield and

lower nutrient requirements, cheaper to implement, uses less space, and offers improved biogas recovery (Chelliapan et al., 2006; Oktem et al., 2008).

Various anaerobic treatment systems have been used for developing a successful strategy for treatment of single antibiotic compounds (Sreekanth et al., 2009; Auerbach et al., 2007). Although research in this area is promising, number of experimental studies investigating the combined effects of antibiotics on anaerobic treatment of pharmaceutical wastewaters is scarce. For example, Massa et al. (2000) investigated the presence of individual and combined antibiotics on the anaerobic digestion of swine manure slurry in SBRs. According to the results, the presence of 55 mg Carbadox/kg, 110 mg Tylosin/kg and 16 mg Penicilin/kg combinations in manure slurries did not have noticeable adverse effect of methane production. However, the presence of individual penicillin and tetracycline (550 mg/kg) in manure slurries reduced methane production by 35% and 25%, respectively. Furthermore, Alvarez et al. (2010) observed the significant inhibition of antibiotic mixtures including oxytetracycline and chlortetrcycline on the anaerobic digestion of pig manure. Similarly, Beneragama et al. (2013) demonstrated the individual oxytetracycline (OTC) and combined OTC and cefazolin (CFZ) at concentrations of 30, 60 and 90 mg/L represented 70.3%, 68.6% and 82.7%, 70.3% reduced methane production, respectively. Aydin et al. (2015) also found that remarkable synergistic effect was observed in various tetracycline, sulfamethoxazole and erythromycin combinations on anaerobic digestion. This study revealed that antibiotic mixtures have an adverse effect on homoacetogenic bacteria and methanogens, which may exert inhibitory effects on propionate (e.g. Syntrophobacter species, Pelotomaculum species) and butyrate-oxidizing syntrophic bacteria (e.g. Syntrophomonas spp. and Syntrophospora spp.), resulting in unfavorable effects on methanogenesis.

This study focuses on tetracycline, sulfamethoxazole and erythromycin antibiotics that are most commonly used within human and veterinary medicine in Turkey (Turkdogan and Yetilmezsoy, 2009). Erythromycin is among the main representative of the macrolide group antibiotics for clinical use. An important difference between erythromycin and other macrolides, such as clarithromycin and roxythromycin, is the sensitivity of erythromycin to pH. Tetracycline are also the cheapest classes of antibiotics available today, making them attractive for use in developing countries with limited health care budgets (Eliopoulos and Roberts, 2003). Erythromycin and tetracycline prevent bacteria from growing by binding irreversibly to the 50S and

30S bacterial rRNA subunits, respectively (Tenson et al., 2003). Sulfamethoxazole is a one of the most frequently detected sulfonamide group antibiotics in a wastewater treatment systems that achieves an inhibitory effect through two main methods. It inhibits the synthesis of nucleic acids or can inhibit the permeability of bacterial cell wall for glutamic acid, which is a necessary element for folic acid synthesis to be successful (McDermott et al., 2003). So, the direct effect of choosing antibiotic combinations would not be expected on methanogens. They are also active against a broad spectrum of Gram-positive and Gram-negative bacteria including species of the genus Syntrophobacter, Pelotomaculum, Syntrophomonas spp., Syntrophospora spp, Streptococcus, Staphylococcus, and Clostridium (Le-Minh et al., 2010).

This research aims to develop an understanding of triple effects of sulfamethoxazole-erythromycin-tetracycline (ETS) and dual effects of sulfamethoxazole-tetracycline (ST) on performance of anaerobic Sequencing Batch Reactor systems (SBRs) throughout a year operation. Chronic joint effects of ETS and ST on the COD removal efficiency, VFA production, antibiotic removal, biogas production and composition in SBRs were investigated.

2.2 Materials and methods

2.2.1 The experimental approach

The experimental set-up consisted of three anaerobic SBRs with identical dimensions and configurations. The anaerobic SBRs were run in a daily "fill and draw" mode using a synthetic substrate mixture including volatile fatty acids, glucose and starch that resembled the wastewater from a pharmaceutical industry are given in the study of Amin et al. (2006). Operation of the anaerobic SBRs included a start-up period of approximately 90 days for acclimation and establishment of steady-state conditions. At steady-state, it exhibited a stable performance, with an average effluent soluble COD (92±19 mg/L) corresponding to a COD removal of around 95% and an average biogas production (1247±3 mL/day). The influent antibiotics concentrations were gradually increased through successive phases each lasted for 30 days until metabolic collapse of the anaerobic batch reactors inferred from no COD removal or biogas production in the SBRs. The antibiotic concentrations used in each stage are given in Table 2.1. During the operation, the ST reactor and ETS reactor lasted until 10th (360 days operation) and 12th stages (420 days operation), respectively. The concentrations of antibiotics used are based on inhibition levels of

antibiotics reported by Cetecioglu et al. (2014) and Aydin et al. (2015). Daily antibiotics dosing were stopped after total collapse of the ST and ETS reactors, which were further operated for next 30 days in order to observe a possible recovery of both reactors. A third anaerobic SBR fed free of antibiotics was operated in parallel for the entire period under identical operating conditions serving as a control reactor.

Table 2.1 : Tested antibiotic concentrations

	Sulfamethoxazole (mg/L)	Erythromycin (mg/L)	Tetracycline (mg/L)
Stage 1	0.5	0.1	0.1
Stage 2	5	0.2	0.2
Stage 3	5	0.5	0.5
Stage 4	10	0.5	0.5
Stage 5	10	1	1
Stage 6	15	1	1
Stage 7	15	1.5	1.5
Stage 8	20	1.5	1.5
Stage 9	20	2	2
Stage 10	25	2.5	2.5
Stage 11	40	2.5	2.5
Stage 12	40	3	3

Evaluation of the anaerobic SBRs performances were predominantly based on measurements of soluble COD and volatile fatty acid (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.

2.2.2 Operation of anaerobic sequencing batch reactor systems

The three anaerobic SBRs with a liquid volume of 1.5L were inoculated using a granular sludge from an anaerobic contact reactor treating raki and fresh grape alcohol wastewaters. The anaerobic SBRs with 24-h cycles (10 min feeding, 23h 40 min reaction, 1 min settling and 9 min liquid withdrawal). The three anaerobic reactors were initially operated to reach a steady state at an OLR of 2.5 kg COD/m^3.d

at which point daily antibiotic additions were started. Throughout the operation, hydraulic retention time (HRT) of 2.5 days, a solids retention time (SRT) of 30 days were used. Reactor temperatures of 35±2˚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 solid (MLVSS) was fixed at 5000 mg/L. The composition of synthetic wastewater constituted 1160 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 (Aydin et al., 2014) as mg/L ($FeCl_2.4H_2O$, 2; $CoCl_2.6H_2O$, 2; $MnCl_2$, 0.32; $CuCl_2$, 0.024; $ZnCl_2$, 0.05; H_3BO_3, 0.05; $(NH_4)Mo_7O_{24}.4H_2O$, 0.09; Na_2SeO_3, 0.068; $NiCl_2.6H_2O$, 0.05; EDTA, 1; resazurine, 0.5; HCl (36%) 0.001 mL), vitamins as 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) solution, and was added to the wastewater. The pH of the SBRs were daily adjusted to 6.8 to 7.2 by addition of 1000 mg/L $CaCO_3$ alkalinity for sustaining operational stability.

2.2.3 Analytical methods

Duplicate samples were collected daily from the influent and effluent streamlines of the SBRs and chemical analyses such as soluble COD, alkalinity, total solids (TS), total volatile solids (TVS) were performed in accordance with Standard Methods (APHA, 2005). Miligas Counter (Ritter Digital Counter, U.S.A.) was used for monitoring the biogas production in ASBRs. Gas compositions and VFA concentrations were carried out using gas chromatographs with a flame ionization detector (Perichrom, France and Agilent Technologies 6890N, USA, respectively). The column used was Elite FFAP (30 m X 0.32 mm). The set point of the oven and maximum temperature of inlet are 100 ˚C and 240 ˚C, respectively. Helium gas was used as a carrier gas at a rate of 0.8 ml min^{-1}.

2.2.4 Erythromycin, tetracycline and sulfamethoxazole assay

Erythromycin, sulfamethoxazole and tetracycline assay was performed using Shimadzu high-performance liquid chromatography (HPLC) instrument (Schimadzu LC-10 AD) equipped with an UV detector (UV-Vis Detector, SPD 10-A) by injecting sample solutions onto a C18 analytical column. Degassing of the solvents

was achieved by sonification in a transonic ultrasonic bath (ELMA D-78224, Singen/Htw) prior to use. All results were analyzed by the system software, LC Solutions (Schimadzu Scientific Instruments Inc., MD, USA).

Triplicate samples were collected from the biomass, influent and effluent streamlines of the ETS and ST reactors at 20^{th} days of every antibiotic stage. Samples preparation were also performed from our previous study (Cetecioglu et al., 2013). Quantification based on peak areas, was performed by external standard calibration. The external standards used for quantification of the compounds were sulfamethoxazole D3, erythromycin and tetracycline. Seven point calibration curves (0.5-100 ppb) were generated using linear regression analysis. The calibration standards were measured three times randomly. The linarites were qualified by linear correlation coefficient, r^2 and seven points calibration curves of the compounds also gave very good fits, r^2 >0.99.

Erythromycin's gradient elution was applied using (A) 32mM potassium phosphate buffer by dissolving 5.57 g dipotassium hydrogen phosphate in 1000 ml water adjusted with concentrated phosphoric acid to pH 8.0 and a mixture (B) of acetonitrile/methanol (75/25). 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. Erythromycin was detected at 215 nm. The flow rate was 1.0 mL/min (Deubel and Holzgrabe, 2007). Sulfamethoxazole 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 (Karci et al., 2009). Tetracycline eluted with 74% 0.1 M okzalic acid and 25% metanol:asetonitril (1:1.5) solution which was delivered at a flow rate was 1 mL/min. Detection was carried out 367 nm (Yuan et al., 2010). Limit of detection (LOD) and limit of quantification (LOQ) of the erythromycin, tetracycline and sulfamethoxazole measurement were 0.58, 0.56 and 0.52; 1.94, 1.85 and 1.75 ng/mL, respectively. The recovery of the erythromycin, tetracycline and sulfamethoxazole were also 110.2±12.0%, 96.8±2.5% and 107±9.3%, respectively.

2.2.5 Statistical analysis

Statistical analyses were conducted in MINITAB (2013, USA). To determine the statistical significance of antibiotic mixtures' inhibition (ETS and ST), COD removal efficiencies of the ASBRs were compared using a one-way ANOVA test, followed by running a student's T- test. Significant differences were determined at $p<0.05$.

2.3 Results and Discussion

2.3.1 Performance of ASBRs

2.3.2 pH

The pH levels were generally stable (pH 6.8-7.2) at all stages of the control reactor. However, with the ST reactor, the pH in stage 8 dropped to 5.9 and the pH in stage 10 dropped to 5.6 in the ETS reactor due to rapid production of VFA created from reduced methanogenic activity and also increased acetogenesis process. From the pH data it can be assumed that the metabolic processes differed between and stages 1-8 of the ST reactor; stages 1-10 of the ETS reactor. Existing research indicates that a short contact time between high antibiotic concentrations and biomass has a positive impact on organic acid producing (acidogens) which have faster growth kinetics and a better rate of adaptation to reduced pH than the methanogens (Ma et al., 2013). A decrease in pH can also be caused by an accumulation of organic acids due to the failure of methanogens to convert the organic acids to methane. An example of this is the study carried out by Ahring et al. (1995) in which reported that the organic acid production effects a decrease in pH, which prevents acetate and hydrogen using methanogenic archaea.

2.3.3 COD removal

Efficient COD removal was observed during stage 1 in the ST reactor and stages 1-3 in the ETS reactor: Soluble COD in the effluent was reduced from an initial COD concentration of 2500 mg/L at the beginning of each cycle to 92 ± 19 mg/L, corresponding to an efficiency higher than 95 % (Figure 2.1). Similar COD removal could be maintained in the control reactor for the entire monitoring period. It is worth noting that synthetic substrate consists of organic compounds that are naturally biodegradable. Based on similar study used as single substrate or substrate mixtures, these compounds would be totally removed and that the low levels of soluble COD

detected in the effluent is essentially residual soluble microbial products that were generated during this biochemical reactions (Cetecioglu et al., 2013).

Soluble COD removal efficiency was not affected significantly ($p>0.05$) by the addition of ST mixtures from stage 2 to stage 3 in the ST reactor influent (days 90-150). The first remarkable effect of ST 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 ST reactor was observed at stage 4, while 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 ST mixtures (30 mg/L) addition between the 330th and 360th days. In contrary of this result, Sponza and Demirden (2007) found that the COD removal efficiency decreased from 87% to 68% when sulfamerazine concentration was increased from 10 mg/L to 90 mg/L. This contradiction could be originated from different anaerobic reactor types, the inoculum used, operation conditions or antibiotic combinations using in the studies.

Semi-continuous ETS dosing of 11 mg/L in stage 4, 12 mg/L in stage 5 and 17 mg/L in stage 6 did not seem to exert a noticeable effect on the overall COD removal. A significant ($p <0.05$) increase in the effluent soluble COD concentration to 600±15 mg/L corresponding to overall COD reduction of 75% in the ETS reactor was observed at stage 7, while effluent soluble COD concentration of the control reactor was 82 ± 21.2 mg/L. ETS mixtures increased to 46 mg/L in the following operation phase (stage12) resulting in a significant decrease in the reactor performance: The soluble COD value in the effluent increased to more than 2000 mg/L corresponding to overall COD reduction of only 10% after the 420th day.

At the end of stage 12 in the ETS reactor and stage 10 in the ST 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 substrate utilization and the reactor operated was terminated on day 390 in the ST reactor and day 450 in the ETS reactor.

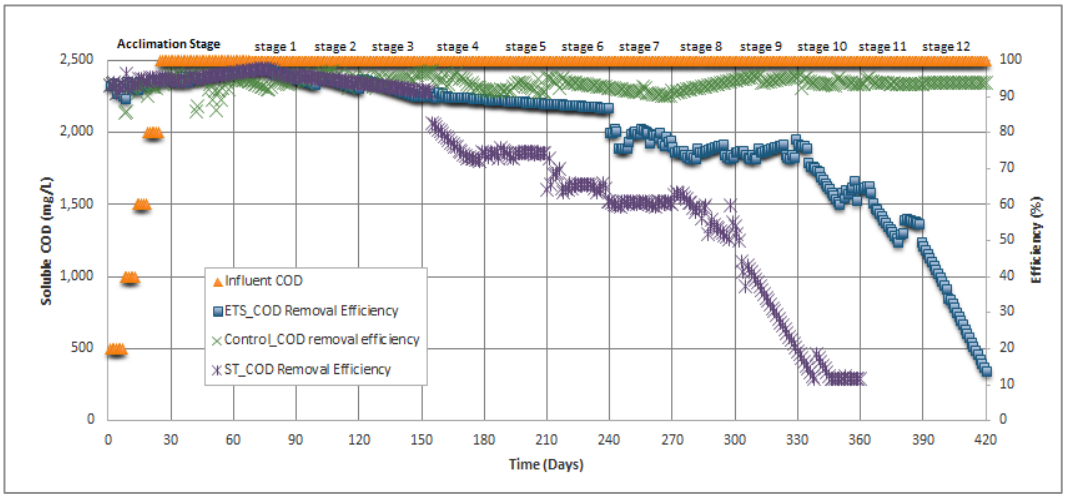

Figure 2.1 : COD removal efficiency in the ST and ETS reactors.

Previous research on the influence of antibiotics on anaerobic treatment systems has not been conclusive. For example, Cetecioglu et al. (2013) observed significant effect of tetracycline during anaerobic systems, however Hu et al. (2011) reported no influence of tetracycline during similar systems. Furthermore, Shimada et al. (2008) mentioned that strong inhibition of anaerobic SBRs in the present of tylosin (from the same group of erythromycin) while Chelliapan et al. (2006) observed negligible effects of the addition of tylosin in similar experiments. Also, Loftin (2005) observed significant inhibition by sulfamethoxazole during anaerobic batch systems but Sponza and Demirden (2007) observed a much less substantial impact of the addition of sulfamerazine (sulfonamide antibiotics). However, there is lack of information combined effect of ST and ETS on anaerobic treatment systems.

2.3.4 Biogas production

Biogas production was monitored in all stages throughout the operation of the reactor, particularly for the assessment of methanogenic activity. Figure 2.2 illustrates the biogas produced in all stages of the ETS and ST reactors. Biogas generation showed a significant ($p<0.05$) reduction in stage 4 for the ST reactor (86±2%) and in stage 6 for the ETS reactor (87±3%). The results of this study indicate that biogas production was parallel to COD removal efficiency in the ETS and ST reactors. Meanwhile, the amount of biogas production in the control reactor was almost constant through the operational time (1247±3 mL/day).

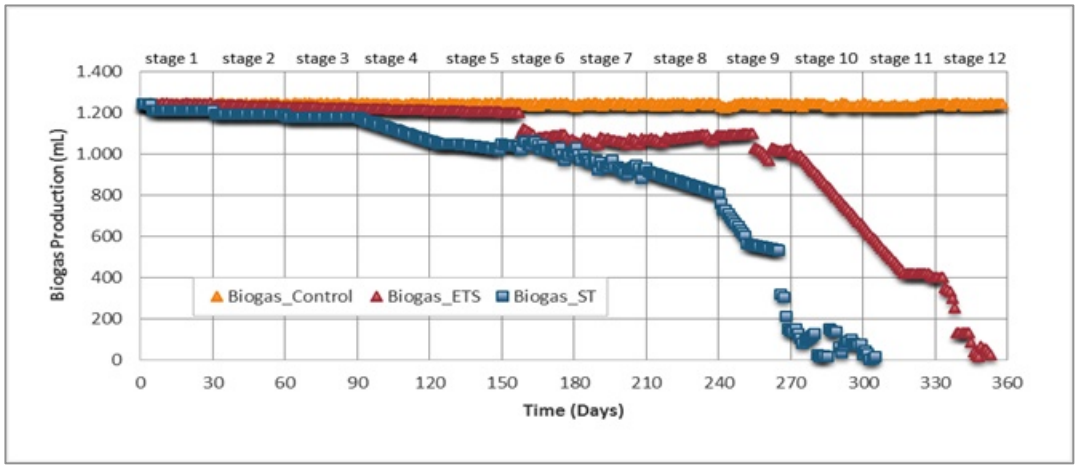

Figure 2.2 : Biogas production in the ETS and ST reactors.

Methane yield (L methane produced per gr COD removed) can be a useful parameter to assess the performance of an anaerobic reactor. Figure 2.3 shows that mean, steady-state methane yield was relatively constant in Stages 1-5 of the Control and ETS reactors, indicating an average specific methane production yield, Y_{CH4} of 0.30 ± L/g COD removed. However, the methane yield dropped dramatically in stage 6 when the ETS dosing changed from 17 to 18 mg/L and in nearly stable level until stage 10. In the following stage 10, with an ETS dose of 45 mg/L, the significant adverse effect of the reactor performance was observed for methane yield. It dropped from 44% to 24 % between days 300 and 322. In the ST reactor, methane yield was at an almost stable level until stage 4 and then decreased dramatically in stage 9. Consequently methane yield decreased substantially after stage 9, with the addition of ST mixtures (22 mg/L) on day 266 (25%). Methane production in all reactors showed a strong correlation ($p<0.05$) with biogas production during the operational time. This also highlights the effect of antibiotic combinations varies among different microbial groups of anaerobic process. The average amount of methane production in the control reactor was 879±4 mL/day. 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.

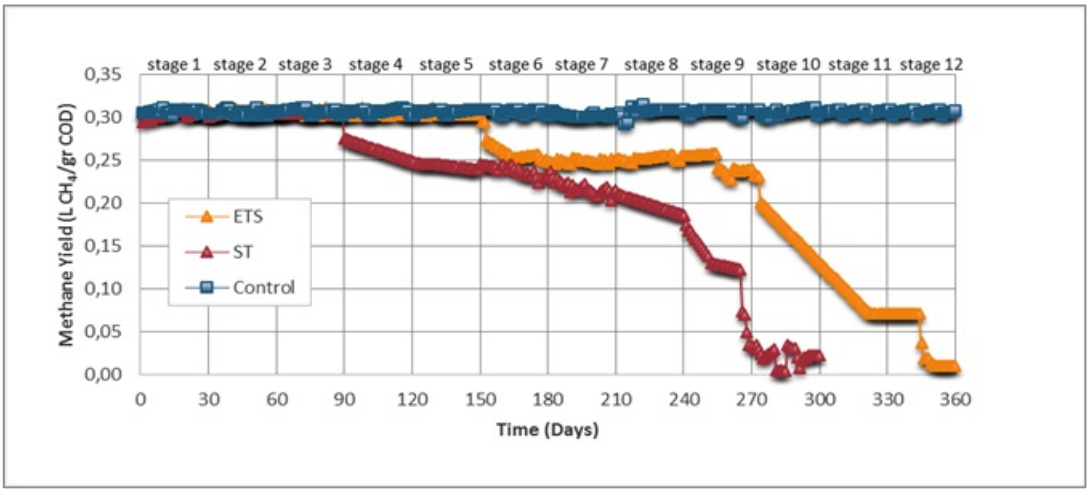

Figure 2.3 : Methane yield in the ETS and ST reactors.

A limited study about combined effect of antibiotic on biogas production in the literature was found. Masse et al. (2000) reported that tetracycline and penicillin reduced the methane production of psychrophilic anaerobic digestion (20 ˚C) of swine manure slurry in sequencing batch reactor by 25% and 35% respectively. Similarly, Alvarez et al. (2010) reported significant inhibition of anaerobic digestion of swine manure containing a combination of chlortetracycline (CTC) and oxyteracycline (OTC) at concentrations of 10, 50 and 100 mg/ L at 35 ˚C, where maximum methane production decreased by 64% in manure containing 100 mg/L of both CTC and OTC. Christensen et al. (2006) 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 biological treatment processes. The impacts of antibiotic mixtures on a mixed culture may be different than pure cultures, due to each group of bacteria may have different kind of response to the different group of antibiotics.

2.3.5 Effluent VFA composition

The presence and composition of the volatile fatty acids concentration in all stages of the ST and ETS reactors are shown in Figure 2.4. During the entire operation period, VFA could not be detected in the effluent of the control reactor. Furthermore, VFAs were not detected in the ST 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. While butyric acid concentration varied between 7 and 49 mg/L, valeric acid concentration increased from 17 mg/L to 105 mg/L slowly until the end of

operation. At stage 10, acetic acid and propionic acid were detected at 1000 and 691 mg/L, respectively. The results indicated that ST antibiotic combination affected the propionic and acetic acid utilization pathways in the higher concentrations. The degradation of propionate is most often utilized by gram-negative bacteria, and combinations of ST antibiotics would be expected to inhibit sensitive strains of this microbial group.

The first VFA in the effluent of the ETS reactor was measured on the 180th day, the first day of stage 7, at 50 mg/L of acetic acid (Figure 2.4b). On the 240th day, while the acetic acid concentration increased to 210 mg/L; valeric, butyric and isobutyric acids were determined for the first time at 20, 140 and 70 mg/L, respectively. Butyric acid concentration increased day to day and reached 710 mg/L at the end of operation. Similar findings have been reported by Beneragama et al. (2013). They have reported an accumulation of VFAs resulting in decreased methane production in the presence of antibiotics cefazolin and oxytetracycline.

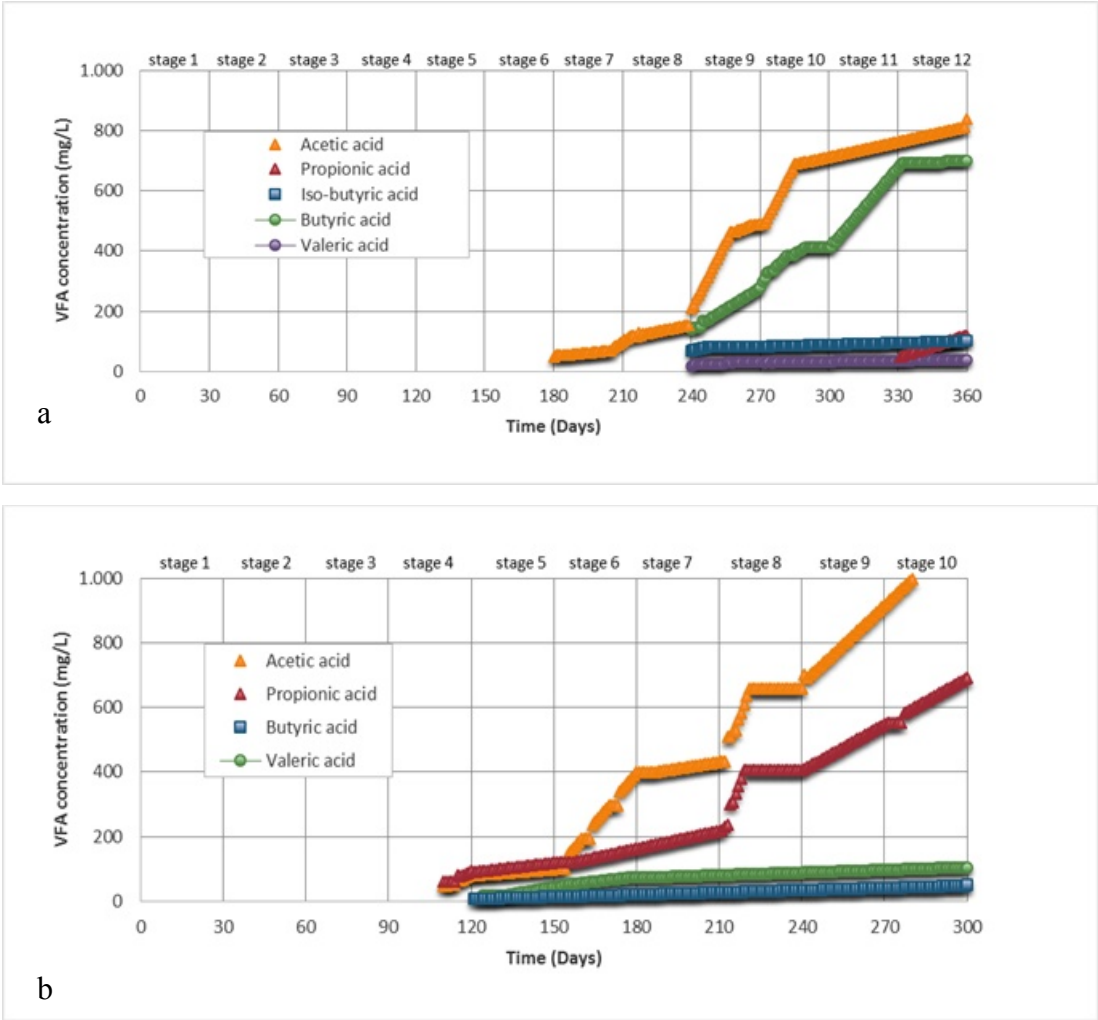

Figure 2.4 : VFA profile in the (a) ETS and (b) ST reactors.

Acetic acid accumulation displayed a similar trend however the concentration was higher than butyric acid and propionic acid. Unlike the ST reactors, butyric acid utilization pathways were inhibited by the ETS reactor. The results indicate that ETS combinations have a more dramatic effect on gram-positive bacteria than gram-negative bacteria (Aydin et al., 2015). The degradation of butyric acid is most often utilized by gram-positive bacteria and ETS combinations would be expected to inhibit sensitive strains of this microbial group.

A clear effect of the ST and ETS reactors on acetoclastic methanogens, which utilize acetate to produce methane, was observed in the VFA results. This is clearly indicated that homoacetogen affect during antibiotic biodegradation. However, there was no measurement of hydrogen in the biogas evaluation, as the study corresponded to that of Shimada et al. (2008). This is a clear indication that hydrogenotrophic methanogens can instantly change hydrogen to CH_4. It should be stated that homoacetogenic bacteria are not expected to perform any vital function in the degradation of acetate. These findings of the current study support the information and these results match those observations in earlier studies (Cetecioglu et al., 2013; Aydin et al., 2015). Similarly, Stone et al. (2010) speculated that chlortetracycline, is a tetracycline antibiotic, might have contributed to the inhibition of acetoclastic methanogens and that the VFA concentration during the operation of anaerobic digester increased.

According to VFA results, the high dosage of antibiotics has an impact on the acetoclastic methanogens in the ETS and ST reactors. The major reason for this can caused by accumulation of organic acids due to the failure of methanogens to utilize acetate to produce methane. Also, ETS combinations have a more dramatic effect on gram-positive bacteria than gram-negative bacteria. Differently from the ETS reactor, propionic acid utilization pathway was inhibited by ST reactor. The degradation of propionate is most often utilized by gram-negative bacteria (e.g. Syntrophobacter species, Pelotomaculum species), and combinations of ST antibiotics would be expected to inhibit sensitive strains of this microbial group (Aydin et al., 2015).

2.3.6 Erythromycin, tetracycline and sulfamethoxazole elimination

The degradation efficiency of erythromycin (ERY), sulfamethoxazole (SMX) and tetracyline (TET) antibiotics is illustrated in Figure 2.5. It indicates a SMX and ERY reduction pattern that started with more than 60%, increased to more than 80% in stage 6, and sustained around 10% at the end of stage 12, where the COD removal efficiency and biogas production were practically stopped. TET removal efficiency started with 60 %, then throughout stages 2-3-4 the TET removal efficiency of the ETS reactor remained constant (95%) and 3% in stage 10. The results indicated that TET was removed from the system in stages 1-9, even though it displayed that TET is not biodegradable under anaerobic conditions. Cetecioglu et al. (2013) also examined tetracycline removal efficiency under anaerobic conditions. According to the results, an average of 80% tetracycline reduction was achieved in the SBR, indicating that this antibiotic could be degraded efficiently in the anaerobic reactor system. Figure 2.5(c) indicates SMX reduction patterns that started at 62%, increased to %80 in stage 5, and sustained at around 20% at the end of stage 10; TET reduction started at 50% then increased to 60% stages 2-3-4, and continued to 12% at the end of stage 12, where substrate/COD utilization and biogas production was practically stopped. Also, a comparison of SMX and TET removal behavior in the ST reactor demonstrated that, SMX had a higher removal efficiency than TET and it was clear that anaerobic treatment is suitable for this compound to remove from wastewater at lower concentration (10.5 mg/L and 16.5 mg/L for the ST and ETS reactor respectively).

It is clear from Figure 2.6, that despite collapsing in stage 12 (46 mg/L) of the ETS reactor, in stage 10 (27.5 mg/L) of the ST reactor, antibiotic removal efficiency in both reactors were not zero. One possible explanation is that the amount of antibiotics and their metabolites were physical removed by means of sorption onto biomass. In fact, these results match those observations in earlier studies that shown sorption as the main mechanism for the removal of antibiotics (Le-Minh et al., 2010; Cetecioglu et al., 2013).

Comparison of the TET removal behavior in the ETS and ST reactors demonstrated that the ETS reactor had higher TET removal efficiency (44%) than the ST reactor (35% TET removal efficiency). It suggests that the significant ($p<0.05$) synergistic effects the of TET and ERY antibiotic combination on anaerobic biodegradation pathway.

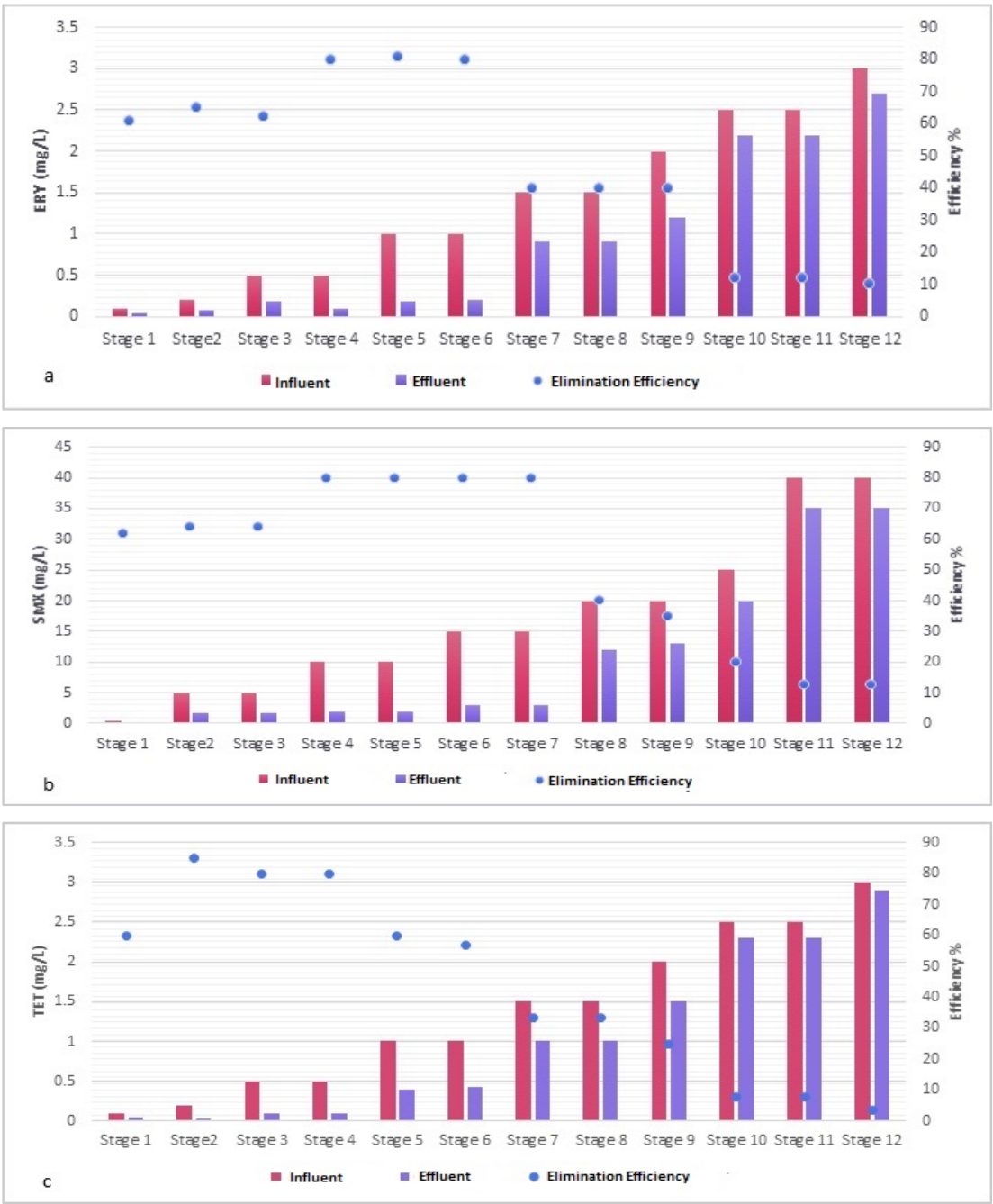

Figure 2.5 : ERY (a), SMX (b) and TET (c) measurement results of the ETS reactor.

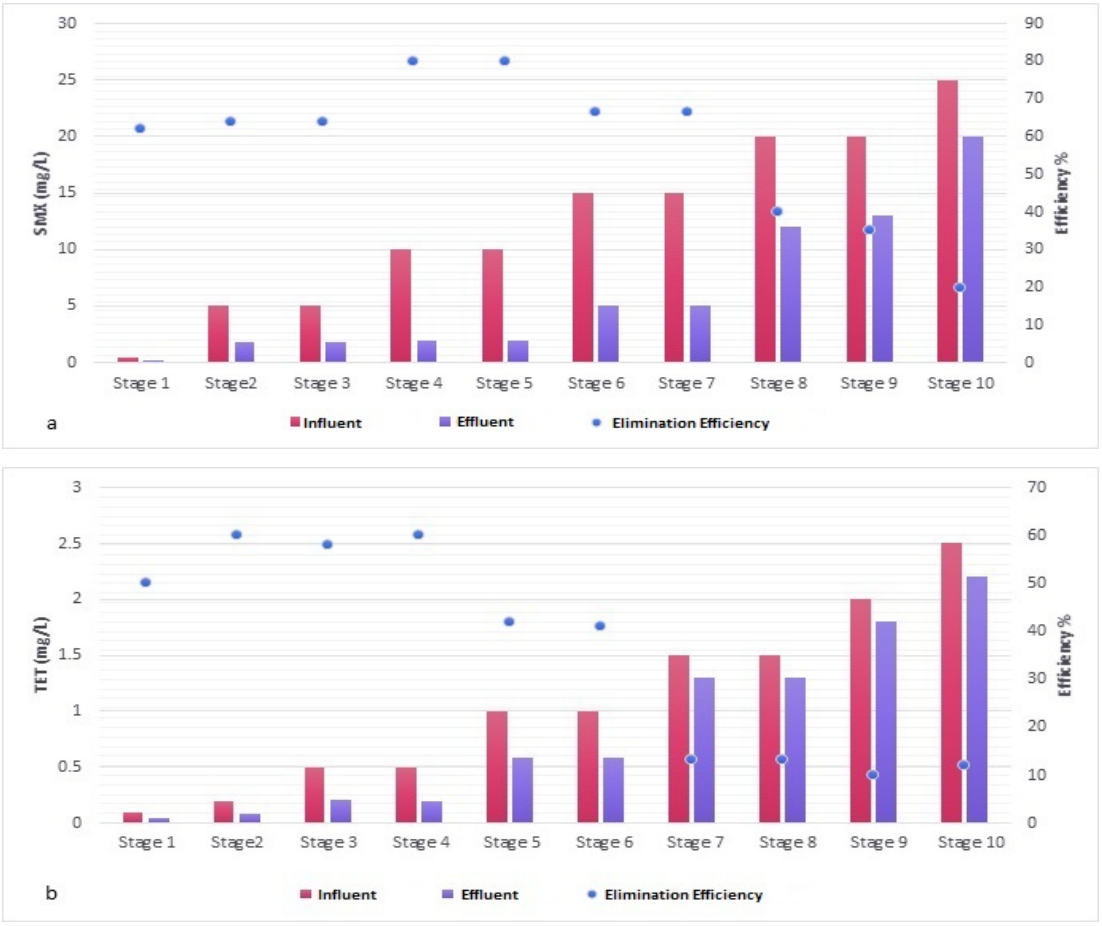

Figure 2.6 : SMX (a) and TET(b) measurement results of the ST reactor.

Results obtained from the ETS and ST reactors showed that the increase of antibiotic combinations dosages caused a decrease in antibiotic removal efficiency, but when repeating a constant dosage in the next stage, the reactor showed a non-significant removal efficiency because of the microorganisms' development resistance to antibiotics. Antibiotic resistance genes can be transferred between bacteria that are found in the environment through plasmids, integrons, and transposons (Resende et al., 2014). 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 and He (2011) established that the existence of erythromycin at concentrations as low as those found in the natural environment can significantly increase antibiotic resistance. Furthermore, Geo 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.

2.3.7 Effect of elevated erythromycin, tetracycline and sulfamethoxazole concentrations

The COD removal efficiency compared with ERY, SMX and TET removal efficiency in the ETS and ST reactors can be seen in Figure 2.7 and 2.8 As shown, COD reduction was higher than antibiotics removal efficiency in all stages. It can occur due to inadequate degradation efficiency in the anaerobic system. Also COD removal efficiency of the ETS and ST reactors decreased with respect to time and antibiotics concentration.

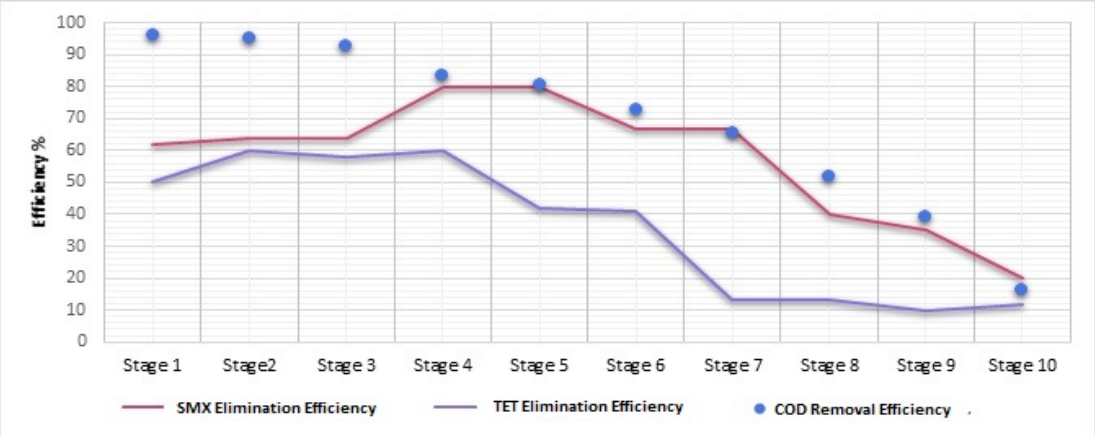

Figure 2.7 : Biodegradation profile in sulfamethoxazole and tetracyline for the ST reactor.

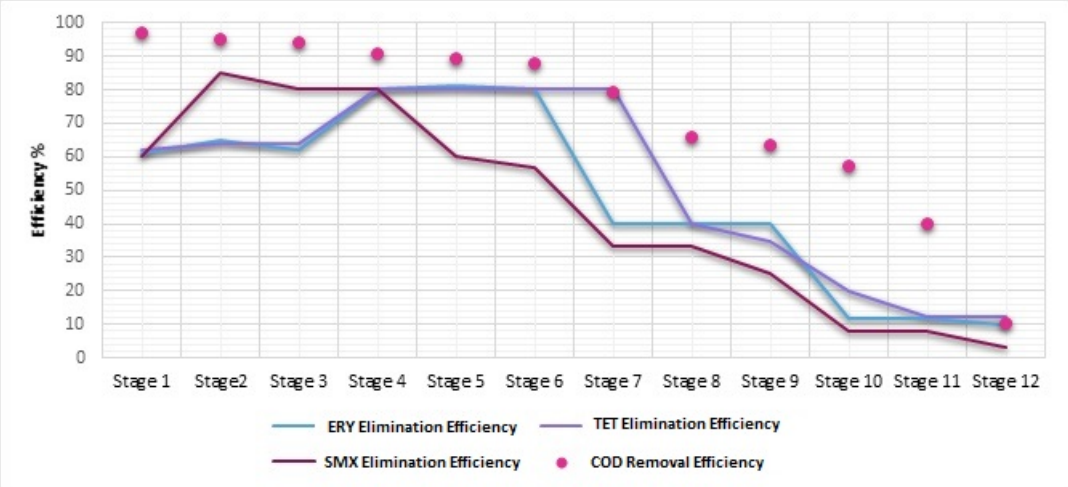

Figure 2.8 : Biodegradation profile in sulfamethoxazole, tetracycline and erythromycin for the ETS reactor.

As seen in Figure 2.7, antibiotics degradation significantly ($p<0.05$) decreased in stage 7. At the same time, removal of antibiotics impaired gradually with COD removal efficiency. There was a high degree of SMX degradation in stages 4-5-6-7

of the ETS reactor. COD reduction was gradually decreased and Figure 2.8 showed that COD removal efficiency was nearly the same as SMX degradation efficiency in stages 4-5. Also, COD removal efficiency and SMX degradation in stage 6 decreased dramatically. It is indicated that the long-term antibiotic combinations feeding to the reactors caused an increased in accumulation of the antibiotics and it directly affected reactors efficiency. It is also required to compare the full chromatograms of the influent, effluent and sludge to see whether the intermediates metabolites have been formed during the treatment process as future study.

2.4 Conclusions

This study indicated that the anaerobic process tolerated antibiotic combinations until stage 3 in the ST and stage 6 in the ETS reactors and following these stages, there is a negative impact of increasing antibiotic concentrations on COD removal and biogas production in the SBRs. The antibiotic removal behavior in the SBRs demonstrated that the ETS reactor had higher antibiotic removal efficiency than ST reactor. The 8th and 10th stages were the critical for the ST and ETS reactors respectively. After these stages, antibiotics and VFA accumulation were increased rapidly and they directly affected the performance of the reactors.

3. APPLICATION OF REAL-TIME PCR TO DETERMINATION OF COMBINED EFFECT OF ANTIBIOTICS ON BACTERIA, METHANOGENIC ARCHAEA, ARCHAEA IN ANAEROBIC SBRS

This chapter has been published in Water Research (Aydin, S., Ince, B., Ince, O., 2015. Water research, 76, 88-98)

3.1 Introduction

Occurrence of antibiotics, one kind of important contaminants due to their potential for long-term adverse effects on the microorganism, have become a growing concern in the aquatic environment (Kümmerer, 2009). These substances are usually poorly degradable contaminants and cannot be removed entirely with using the wastewater treatment plants (WWTPs) and they can be found in wastewater effluents. Although the concentration of these antibiotics is relatively low in wastewater, they can be significantly higher in hospital and pharmaceutical industry effluents, reaching around the 100-500 mg/L level (Kummerer, 2001; Amin et al., 2006). This accumulation play a role in the dissemination and the development of antibiotic resistance genes and resistant bacteria, which may pose health risk to public health and can cause the changes in the native microbial population in the ecosystem (Selvam et al., 2012; Fan et al., 2011; Resende et al., 2013; Rodríguez-Mozaz et al., 2014). At high levels antibiotics also have an effect on WWTPs performance, such as carbon removal when WWTPs have been contaminated with these substances (Sanz et al., 1996; Shimada et al., 2008; Cetecioglu et al., 2013). Because all of these reasons, quantitative detection of the microbial community inside bioreactors is so important for maintaining efficiency and stable reactors operation (Kindaichi et al., 2006; Wang et al., 2012).

Anaereobic treatment processes have been reported effective at treating some by/end products of pharmaceutical manufacturing wastewaters (Chelliapan et al., 2006; Oktem et al., 2008). However, the anaerobic treatment is unique process, and involves several bacterial and archaeal groups and a number of phases that must be followed in a sequential and parallel manner by four major steps: hydrolysis,

acidogenesis (fermentation), acetogenesis and methanogenesis as seen in Figure 3.1 (Narihiro and Sekiguchi, 2007). Acetogenic bacteria is an important step responsible for converting simple products such as acetate, H_2, CO_2 and a series of other fermentation products like propionate, butyrate and alcohols. These microorganism s is so important because complex substrates are not used by archaea (Stams et al., 1994; Stams et al., 2012; Town et al., 2014). In order to ensure that the process is successful and the performance of the systems in a stable manner, a special group of archaea called methanogens need to be maintained. This population, which is responsible for catalyzing the terminal and most sensitive step in the anaerobic process (methanogenesis), is generally categorized as two main groups (acetotrophic and hydrogenotrophic methanogens) according to their substrate conversion capabilities. Acetotrophic methanogens have an extremely important role in CH_4 production, as 70% of the methane that is produced as an output of the process is derived from acetate (Ince et al., 2011; Kim et al., 2013). The relation between species in the microbial community and distribution in the anaerobic process are not well understood during treatment of wastewater which contains antibiotic combinations. So, necessity of comprehensive researches on this subject, particularly based on 16S rRNA gene and several functional genes, is available for understanding of population dynamics and its effects on WWTPs performance (Lee et al., 2009).

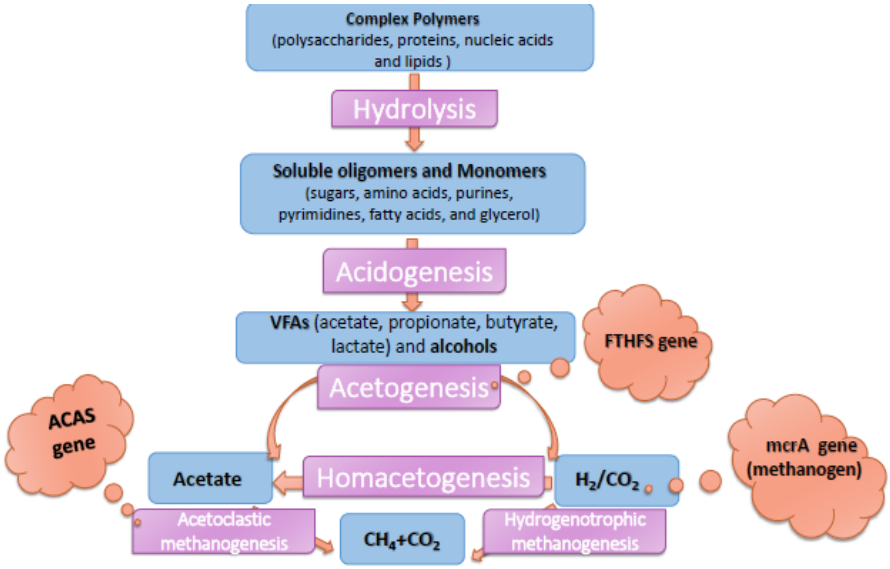

Figure 3.1 : Anaerobic process and targeting metabolic genes.

Quantitative real-time PCR (qPCR), or real-time PCR, is highly important in the development of culture independent approaches in microbial ecology researches to understand wastewater treatment systems because of its sensitivity, accuracy and specific quantification capacity (Kim et al., 2013; Cater et al., 2013). Moreover, q-PCR is a more suitable approach to determine the composition of functional group based on the analysis of the abundance gene and/or transcript numbers present in wastewater samples for measurement of specific microbial activities (Kim et al., 2013). Traversi et al. (2011) conducted q-PCR assays targeting mcrA gene to quantititative changes in methanogenic communities in two pilot-scale anaerobic digesters. The q-PCR results revealed that aceticlastic methanogens (especially Methanosarcina and Methanosaeta) were predominant and significantly different in two anaerobic digesters. Methanosarcina and Methanosaeta were also positively correlated with biogas production. Wang et al. (2012) also revealed that both of 16S rRNA gene and amoA gene quantification have been widely used to characterize ammonia-oxidizing bacteria in activated sludge samples. Furthermore, Kindaichi et al, (2006) investigated ammonia-oxidizing bacteria and uncultured Nitrospira-like nitrite-oxidizing bacteria in biofilms by using fluorescence in situ hybridization (FISH) and q-PCR techniques. According to the results, q-PCR techniques were easier and faster than FISH in act.

16S rRNA gene has been the most commonly used phylogenetic approaches in detailing target microorganisms of anaerobic process, but also functional genes have been searched as an alternative phylogenetic tool in the specific detection and identification of anaerobic microorganisms. Targetting an functional genes also provide the potential for activity-based detection in biological process. For instance, the FTHFS gene that encode the formyltetrahydrofolate synthesis in all homoacetogens, a key enzyme of acetyl-CoA pathway. This enzyme is thought to be unique to homoacetogen that are essential for maintain efficient and stable in operation of the anaerobic process (Leaphart et al., 2001; Xu et al., 2009). The mcrA gene that encodes the methyl CoM reductase of the methanogenic pathway and is thought to be unique and universal in methanogens, making it has been widely used in specific detection of methanogen in anaerobic process. However the mcrA gene highly similar sequence to the mrtA gene fragment. This means that quantification based on mcrA primers for qPCR is also likely to quatify mrtA sequences. However, mrtA gene sequences have only single methanogens family and also Luton et al

(2002) confirmed the validity of mcrA gene was achieved successfully for specific detection and identification of methanogens. Acetyl-coA synthetase (ACAS) of Methanosaeta concilii which is present in acetotrophic methanogens, is also a key enzyme that stimulates acetate to acetyl-CoA (Smith and Ingram-Smith, 2007; Ince et al., 2011).

Human and veterinary antibiotics of sulfamethoxazole (S), erythromycin (E) and tetracycline (T) are widely used. Erythromycin is macrolide groups of antibiotic for treatment of bacterial disease. Tetracyclines are also a group of broad-spectrum antibiotics in the treatment of different diseases, but now, their use for most disease have become less common due to occurrence of tetracycline resistance. Erythromycin and tetracyline prevent bacteria from growing by binding irreversibly to the 50S and 30S subunit of bacterial ribosomal, respectively (Tenson et al., 2003; Chopra et al., 2001).The ribosomes of Archaea are not similar size and shape to bacterial parts and consist of three RNA molecules, 16S, 23S and 5S RNA and 50-70 proteins depending on the species. Some archaeal r-proteins are also closer to eukaryote ribosamal proteins (Ramirez et al., 1993). Sulfamethoxazole is a sulfonamide group that compete with para-aminobenzoic acid (PABA) and inhibit normal bacterial utilization of PABA for the synthesis of folic acid (Baran et al., 2011). This antibiotics work by affecting things that bacterial ribosomal proteins and cell walls. So the direct effect of chosen antibiotic combinations would not be expected on Archaea.

The goal of this research was to understand how the microbial community changes in an anaerobic sequencing batch reactors (SBRs) for the treatment of wastewaters that contains erythromycin-tetracycline-sulfamethoxazole (ETS) and sulfamethoxazole-tetracycline (ST) as antibiotic combinations and how they are effected on the performances of reactors throughout a year operation. Antibiotics were selected from three different antibiotic structural classes which have widely usage area on human and veterinary consumption. qPCR quantification gives us strong understanding ability of anaerobic pathways enzymes. Three different primer sets are targeted to metabolic genes encoding FTHFS, mcrA and ACS which were used to determination of the inhibition level on mRNA expression of the homoacetogenesis, methanogenesis and acetoclastic methanogenesis, respectively. This approach may be helpful for understanding of microbial relationships between acetogens and

methanogens that hinders improvement of both stability and efficiency of the anaerobic SBRs.

3.2 Materials and methods

3.2.1 Start-up and operation of anaerobic SBRs

Detailed information on the reactors setup and operation has been reported in the previous studies (Cetecioglu et al., 2013; Aydin et al., 2014). In brief, the experimental set-up consisted of three anaerobic SBRs with identical dimensions and configurations. The anaerobic SBRs were run in a daily "fill and draw" mode (10 min feeding, 23h 40 min reaction, 1 min settling and 9 min liquid withdrawal) using a synthetic substrate mixture including volatile fatty acids, glucose and starch that resembled the wastewater from a pharmaceutical industry. Operation of the anaerobic SBRs included a start-up period of approximately 90 days for acclimation and establishment of steady-state conditions. The three anaerobic reactors were initially operated to reach a steady state at an organic loading rate (OLR) of 2.5 kg COD/m^3.d at which point daily antibiotic additions were started. Throughout the operation, hydraulic retention time (HRT) of 2.5 days, a solids retention time (SRT) of 30 days were used. Reactor temperatures of 35±2 ˚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 solid (MLVSS) was at 5000 mg/L. The composition of synthetic wastewater and the trace element solution were adopted from a previous study (Aydin et al., 2014). The pH of the SBRs were daily adjusted to 6.8 to 7.2 by addition of 1000 mg/L $CaCO_3$ alkalinity for sustaining operational stability. The influent antibiotics concentrations were gradually increased through successive phases each lasted for 30 days until metabolic collapse of the anaerobic batch reactors. During the operation, the ST reactor and ETS reactor lasted until 10th (360 days operation) and 12th stages (420 days operation), respectively. The concentrations of antibiotics used are based on inhibition levels of antibiotics reported by Gartiser (2007) and Cetecioglu (2013). Daily antibiotics dosing were stopped after total collapse of the ST and ETS reactors which were further operated for next 30 days in order to observe a possible recovery of both reactors. A third anaerobic SBR fed free of antibiotics was operated in parallel for the entire period under identical operating conditions serving as a control reactor.

3.2.2 Sample collection

Triplicate samples were collected from the Control, ETS and ST reactor sludges on the 10^{th}, 20^{th} and 30^{th} days of every antibiotic stage for gDNA and total RNA isolation.

3.2.3 Genomic DNA (gDNA) extraction

Genomic DNAs were extracted from 1 mL sludge samples using PureLink Genomic DNA Kits (Invitrogen, U.K.) by following the manufacturer's instructions. The gDNA concentrations that were extracted were determined using Qubit 2.0 Flouremeter (Invitrogen, UK) and diluted to 25 ng/µl by DNase-free water. The extracted gDNAs were stored at -20 °C until required for further analysis.

3.2.4 Total RNA extraction and cDNA synthesis

The total RNAs were isolated from 1 mL sludge sampled using a PureLink RNA extraction kit (Invitrogen, UK) that was used according to recommended procedures. The concentration of the isolated RNAs was measured using the Quant-It RiboGreen RNA Assay Kit with Qubit 2.0 Flouremeter (Invitrogen, UK). cDNAs were synthesized from isolated RNAs immediately. The rest of the isolated RNAs were stored at -80°C.

cDNAs were synthesized using a SuperScript cDNA synthesis kit (Invitrogen, UK) through a process of reverse transcription polymerase chain reaction (RT-PCR) using hexamer primers. cDNA synthesis 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 required for further analysis.

3.2.5 Quantitative real time polymerase chain reaction (qPCR)

Three primer sets that were designed to target Bacteria, Archae and Methanogens were used to quantify the existing and active microbial community through the application of the by template-extracted gDNAs and synthesized cDNA respectively. Furthermore, three primer sets that targeted metabolic genes encoding formylterahydrofolate synthetase (FTHFS), methyl-coenzyme M reductase (mcrA) and acetyl-coA synthetase (ACAS) were used to determining of the inhibition levels on mRNA expression of the homoacetogenesis, methanogenesis and, specifically, acetoclastic methanogenesis, respectively. A qPCR analysis of all primers is provided in Table 2.

Table 3.1 : Primers used for PCR amplifications in this study.

Target Microorganism	Primer	Sequence of the Primer	Target Gene	Annealing (°C)	Reference
Bacteria	Bac519f	5'-CAGCMGCCGCGGTAANWC-3'	16S rRNA	53	Lane, (1991)
	Bac907r	5'-CCGTCAATTCMTTTRAGTT-3'			
Archaea	Arc349f	5'-GYGCASCAGKCGMGAAW-3'	16S rRNA	60	Takai and Horikoshi, (2000)
	Arc806r	5'-GGACTACVSGGGTATCTAAT-3'			
Methanogen	Met348f	5'-GYGCAGCAGGCGCGAAA-3'	16S rRNA	55	Sawayama et al. (2006)
	Met786r	5'-GGACTACVSGGGTATCTAAT-3'			
Authentic Acetogens	FTHFS_f	5'-TTYACWGGHGAYTTCCATGC-3'	FTHFS	53	Lovell and Leaphart (2005)
	FTHFS_r	5'-GTATTGDGTYTTRGCCATACA-3'			
Methanogenic Archae	mcrA_f	5'-TGTAAAACGACGGCCAGTGGTGGTGTMGGATTCACACARTAYGCWACAGC- 3'	mcrA	51	Luton et al. (2002)
	mcrA_r	5'-CAGGAAACAGCTATGACCTTCATTGCRTAGTTWGGRTAGTT-3'			
Acetoclastic Methanogen	MSaeta_Aco-A _f	5'-TAATCCGCCAAAAGAGTTGG-3'	ACAS	56	Ince et al. (2011)
	MSaeta_Aco-A _r	5'-TCTTCTGGACTGGCTGGTCT-3'			

The recommended procedure 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 $MgCl_2$ 1.0 µl Primer F and R, 13.4 µl H_2O, 1 µl sample). Absolute quantification analysis of the gDNA was carried out using a LightCycler 480 Instrument (Roche Applied Science, Switzerland). The amplification protocol was as follows: initial denaturation for 10 min at 94 °C followed by 45 cycles of 10 s at 94 °C, 5 s at specific annealing temperature as given in Table 5.5, 16 s at 72 °C.

The standard curves for qPCR were constructed from an anaerobic sludge. The PCR products were purified using ethanol precipitation and then sequenced with using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA) using with the primers described in Table 2. After the primer specification was confirmed by the sequence analysis, these amplicons were used as standards. The dilution series of the purified PCR products 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 copied numbers of the genes in each sample were interpolated using these standard curves.

A melting curve analysis was performed for each PCR run with SYBR Green I detection. This was designed to verify specificity in each reaction tube by identifying the absence of primer dimers and other nonspecific products. The reactions for all samples were shown to have only one melting peak, which indicated a specific amplification that was suitable for accurate quantification. qPCR inhibition was detected in the control, ETS and ST reactors. The experiment concludes that qPCR inhibition was only observed for stage 12 in the ETS reactor.

3.2.6 Fluorescence in situ hybridization (FISH)

FISH was used to determine the total number of active cells of Archaea and Methanogens in the control, ETS and ST reactors. 16S rRNA specific probes targeting Archaea (ARC915) and different methanogens (Metahanobacteriales; MB311; Methanomicrobiales; MG1200b and Methanosarcinales; MSMX860) were used for hybridization (Crocetti et al., 2006). Samples were initially stained using DAPI before hybridization to enable the observation of the intact cell concentration. The FISH analysis was described in a previous study (Amann, 1995 and Sakai et al., 2004).

3.2.7 Determination of antibiotic resistance genes

qPCR were also used to detect anaerobic sludge taken from the beginning of the research on the development of antibiotic resistance genes (ARGs). For this purpose, nine tetracycline resistance genes were selected in Table 3.2. These included three different mechanisms of ARGs: the efflux pump genes tetA, tetB, tetC, tetD and tetE; the ribosomal protection genes tetM, tetS and tetQ; and a single enzymatic modification gene tetX. Three sulfamethoxazole resistance genes (sul1, sul2, sul3) and five erythromycin resistance genes (the efflux pump genes msrA; the ribosome protected genes ermA, ermF and ermB; and the enzymatic inactivation genes ereA) were also selected according to different resistance mechanisms. The quantitative analysis demonstrated that these ARGs were not present above detection limits in inoculated sludge's in ETS and ST reactors. Triplicate samples were collected from

the ETS and ST reactors' sludge on the 10th days of every antibiotic stage and these were used to quantify the antibiotic resistance genes.

Table 3.2 : Primer pairs used in qPCR assays for specific detection and quantification of ARGs.

Target gene	Mechanism	Sequence (5'–3')	Annealing temp (°C)	References
tetA	Efflux pump	F: GCGCGATCTGGTTCACTCG R: AGTCGACAGYRGCGCCGGC	61	Aminov et al. (2002)
tetB	Efflux pump	F: TACGTGAATTTATTGCTTCGG R: ATACAGCATCCAAAGCGCAC	61	Aminov et al. (2002)
tetC	Efflux pump	F: GCGGGATATCGTCCATTCCG R: GCGTAGAGGATCCACAGGACG	68	Aminov et al. (2002)
tetD	Efflux pump	F: GGAATATCTCCCGGAAGCGG R: CACATTGGACAGTGCCAGCAG	68	Aminov et al. (2002)
tetE	Efflux pump	F: GTTATTACGGGAGTTTGTTGG R: AATACAACACCCACACTACGC	61	Aminov et al. (2002)
tetM	Ribosomal protection	F: ACAGAAAGCTTATTATATAAC R: TGGCGTGTCTATGATGTTCAC	55	Aminov et al. (2001)
tetQ	Ribosomal protection	F: AGAATCTGCTGTTTGCCAGTG R: CGGAGTGTCAATGATATTGCA	63	Aminov et al. (2001)
tetW	Ribosomal protection	F: GAGAGCCTGCTATATGCCAGC R: GGGCGTATCCACAATGTTAAC	64	Aminov et al. (2001)
tetS	Enzymatic inactivation	F: GAAAGCTTACTATACAGTAGC R: AGGAGTATCTACAATATTTAC	50	Aminov et al. (2001)
ermA	Ribosomal protection	F:TCTAAAAAGCATGTAAAAGAA R:CTTCGATAGTTTATTAATATTAGT	45	Hungb et al. (2008)
ermF	Ribosomal protection	F: TCGTTTTACGGGTCAGCACTT R: CAACCAAAGCTGTGTCGTTT	50	Knapp et al. (2010)
msrA	Efflux pump	F: GGCACAATAAGAGTGTTTAAAGG R: AAGTTATATCATGAATAGATTGTCCTGTT	50	Faria et al. (2009)
ermB	Ribosomal protection	F: AAACTTACCCGCCATACCA R: TTTGGCGTGTTTCATTGCTT	60	Knapp et al. (2010)
ereA	Enzymatic inactivation	F: ACACCCTGAACCCAAGGGACG R: TTCACATCCGGATTCGCTCGA	50	Sutcliffe et al. (1996)
sul1	Enzymatic inactivation	F: CGGCGTGGGCTACCTGAACG R: GCCGATCGCGTGAAGTTCCG	56	Kerrn et al. (2002)
sul2	Enzymatic inactivation	F: GCGCTCAAGGCAGATGGCATT R: GCGTTTGATACCGGCACCCGT	55	Kerrn et al. (2002)
sul3	Enzymatic inactivation	F: TCAAAGCAAAATGATATGAGC R: TTTCAAGGCATCTGATAAAGAC	51	Heuer and Smalla (2007)

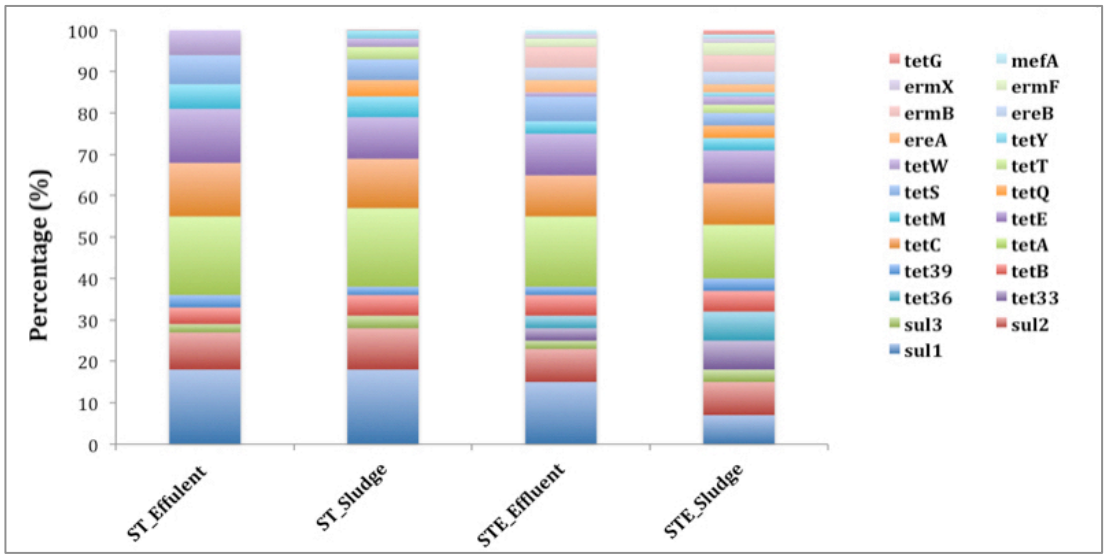

Figure 3.2 : Composition of antibiotic resistance genes in ST and ETS reactor.

3.2.8 Statistical analysis

Data normality was assessed by examining histogram and q-q plots and through the use of the Shapiro-Wilk's test. The Levene's test was also applied to test variance homogeneity. To compare the differences in gene expression between control and antibiotic-added reactors (ETS and ST), either a one-way analysis of variance (ANOVA) or independent samples t test was used. The Tukey's test was applied for multiple comparisons. Values are expressed as mean and standard deviation. Analyses were conducted using R 3.1.1 (www.r-project.org). A p value less than 5% was considered to be statistically significant. The outputs of all the statistical analysis are provided in the supporting information (Table A1).

3.3 Results

3.3.1 Performance of Anaerobic SBRs

Soluble COD removal and methane generation were not affected significantly ($p<0.05$) during all stages in the control, Stage 1 in the ST and Stage 1-3 in the ETS reactors. The first remarkable effect of ST and ETS mixtures on the COD utilization and methane production was detected in stage 4 and stage 7 respectively as per the information. In the presence of 20+1.5 mg L^{1} (Stage 4) of ST and 2.5+2.5+25 mg L^{-1}

of ETS (Stage 6) the concentration in the anaerobic SBRs became critical. After these stages, performance of the reactor substantially decreased.

At the end of Stage 12 in the ETS reactor and Stage 10 in the ST reactor, antibiotics dosing was stopped in order to observe any possible recovery in the performance of the reactor. However, the metabolic activity of the biomass could not be re-activated to induce noticeable substrate utilization, and the operation of the ST and ETS reactors was terminated on day 390 and day 450 respectively.

During the entire operation period, no VFA was detected in the effluent of the control reactor, as per the supporting information. VFAs were also not detected in the ST and ETS reactor's effluent until the 110^{th} day and 180^{th} days, respectively, at which point acetic acid accumulation started in the ST and ETS reactors. Acetic acid accumulation displayed similar trends and the concentration of butyric acid and propionic acid observed was higher during all stages in the anaerobic SBRs. The results indicated that antibiotic combination did have a dramatic effect on the acetoclastic methanogens present in the ST and ETS reactors.

3.3.2 Quantification of total Bacteria, Methanogenic Archaea, Archaea

Quantitative changes in the concentrations of 16S rRNA in terms of Bacteria, Methanogenic Archaea, Archaea were determined through the use of qPCR in the ETS and ST reactors. The concentration profiles indicated that there were temporal variations in the number of total genes at different stages of the anaerobic SBRs.

The number of total Bacteria, Methanogenic Archaea, Archaea in the ETS and ST reactors are presented in Figures 3.2, 3.3 and 3.4. The total population of Bacteria and Archaea were also not significantly ($p<0.05$) affected by the addition of ST and ETS combinations from Stages 1-2-3, Stages 1-2-3-4-5 in the ST and ETS reactors, respectively. The first remarkable effect of ST mixtures on the bacterial populations was detected in 10+0.5 mg L^{-1} (Stage 4). The efficiency of COD removal also started to significantly ($p<0.05$) decrease during the same stage in the anaerobic SBR. A significant effect on the total bacteria, methanogenic archaea and archaea was observed in the ETS reactor during Stage 6. These results could also reflect the performance of the anaerobic SBRs.

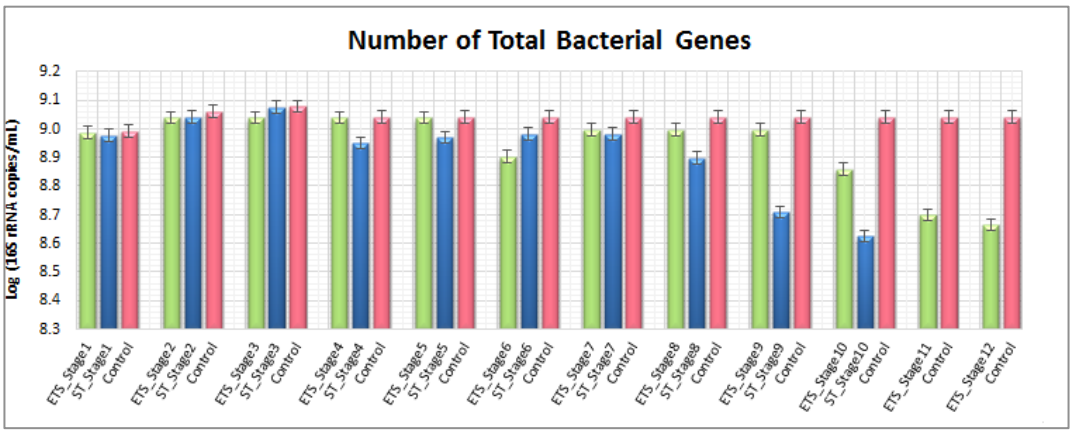

Figure 3.3 : Quantitative changes in bacterial 16S rRNA gene analyses at all stage of the ST and ETS reactors.

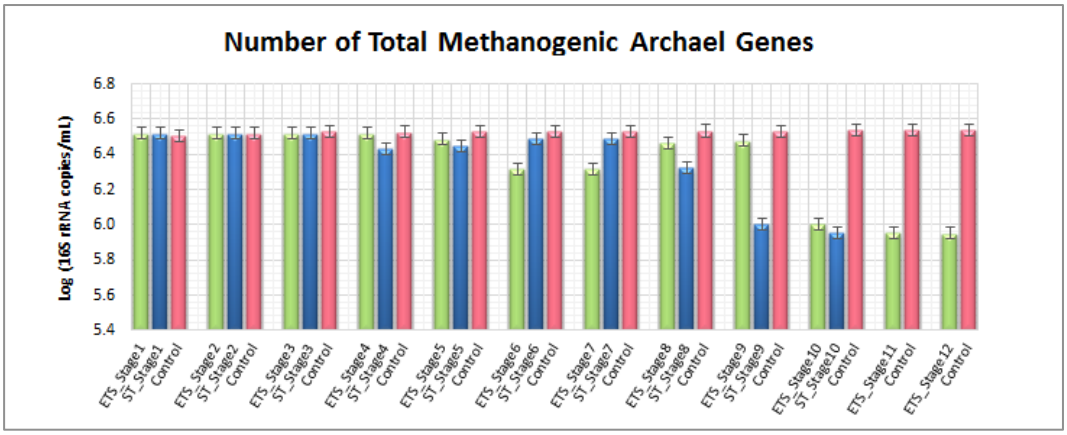

Figure 3.4 : Quantitative changes in methanogenic archaeal 16S rRNA gene analyses at all stage of the ST and ETS reactors.

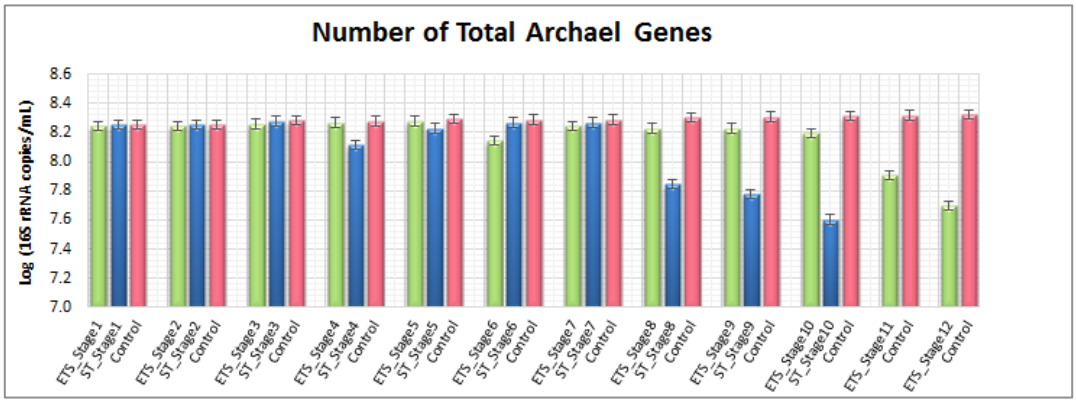

Figure 3.5 : Quantitative changes in archaeal 16S rRNA gene analyses at all stage of the ST and ETS reactors.

3.3.3 Quantification of Active Bacteria, Methanogenic Archaea, Archaea

qPCR was used for quantification of active Bacteria, Methanogenic Archaea and Archaea using 16S rRNA during a year operation of the ST and ETS reactors as seen in Figures 3.5, 3.6 and 3.7. The qPCR assay showed a significant decrease ($p < 0.05$) in the number of active genes in the presence of 10+0.5 mg L^{-1} (Stage 4) of ST and 1+1+15 mg L^{-1} (Stage 6) of ETS combination in the anaerobic SBRs. 16S rRNA concentration also increased at Stage 6-7 in the ST reactor and Stage 7-8-9 in the ETS reactor, compared to early stages. The reason for the increase in the active population of anaerobic SBRs could be due to adaptation of using antibiotic combination. The active community decreased dramatically at the beginning of Stage 10 and 8 in both the ETS and ST reactors.

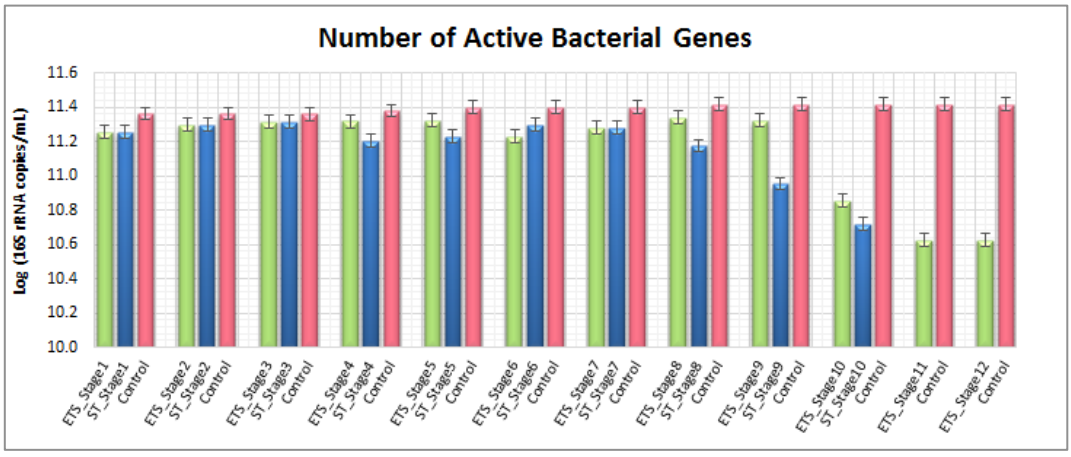

Figure 3.6 : Quantitative changes in bacterial 16S rRNA gene analyses at all stage of the ST and ETS reactors.

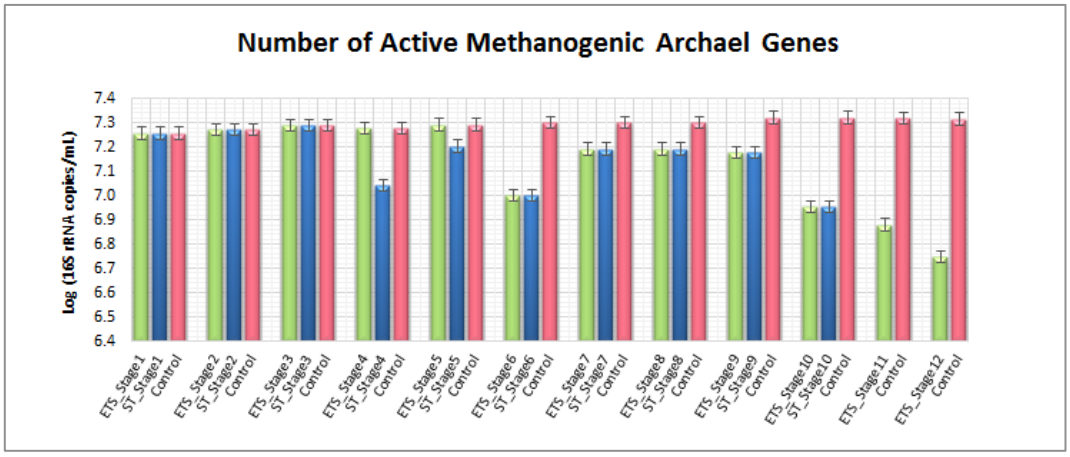

Figure 3.7 : Quantitative changes in methanogenic archaeal 16S rRNA gene analyses at all stage of the ST and ETS reactors.

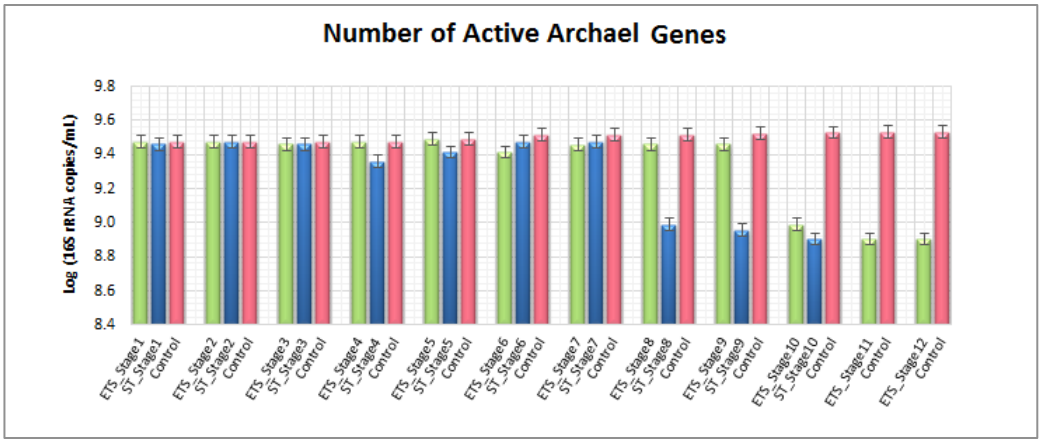

Figure 3.8 : Quantitative changes in archaeal 16S rRNA gene analyses at all stage of the ST and ETS reactors.

3.3.4 Quantification of gene expression

Three enzymes (FTHFS, ACAS, mcrA) were selected for the purpose of detecting for the extent to which the ST and ETS combinations inhibited the anaerobic pathway. This was assessed using the mRNA level from the different metabolic pathways of the anaerobic degradation. The expression level of FTHFS gene was detected at every stage of the ST and ETS reactor, with the exception of Stage 1 and Stage 2, as seen in Figure 3.8a. There was an increase in FTHFS genes expression for the ST and ETS reactors during Stages 3 and 7 respectively. The efficiency of the COD removal in the ST and ETS reactors decreased dramatically during same stages. The expression of mcrA and ACAS genes was detected during every stage of the operation of the ETS and ST reactors, as seen in Figures 3.8 b, c. The expression of the mcrA gene was remarkably decreased after Stage 4 and Stage 5 in the ST and ETS reactors respectively.

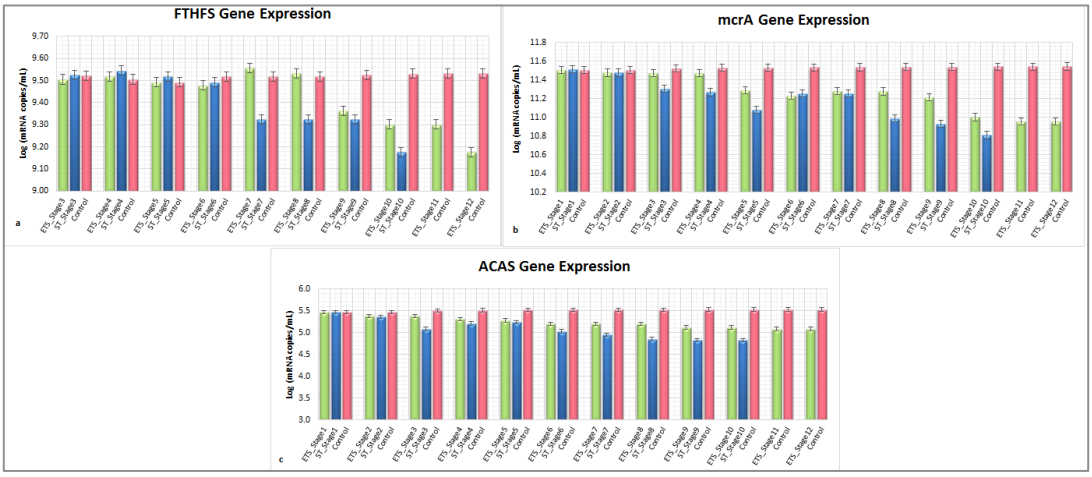

Figure 3.9 : Quantitative changes in targeting genes at all stage of the Control, ST and ETS reactors.

3.3.5 Quantification of Archaea and Methanogenic Archaea using FISH

The changes in the number of archaeal cells observed during the one-year operation of the reactor. According to the results of the FISH, the number of Archaea and Methanogenic Archaea in the ETS and ST reactors was negatively impacted by the increase in the concentration of antibiotics. Most of the Archaea consisted of Methanogens, which have similar expression levels as the qPCR results in the ETS and ST reactors. The situation was also similar for the total number of Archaea.

3.4 Discussion

Whereas many groups have used the quantification of 16S rRNA genes to unravel the complexities of microbial populations in anaerobic SBRs, the use of qPCR to generate functional genes, such as mcrA, ACAS and FTHFS were essential for maintaining the efficient and stable operation of the anaerobic process. For example, metabolic parameters, such as acetate concentration and biogas/methane production, are determined as a function of the whole microbial community. The VFA data revealed that the concentration of acetic acid concentration increased on a daily basis and reached high levels in the both the ETS and ST reactors by the end of the operation. Perhaps the most serious disadvantage of this method was that the results do not reveal which species of acetogens is most effect during anaerobic treatment. 16S rRNA gene or functional genes are used to investigate the diversity and populations of certain syhrophic acetogens. This also highlights the need to establish optimized growth conditions for syntrophic acetogens and the hydrogenotrophic partner in order to optimize methane production during antibiotic degradation.

However, data pertaining to VFA and methane about synthrophic acetate oxidation, the microorganism responsible, and their role in the methanogenic environment is currently limited. Evaluation of qPCR data is also useful for suggesting the potential to control ultimate microbial community composition via bioaugmentation to successful for antibiotic biodegradation.

The results of the Methanogen-spesific qPCR approach (mcrA and ACAS genes quantification) indicated that the combination of antibiotics has a more dramatic effect on Methanogenic Archaea than total Archaea combined. A decrease in the expression of ACAS genes was also found to be closely related to the decreased in the number of acetoclastic methanogens represented by the Methanosarcinales in the ETS and ST reactors. These results are consistent with those of previous studies and suggest that methanogens are more sensitive to changes in antibiotic combinations than the other microorganisms present in anaerobic SBRs. As such, the effective control of mcrA and ACAS genes is required for the successful operation of anaerobic systems (Yu et al., 2006; Stone et al., 2009; Cetecioglu et al,. 2013; Aydin et al., 2014; Ozbayram et al., 2014) and the maintenance of active methanogenic populations in anaerobic reactors is critical for the stable performance of ETS and ST reactors. The quantification of active bacteria, Archaea and Methanogenic Archaea results could be more reflective of performances of the ETS and ST reactors than they are of the quantification of total gene abundance. Thus, it is possible to target the active 16S rRNA gene for better sensitivity to develop a further understanding of the syntrophic interaction of the microbial community that is present in anaerobic SBRs.

The effect of antibiotic combinations varies among different microbial groups of anaerobic process. 16S rRNA quantification in the microbial community was strongly impacted by antibiotic combinations and the composition of the microbial community altered according to the efficiency with which COD was removed. The analysis of the results pertaining to the 16S rRNA genes and the functional genes also revealed that the concentration of a number of genes increased significantly ($p<0.05$) at Stages 6-7 and Stages 7-8-9 in the ST and ETS reactors when antibiotic doses from one phase to another were repeated. qPCR analysis demonstrated that ARGs were not present in the inoculated sludges that were produced in the ETS and ST reactors. Under these circumstances, antibiotics inhibit the growth of sensitive species allowing resistant bacteria to survive and proliferate. This adaptation was

also observed in the efficiency of the ST and ETS reactors because of bacteria development resistance to antibiotics (Figure S3 and Figure S4), and during these stages the efficiency of the COD removal did not exhibit a decline. Antibiotic resistant genes can be transferred between the microorganisms that are present in a given 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 transfer bacteria (Baquero et al., 2008). The findings of this current study support the outcomes of previous research in this area and are aligned with the observations made in earlier studies. Stone et al. (2009) reported that chlortetracycline might have contributed to the inhibition of the acetoclastic methanogens and resulted in an increased in the concentration VFA during the operation of anaerobic digesters. Furthermore, Amin et al. (2006) suggested that anaerobic biomass can develop erythromycin resistance during seven weeks of SBRs operation.

Analysis of FTHFS gene indicated that contact between antibiotic combinations and biomass have a positive impact on acetogens. This was demonstrated through faster growth kinetics and a better adaption rate of antibiotics (Ma et al., 2013). Moreover, compared to the control reactor, the lowest FTHFS was detected at Stage 12 in the ETS reactor and Stage 10 in the ST reactor. According to these results, it could be argued that the inhibitory effect of FTHFS is indirect effect, and it could be the result of the accumulation of toxic levels of intermediates and the accumulation of VFAs upon the inhibition of acetogenic bacteria.

The expression level of the mcrA gene was also higher than the ACAS during all stages in the ST and ETS reactors. The main reason for the difference in the ACAS and msrA genes expression results could be due to the response fo antibiotic combinations. When compared to hydrogenotrophic methanogens and acetoclastic methanogens, hydrogenotrophic methanogens have a higher substrate utilization rate, growth rate and cell yield when exposed to high antibiotic concentrations (Leaphart et al., 2001; Xu et al., 2009). FISH analysis also supported this finding and revealed that Methanosarcinales were the most affected group in the Methanogens present in the ETS and ST reactors. On the other hand, the dominance of the Methanobacteriales (hydrogenotrophic methanogens) could be pronounced in the SBRs, which was also in accordance with the results of the qPCR. Furthermore, the

tolerance of Methanobacteriales to toxic substances has been mentioned in previous literature (Xu et al., 2009).

3.5 Conclusions

The results of this study indicated that the anaerobic process tolerated lower antibiotic combinations (until Stage 3 in the ST and Stage 6 in the ETS reactors) during long-term operation and also revealed that increasing antibiotic concentrations has a negative impact on the microbial community structure and function in anaerobic wastewater treatment. Furthermore, the effects of the ETS mixtures were different from the ST mixtures; ertyhtromycin can have an antagonistic effect on sulfamethoxazole and tetracycline. The investigation of the active microbial community also revealed that the concentration of 16S rRNA genes increased at Stages 6-7 in the ST reactor and Stages 7-8-9 in the ETS reactor when the compared to the control reactor. This is due to the fact that they had acquired antibiotic resistance.

According to the results from the FISH and qPCR analysis, Methanogenic Archaea are very sensitive to small changes and this was ultimately reflected in the performance of the reactors. For this reason, the qPCR analysis of active Methanogens (especially acetoclastic methanogens) could be useful for the purposes of monitoring microbial communities that are present in wastewater treatment systems and assessing the extent to which the reactor can control and improve such systems.

4. USE OF PCR-DGGE BASED MOLECULAR METHODS TO ASSESSMENT OF MİCROBIAL DIVERSITY DURING ANAEROBIC TREATMENT OF ANTIBIOTIC COMBINATIONS

This chapter has been published in Bioresource Technology (Aydin, S., Shahi, A., Ozbayram, E.G., Ince, B., Ince, O., 2015. Bioresour. Technol. 192, 735-740)

4.1 Introduction

Antibiotics have proven to be very effective in the treatment and prevention of microbial infections and, as such, they are commonly prescribed in both human and veterinary medicine. This, combined with the increasing worldwide population and the utilization of antibiotics to improve the growth of livestock, has led to a significant increase in the global use and production of antibiotics. However, this has serious implications for the environment (Johnson et al., 2015). After use, the human body cannot metabolize the active compounds that are present in antibiotics and, as such, they enter the environment via sewage (Tao et al., 2014). Wastewater treatment systems are incapable of removing the compounds from wastewater and the antibiotics, therefore, pollute the environment. The extent to which they can impact the microbial community that is present in the environment has been observed in the acceleration of the resistance of species (Threedeach et al., 2012; Naquin et al., 2015). A number of studies have assessed the impact that antibiotic residues have on microbial communities. However, the majority of these have focused on non-industrial environments e.g. in soil and aquatic sediments and there is a distinct lack of published research that examines the bacterial communities that are found in the engineering biomass that is activated and anaerobic sludge, or biofilm (Ho et al., 2013; Aydin et al., 2015a, Aydin et al., 2015b).

The high amount of COD that is present in the wastewaters produced by pharmaceutical manufacturing plants makes them a favourable alternative for anaerobic processes (Oktem et al., 2008; Sreekanth et al., 2009; Selvam et al., 2012; Aydin et al., 2014; Aydin et al., 2015a; Aydin et al., 2015b). However, the anaerobic process is complicated, Bacteria and Archaea work together to convert complex

polymers into methane through a number of steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) that must be followed in a sequential and parallel manner (Aydin et al., 2015b). Due to these reasons, it is important to understand how antibiotic combinations impact anaerobic microbial communities dynamics; as well as how microbial communities can impact the fate of antibiotics in sequencing batch reactor (SBRs). However, conventional culture-dependent method is not also a time consuming and arduous technique but also detects very low amount of microorganisms present in the environmental samples (Zhang et al., 2013; Li et al., 2014; Hu et al., 2014).

PCR-DGGE as a powerful molecular method for rapid detection of microbial community changes or comparative analysis of environmental samples offers more accurate information about distribution and composition of microbial species. For examples, Dong et al. (2010) have used successfully the PCR-DGGE for comparing of Shannon diversity index and richness between influent to effluent of constructed wetlands treated with swine wastewater. Juang (et al., 2010) have also analyzed the Arthrobacter sp. corresponded to internal biofilm by using PCR-DGGE. Furthermore, Piterina and Pembroke (2013) with using suitable molecular target and electrophoresis condition have optimized PCR-DGGE technique autothermal thermophilic aerobic digestion (ATAD). They also observed that amplifying of V6-V8 region of 16S rDNA was more effective than rpoB gene profiles and this technique can be use as a monitoring method for assessment of the ATAD process efficiency. Zhang et al (2013) have been used DGGE for detection of the structural changes of the microbial community in sequencing batch reactor during the treatment of trace amount of tetracycline. Li et al. (2014) also reported DGGE for studying of the functional microbial community in composting by designing of three sets of PCR primers for identifying b-glucosidase. Hu et al. (2014) have developed specific primers for analyzing of the clostridial diversity in fermentation mud using DGGE technique.

Not only do these multi-component mixtures further threaten the environment, their joint toxic effect can also be a major issue for hazard and risk assessment. This is because the total ecotoxicity of a given mixture will be higher than the impact of its individual components. Furthermore, mixtures can exhibit significant ecotoxicity, even if the various components are only present in low concentrations that do not result in toxic effects on those microorganisms that are exposed to them

(Beneragama et al., 2013; Mitchell et al., 2013; Aydin et al., 2015; Aydin et al., 2015a, Aydin et al., 2015b). However, while it is a well-known fact that antibiotics have a combined effect on the anaerobic microbial community, the nature and extent of this effect is not fully understood. Therefore, the purpose of this study is to examine how sulfamethoxazole, erythromycin, and tetracycline combinations impact anaerobic processes. Each of these compounds is common components of the pharmaceuticals that are used in human and veterinary medicine.

The aim of this research was to determine how the Bacterial and Archaeal communities changes in anaerobic SBRs for the treatment of pharmaceutical wastewaters that contains sulfamethoxazole-erythromycin-tetracycline (ETS) and dual effects of sulfamethoxazole-tetracycline (ST), erythromycin- sulfamethoxazole (ES) and erythromycin-tetracycline (ET) throughout a year operation. In the current study, cloning and polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) were used to detect the effect of different concentration and combinations of antibiotics in the anaerobic SBRs. This approach may help to understanding of the microbial relationships and allow in further studies to model the inhibition of anaerobic processes by certain antibiotics.

4.2 Materials and Methods

4.2.1 Start-up, Operation and Performance of Anaerobic SBRs

Detailed information on the setup, operation and performance of the reactors has been reported in the previous studies (Aydin et al., 2014; Aydin et al., 2015a, Aydin et al., 2015b). Briefly, five 1.5 L anaerobic SBRs were set up and operated under exactly the same conditions, including identical seed sludge obtained from an anaerobic contact reactor treating of wastewater produced from Raki and Fresh Grape alcohol companies. After steady-state conditions, the influent antibiotics concentrations were gradually increased through successive stages each lasted for 30 days until metabolic collapse of the SBRs. Performances of reactors were observed during operational period, which was 360 days (10th Stages) for ST reactor, 440 days (13^{th} Stages) for ET reactor, 360 days (10^{th} Stages) for ES reactor, and 420 days (12^{th} Stages) for ETS reactor (Aydin et al., 2014; Aydin et al., 2015a; Aydin et al., 2015b). The results of the VFA measurement indicated that all antibiotic combinations had the highest inhibition effect on acetate degradation pathways, leading to the accumulation of acetic acid. Furthermore, ETS and ET antibiotic combination

affected butyric acid utilization pathway, leading to accumulation of butyric acid. Differently from ETS and ET reactors, ST and ES combinations inhibited the degradation of propionate (Aydin et al., 2014; Aydin et al., 2015a; Aydin et al., 2015b).

4.2.2 Genomic DNA (gDNA) extraction, total RNA extraction and cDNA synthesis

Triplicate samples were collected from all the anaerobic SBRs on the 10th day of every antibiotic stage for RNA and DNA isolation. A PureLink RNA extraction and a SuperScript cDNA synthesis kits (Invitrogen, U.K.) were used in accordance with recommended procedures to isolate the total RNAs and DNA from the 1 mL sludge sample respectively. NanoDrop spectrophometer (NanoDrop Technologies; Wilmington, DE, USA) was used to measure the concentration of the isolated RNAs and DNAs, and the cDNAs were synthesized from the isolated RNAs using Superscript Vilo cDNA synthesis kit (Invitrogen, UK) immediately. The isolated DNAs and cDNA samples were stored at -80 °C, -20°C until required for further analysis respectively.

4.2.3 Cloning, sequencing and phylogenetic analysis

The bacterial and archaeal PCR products of the seed sludge of reactors obtained using Bact8f-Bact1541r and Arch344f-Arch855r primers is given in Table S2, which were cloned by TOPO TA Cloning Kit (Invitrogen, USA) in accordance with the manufacturer's instructions. Before cloning, all PCR products were purified by PCR Clean-up Kit (GeneOn, Germany). To select the positive ones, 75 clones were collected from bacteria clone library and 83 clones were collected from Archaea clone library. Colony PCR were applied using the vector-specific primers M13f and M13r. Bact341f_GC-Bact534r and Arch344f_GC-Univ522r primers were used to re-amplify positive bacterial and archaeal inserts respectively. The re-amplified PCR products were analyzed by high-resolution melt (HRM) to select the representative OTUs. Then, the PCR products to be sequenced were purified by ethanol precipitation and sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA) using primers Bact342f and Arc344f generating 500bp and 800bp of bacterial and archaeal sequence data respectively.

4.2.4 DGGE analysis

A PCR-DGGE analysis was used to determined the Archaea and Bacteria communities dynamics throughout all stages in the control, ETS, ET, ES and ST reactors using specific primers as given in Table 4.1. A 500 ng sample of the V3 and V6 area PCR product for domain Bacteria and Archaea were evaluated using the D-code mutation detection system (Bio-Rad, USA). The PCR-DGGE analysis was described in a previous study (Zhang et al., 2013).

Table 4.1 : Primers used for PCR amplifications.

Primer	Target	Experimental Stage	Annealing (°C)	Position[1]	Reference
Bact341f_GC[2]	Bacterial 16S rDNA	DGGE	55	341-357	Muyzer et al., 1993
Bact534r				534-518	
Bact8f		Cloning		8-27	Lane, 1991
Bact1541r				1541-1522	
Bact342f		Sequencing		342-361	Edwards et al., 1988
Arch46f	Archaeal 16S rDNA	First round of nested PCR	40	46-61	Øvreas et al., 1997
Arch1017r				1017-999	Barns et al., 1994
Arch344f		Cloning	53	344-358	Raskin et al., 1994
Arch855r				855-836	Shinzato et al., 1999
Arch344f_GC[2]		DGGE		344-358	Raskin et al., 1994
Univ522r				522-504	Amann et al., 1995
M13f	B-galactosidase	Clone screening	54	–	Schrenk et al., 2003
M13r					

[1] *Escherichia coli* numbering.

[2] 5'-GC clamp on Arch344f and Bact341f (GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGG).

4.2.5 Statistical Analysis

Partial 16S rRNA gene sequences were analyzed and manually edited in Amplify 3X software package version 3.14 (http://engels.genetics.wisc.edu/amplify). The sequences were checked for reading errors with the alignment programs of the ARB package, which are based on secondary structures of rRNA. The 16S rRNA sequences were checked for chimerical constructs by using the CHECK-CHIMERA program of the Ribosomal Database Project and the ARB software package. Homology searches of the EMBL and GenBank DNA databases for the 16S rRNA gene sequences were performed with BLAST provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html) to identify

putative close phylogenetic relatives. Sequence data were aligned by ClustalW tool at website of European Bioinformatics Institute. Distance analyses using the Jukes and Cantor correction and bootstrap resampling (1000 times) were done using the MEGA Software package version 5.1 (http:// http://www.megasoftware.net/) and trees were generated from distance matrices using the neighbour-joining method.

16S rRNA gene sequences showing 99% similarity or higher was considered to belong to the same phylotype. Related 16S rRNA gene sequences were placed within tentative taxa (between Phylum and Order) by determining the taxonomic class (using the NCBI taxonomy database) of the closest relative in GenBank of sequences that formed a phylogenetic clade. Sequences that showed no or low (below 70%) relatedness with known bacterial or archaeal phylogenetic groups were listed as unclassified. The distribution of clone types present in the clone libraries was determined and used to calculate the Shannon-Weaver index ($H = -\Sigma[pi \cdot \ln(p_i)]$), where p_i is the relative contribution of clone type i to the whole library ($n_1/N\}$. Coverage was calculated as $1 – (n_1/N)$, where n_1 is the number of clone types that was encountered only once in the library and N is the total number of clones analyzed. The Chao1 estimator of species (here, clone type) richness (Schao1) was calculated as; $S_{obs} + ni^2 /2n_2$.

DGGE images were converted, normalized and analyzed by using the Bionumerics 5.0 software (Applied Maths, Kortrijk, Belgium). Similarities of the community fingerprints between each sample were calculated by using the Dice coefficient (S_D) (unweighted data based on band presence or absence) and UPGMA clustering. For analysis using Dice coefficient a band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at 100%.

4.3 Results and discussion

4.3.1 Bacterial and Archaeal 16S rRNA clone libraries

Bacterial and Archaeal 16S rRNA clone library were constructed for the seed sludge of anaerobic SBRs using specific primers for characterization of microbial community structure. All clones were screened by HRM analysis and this analysis presented that 12 different OTUs were obtained from 75 bacterial clones and 22 different OTUs were found in 83 archaeal clones. Table S1 and S2 illustrate all

different OTUs were sequenced and the closest relatives of bacterial and archaeal sequencing results respectively.

The dominant bacterial clone phyla belong to Firmicutes (21%), Actinobacteria (11%), Cyanobacteria (4%) and unclassified Bacteria (64%) as seen in Figure 4.1. Clostridium sp., were represented 93% of Firmicutes members in the seed sludge and which is Gram-positive bacteria and responsible for degradation of organic compounds. Actinobacteria are also Gram-positive microorganism including Bifidobacterium, Mycobacterium and Corynebacterium.

The most abundant Archael phyla in seed sludge were Methanosarcinales (27%), Euryarchaeota (8%), Methanomicrobiales (7%) and unclassified Archaea (58%) as seen in Figure 4.2. Maintenance of Methanosarcinales (acetoclastic methanogens) in anaerobic reactor is critical for stable performance. Prior studies indicated the importance of Methanomicrobiales (hydrogenotrophic methanogens), which was the most resistant group in Methanogens to toxic substances. The uncultured archaeal and bacterial 16S rRNA gene sequences were deposited in the GenBank database under the accession nos. KJ018671-KJ018699.

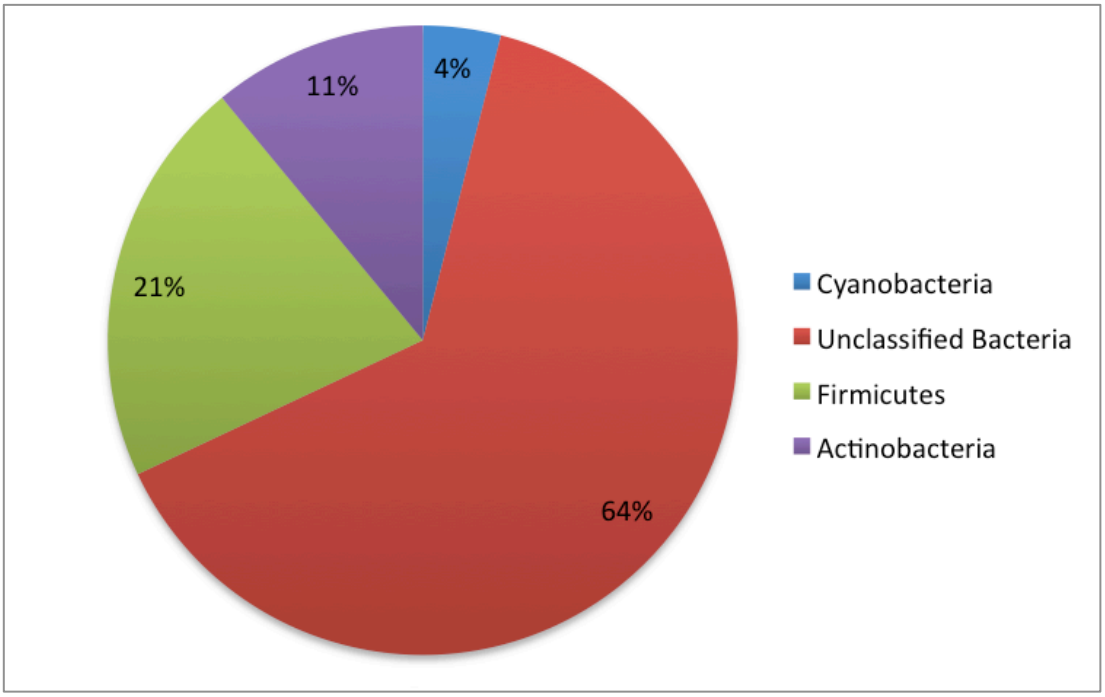

Figure 4.1 : Distribution of phylogenetic phylum from clone sequence of bacterial 16S rRNA clone library.

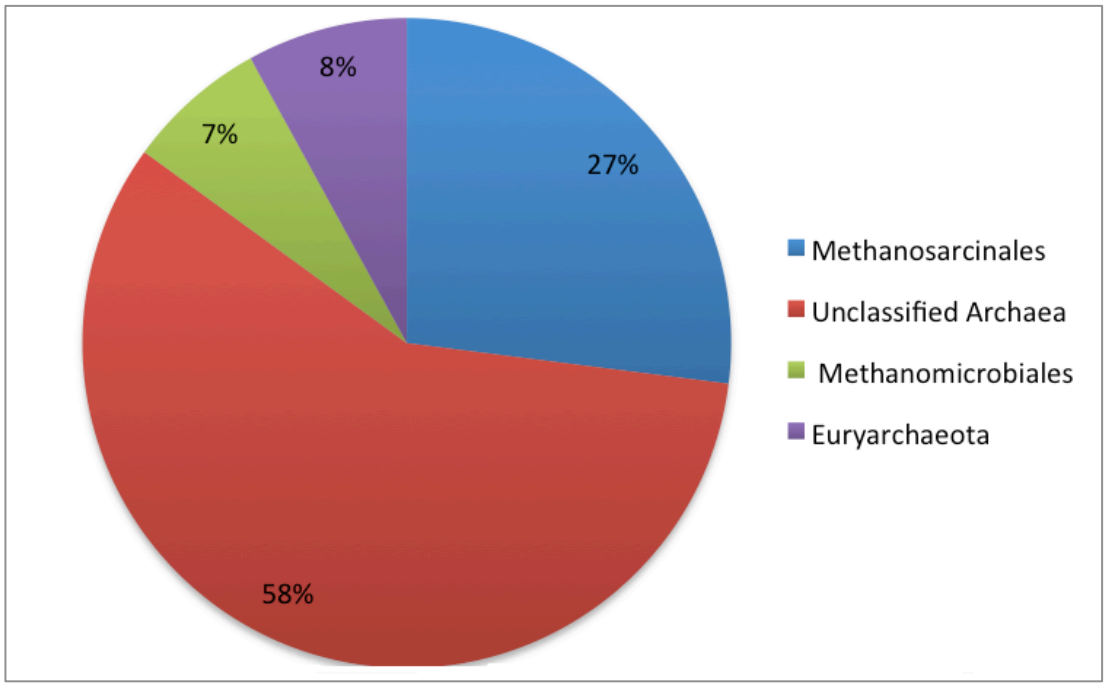

Figure 4.2 : Distribution of phylogenetic phylum from clone sequence of archael 16S rRNA clone library.

4.3.2 The influence of ETS and ET combinations on the Bacterial and Archael community dynamics in the SBR

The clones were compared with the samples' DGGE bands. Using band intensities, canonical correspondence analyses were applied to understand the relations of species by digestion time, biogas/methane production, VFA accumulation and ETS and ET concentration is given in Figure 4.3. From the results of canonical correspondence analysis, it can be said that all of the microbial groups in the SBRs were negatively affected by ETS and ET toxicity and these results were similar compared to each other. Decrease in methane and biogas productions were found out to be closely related with the disappearance of the acetoclastic methanogens represented by the Methanosarcinales order in the ETS reactor. There, it can be said that Methanosarcinales order was the most affected group from ETS toxicity during the operation. The case is also reported in other previous studies regarding ETS antibiotics (Aydin et al., 2015b).

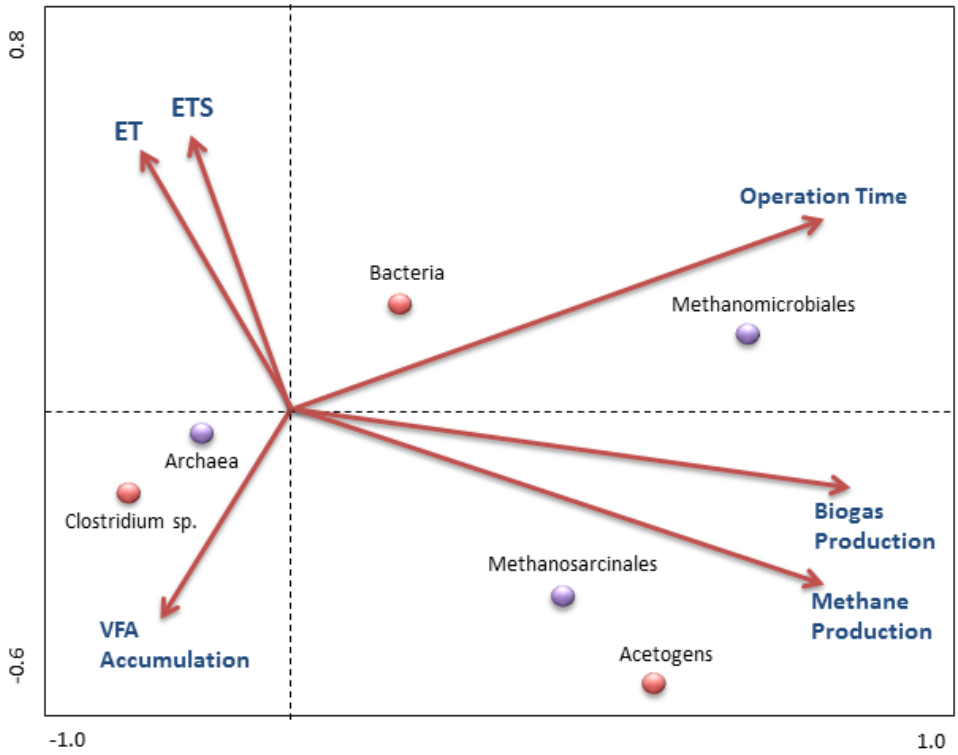

Figure 4.3 : Canonical correspondence analyses of DGGE results in the ETS and ET reactors.

According to canonical correspondence analysis, the number of total active bacteria seemed not to effect biogas and methane production directly; however, when apply for the subgroups (Clostridium sp., Propionibacterium sp., Acetogens), which was Acetogens were effective on ETS and ET combinations due to their syntrophic relations with the methanogens. The situation was also similar for the total number of Archaea, meaning that changes in subgroups were more effective on explaining ETS inhibition. Additionally, Methanomicrobiales (Hydrogenotrophic methanogens) in these reactors was positively correlated with the operation day, which means that its abundance significantly increased through the operation. As mention in the previous researches, hydrogenotrophic methanogens are higher in substrate utilization rate; growth rate and cell yield to exposed toxic substances than compare to acetoclastic methanogens (Aydin et al., 2015b).

4.3.3 The influence of ST and ES combinations on the Bacterial and Archael community dynamics in the SBR

All the archaeal and bacterial groups were significantly ($p<0.05$) negatively affected with ES and ST concentration according to canonical correspondence analysis in the

SBR. Canonical correspondence analysis also display that the influence of an ST and ES combination produces a comparable effect between bacterial and archael community dynamics as seen in Figure 4.4.

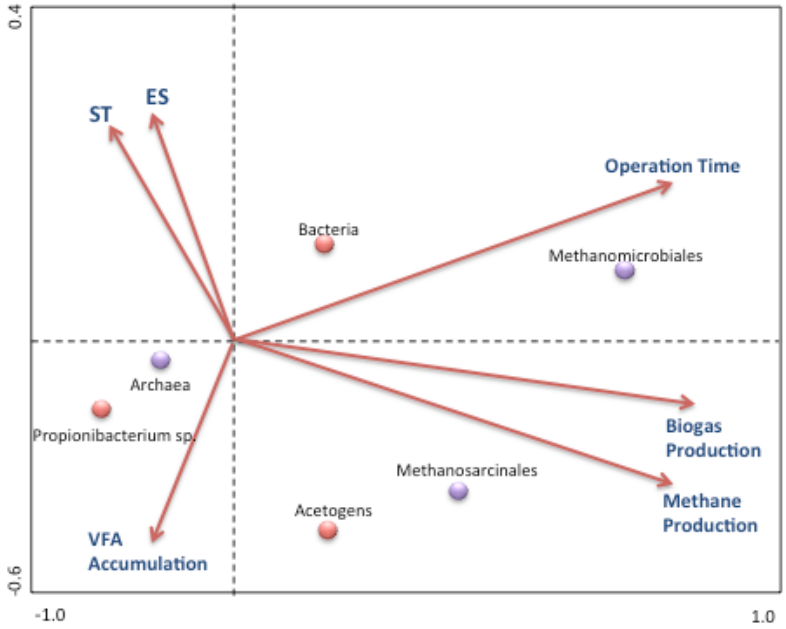

Figure 4.4 : Canonical correspondence analyses of DGGE results in the ST and ES reactors.

Furthermore, the number of total active Bacteria and Archaea seemed not to effect biogas and methane production directly as well as ETS and ET combinations; however, biogas/methane productions and COD removal were found correlated very largely with Methanosarcinales sp. and Acetogens. Archaea did not show a significant change with operation time. This can be explained by the slower grow rates of archaeal cells. In the degradation of propionic acid is most often utilized by Propionibacterium sp. via methylmalonyl coenzyme A (MMC) pathway, and combinations of ST and ES combination inhibited these sensitive strains of this microbial community (Aydin et al., 2015).

Compare ETS, ST, ES and ET reactors revealed that Gram-Negative bacteria are much important than Gram-Positive bacteria during the operation. There was also a significant positive correlation between decrease numbers of Gram-negative bacteria and metabolic collapse of reactors. Gram-negative bacteria are distinct in that there is a double membrane surrounding each bacterial cell. In addition to the inner cell membrane that is present in all bacteria, gram-negative bacteria also have an outer membrane that prevents certain antibiotics from penetrating the cell. This entails that gram-negative bacteria are typically more resistant to antibiotics than gram-positive

bacteria. Gram-negative bacteria have demonstrated the ability to exchange DNA among strains of the same species and, in some cases, between different species. As such, if a gram-negative bacterium undergoes any mutation or acquires genetic material that has antibiotic resistant properties, it may later pass on these resistant properties to other strains of bacteria through the sharing of DNA (Pagès and Amaral, 2009).

The most obvious finding to emerge from the DGGE analysis is that Gram-negative bacteria was affected in the earlier stage of the reactors and then cannot acquire of antibiotic resistance until metabolic collapse of the anaerobic reactors. Moreover, Methanosarcinales in these reactors was negatively correlated with the biogas/methane production, which means that its abundance significantly decreased through the operation. As mention in the ETS and ET reactors, acetoclastic methanogens were the most sensitive compared with Methanomicrobiales, and toxins would directly inhibit this group in Methanogens.

4.3.4 Cluster analysis of DGGE banding pattern of the anaerobic SBRs

Microbial diversity shifts in Bacterial and Archaeal communities, which presented in the anaerobic SBRs during all the stages were estimated based on the DGGE patterns of the partial 16S rRNA amplicons. The results of this study are in keeping with previous observational studies, which significant changes in 16S rDNA and 16S rRNA profile of bacterial and archaeal communities were detected in the SBRs after Stage3 for the ST and ES reactors; stage 6 for the ETS and ET reactors (Aydin et al., 2015b).

4.3.5 Assessment of DGGE for monitoring of microbial communities

In summary, for the informants in this study, PCR-DGGE approach gives a reasonable comparison of the combined effects of antibiotic substances on microbial community structures as well as displays the likely effect on SBRs operation. With the use of a gradient pump for DGGE gels and Bionumerics software, it is possible to analyze differences in the amount of bacteria and their diversity between many samples, so long as enough ladders in the gel are used for normalization. One main difficulty, the method requires to establish the gradient marker from one gel to another, so that it is difficult to compare fingerprints of more than 20 samples (Li et al., 2013; Hu et al., 2014). qPCR will also provide an important molecular method toward the quantitative detection of influence of the antibiotic combinations on the

anaerobic microbial community and expression of functional genes in the SBRs (Yu et al., 2006; Smith and Osborn, 2009; Li et al., 2013; Aydin et al., 2015b). Briefly, the method requires establishing calibration curves with target bacterial and archael genera (decimal dilutions of target DNA/RNA from pure culture or from cloned target DNA/RNA), control for false positive (non target genera) and control for false negative (precise melting temperature and/or sequencing of some qPCR products). Furthermore, A complex sample matrix, such as in reactors that had different amounts of COD removal that the sample matrix was not the same, can hinder the efficiency of qPCR, this result is known as "qPCR inhibition." To be need check for inhibition, samples are often diluted 10x, 100x, and 1000x, qPCR is performed on each dilution, and the final result should be the same for all samples. If the sample that was not diluted has a lower value than the diluted samples then the matrix is attributed to inhibiting the PCR. For instance, Aydin et al. (2015b) have observed qPCR inhibition at Stage 12 in the ETS reactor. On the other hand, once the method is established in the routine, hundreds of samples may be run but it becomes however very expansive with an increasing number of sample compare to the PCR-DGGE analysis. qPCR and PCR-DGGE analysis also support this same results and demonstrate that Acetoclastic methanogens (Methanosarcinales) was the most affected group in Methanogens in the anaerobic SBRs. The results reported in this research are valuable as they may allow in further studies to model the inhibition of anaerobic process by certain antibiotics.

4.4 Conclusion

The results of the study indicated that increasing antibiotic concentrations negatively impact on microbial community structure and function in anaerobic bioreactor technology. The findings of this research provide insights for importance of Gram-negative bacteria, which was essential to anaerobic biodegradability of antibiotic combinations in the SBRs. PCR-DGGE could also be useful for examining of microbial communities in anaerobic systems and assess the condition of the reactor for control and improvements of such systems.

5. CONCLUSIONS AND RECOMMENDATIONS

The results of the study indicated that increasing antibiotic concentrations negatively impact on microbial community structure and function in anaerobic bioreactor technology. The second major finding was that anaerobic treatment processes do result in the occurrence and dissemination of antibiotic resistance genes in the presence of high non-lethal concentrations of ETS, ES, ET and ST combinations. According to the occurrence of ARGs in the SBR, the activity of antibiotic combinations is greater than the sum of their independent activities. A comparison of the prevalence of ARGs in the ETS, ES, ET and ST reactors revealed that there was a larger number of ARGs in the ETS and ET reactors than in the ST and ES reactor. By applying qPCR assays that targeted ARGs, the research revealed that anaerobic treatment increased the concentrations of tetA, tetB, tetC, tetE, tetM, tetS, tetQ, tetX, msrA, ermA, ermF, ereA, sul1, sul2 and sul3 genes encoding tetracycline, sulfamethoxazole and erythromycin over the course of one-year operation of the anaerobic SBRs. The anaerobic treatment was unable to completely remove ARGs from wastewater effluents. Additional study is required to investigate methods of increasing the efficiency with which anaerobic treatment processes reduce ARGs. As shown, sorption of antibiotic and TPs in the biomass increased with respect to time, types and concentration of antibiotics, which is important to increase of resistance genes in the anaerobic SBRs. Further studies with more focus on prevent of antibiotic and TPs sorption in the sludge is therefore suggested.

Long-term observation of microbial community may support the hypothesis that the Gram-negative Bacteria is critical to the anaerobic biodegradation of antibiotics. Failure to maintain the stability of these microorganisms resulted in a decrease in the antibiotic biodegradation and stability of the anaerobic reactors. Assessment of DGGE data is also useful for suggesting the potential to control ultimate microbial community structure, especially derived from Gram-negative bacteria, through bioaugmentation to successful for antibiotic biodegradation.

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- Sengul, R., **Aydin, S.**, Turken, T., Genceli, E. A., Koyuncu, I. (2015). Biomimetic Approaches for Membrane Technologies, Separation & Purification Reviews, DOI: 10.1080/15422119.2015.1035443