**ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY**

# DETERMINATION OF THE SYNERGISTIC ACUTE EFFECTS OF ANTIBIOTICS ON METHANOGENIC PATHWAY

**M.Sc. THESIS**

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**Department of Environmental Engineering**

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**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# ANTİBİYOTİKLERİN METANOJENİK YOLİZLERİ ÜZERİNDE ÇOKLU AKUT ETKİLERİNİN BELİRLENMESİ

**YÜKSEK LİSANS TEZİ**

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**HAZİRAN 2012**

**Emine Gözde Özbayram**, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 501091829, successfully defended the **thesis** entitled "**DETERMINATION OF THE SYNERGISTIC ACUTE EFFECTS OF ANTIBIOTICS ON METHANOGENIC PATHWAY**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

| | | |
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**FOREWORD**

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**ABBREVIATIONS**

**ASBR** : Anaerobic Sequencing Batch Reactor
**CBP** : Cumulative Biogas Production
**CMP** : Cumulative Methane Production
**COD** **:** Chemical Oxygen Demand
**DGGE** **:** Denaturing Gradient Gel Electrophoresis
**DNA** **:** Deoxyribonucleic Acid
**ERY** **:** Erythromycin
**ES** **:** Erythromycin and Sulfamethoxazole mixture
**ET** **:** Erythromycin and Tetracycline mixture
**ETS** **:** Erythromycin, Tetracycline, and Sulfamethoxazole mixture
**FISH** **:** Fluorescence *in situ* Hybridization
**GC** **:** Gas Chromatograph
**HRM** **:** High Resolution Melt
**HRT** **:** Hydraulic Retention Time
**PCR** **:** Polymerase Chain Reaction
**PMP** **:** Potential Methane Production Rate
**Q-PCR** **:** Quantitative Real-Time PCR
**RNA** **:** Ribonucleic Acid
**SMA** **:** Specific Methanogenic Activity
**SMX** **:** Sulfamethoxazole
**SS** **:** Suspended Solid
**ST** **:** Sulfamethoxazole and Tetracycline mixture
**TET** **:** Tetracycline
**TS** **:** Total Solid
**TVS** **:** Total Volatile Solid
**VFA** **:** Volatile Fatty Acid
**VSS** **:** Volatile Suspended Solid

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## DETERMINATION OF THE SYNERGISTIC ACUTE EFFECTS OF ANTIBIOTICS ON METHANOGENIC PATHWAY

## SUMMARY

Antibiotics are defined the chemotherapeutic agents that inhibit or abolish the growth mechanisms of microorganisms. Large quantities of antibiotics are used in human and veterinary medicine. Approximately 500 ton of antibiotics are produced and consumed every year. After consumption, antibiotics and/or their metabolites are excreted into the effluent and reach the sewage treatment plants, and, if they are not degraded, will eventually enter the environment. Antibiotics have been detected in the ng/L and μg/L range in municipal sewage, effluents of treatment plants, surface water and groundwater. On the other hand, emissions from production plants have major importance. It has been found that, the concentration of antibiotics in pharmaceutical wastewater is in mg/L level. The presence of antibiotic in wastewaters may negatively effect biological processes in treatment plants either by influencing the composition of the microbial community or direct inhibition of pollutant degradation. Furthermore, antibiotics do not occur as pure substances in environment. They are present as multi-component mixtures. Mixtures generally cause higher effects than each of their comprising substances alone.

In this thesis, the mixture short-term effects of selected antibiotics on methanogenic activity of a microbial community under anaerobic conditions were determined. In this scope, acute tests were designed based on a three-way factorial design where one factor was the composition of the substrate solution (acetate, butyrate, and VFA mixture), another factor was the composition of antibiotic mixtures (Sulfamethoxazole, Erythromycin, and Tetracycline) and the last factor was concentration of antibiotics added (1 – 250 mg/L). Substrate removal was monitored by VFAs measurements, together with daily measurements of biogas and methane generation. In addition, the microbial community structure of seed sludge which used in acute tests were identified for determining the microorganisms which associated with degradation of selected substrates.

Statistical analyses showed that, there is a strong correlation between antibiotic mixture concentration and methane generation. For all substrate conditions, 250 mg/L ST mixture had the highest inhibitory effect on total methane production. After 100 mg/L, short-term inhibitory effect of antibiotic mixtures was seen clearly in all test bottles. The results showed that, mixtures had higher inhibition effect on total methane production of acetoclastic methanogens than single compounds.

# ANTİBİYOTİKLERİN METANOJENİK YOLİZLERİ ÜZERİNDE ÇOKLU AKUT ETKİLERİNİN BELİRLENMESİ

## ÖZET

Antibiyotikler mikroorganizmaların büyüme mekanizmalarını inhibe eden ya da durduran kemoterapötik ajanlar olarak tanımlanır. Tıp ve veterinerlik alanlarında büyük miktarda antibiyotik kullanılmaktadır. Her yıl yaklaşık olarak 500 ton antibiyotik üretilmekte ve tüketilmektedir. Tüketim sonucunda, antibiyotikler ya da onların metabolitleri atıksuya karışarak atıksu arıtma tesislerine ulaşmakta, burada parçalanmama halinde ise çevreye karışmaktadırlar. Yüzeysel sular ve atıksularda antibiyotik konsantrasyonları ng/L ve µg/L seviyelerinde tespit edilmişken, ilaç endüstrisi ve hastane atıksularında antibiyotik konsantrasyonları mg/L düzeyindedir. Yapılan çalışmalar antibiyotiklerin düşük konsantrasyonlarda dahi çevrede olumsuz etkilere sebep olabildiklerini göstermektedir. bu maddelerin su ve toprakta birikimi, doğada antibiyotiğe dirençli patojenlerin gelişmesine neden olmaktadır. Bu durum halk sağlığını tehdit etmektedir. Antibiyotik arıtımında ozonlama, fenton prosesi ile oksidasyon, UV ile parçalama ve fotodegredasyon gibi kimyasal teknolojiler kullanılmaktadır. İlaç endüstrisi atıksuları yüksek organik madde içerikleri nedeniyle anaerobik arıtıma uygundur. Atıksuda bulunan antibiyotikler, arıtma tesislerindeki biyolojik üniteler üzerinde mikrobiyal kompozisyonun değişmesine sebep olarak ya da direk olarak kirletici degradasyonunu inhibe ederek negatif etkide bulunabilirler. Antibiyotikler çevrede saf maddeler olarak değil, çok bileşenli karışımlar halinde bulunular. Karışım halinde genellikle tekli olarak bulunduklarından daha fazla etkiye sebep olurlar. Literatürde antibiyotikler ile ilgili çalışmalar genellikle tekli etkiler üzerine yoğunlaşmıştır, antibiyotik karışımlarının biyolojik sistemler üzerindeki bütünleşik etkilerini inceleyen sınırlı sayıda çalışma bulunmaktadır.

Bu tezde, seçilmiş olan antibiyotik karışımlarının havasız koşullarda mikrobiyal komünitenin metanojenik aktivitesi üzerindeki kısa süreli inhibisyon etkisi incelenmiştir. Bu testlere ek olarak, akut testlerde kullanılan aşı çamurunun mikrobiyal komünite yapısı, antibiyotiklerin farklı yolizleri üzerindeki mikrobiyal komüniteye etkisinin belirlenebilmesi için tanımlanmıştır.

Bu kapsamda, üç değişkenli akut testler tasarlanmıştır. Birinci değişken substrat çözeltisinin içeriğidir. Asetoklastik metanojenler üzerindeki etkilerin belirlenebilmesi için asetat, tüm metanojenler ve homoasetojenler üzerindeki etkilerin belirlenebilmesi için uçucu yağ asidi karışımı ve bütirat karbon kaynağı olarak belirlenmiştir. Diğer değişken antibiyotik karışımının kompozisyonudur. İlaç endüstrisi atıksularında yüksek konsantrasyonlarda bulunan, ekosisteme başta evsel atıksular olmak üzere birçok kaynaktan giriş yapan ve insanlarda görülen enfeksiyonel hastalıkların tedavisinde sıklıkla kullanılan, aynı zamanda üç farklı antibiyotik mekanizmasını temsil eden antibiyotiklerden Sulfametoksazol, Eritromisin ve Tetrasiklin seçilmiştir. Son değişken ise antibiyotik

konsantrasyonudur (1 – 250 mg/L). Akut testlerde kullanılacak olan stok çamurunu hazırlamak için toplam hacmi 10 litre olan ve 8 litre aktif hacimde işletilen anaerobik ardışık kesikli reaktör (AAKR) kurulmuştur. Sistem iki aşamalı anaerobik-aerobik biyolojik arıtma sistemleri uygulanan bir alkollü içki endüstrisinin atıksularını arıtan yukarı akışlı anaerobik çamur yatağının 1.aşamasından alınan aşı çamuru ile inoküle edilmiştir. Reaktörde UAKM konsantrasyonu 12250 mg/L tutulmuş, organik yükleme 1250 mg/L.gün KOI'den 6250 mg/L.gün KOI'ye kademeli olarak çıkarılmıştır. Reaktör stabil hale geldikten sonra, öncelikle testlerde kullanılacak optimum substrat konsantrasyonunu belirlemek için 500 – 1000 – 1500 – 2000 mg/L konsantrasyonlarda substrat içeren akut testler kurulmuştur. Üç substrat için de 1500 mg/L optimum konsantrasyon olarak belirlenmiştir. Akut testlerde 100 mL aktif hacimli, 120 mL'lik serum şişeleri kullanılmıştır. Şişelere son konsantrasyon 1000 mg/L TUKM olacak şekilde AAKR'den alınan aşı çamurundan eklenmiştir. Testlerde kullanılan besiyeri OECD 311 protokolünde belirtilen şekilde hazırlanmıştır. Test süresi substrat cinsine bağlı olarak 8 ile 12 gün arasında değişmiştir. Test süresince serum şişeleri 35±2°C'de tutulmuş ve her gün el ile çalkalanmıştır. Basınç değişimi 5-7000 mbar arasında ölçüm yapabilen manometre ile ölçülmüştür. Aşı çamurunun mikrobiyolojik karakterizasyonu için bakteriyel ve arkeal klon kütüphaneleri kurulmuş, mikrobiyal çeşitlilik HRM analizi ile belirlenmiştir. Analiz sonucunda 12 farklı bakteriyel ve 22 farklı arkeyal tür tespit edilmiştir. Bu türleri temsil eden numunelere dizileme analizi yapılmıştır. Bakteriyel klon kütüphanesinin %21'i bütirat ve propiyonat degradasyonundan sorumlu türleri içeren Firmicutes filumu oluşturmaktadır. Toplam metan üretiminin yaklaşık %70'inden sorumlu asetoklastik metanojenleri içeren Methanosarcinales filumu ise arkeyal klon kütüphanesinin %27'sini oluşturmaktadır.

Test şartlarında, hiçbir koşulda tam metan inhibisyonu görülmemiştir. Yapılan istatistiksel analizler antibiyotik karışımının konsantrasyonu ile toplam metan üretimi arasında anlamlı bir ilişki olduğunu göstermektedir. Kurulan test şişelerinin tümünde, antibiyotik konsantrasyonu arttıkça, metan üretiminde düşüş gözlenmiştir. Tüm setlerde en yüksek inhibisyon etkisini 250 mg/L ST içeren test şişeleri göstermiştir. Her üç substrat için toplam metan üretimindeki düşüş 100 mg/L'den sonra görülmüştür, Asetat setinde, antibiyotik karışımı içermeyen kontrol test şişelerinde toplam metan üretimi 64 mL olarak ölçülmüştür. Bu değer, ST içeren test şişelerinde 1 mL'ye düşmüştür. $EC_{50}$ değerleri ETS için 50 mg/L, ET 65 mg/L, ST için 102 mg/L ve ES için 138 mg/L olarak hesaplanmıştır. Karışımların $EC_{50}$ değerleri, bu karışımı oluşturan antibiyotiklerin herbiri için hesaplanan değerden daha düşüktür. Antibiyotik karışımları asetoklastik metanojenler üzerinde sinerjistik etki göstermektedir. Bütirat setinde, kontrol şişelerinde toplam metan üretimi ortalama 103 mL'dir. 250 mg/L ST içeren test şişelerinde bu değer 49 mL'ye düşmüştür. Bütirat setinde $EC_{50}$ değeri ST karışımı için 96 mg/L olarak hesaplanmış, diğer antibiyotik karışımlarında $EC_{50}$ değerinin test edilen konsantrasyonların üzerinde olduğu tahmin edilmektedir. Antibiyotik karışımlarının bütirat degradasyonundan sorumlu bakteriler üzerindeki etkisi iki şekilde açıklanabilir. Karışımlar, mikrobiyal popülasyon üzerinde antagonistik etki göstermiş olabilirler, ya da bütirat degradasyonundan sorumlu bakteriler bu antibiyotiklere karşı dirençlidirler. Karbon kaynağı olarak UYA karışımını içeren deney setinde, kontrol şişelerinde toplam metan üretimi 80 mL, metan üretiminde en yüksek inhibisyon etkisi gösteren 250 mg/L ST içeren test şişelerinde ise bu değer 31 mL'dir. $EC_{50}$ değerleri ETS için 88 mg/L, ET 106 mg/L, ST için 52 mg/L olarak hesaplanmış, ES karışımı için ise $EC_{50}$

değerinin 250 mg/L'nin üzerinde olduğu düşünülmektedir. UYA setinde ETS, ET ve ES karışımlarının $EC_{50}$ değerleri karışımı oluşturan bileşenlerin $EC_{50}$ değerlerinden yüksek olduğu görülmüş, bu karışımlarda antagonistik etki gözlenmiştir. ST karışımında ise $EC_{50}$ değeri, SMX'in $EC_{50}$ değerinden düşüktür. UYA degradasyonunda ST karışımı sinerjistik etki göstermiştir. Sonuç olarak, antibiyotik karışımlarının farklı metanojenik yol izleri üzerindeki etkilerinin aynı olmadığı tespit edilmiştir.

## 1. INTRODUCTION

Antibiotics are defined as chemotherapeutic agents that inhibit or abolish the growth mechanisms of microorganisms (Kümmerer, 2009a). They are the most intensively used and important groups of pharmaceuticals. Large quantities of antibiotics are used in human and veterinary medicine. They have also been used for treating and preventing plant infections and growth promotion in animal farming (Martinez, 2009). Substantial amounts of antibiotics release in natural ecosystem because of these applications.

Antibiotics have been detected in the ng/L and µg/L range in municipal sewage, effluents of treatment plants, surface water and groundwater (Kümmerer, 2008). On the other hand, emissions from production plants have minor importance. It has been found that, the concentration of antibiotics in pharmaceutical wastewater is in mg/L level (Amin et al., 2006). Antibiotic consumption varies between countries. While in the United States, the antibiotic consumption in human medicine is 17 g per capita and year, in various European countries the consumption of antibiotics for medical purposes ranges between 4 – 8 g per capita and year (Alexy, 2006).

Generally, antibiotics are not metabolized after their application. They are released into the effluent and they reach sewage treatment plants. If they are not degraded in the treatment plant, they can reach in the environmental compartments (Alexy *et al*., 2004).The presence of antibiotic in wastewaters may negatively affect biological processes either by influencing the composition of the microbial community or direct inhibition of pollutant degradation. Besides these effects, release of antibiotics into the environment increases the risk of bacterial resistance development (Kümmerer, 2009b).

Inhibition effects of a selected chemical can be evaluated with short-term (acute) and long-term (chronic) tests. Acute experiments comprise a microbial community selection and sustainability of the selected organic substrate in the system and not previously exposed to the inhibitor. Short-term tests represent the effect of pulse

discharge of inhibitors, possibly at high concentrations to the joint treatment systems, while long-term tests may be useful in assessing the continuous impact of lower antimicrobial concentrations, similar to those found in domestic wastewater (Cetecioglu et al., 2012). Different opinions were expressed in the literature. According to Kummerer (2009a), acute tests would be inappropriate for determining the effects of antibiotics on bacteria, because of short incubation period. On the other hand, Alighardashi and his colleagues (2009) state that the microbial community becomes adapted to a synthetic substrate in chronic tests which is a different situation in a full-scale treatment plant.

Most risk assessment is based on single compounds in the literature. Antibiotics are present as multi-component mixtures in environment. It has been found that mixtures might exhibit different effects than single compounds (Silva *et al*., 2002; Cleuvers, 2004, 2008; Backhause *et al*., 2008; Pomati *et al*., 2007). Knowledge about the mixtures toxicity is limited in the literature.

### 1.1 Aim and Scope

Approximately 500 ton of antibiotics are produced and consumed every year. Manufacturing plants has the major importance on the emissions of antibiotics to the environment. Pharmaceutical wastewaters have antibiotic concentrations, on the order of a few mg/L. Generally, pharmaceutical wastewater is characterized by high COD concentration, and some pharmaceutical wastewaters can have COD concentration as high as 80,000 mg/L. Effluent from pharmaceutical wastewater is usually treated using ozonation, fenton oxidation, sorption etc. However, due to high COD content, pharmaceutical wastewaters could be treated by anaerobic biological systems (Chelliapan & Sallis, 2011).

There are many studies in literature, which evaluate inhibition effect of the individual antibiotics on treatment systems (Fountoulakis *et al*., 2004; Amin *et al*., 2006; Gartiser *et al*., 2007; Cetecioglu *et al*., 2012). As it is known that antibiotics are not present as single compound substances in environmental compartment. The information on inhibition effects of antibiotics mixtures is very limited in the literature.

The aim of this thesis is to determine the mixture short-term effects of selected antibiotics on methanogenic activity of a microbial community under anaerobic conditions.

In this scope, acute tests were constructed to evaluate the short-term effect of antibiotic mixtures under three different carbon sources (acetate, butyrate, and VFA mixture). Three widely used antimicrobials, sulfamethoxazole, erythromycin and tetracycline were selected for the study. The antibiotic dosages were in the range of 1.0–250 mg/L. Control tests, which had not contained antibiotic, were set-up for the reliable evaluation. Biogas generation/composition and substrate removal were monitored during the test period. The seed sludge was acclimated to a synthetic wastewater for reliability of tests. In addition, the microbial community structure of the seed sludge used in acute tests identified to determine the microorganisms, which had role on the degradation of the selected substrates.

# 2. THEORETICAL BACKGROUND

## 2.1 Antibiotics

Antibiotics are the compounds, which occurred naturally by microorganisms or produced by synthetically such as sulfa drugs or semi synthetically with modification of natural origin compounds (Kemper, 2008; Kümmerer, 2009a). The first antibiotics were natural origin, e.g. penicillin produced by fungi in the genus Penicillium, or streptomycin from bacteria of the genus Streptomyces (Kümmerer, 2009a).

Antibiotics are apparently the most effective drugs in human medicine so far. In addition to the applications in human and animal medicine, antibiotics are used as a growth promoter in animal farming. They are also used for preventing and treating plant infections. Substantial amounts of antibiotics are released in environmental compartment because of these applications (Martinez, 2009).

### 2.1.1 Classification of antibiotics

There are various ways to classify antibiotics. They could classify according to their chemical structure or modes of action. Most antibiotics fall into seven main categories, based on their site activity. These groups consist of inhibition of cell wall synthesis, protein synthesis, DNA and RNA synthesis or competitive inhibition of folic acid synthesis and membrane disorganizing agents (Alanis, 2005). Table 2.1, shows the mechanism of actions of antibiotics.

**Table 2.1** The mechanism of actions of antibiotics

| Mechanism of Action | Antibiotic families |
|---|---|
| Inhibition of cell wall synthesis | Beta-lactams(penicillins, carbapanems); glaycopepdides; cyclic lipopeptides(daptomycin) |
| Inhibition of protein synthesis | Tetracyclines; aminoglycosides; oxazolidonones; streptogramins;ketolides;macrolides;lincosamides |
| Inhibition of DNA synthesis | Fluoroquinolones |
| Inhibition of RNA synthesis | Rifampin |
| Competitive inhibition of folic acid synthesis | Sulfonamides; trimethoprim |
| Membrane disorganizing agents | Polymyxins (Polyxin-B, Colistin) |
| Other mechanisms | Metronidazole |

Modes of antibiotic action are shown schematically in Figure 2.1.

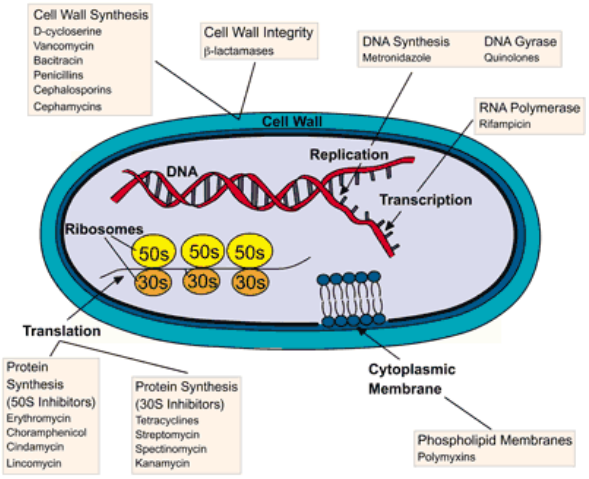



**Figure 2.1 :** Targets for antibiotics (John Wiley & Sons, Inc., 2004).

Tetracycline (TET), sulfamethoxazole (SMX) and erythromycin (ERY) were chosen in this study because these antibiotics are the most commonly used antibiotics in human and veterinary medicine as well as they are represent different modes of action (Chopra & Roberts, 2001; Baran, 2011).

### 2.1.1.1 Tetracycline

The tetracyclines, which were discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites (Chopra & Roberts, 2001). Tetracyclines are toxic to both bacterial and mammalian ribosomes, although tetracyclines reach much higher concentrations within bacterial cells (McDermott *et al*., 2003).

Tetracyclines are characterized by a partially conjugated four-ring structure with a carboxyamide functional group (Figure 2.2). The molecule of tetracycline has several ionizable functional groups of a rather unusual type, and the charge of the molecule depends on the solution pH. Tetracyclines are relatively stable in acidic media, but not in alkaline conditions, and form salts in both media. In general, these compounds are sparingly soluble in water; however, solubility of the corresponding hydrochlorides is reported to be much greater (Sarmah, Meyer, & Boxall, 2006).

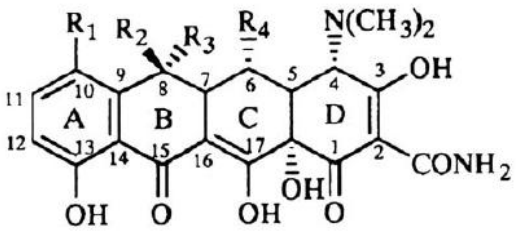



**Figure 2.2 :** Molecular Structure of Tetracycline

Tetracyclines are one of the cheapest classes of antibiotics available today, making them attractive for use in developing countries with limited health care budgets (Roberts, 2003).

The tetracyclines have applications for the treatment of infections in human and veterinary medicine. They are used in animal farming as growth promoters for food animals. Besides these applications, there are also used in aquaculte to control infenctions in salmon, catfish and lobster (Chopra & Roberts, 2001).

### 2.1.1.2 Sulfamethoxazole

Sulfonamides were the first drugs with a selective effect on bacteria, and which, could be used systemically against bacterial infections (Sköld, 2000). Sulfamethoxazole is one of the sulfonamide group antibiotics, which is the inhibitor of nucleic acid metabolism.

Sulfonamides are structural analogues of p-amino benzoic acid (PABA). PABA is a substrate in the synthesis of tetrahydrofolic acid, a donor of one-carbon units in the synthesis of purine and pyrimidine nucleotides. Sulfonamides competitively compete for the (dihydropteroate synthetase) enzyme active site, and block the formation of nucleotide precursors. These drugs can also be incorporated into a compound that resembles dihydrofolate and that in turn can inhibit another enzyme in the pathway, dihydrofate reductase (McDermott et al., 2003). Figure 2.3 shows the chemical structure of sulfamethoxazole.

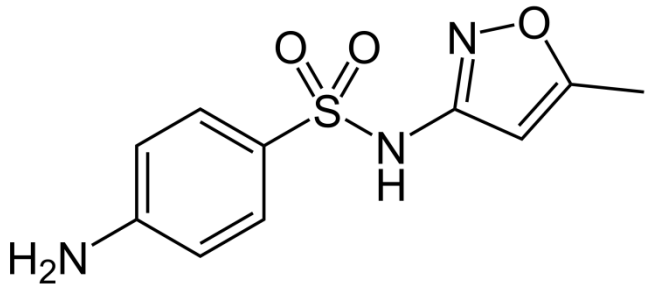



**Figure 2.3 :** Chemical structure of Sulfamethoxazole

Sulfamethoxazole is among the most frequently detected sulfonamides in municipal sewage (Choi *et al.*, 2007; Brown *et al.*, 2006; Gobel *et al.*, 2007; Levine *et al.*, 2006). However, concentrations of this drug in wastewater treatment plant influents and effluents vary significantly, depending on antibiotic consumption patterns and the types of wastewater treatment processes employed (Le-Minh *et al.*, 2010).

### 2.1.1.3 Erythromycin

The RNA and protein machinery of the prokaryotic ribosomes is sufficiently distinct from the analogous eukaryotic machinery that there are many inhibitors of protein synthesis, targeting different steps in ribosome action, with selective antibacterial action. These include such important antibiotics as the macrolides of the

erythromycin class (Walsh, 2000). Figure 2.4 shows the chemical structure of erythromycin.

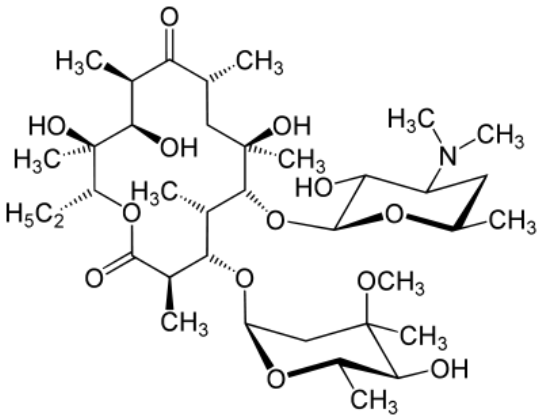



**Figure 2.4 :** Chemical structure of Erythromycin

Erythromycin is among the principal representatives of the macrolide antibiotics for clinical use. An important difference between erythromycin and other macrolides, such as clarithromycin and roxythromycin, is the sensitivity of erythromycin to pH. Under acidic conditions, erythromycin is unstable and is transformed into an inactive anhydro-form by the loss of one $H_2O$ molecule. At the ambient operational pH ranges (6.5-8) of most municipal wastewater treatment plants, erythromycin can exist in both its active original form and as the inactive erythromycin-H2O (Le-Minh *et al.*, 2010).

### 2.1.2 Sources of antibiotics in the environment

The presence of pharmaceuticals in the environment generally results from human and veterinary medicine, as well as in aquaculture, for the purpose of preventing (prophylaxis) or treating microbial infections. The excretion of incompletely metabolized antibiotics by humans and animals is the primary source of antibiotics in the environment (Figure 2.5). Other sources include the disposal of unused antibiotics and waste from pharmaceutical manufacturing processes. Residential (private residences, dormitories, hotels, and residential care facilities) and commercial facilities (including hospitals) are known contributors of antibiotics to municipal wastewater. Other potential contributors of antibiotics to surface and ground water are effluent from wastewater treatment plants (Golet *et al.*, 2002;

Hirsch *et al.*, 1999; McQuillan *et al.*, 2002; Alder *et al.*, 2003) and industrial facilities (including pharmaceutical plants) (Guardabassi *et al.*, 1998).

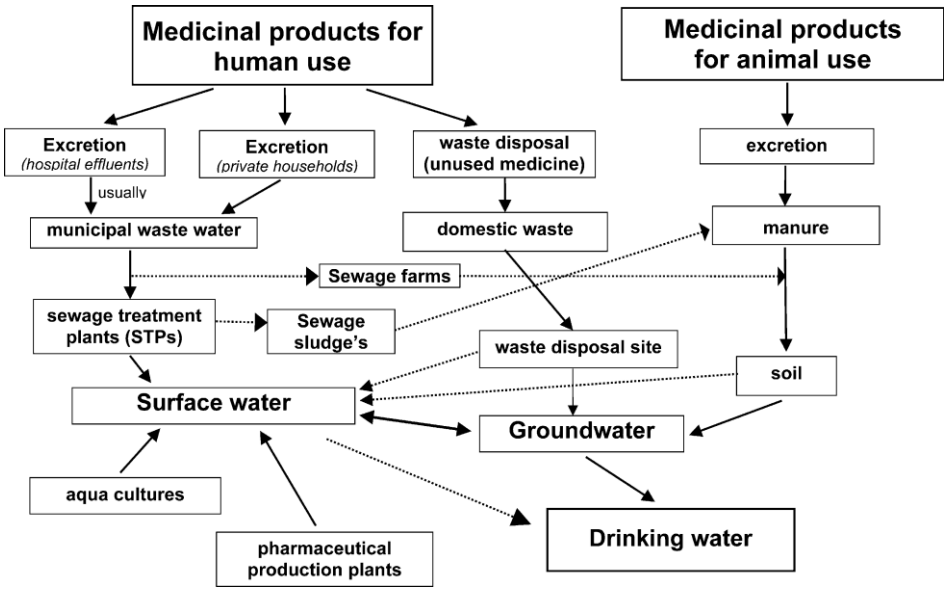



**Figure 2.5 :** Scheme showing possible sources and pathways for the occurrence of pharmaceutical residues in the aquatic environment (Heberer, 2002).

Wastewater treatment processes achieve variable and often incomplete removal of antibiotics, resulting in discharge of antibiotics into surface waters (Brown, 2006 ).

### 2.1.2.1 Natural background

The question of natural background concentrations of antibiotics is important for the risk assessment of antibiotics. Soil bacteria produce several antibiotics such as some ß-lactams, streptomycins, aminoglycosides and others. The group of Actinomycetes includes many soil bacteria such as Streptomycetes. Streptomycetes produce antibiotics. The antibiotic activity from local soil samples is variable and requires the examination of several samples to find a few that produce zones of inhibition (Kümmerer, 2009a).

### 2.1.2.2 Animal livestock and aquaculture

Although most antibiotics are used for the treatment of infections in animals, a significant portion of these are also used in animal feed as a supplement to promote growth in food animals. The use of antibiotics for animal growth promotion is not new; these pharmaceuticals were approved in the United States and United Kingdom in 1949 and 1953, respectively (Kumar *et al.*, 2005). Some drugs are considered feed

additives, often improving and thereby allowing animals to be brought to market faster at lower cost (Boxall, 2003). Since most growth promoters have been banned within the EU, only four compounds remained in this group of feed additives, and these were banned in 2006. The World Health Organization advises abandonment of use of antibiotics as growth promoters, as data show that there is no need for this (Alexy & Kümmerer, 2006).

During livestock production, veterinary drugs enter the environment through removal and subsequent disposal of waste material (including manure/slurry and "dirty" waters), via excretion of faeces and urine by grazing animals, through spillage during external application, via wash-off from farmyard hard surfaces, or by direct exposure/discharge into the environment (Boxall, 2010).

For aquaculture treatments, the drug can be added directly to the aquatic environment. The current definition of aquaculture, according to FAO, is ''the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants". Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding and protection from predators (Kümmerer, 2009a). After the application of antibiotics in aquaculture, the drugs can settle into the sediments (directly, via fish feed, or via fish excrement). In this particular form of application, substantial amounts of the drugs are neither metabolized nor degraded. Thus, oxytetracycline, a drug frequently used in aquaculture worldwide, has been detected at a very high concentration (285 mg/kg) in the sediment of a fish farm (Hamscher, 2006).

#### 2.1.2.3 Agriculture

Antibiotics have been used since the 1950s to control certain bacterial diseases of high-value fruit, vegetable, and ornamental plants (Kümmerer, 2009a). Antibiotics can also excrete in the environment via land application of manure, which is the common practice in many countries. Because of the persistence of various veterinary pharmaceuticals in liquid manure, these compounds reach soil after the fertilization process. Recently, tetracyclines were detected on farmed land at concentrations of up to 300 μg/kg soil, demonstrating that, this class of antibiotics is only slowly degraded

under field conditions and may accumulate in soil after repeated fertilization with liquid manure from intensive pig farming (Hamscher, 2006).

#### 2.1.2.4 Human medicine/hospital

It is widely accepted that patient use is the primary source of human medicines in the environment. In the EU, consumption of antibiotics for medical purposes amounths to a total of 22 g per capita and year, while in the United States the available data give a figure of about 17 g (Alexy, 2006). Kümmerer and Henninger (2003) reported that, in Germany during 1999, hospitals were responsible for 26% (105 t) of the country's antibiotic consumption and estimated that 86 t of antibiotic agents were released into the aquatic environment from hospitals.

The emission of antibiotics from hospitals and households into municipal sewage results in an annual average expected concentration nearly 71 μg/L in Germany. In a study carried out by Ohlsen and his friends, showed that some compounds such as siprofloxacin (51 μg/L), erythromycin (27 μg/L) and dehydratoerythromycin (83 μg/L) were found at concentrations > 10 μg/L. Separate collection of patients' excretions is often impossible for organizational, economic, and technical reasons. In addition, the antibiotics, which are emitted from hospitals to the sewage treatment plants, represent only 30% of the total antibiotic influent to Sewage treatment plants (Alexy & Kümmerer, 2006).

#### 2.1.2.5 Wastewater treatment plant

Sewage treatment plants play an important role in the life cycle of antibiotics in modern society. The main transport pathways of antibiotics into the ambient environment are via Sewage treatment plants, where they may be only partially eliminated (Xu *et al*., 2007).

In general, sulfonamides (sulfamethoxazole), macrolides (roxythromycin and a decomposition product of erythromycin: dehydrato-erythromycin), tetracyclines, andfluoroquinolones (ciprofloxacin) were the most frequently analysed antibiotics in municipal wastewater (Alexy & Kümmerer, 2006). The concentrations of antibiotics in the influent/effluent from the Sewage trematment plants ranged from several hundred ng/L to several μg/L (Xu *et al*., 2007).

Studies have shown that most antibiotics are not fully eliminated in conventional wastewater treatment and are then released into the environment. The overall removal efficiency of a Spanish sewage treatment plant was 60% for the antibiotic sulfamethoxazole (Alexy & Kümmerer, 2006).

#### 2.1.2.6 Pharmaceutical industry

Emissions from production plants have minor importance. Production of pharmaceuticals involves series of steps, often involving sevaral different companies and production sites, in order to produce a finished pharmaceutical product. At each stage in the production process, as with most types of chemical production, there is a risk that chemical waste enters the environment (Larsson *et al.*, 2008).

Larsson *et al*. (2007) reported the occurrence of "extremely high levels of pharmaceuticals" in effluent from a wastewater treatment plant serving 90 bulk drug manufacturers in Patancheru, near Hyderabad, India. In addition to this study, Li *et al.* (2008) reporting the occurrence of high concentrations of oxytetracycline (19,500 ± 2,900 μg/L) in the treated outflow effluent from a wastewater treatment works in Nebei Province.

### 2.1.3 Effects

Antibiotics are designed to affect microorganisms such as bacteria and fungi. If a substance is not eliminated in any way, it can reach the environment with the potential of adversely affecting aquatic and terrestrial organisms. It might reach humans again via drinking water (Kümmerer, 2008).

Once antibiotics enter the ecosystems, they can be treated as an ecological factor, driving the evolution of the community structure (Aminov & Mackie, 2007). Antibiotics are affected in two ways: on the one hand, the microbial community can be severely disturbed by antibiotic activities; on the other hand, these environmental bacteria can acquire and provide gene-encoding resistance (Kemper, 2008). Since antibiotics are efficient inhibitors of bacterial growth produced by environmental microorganisms, it has been widely accepted that their role in nature will be to inhibit microbial competitors. Antibiotic resistance determinants serve to avoid the activity of antibiotics. Besides selecting antibiotic-resistant mutants and favouring the

acquisition of antibiotic resistance determinants by gene-transfer elements that can spread among the environmental microbiota, antibiotic pollution can enrich the population of intrinsically resistant microorganisms, and reduce the population of susceptible microbiota (Martinez, 2009).

#### 2.1.3.1 Effects on treatment plants

Antibiotics have the potential to affect the microbial community in sewage systems. The inhibition of wastewater bacteria may seriously affect organic matter degradation; therefore, effects of antibacterial agents on the microbial population are of great interest (Kümmerer, 2008).

Generally, pharmaceutical wastewater is characterized by high COD concentration, and some pharmaceutical wastewaters can have COD concentration as high as 80,000 mg/L, due to high COD content, pharmaceutical wastewaters could be treated by anaerobic biological systems. There are many studies in the literature, which investigated the effects and treatment potential of pharmaceutical wastewater on anaerobic reactors (Ince *et al*., 2002; Oz *et al*., 2002; Oktem *et al*., 2006; Oktem *et al*., 2008).

A few studies have reported observed inhibition of wastewater microbial activities at elevated antibiotics concentrations (Amin et al., 2006; Gartiser et al., 2007; Ingerslev & Halling-Sørensen, 2000). For sulfonamide antibiotics, concentrations of 10-400 mg/L were reported to inhibit microbial activities in activated sludge by more than 20% (Ingerslev & Halling-Sørensen, 2000). A study by Amin *et al.* (2006) showed that the presence of erythromycin at concentration of 1 mg/L reduced COD removal efficiency and biogas production in anaerobic treatment by about 5%. A study of antibiotic biodegradability using the ISO closed bottle test revealed that a metronidazole concentration of 6 mg/L could reduce anaerobic activity by 69% (Gartiser *et al.*, 2007).

A reduction in the number of bacteria together with alterations in microbial populations were observed in a model sewage purification system when different commonly applied antibiotics were added in concentrations that may occur in hospital waste water (Stanislawska, 1979; Kümmerer *et al.*, 2000).

Nitrification is an important step in wastewater purification, eliminating toxic ammonia. Several antibiotics proved to have low toxicity in relation to nitrifying bacteria in acute tests. These substances showed no effects upon nitrification in concentrations even higher than what might be environmentally expected (Tomlinson *et al.*, 1966; Gomez *et al.*, 1996). An antimicrobial was found to require high concentrations in order to inhibit the nitrification process in a short term test (2–4 h), but a prolonged test period over 5 d showed effects one order of magnitude below the inhibitory concentrations of the acute test (Tomlinson *et al.*, 1966). In a study by Dokianakis *et al.* (2004) the effects caused by the presence of seven different pharmaceuticals on a culture of nitrite-oxidizing bacteria isolated from activated sludge were reported.

Acetoclastic methanogenes are the most sensitive group of microorganisms participating in the anaerobic digestion process. Tests showed that the pharmaceuticals tested (among them sulfamathoxazole) caused mild inhibition of the methanogenes in most cases, which was in turn directly related to the tendency of the compounds to adsorb on the anaerobic biomass (Fountoulakis, 2004).

#### 2.1.3.2 Antibiotic resistance

The capacity of bacteria to adapt to changes in their environment and thus survive is call resistance. In other words, wherever there is a change in susceptibility that renders an agent ineffective against a certain organism, this organism is refer to as resistant. Some organisms have always been resistant to a particular agent by nature of their physiology or biochemistry (inherent or intrinsic resistance), while others have acquired resistance as a result of the selective effects due to the application of antibiotics by humans (acquired resistance) (Kümmerer, 2009b).

It was suggested that antibiotic resistance gene could be considered as environmental pollutants, since they are widely distributed in various environmental compartments, including wastewater and STPs, surface water, lagoon water of animal production areas, aquaculture water, sediments and soil, groundwater, and drinking water (Rysz & Alvarez, 2004). The rapidly growing number of antibiotic-resistant bacterial pathogens severely undermines the ability to control infectious diseases and currently

it is one of the most challenging problems in public health care (Aminov & Mackie, 2007).

Antibiotic resistance genes (ARGs) are well-known 'easy-to-get, hard-to-lose' pollutants. Usually, antibiotic resistance bacteria and genes emerge in the environments under the selection pressure of some antibiotics, but the ARGs could not be easily removed from the polluted areas, even when the pressure has disappeared (Aminov & Mackie, 2007). This could be an explanation, why antibiotic resistance genes were often detected in antibiotic-free environments (Rahman et al, 2008).

### 2.1.4 Fate and removal

Organic compounds may be eliminated by biodegradation, by non-biotic degradation processes such as hydrolysis and photolysis, or by sorption onto suspended particles, sediments and sludge in the aquatic environment (Alexy & Kümmerer, 2006 ).

#### 2.1.4.1 Sorption

Binding to particles or the formation of complexes with metal cations may cause a loss in detectability, as well as a loss in antibacterial activity. Christian and his collegues (2003) demonstrated the loss of antibacterial activity for an aquaculture antimicrobial in seawater driven by the formation of complexes with the magnesium and calcium ions naturally present in marine water.

In general, the sorption behavior of an organic compound strongly depends upon its chemical structure. Most antibiotics are complex chemical molecules, which may contain acidic and basic groups within the same molecule. Therefore, the distribution of such antibiotics between water and particulate matter (log $K_D$) depends on pH (Alexy & Kümmerer, 2006).

Humic substances may alter the surface properties and sites available for sorption and reactions. They can either suppress or promote sorption of organic compounds to mineral surfaces (Kümmerer, 2009a). Antibiotics can also diffuse into biofilms, present in sewage pipes, sludge flocks, or on stones in rivers and lakes. This may result in a biased risk estimate, as concentration in such "reservoirs" may be much higher than in the free water phase. It is not known how strongly the antibiotics are

sorbed onto sludge, particulate matter, bio-solids such as sewage sludge, and sediments, and under what circumstances they are (bio) available and active after sorption (Alexy & Kümmerer, 2006).

### 2.1.4.2 Photolysis

Lunestad (1995) reported that, if a substance is sensitive to light, photodecomposition might be crucial sicnificance in the elimination process. Some antibiotics such as quinolones, tetracyclines, metronidazole, and sulfonamides are sensitive to light. Fluoroquinolones are insensitive to hydrolysis and increased temperatures, but are degraded by UV light (Alexy & Kümmerer, 2006). However, not all compounds are photodegradable. The significance and extent of direct and indirect photolysis of antibiotics in the aquatic environment are different for each compound (Kümmerer, 2009a).

Photochemical decomposition can play an important role in surface water as an additional elimination pathway or for effluent treatment. The effectiveness of the process depends on light intensity and frequency. Photodecomposition may not occur when the compounds are present in turbid water, if the surface of the water is shadowed by trees, or if the compounds are in soil, sewage and sewage pipes since they have low light exposure. Frequency relates to the absorption spectrum of a compound, and the absorption spectrum may be affected by sorption and complexation (Kümmerer, 2009a).

Samuelsen (1989) studied the persistence of oxytetracycline and its sensitivity against the light, both in seawater and on the surface of sediments. The results showed that the compound was unstable in seawater but stable in sediments.

### 2.1.4.3 Hydrolysis

Hydrolysis is another major elimination type of organic substances in the environment. In general, the hydrolysis rates for oxytetracycline increase as the pH deviates from pH 7 and as temperature increases. The half-lives of oxytetracycline under investigation varied due to differences in temperature, light intensity and flow rate from one test tank to another. However sulphonamides and quinolones are resistant to hydrolysis (Kümmerer, 2009a).

#### 2.1.4.4 Biodegradation

Biologically mediated processes (i.e. biotransformation) can result in the partial transformation or total mineralization of chemicals in the aquatic environment. Microorganisms may utilize organic compounds to gain energy. Sometimes total degradation does not take place and the process stopped before mineralization has completed. These biodegradation intermediates, i.e. the products of biotransformation, can be even more stable than the parent compounds. Bacteria and fungi are the two major groups of organisms degrading organic compounds. Therefore, in sewage treatment plants, surface water, groundwater, and seawater bacteria are assumed to be responsible for most biodegradation processes (Alexy & Kümmerer, 2006).

Most antibiotics tested to date have not been biodegradable under aerobic conditions. No evidence of biodegradation for tetracycline was observed during a biodegradability test (sequence batch reactor), and sorption was found to be the principal removal (Kim, 2005). Some antibiotics occurring in soil and sediment proved to be quite persistent in laboratory testing as well as in field studies. Some do not biodegrade well under anaerobic conditions (Gartiser *et al*., 2007) others did (Maki *et al.*, 2006).

It could be assumed that microbial degradation of trace pollutants will be slower in surface water than in the sewage system because of its lower bacterial density and lower bacterial diversity. In a study examined the biodegradability of 18 clinically important antibiotics by Alexy and his friends (2004), showed that none of these antibiotics was readily biodegradable.

#### 2.1.4.5 Technical oxidation processes

Antibiotic formulation effluents are well known for the difficulty of their elimination by traditional bio-treatment methods and their important contribution to environmental pollution is due to their fluctuating and recalcitrant nature. For advanced effluent treatment, oxidation processes are usually applied. However, ozonation will not work well for all types of molecules. The presence of carbon–carbon double bonds, aromatic bonds or nitrogen is a prerequisite.

Li *et al*. (2008) investigated the effect of ozonation on the degradation of oxytetracycline in aqueous solution at different pH values (3, 7 and 11). The results demonstrate that ozonation, as a partial step in a combined treatment concept is a potential technique for biodegradability enhancement for effluents from pharmaceutical industries containing high concentrations of oxytetracycline. Sulfamethoxazole was efficiently degraded by ozonation (Dantas *et al.*, 2007).

## 2.2 Inhibition and Toxicity

Inhibitors are compounds, which interact with an enzyme to slow down its rate of reaction. They may occur naturally in cells, where they might be used for controlling metabolic reaction rates, or artificially, where they might be used as experimental tools in the study of enzyme reactions.

Inhibition can take place in irreversable or reverseble types. Reversible inhibitors bind to the enzyme using weak bonds, similar to those used in binding the substrate. These bonds form rapidly, but also break easily. Irreversible inhibitors are also known as enzyme inactivators. They combine with the enzyme by forming a strong, usually covalent bond.

Competitive inhibition is one of the reversible types of inhibition. The simplest explanation for the competitive inhibition is that inhibitor binds to the same site on the enzyme as the substrate, forming an abortive, nonproductive complex; inhibitor and substrate are mutually exclusive (Leskovac, 2003). In other words, the substrate and inhibitor compete for the same site, so that only one enzyme– inhibitor complex is possible.

A noncompetitive inhibitor binds to an inhibitor site on the enzyme, which is remote from the active site and brings about a conformational change in the active site. A classical noncompetitive inhibitor has absolutely no effect on substrate binding. In fact, a change to the shape of the active site is almost certain to alter the ability of the substrate to bind. In this situation, inhibitors are often called mixed inhibitors, as they appear to have some of the properties of competitive and noncompetitive types.

Uncompetitive inhibitors are incapable of binding to free enzyme. They can only bind to the enzyme-substrate complex. This could be because the substrate is itself

directly involved in binding the inhibitor or because it brings about a conformational change in the inhibitors binding site, which was previously incapable of binding the inhibitor. Once the inhibitor has bound, it prevents the enzyme from turning the substrate into product.

Normally an increase in substrate concentration increases the velocity of the enzyme reaction. Some enzymes, however, display the phenomenon of excess substrate inhibition. This means that large amounts of substrate can have the opposite effect and actually slow the reaction down. Substrate inhibition happens with this enzyme when two substrate molecules bind to the active site at the same time. As long as both substrate molecules are attached to, the active site of the enzyme is effectively inactive, and therefore inhibited. For this process to occur the second substrate must approach the active site very rapidly after the first, otherwise the first substrate would quickly attain the correct catalytic placement.

Inhibitory action of a selected chemical may be experimentally evaluated in two different approaches: Short-term (acute) and long-term (chronic) tests: Acute experiments involve a microbial community selected and sustained by the selected organic substrate in the system and not previously exposed to the inhibitor. There are many different opinions about advantages and disadvantages of these approaches, despite that the two inhibition tests complement one another and reflect real-life inhibition schemes encountered in wastewater treatment: Short-term tests represent the effect of pulse discharge of inhibitors, possibly at high concentrations to the joint treatment systems, while long-term tests may be useful in assessing the continuous impact of lower antimicrobial concentrations, similar to those found in domestic wastewater (Cetecioglu *et al.*, 2012).

### 2.2.1 Mixture toxicity

Pharmaceuticals do not occur as isolated, pure substances in an environmental compartment. As a broad range of different substances is used simultaneously in human and veterinary medicine in any given area, pharmaceuticals are present as multi-component mixtures in the environment. Two characteristics also make their joint toxic effects a major issue for hazar and risk assesment. Firstly, the ecotoxicity of a mixture is always higher than the effects of its individual components. Secondly, a mixture can have a considerable ecotoxicity, even if all components are present

only in low concentrations that do not provoke significant toxic effects if acting signly on the exposed organisms (Backhause, 2008).

Backhouse (1999, 2000) reported that the resulting mixture effect is even higher than 50% in the case of the ten-component mixture of quinolone antibiotics and higher than 15% in the case of the fourteen compound mixture (twelve pharmaceuticals plus two additional toxicants).

Even mixtures of only comperatively few compounds often show a similar pattern. A mixture of fluoxetine and cloribric acid killed more than 50% of a water – flea (Daphnia) population after an exposure of six days, although a components were present at concentrations that did not provoke significant effects individually (Flaherty & Dodson, 2005). In the same study, a significant shift in the sex ratio was observed after an exposure to a three-component mixture of erythromycin, triclosan and trimethoprim again at a mixture concentration at which all components were present at concentrations that did not provoke significant individual effects.

In another study, Wilson and coworkers (2004) documented the impact of a mixture of four tetracyclines on plankton structure and function. Effects on algal communities were observed only in concentrations greater than 200 nmol/L, which is actually well above environmentally realistic concentrations. Zooplankton was not affected significantly at the tested concentrarions. However, it should be pointed out that, the effects on the bacterial populations in the microcosms were not recorded, although these organisms are vastly more sensitive to tetracycline than algea.

### 2.3 Anaerobic Degradation Pathway

The term anaerobic process refers to a divers array of biological wastewater treatment systems from which dissolved oxygen and nitrate-N are excluded. In most instances, they are operated to convert biodegradable organic matter, both soluble and particulate, to methane and carbon dioxide (Grady *et al*., 2011).

The transformation of complex macromolecules into biogas requires the mediation of severak groups of microorganisms (Haandel & Lettinga, 1994). These grpups work in sequence, with the products of one group serving as substrates of another group. Therefore, each group is lenked to other groups in chainlike fashion, with the

weakest links being acetate production and methane production (Gerardi, 2003). Four different phases can be distinguished in the overall conversion proses. The biochemical reactions within these stages are presented in Figure 2.6.

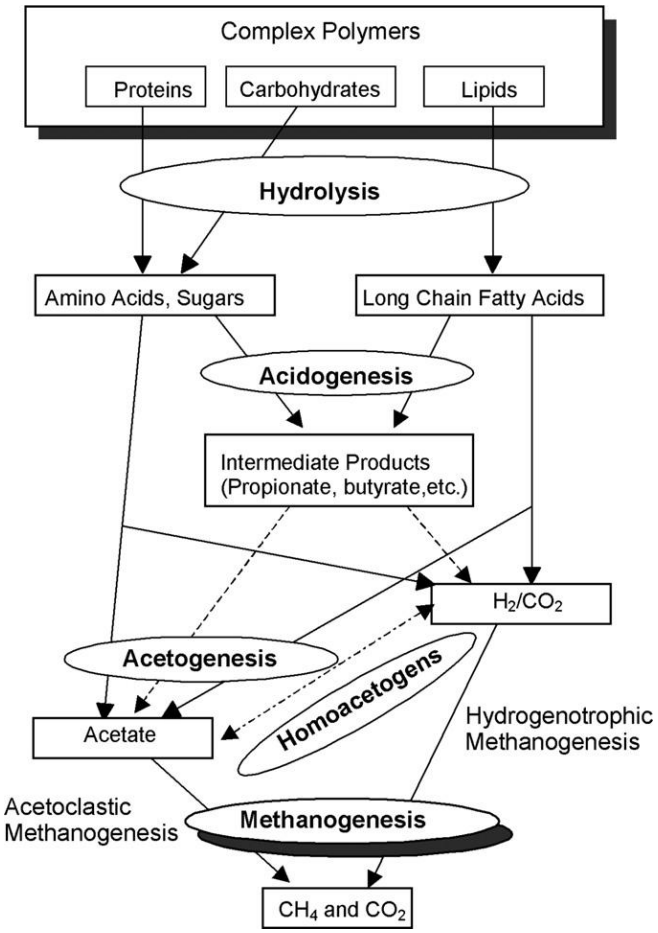



**Figure 2.6 :** Anaerobic degradation pathway (Gujer & Zehnder, 1983).

### 2.3.1 Hydrolysis

Hydrolysis is the first step of the anaerobic degradation. In this prosess, complex particulate matter is converted into dissolved compounds with a lower molecular weight (Haandel & Lettinga, 1994). Particular organic wastes consist of carbohydrates, proteins, and lipids which are polymers. Polymeric substances are large molecules consisting of many small molecules joined together by unique chemical bonds. The small molecules are soluble and quickly go into solution once the chemical bonds are broken. Hydrolytic bacteria or facultatvie anaerobes that are capable of performing hydrolysis achieve breakage of these unique bonds (Gerardi, 2003). Hydrolysis of organic polymers is usually carried out by extracellular

enzymes (hydrolases) and it may or may not be the rate-limiting step of their bioconversion under anaerobic conditions (Ahring, 2003).

### 2.3.2 Acidogenesis

Dissolved compounds, generated in the hydrolysis step, are taken up in the cells of fermentative bacteria and after acidogenesis are excreted as simple organic compounds such as volatile fatty acidsi alcohols, lactic acid and mineral compounds such as carbon dioxide, hydrogen, ammonia and hydrogen sulphide gas. It is mainly the obligatory anaerobic bacteria that carry out fermentative conversion of substrate to product (Haandel & Lettinga, 1994).

### 2.3.3 Acetogenesis

The products of acidogenesis are converted into the final products for methane production: acetate, hydrogen and carbondioxide. A fraction of approximately 70 per cent of the COD originally present in the influent is converted into acetic acid and remainder of the electron capacity concentrated in the formed hydrogen (Haandel & Lettinga, 1994).

In general, two different types of acetogenic mechanisms can be distinguished: (a) acetogenic hydrogenations and (b) acetogenic dehydrogenations. Acetogenic hydrogenations include the production of acetate as a sole end product either from fermentation of hexoses or from $CO_2$ and $H_2O$. Usually the step of acetogenesis in the anaerobic digestion refers to acetogenic dehydrogenations and specifically to the anaerobic oxidation of long and short (volatile) fatty acids (Ahring, 2003).

### 2.3.4 Methanogenesis

Methanogenesis is often the rate limiting step in the overall digestion process, although at lower temperatures this may be hydrolysis. Methane is produced from acetate or from the carbondioxde and hydrogen gas by acetoclastic methanogens and hydrogenotrophic methanoges.

Acetoclastic methanogens:

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (2.1)$$

Hydrogenotrophic methanogens:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (2.2)$$

The bacteria producing methane from hydrogen and carbon dioxide grow faster than those utilizing acetate, so that the acetoclastic methanogens are usually rate limiting with respecct to the trasnformation of complex macromolecules to biogas.

Two important points must be emphasised with the respect to the differet prcesses that occur during anaerobic digestion:

1) The removal of organic matter – COD during the acid fermentation is limited to the release of hydrogen. Only 30 per cent of the organic matter is converted into methane via the hydrogenotrophic pathway. Thus, a necessary condition for efficient organic matter removal in an anaerobic treatment system is that sufficient amounts of acetoclastic methanogens develop.

2) Acid fermentation tends to cause a decrease in the pH because of the production of volatile fatty acids and other intermediates that dissociate and produce protons. As methanogenesis only develop well at neutral pH values, instability may arise if, the acid removal rate by methane production falls behind the acid production rate: the net production of acid will tend to decrease the pH, and thus may reduce the methanogenic activity further. In practice, this situation called souring of anaerobic reactor contents is the most common cause of operational failure of anaerobic systems. The danger of souring can be avoided by maintaining the proper balance between acidic and methanogenic digestion capacity and buffer capacity of the system should be sufficiently high (Haandel & Lettinga, 1994).

### 2.3.5 Microbiology of anaerobic degradation

The anaerobic degradation of complex organic matter is carried out by various groups of bacteria where a coordinated interaction exists (Khanal, 2008).

#### 2.3.5.1 Fermentative bacteria

Fermentative bacteria are responsible of hydrolysis and acidogenesis steps of anaerobic degradation. In these steps, the anaerobic species belonging to the family of Streptococcaceae and Entrobacteriaceae and the genera of Bacteroides, Clostridium, Butyrivibrio, Eubacterium, Bifidobacterium and Lactobacillus are involved generally (Novaes, 1986).

The end products are differed by the types of substrate and environmental conditions. Only at low hydrogen partial pressure, the formation of acetate, $CO_2$, and $H_2$, which are the major substrates for methanogens, is thermodynamically favorable. If the partial pressure of $H_2$ is high, formation of propionate and some other organic acids occurs (Novaes, 1986).

#### 2.3.5.2 Hydrogen-producing acetogens

Hydrogen-producing acetogens metabolizes C3 or higher organic acids (propionate, butyrate, etc.), and ethanol into acetate, $H_2$, and $CO_2$. The anaerobic oxidation of these compounds is not favorable thermodynamically by hydrogen-producing bacteria in pure culture. On the other hand, there is a symbiotic relationship between hydrogen-producing acetogens and hydrogen-consuming methanogens in coculture. The hydrogen-consuming methanogens use the hydrogen rapidly and keep the level of hydrogen partial pressure extremely low. This provides a thermodynamically favorable condition for the hydrogen-producing acetogens to break down the organic compounds into acetate, $H_2$, and $CO_2$ (Khanal, 2008).

The propionic acid oxidation to acetate becomes thermodynamically favorable only at hydrogen partial pressure below $10^{-4}$ atm and for butyrate and ethanol oxidation below $10^{-3}$ and 1 atm, respectively (Pohland, 1992).

#### 2.3.5.3 Homoacetogens

The end product of homoacetogenesis is acetate, which is an important precursor to methane generation. Homoacetogens can be either autotrops or heterotophs. While heterotrophic homoacetogens use organic substrates such as formate and methanol as a carbon source, autotrophic homoacetogens utilize a mixture of hydrogen and carbon dioxide (Khanal, 2008).

$$CO_2 + H_2 \rightarrow CH_3COOH + 2H_2O \quad (2.3)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (2.4)$$

$$4HCOOH \rightarrow CH_3COOH + 2CO_2 + 2H_2O \quad (2.5)$$

$$4CH_3OH + 2CO_2 \rightarrow 3CH_3COOH + 2CO_2 \quad (2.6)$$

*Clostridium aceticum* and *Acetobacterium woodii* are the two mesophilic homoacetogenic bacteria isolated from sewage sludge (Novaes, 1986).

### 2.3.5.4 Methanogens

Methanogens, which were previously classified as bacteria, are now classified as archaea. Archaea are unique group of microorganisms that are distinguished from true bacteria by presence of membrane lipids, absence of basic cellular characteristics (e.g., peptidoglycan), and distinctive ribosomal RNA (Boone *et al.*, 1993). Classification of methanogens is outlined in Table 2.2.

**Table 2.2:** Classification of methanogenic bacteria (Whitman *et al.*, 2001; Garrity *et al.*, 2004)

| |
|---|
| Class I. Methanobacteria (known to grow on $H_2/CO_2$ and formate as C source) |
| Order I. Methanobacteriales |
| Family I. Methanobacteriaceae |
| Genus I. Methanobacterium |
| Genus II. Methanobrevibacter |
| Genus III. Methanosphaera |
| Genus IV. Methanothermobacter |
| Family II. Methanothermaceae |
| Genus I. Methanothermus |
| Class II. Methanococci (known to grow on $H_2/CO_2$ and formate as C source) |
| Order I. Methanococcales |
| Family I. Methanococcaceae |
| Genus I. Methanococcus |
| Genus II. Methanothermococcus |
| Family II. Methanocaldococcaceae |
| Genus I. Methanocaldococcus |
| Genus II. Methanotorris |
| Class III. Methanomicrobia (known to grow on $H_2/CO_2$ and formate as C source) |
| Order I. Methanomicrobiales |
| Family I. Methanomicrobiaceae |
| Genus I. Methanomicrobium |
| Genus II. Methanoculleus |
| Genus III. Methanofollis |
| Genus IV. Methanogenium |
| Genus V. Methanolacinia |
| Genus VI. Methanoplanus |
| Family II. Methanocorpusculaceae |
| Genus I. Methanocorpusculum |
| Family III. Methanospirillaceae (known to be hydrogenotrophic) |
| Genus I. Methanospirillum |
| Order II. Methanosarcinales (known to be acetato- and methylotrophic) |
| Family I. Methansarcinaceae |
| Genus I. Methanosarcina |
| Genus II. Methanococcoides |
| Genus III. Methanohalobium |

**Table 2.2 (continued):** Classification of methanogenic bacteria (Whitman *et al.*, 2001; Garrity *et al.*, 2004).

| |
|---|
| Genus IV. Methanohalophilus |
| Genus V. Methanolobus |
| Genus VI. Methanomethylovorans |
| Genus VII. Methanimicrococcus |
| Genus VIII. Methanosalsum |
| Family II. Methanosaetaceae |
| Genus I. Methanosaeta |

Methanogenesis occur through three major pathways: $CO_2$-reducing or hydrogenotrophic methanogenesis, acetotrophic or acetoclastic methanogenesis, and methylotrophic pathways (Boone *et al.*, 1993).

Hydrogenotrophic pathway contributes up to 28% of the methane generation in anaerobic systems. The acetoclastic pathway is a major catabolic process contributing up to 72% of the total methane generation (Gujer & Zehnder, 1983).

In acetoclastic pathway acetate is converted into methane. *Methanosarcina* and *Methanosaeta* (previously known as *Methanothrix*) are the two major genera of acetoclastic methanogens. *Methanosarcina* uses several methanogenic substrates such as methanol, methylamines, and sometimes $H_2/CO_2$. *Methanosaeta* can only grow on acetate. While Methanosarcina has a typical doubling time of 1–2 days on acetate, Methanosaeta has a doubling time of 4–9 days (Khanal, 2008).

## 2.4 Molecular Methods Used for Establishing the Interactions and Functions of Microorganisms in Anaerobic Bioreactors

Anaerobic digestion has been applied in wastewater treatment with success over the last 20 years (Talbot, 2008). Molecular techniques have recently been applied to the analysis of communities in anaerobic digesters (Godon,1997; Sekiguchi, 1998). As research in molecular microbiology progresses in this area, the scientific community has now pointed out that microorganisms found in anaerobic environments, including bioreactors, are extremely diverse and that their interactions and metabolic functions and regulation remain to be elucidated in detail. Biomonitoring digesters with high throughput molecular tools would be useful to predict eventual stability problems (Talbot, 2008). Molecular techniques can provide detailed information about the structure, function, dynamics, and diversity of microbial communities. Molecular

analysis can chracterize genes, proteins (enzymes), and metabolic products associated with bioenergy production at extremely low concentrations without any difficulties of culturability. Molecular techniques of microbial cells and communities can, therefore, furnish useful information about structure (who they are), function (what they do), and dynamics (how they change through space and time) (Dhamwichukorn, 2008). Figure 6 illustrates how different molecular techniques can be used to analyze microbial community structure, function, and metabolic transformation seperately or together.

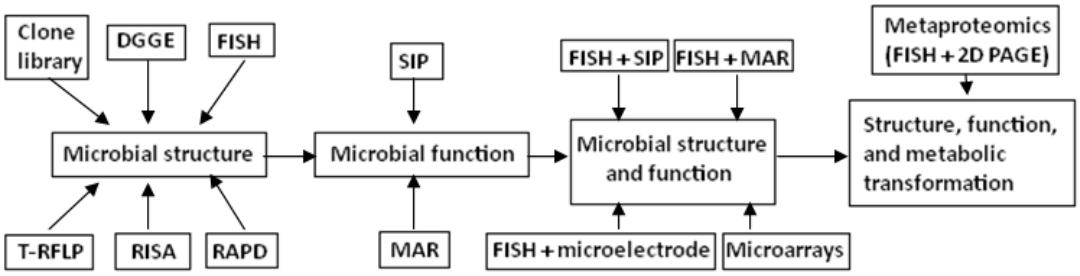



**Figure 2.7 :** Molecular techniques for analysis of microbial community structure, function, and metabolic transformation (Dhamwichukorn, 2008).

According to Zuckerkandl and Pauling (1965), evolutionary history could be documented by nucleic acid sequences. The techniques are based on the RNA of the small ribosomal subunit (16S rRNA for prokaryotes) or their corresponding genes, considering it as a "molecular clock" or "evolutionary chronometer". This molecule was chosen because of its universality and abundance in all living beings (103-105 ribosomes/cell) (Sanz & Köchling, 2007).

The prokaryotic small subunit (SSU) rRNA gene (16S rRNA) gene is highly conserved but it contains variable regions (Woese, 1990). The highly conservative regions facilite PCR amplification using only once universal primer set to amplify the 16S rRNA gene sequences for all eubacteria from environmental samples. The variable regions are different for each bacterial species, allowing accurate bacterial detection and identification. From these characteristics, the 16S rRNA genes help to determine the genetic diversity of a microbial community as well as to assist in identifying the phylogenetic affiliation of its members (Dhamwichukorn, 2008).

### 2.4.1 Nucleic acid extraction

The quality of nucleic acids (DNA and RNA) extracted from digesters is crucial to subsequently obtain informative results. The extraction procedure has to be adapted and optimized for the type of samples under study. The first step in RNA and DNA extraction involves enzymatic digestion, or mechanical disruption of cells with a bead beater. Mechanical disruption in the presence of phenol is more convenient than enzymatic lysis of bacteria because nucleases are immediately destroyed. Many protocols have been published for the extraction of DNA from environmental samples and for co-extraction of DNA and RNA (Raskin, 1995; Griffin, 1998; Ibrahim & Ahring, 1999). Extracted RNA should be stored at -80°C and extracted DNa may be stored at -20°C (Talbot, 2008).

### 2.4.2 Polymerase chain reaction (PCR)

Copies of a specific DNA sequence are necessary for many molecular procedures, and the polymerase chain reaction (PCR) is the method by which copies are made in vitro. The polymerase chain reaction can copy segments of DNA by a process called amplification, yielding large amounts of specific genes or other DNA segments for a host of applications in molecular biology. PCR makes use of the enzyme DNA polymerase, which naturally copies DNA molecules. PCR does not actually copy whole DNA molecules but amplifies stretches of up to a few thousand base pairs (the target) from within a larger DNA molecule (the template) (Madigan, et. al, 2009). Figure 2.8 summarized the steps in PCR amplification of DNA.

### 2.4.3 Denaturing gradient gel electrophoresis

The variable regions of the 16S rRNA genes of bacteria can be amplified by PCR from total genomic DNA and analyzed by DGGE. The DGGE technology is based on electrophoresis of PCR-amplified rRNA gene fragments in polyacrylamide gels containing a linearly increasing concentration of denaturant (Muyzer, 1993). Amplified DNA fragments may have the same length but with different basepair sequence and can be electrophoretically resolved, revealing the diversity of the microbial community. Amplified DNA fragments are separated by DGGE according to the sequence of the different DNA. This technology has been used widely in environmental microbiology to study diversity and populations' relative abundance shifts (Muyzer, 1999) in complex systems, including manure (Leung and Topp,

2001) and anaerobic bioreactors (Liu *et al.*, 2002; Roest *et al.*, 2005; Connaughton *et al.*, 2006; Miura *et al.*, 2007).

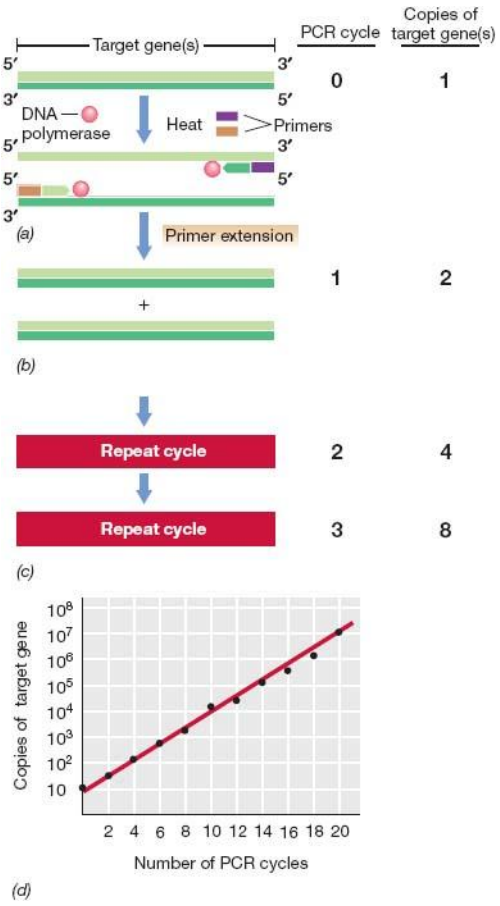



**Figure 2.8 :** The polymerase chain reaction (PCR) (Madigan et. al., 2009).

### 2.4.4 Quantitative real-time PCR

Once the genetic identification of key Bacteria and Archeae are done, or if candidate bacterial species that are suspected to be of interest in the study are characterized, the design of specific primers is possible for a quantitative monitoring of these microorganisms by real-time PCR technology. Assays quantifying functional microbial genes, such as mcrA gene coding for an enzyme found in all methanogens and responsible for the production of methane, can as well be developed to determine the presence of bacteria having a common role in a community (Talbot, 2008).

In contrast to the conventional end-point detection PCR, quantitative real-time PCR (Q-PCR) technology is based on the detection of fluorescence during amplification of target DNA (Higuchi, 1993).

### 2.4.5 Cloning and sequencing

Of all the molecular techniques, cloning and sequencing of the gene that codes for 16S rRNA is still the most widely used in the field of microbial ecology. The microbial community composition has been commonly determined by constructing 16S rRNA clone libraries (Roest, 2005). This methodology implies the extraction of nucleic acids, amplification and cloning of the 16S rRNA genes, followed by sequencing and finally identification and affiliation of the isolated clone with the aid of phylogenetic software (Figure 2.9).

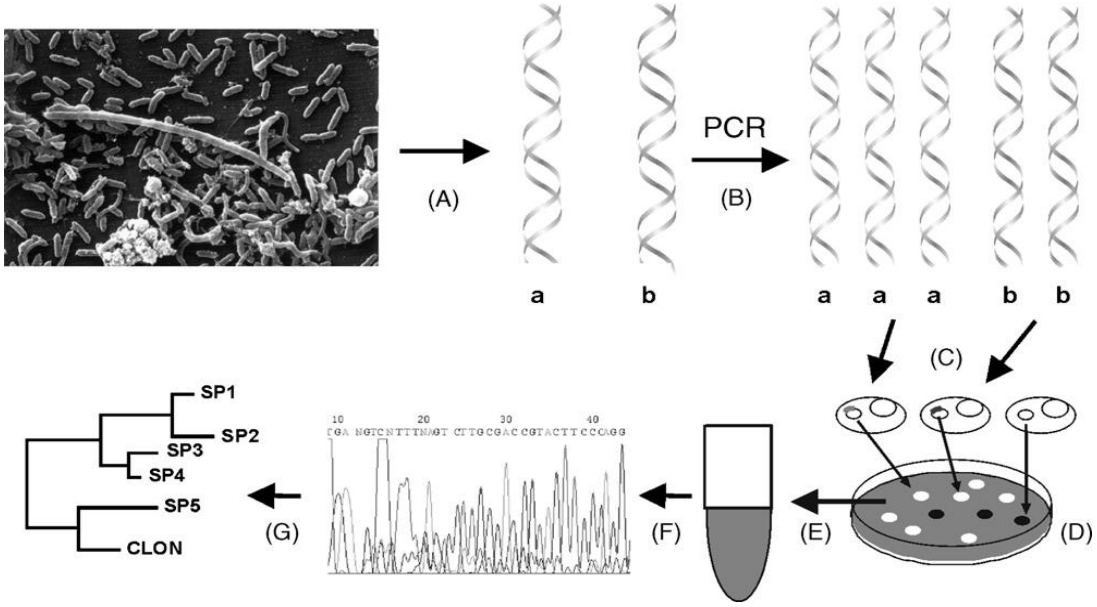



**Figure 2.9 :** Outline of the cloning procedure for studying a microbial community. The work cycle is as follows: (A) direct nucleic acid extraction, without the need for previous isolation of microorganisms; (B) amplification of the genes that code for 16S rRNA by polymerase chain reaction (PCR), commonly using universal primers for bacteria or archaea, resulting in a mixture of rDNA copies corresponding to the microorganisms present in the sample; (C) cloning of the PCR products obtained into a suitable high copy number plasmid and transformation of competent E. coli cells with this vector; (D) selection of transformed clones with an indicator contained in the plasmid (the white colonies in the figure); (E) extraction of plasmid DNA; (F) sequencing of the cloned gene, creating a clone library; (G) determination of the phylogenetic affiliation of the cloned sequence with the help of dedicated computer programs (ARB, Seqlab, PAUP, PHYLIP) (Sanz & Köchling, 2007).

16S ribosomal DNA clone libraries are made from environmental samples by cloning PCR products from extracted DNA into plasmid vectors. In the anaerobic digestion research area, *Archaea* and *Bacteria* are both important, and both corresponding

clone librairies are made separately by choosing domain-specific PCR primer sets, during the initial PCR amplification step. Each clone then contains one rRNA gene that is present in the environmental sample (Talbot, 2008). This cloning and sequencing approach was first reported by Giovannoni (1990) in an analysis of the diversity of bacterioplankton in Sargasso Sea.

# 3. MATERIALS & METHODS

## 3.1 Experimental approach

The aim of this thesis is determination the acute inhibitory effects of antibiotic mixtures on methanogenic pathway. Within this framework, in the first step a stock anaerobic sequencing batch (ASBR) reactor was operated for acclimation of biomass.

In the second step, a series of batch tests were set-up to examine the short-term effects of antibiotic mixtures. Acclimated microbial culture was used as a seed for batch tests. In addition, a different concentration (1 – 250 mg/L) and combination of the selected antibiotics were used to inoculate the test bottles. Serum bottles were kept running for an observation period of eight – twelve days (according to the carbon source) for assessing short-term (acute) inhibitory impact. VFA concentrations measured at the beginning and at the end of the tests for monitoring substrate removal. Biogas productions were monitored during the test period. Additionally microbial characterization of the seed sludge was done by cloning-sequencing.

## 3.2 Seed sludge Characteristics

Seed was obtained from a full-scale UASB reactor (with a volume of 490 $m^3$) from a local alcohol distillery. The temperature and pH in the UASB reactor were maintained within the ranges of 32-35°C and 7.2-7.7 respectively. Total solid (TS) and total volatile solid (TVS) concentrations of the granular sludge was 42724 mg/L and 27211 mg/L, respectively.

### 3.3 Laboratory scale anaerobic sequencing batch reactor operation

An anaerobic sequencing batch reactor (ASBR) with a total volume of 10 L was operated at 35°C (Figure 3.1). The reactor was operated with a 24-hour cycle consisting of fill (10 min), react (22 h 45 min), settle (1 h) and decant (5 min) phases. The rector was mixed using a magnetic stirrer at 90 rpm.

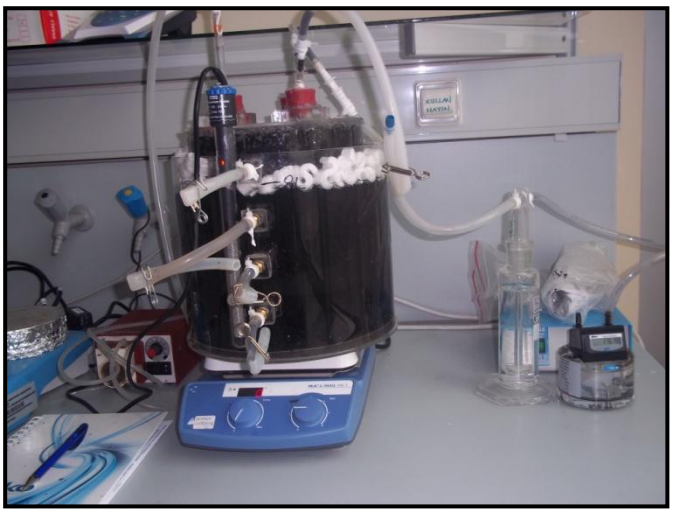


**Figure 3.1 :** Lab-scale ASBR

The system was inoculated with an anaerobic sludge obtained from a full-scale mesophilic anaerobic contact reactor treating the wastewater of raki and fresh grape alcohol. The MLVSS concentration of the reactor was 12250 mg/L. The ASBR was fed a synthetic wastewater with a total chemical oxygen demand (COD) 6250 mg/L of COD equivalent substrate mixture. The organic components and concentration of model wastewater was given in Table 3.1.

**Table 3.1 :** The components and concentrations of model wastewater

| Components | COD (mg/L) |
|---|---|
| Starch | 2915 |
| Glucose | 1865 |
| NaAcetate | 330 |
| NaButyrate | 460 |
| NaPropionate | 680 |
| **Total COD** | **6250** |

The pH of the model wastewater was adjusted 7 through addition of NaOH and the pH in the reactor varied from 6.8 to 7.2.

The reactor was operated with an organic loading rate (OLR) of 1250 mg COD/L.d for the first 3 days of operation and then increased to 6250 mg/L.d in a stepwise manner. The solid retention time was approximately 30 days throughout the study and was calculated based on VSS loss in the effluent and removed during sampling with excess sludge. The hydraulic retention time was 2.7 days.

Temperature, pH and gas production were monitored daily *in situ*. Duplicate samples were collected from the reactor for chemical and microbiological analysis.

### 3.4 Acute Tests Set-Up

Acute tests were performed based on specific methanogenic activity (SMA) principles were carried out by using the pressure transducer technique (Colleran, 1992). Increase in headspace pressure was monitored in sealed bottles by the hand-held pressure transducer (Lutron PM-9107, U.S.A.) which was capable of measuring the pressure in a range of 5-7000 mbar daily. The test medium and the trace element solution were prepared according to the OECD311 protocol under strict anaerobic conditions (2006). The constituents of the medium is given in Table 3.2 while the constituents of trace element solution is given in Table 3.3.

**Table 3.2 :** The constituents of test medium

| **Component** | **Amount (g)** |
|---|---|
| Anhydrous potassium dihydrogen phosphate ($KH_2PO_4$) | 0.27 |
| Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$) | 1.12 |
| Ammonium chloride ($NH_4Cl$) | 0.53 |
| Calcium chloride dihydrate ($CaCl_2.2H_2O$) | 0.075 |
| Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) | 0.10 |
| Iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$) | 0.02 |
| Resazurin (oxygen indicator) | 0.001 |
| Sodium sulphide nonahydrate ($Na_2S.9H_2O$) | 0.10 |
| Stock solution of trace elements | 10 ml |
| Add de-oxygenated water | to 1 liter |

**Table 3.3 :** The constituents of trace element solution

| **Component** | **Amount (mg)** |
|---|---|
| Manganese chloride tetrahydrate ($MnCl_2.4H_2O$) | 50 |
| Boric acid ($H_3BO_3$) | 5 |
| Zinc chloride ($ZnCl_2$) | 5 |
| Copper (II) chloride ($CuCl_2$) | 3 |
| Disodium molybdate dihydrate ($Na_2MoO_4.2H_2O$) | 1 |
| Cobalt chloride hexahydrate ($CoCl_2.6H_2O$) | 100 |
| Nickel chloride hexahydrate ($NiCl_2.6H_2O$) | 10 |
| Disodium selenite ($Na_2SeO_3$) | 5 |
| Add de-oxygenated water | To 1 liter |

Glass serum bottles with 120 ml total volume sealed with butyl rubber septa and aluminium crimped were used in the tests. Liquid volume was 100 ml and the headspace volume was 20 ml (Figure 3.2). 1000 mg/L VSS were added to the serum bottles. Acclimated biomass was sampled from the ASBR. Acute tests were organized based on a three-way factorial design where one factor was the composition of the substrate solution, another factor was the composition of antibiotic mixtures and the last factor was concentration of antibiotics added. Three different substrate solutions which were acetate, butyrate, and volatile fatty acid (VFA) mixture were used in the tests. Test conditions are given in Table 3.4. Firstly, substate concentrations in different concentrations (500, 1000, 1500, and 2000 mg/L) were tested in order to reach maximum potential methane production (PMP) rate during the batch tests. Thus the optimum substrate concentrations were determined.

**Figure 3.2 :** 120 mL serum bottle with 100 mL active volume used for tests.

Among those 1500 mg/L were found to be optimum for each substate solutions (acetate, butyrate and VFA mixture). Acute tests were performed in duplicate bottles. The control bottles, which only included acetate, butyrate, or VFA mixture, were also set-up. During the test period, the bottles were stored at 35±2°C. Headspace pressure was measured by hand-held pressure transducer (Lutron PM-9107, U.S.A.) and the bottles were shaken by hand everyday. The percentage of inhibition of biogas production was calculated from the amount produced in the respective test and control bottles.

**Table 3.4 :** Test conditions for determining the acute effects of two or three component antibiotic mixtures

| | **ANTIBIOTIC CONCENTRATIONS (mg/L)** | | |
|---|---|---|---|
| **SUBSTRATE** | **TET** | **ERY** | **SMX** |
| Acetate (1500 mg/L)/ Butyrate (1500 mg/L)/ VFA mixture (1500 mg/L) | 1 | 1 | x |
| | 1 | x | 1 |
| | x | 1 | 1 |
| Acetate (1500 mg/L) / Butyrate (1500 mg/L) / VFA mixture (1500 mg/L) | 10 | 10 | x |
| | 10 | x | 10 |
| | x | 10 | 10 |
| Acetate (1500 mg/L) / Butyrate (1500 mg/L) / VFA mixture (1500 mg/L) | 25 | 25 | x |
| | 25 | x | 25 |
| | x | 25 | 25 |
| Acetate (1500 mg/L) / Butyrate (1500 mg/L) / VFA mixture (1500 mg/L) | 50 | 50 | x |
| | 50 | x | 50 |
| | x | 50 | 50 |
| Acetate (1500 mg/L) / Butyrate (1500 mg/L) / VFA mixture (1500 mg/L) | 100 | 100 | x |
| | 100 | x | 100 |
| | x | 100 | 100 |
| Acetate (1500 mg/L) / Butyrate (1500 mg/L) / VFA mixture (1500 mg/L) | 250 | 250 | x |
| | 250 | x | 250 |
| | x | 250 | 250 |
| Acetate (1500 mg/L) / Butyrate (1500 mg/L) / VFA mixture (1500 mg/L) | 1 | 1 | 1 |
| | 10 | 10 | 10 |
| | 25 | 25 | 25 |
| | 50 | 50 | 50 |
| | 100 | 100 | 100 |

In the scope of the study 144 test bottles were set-up having three different substrate solutions, various combinations and concentrations of antibiotic mixtures. After the test period, samples were taken for chemical and microbial analysis.

### 3.5 Chemical analysis and Analytical techniques

Suspended solids (SS), volatile suspended solids (VSS), total suspended solids (TS), total volatile suspended solids (TVS), alkalinity and soluble COD were carried out according to American Public Health association APHA, 2005. Miligas Counter (Ritter Digital Counter, U.S.A.) was used for monitoring the biogas production in the stock reactor. Gas compositions and VFA concentrations were determined using gas chromatograph (Perichrom, France and Agilent Technologies 6890N, USA, respectively).

### 3.6 Microbial Characterization

The samples were taken from seed sludge for DNA extraction as triplicate. The samples were stored at -20ºC until DNA extraction.

#### 3.6.1 Genomic DNA (GDNA) extraction

Genomic DNAs were extracted using GF-1 Bacterial DNA Extraction Kit according to the manufacturer's instructions (GeneON, Germany). The concentrations of extracted GDNA were determined Qubit 2.0 Flouremeter (Invitrogen, UK). Then GDNA were diluted to 25 ng/µl by DNase free water. and stored at -20 ºC until the further analysis.,

#### 3.6.2 Polymerase chain reaction (PCR) of 16S rDNA

Amplification of 16S rDNA was performed by PCR using bacterial and archaeal specific primers. Bac8f-Bac1541r primers were used for the amplification 16S rDNA of bacteria, while Arch07f- Arch1384r primers were used for the amplification of Archaea. The primers and annealing temperatures are given in Table 3.5. Extracted GDNA was used as a template.

**Table 3.5 :** Bacterial and archaeal primers used for PCR amplification

| Primer | Target | Experimental Stage | Annealing (°C) | Position[1] | Reference |
|---|---|---|---|---|---|
| Bact8f | Bacterial 16S rDNA | Cloning | 55 | 8-27 | Lane, 1991 |
| Bact1541r | | | | 1541-1522 | |
| Arch7f | Archaeal 16S rDNA | Cloning | 53 | 07-24 | Raskin *et al.*, 1994 |
| Arch1384r | | | | 1384-1368 | Lueders *et al.*, 2004 |
| M13f | B-galactosidase | Clone screening | 54 | – | Schrenk *et al.*, 2003 |
| M13r | | | | | |

PCR reactions were performed in a 50 µl total volume including 25ng of DNA, 10 pmol of each primer , 40 mM of each deoxynucleoside triphosphate, 5 µl of 10×*Taq* buffer and 10 U of Maximo Taq DNA polymerase (GeneON, Germany). Amplification was performed with a TECHNE-TC 412 thermal cycler. Reactions had an initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation step at 94°C for 45 sec, annealing step at 53°C for 1 45 sec, and extension step at 72°C for 2 min and a final extension step at 72°C for 10 min. All PCR products were run on the %1 (w/v) agarose gel stained with sybrgreen in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8) at 7 V cm-1 by electrophoresis (Invitrogen, UK). Gels were visualized by using a gel documentation system, Vilber Lourmat.

### 3.6.3 Cloning, sequencing and phylogenetic analysis

The bacterial and archaeal PCR products of the seed sludge of acute tests obtained using Bact8f-Bact1541r and Arch344f-Arch855r, respectively, were cloned by TOPO TA cloning kit (Invitrogen Ltd., UK) according to manufacturer's instructions. Before cloning, the 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 analysed by high resolution melt (HRM) to select the respresentative 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.

Partial 16S rRNA gene sequences were analyzed and manually edited in Amplify 3X software package version 3.14 (http://engels.genetics.wisc.edu/amplify). 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 (1969) 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 (Saitou and Nei, 1987).

16S rRNA gene sequences showing 99% similarity or higher were 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%) relateness with known bacterial or archaeal phylogenetic groups were listed as unclassified. Coverages of the clone libraries were calculated as 1 – (n1/N), where n1 is the number of clone types that was encountered only once in the library and N is the total number of clones analyzed (Good 1953).

### 3.7 Statistical Analysis

Statistical analyses were made by using SPSS programme. One-way ANOVA test was applied to determine the significant inhibition of antibiotic mixtures on the total methane production and VFA utilization.

# 4. RESULTS AND DISCUSSION

## 4.1 Stock Anaerobic Sequencing Batch Reactor

The ASBR was operated as a stock reactor for further analysis, fed a synthetic wastewater including starch, glucose, butyrate, propionate and acetate with an organic loading rate 6250 mg COD/L. Each step of the anaerobic treatment was set to run with this substrate solution, thus the specific inhibition step could easily investigate.

The reactor was operated with 2.7 day HRT and short settling time (5 min) resulted in good biomass settling conditions and low effluent suspended solids. Prior the steady-state condition, the influent COD was increased in a stepwise manner from 1250 mg/L COD to 6250 mg/L COD. At steady-state, it exhibited a stable performance, with an average effluent soluble COD of 475±50 mg/L corresponding to a COD removal of around 93% (Figure 4.1) and an average biogas production of 13,01±1,27 L/d.During this period, the methane content in the biogas averaged 75±1.4%.

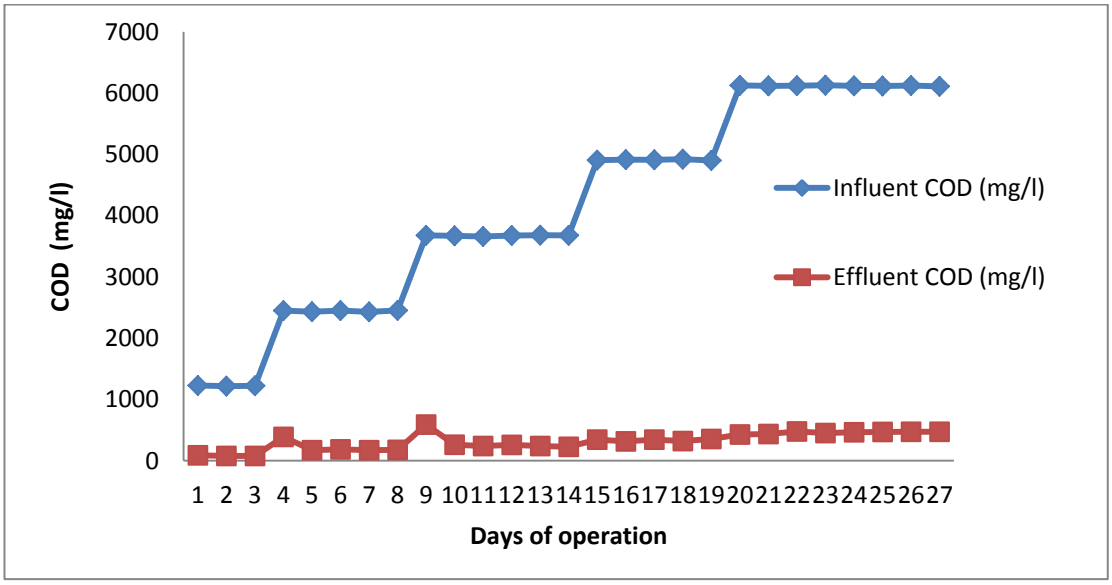



**Figure 4.1 :** Influent and effluent soluble COD concentration of stock ASBR.

## 4.2 Characterization of Seed Sludge

Seed sludge which used in acute tests was characterized in terms of potential methane production capacity and dominant microbial species. Spesific methanogenic activity (SMA) test was applied to determine the potential methane production capacity of the seed sludge. For the microbial characterization, bacterial and archaeal 16S rDNA clone libraries were constructed. HRM analysis was applied to compare the detected microbial species by clone libraries to all OTUs in the sludge samples.

### 4.2.1 Specific methanogenic activity (SMA) test

Methanogenic characteristics of seed sludge were investigated by specific methanogenic activity (SMA) tests. SMA tests with acetate concentration between 1000-5000 mg/L were set up for determination of acetoclastic methanogenic activity. To determine the maximum methane production capacity of seed sludge, VFA mixtures (acetate, propionate, butyrate) were used as substrate for SMA tests. 3000 mg/L of acetate and 4000 mg/L of VFA mixture were found as optimum concentrations for the next analysis.

### 4.2.2 Bacterial and archaeal 16S rDNA clone libraries

Bacterial and archaeal 16S rDNA gene clone libraries were constructed from the seed sludge for characterization of microbial community structure.

All clones were screened by HRM analysis and this analysis showed that 12 different OTUs were obtained from 75 bacterial clones and 22 different OTUs were found in 83 archaeal clones. Table 4.1 and 4.2, shows all different OTUs were sequenced and the closest relatives of bacterial and archaeal sequencing results respectively.

As seen in Figure 4.2. 64% of the bacterial clone library were composed of unclassified bacteria. The most abundant classified phylum was Firmicutes (21%). The members of Actinobacteria and Cyanobacteria were distributed as 11% and 4%, respectively. Clostridium sp., were represented 93% of Firmicutes members in the seed sludge.

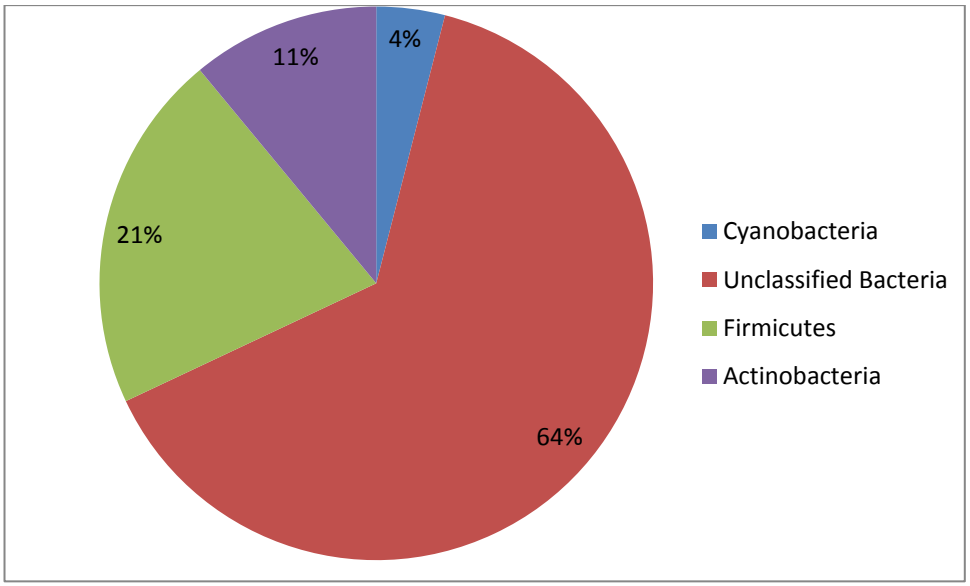



**Figure 4.2 :** Distribution of phylogenetic phylums from clone sequence of bacterial 16S rDNA clone library.

The dominant Firmicutes OTU was 07a (JF946895) and it represented %12 of all bacterial clones. It is most closely related to the Clostridium sp. L35B_99 isolated from mesophilic and thermophilc methanogenic enrichment that degrades petroleum hydrocarbons (Cheng & Lu, 2011). Other OTU related to Firmicutes phylum was 05a, mostly closed to isolate from oil well by Pham *et al.* (2009).

Actinobacteria was the second abundant phylum (11%) of the bacterial clone library which are responsible to produce of propionic acid, acetic acid and carbon dioxide (Madigan *et al.*, 2009). The dominant OTU was 09c (HM480642), represents 8% of all bacterial clones. It is mostly related to an uncultured actinobacterium clone O_1H06 obtained from meadow soil (Sagova-Mareckova, *et al.*, 2011). Other OTU in Actinobacteria phylum was 10b (JQ217228). It is most closely related to an uncultured Brachybacterium sp. clone OTU116 obtained from marine sediment (Yan *et al.*, 2011).

Cyanobacteria phylum represented 4% of the bacterial clone library. It was most closely related to an uncultured Plectonema sp. clone 2K79 which was isolated from groundwater (Aizenberg-Gershtein *et al.*, 2012).

The phylogenetic tree constructed by bacterial clones was given in Figure 4.3.

**Table 4.1 :** Affiliation of bacterial clones and their abundance in the seed sludges.

| Clon Name | Clone Frequency (%) | Accession No | Closest Relative | Similarity |
|---|---|---|---|---|
| 01a | 0,0135 | GU074264 | Uncultured Plectonema sp. clone 2K79 | 100 |
| 01b | 0,0135 | GQ091211 | Uncultured bacterium clone nbw343d12c1 | 97 |
| 01c | 0,0135 | GU074264 | Uncultured Plectonema sp. clone 2K79 | 95 |
| 02a | 0,0135 | GU074264 | Uncultured Plectonema sp. clone 2K79 | 99 |
| 03a | 0,0270 | GQ091211 | Uncultured bacterium clone nbw343d12c1 | 91 |
| 03b | 0,0135 | GQ091212 | Uncultured bacterium clone nbw343d12c1 | 89 |
| 04a | 0,0270 | JF946895 | Uncultured Clostridia bacterium clone L35B_99 | 94 |
| 05a | 0,0135 | GU179924 | Uncultured Firmicutes bacterium clone D120231B11 | 97 |
| 06a | 0,0135 | JF946895 | Uncultured Clostridia bacterium clone L35B_99 | 94 |
| 07a | 0,1216 | JF946895 | Uncultured Clostridia bacterium clone L35B_99 | 96 |
| 07b | 0,0135 | JF946894 | Uncultured Clostridia bacterium clone L35B_105 | 94 |
| 08a | 0,0135 | JN680632 | Uncultured Clostridiaceae bacterium clone SL89 | 100 |
| 08b | 0,0135 | DQ125557 | Uncultured bacterium clone AKAU3538 | 97 |
| 09a | 0,1757 | FQ658959 | Uncultured bacterium clone M1CB122 | 95 |
| 09b | 0,2838 | HM823586 | Uncultured bacterium clone nby472e12c1 | 94 |
| 09c | 0,0811 | HM480642 | Uncultured actinobacterium clone O_1H06 | 97 |
| 09d | 0,0135 | JN397736 | Uncultured bacterium clone BSB0103-03 | 96 |
| 10a | 0,0405 | HQ393224 | Uncultured bacterium clone 4.19 | 95 |
| 10b | 0,0270 | JQ217228 | Uncultured Brachybacterium sp. clone OTU116 | 96 |
| 10c | 0,0135 | JN521901 | Uncultured organism clone SBZI_3837 | 96 |
| 11a | 0,0405 | FN563168 | Uncultured bacterium clone HAW-RM37-2-B-650d-A3 | 100 |
| 12a | 0,0135 | AB291266 | Uncultured bacterium clone: SmB7 | 95 |

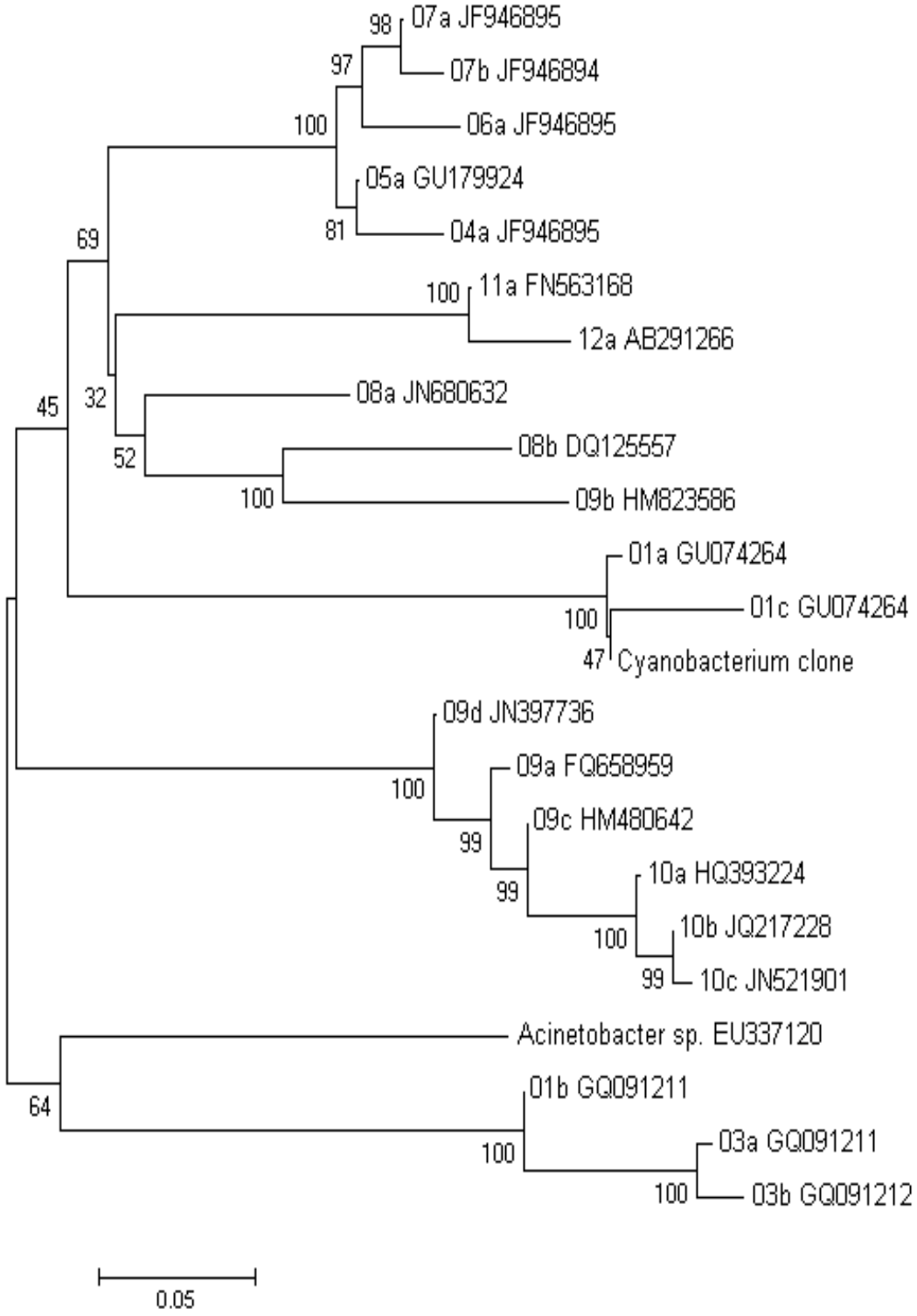



**Figure 4.3 :** Neighbor-joining full-length 16S rDNA trees showing the genetic distances among individual clones from bacterial 16S rDNA clone library. Tha *bar scale* represents 5 nucleotide substitutions per 100 nucleotides.

Methanosarcinales (27%) is the most abundant classified phylum of archaeal clone library of seed sludge. This rate is important cause, nearly 70% of total methane produce from acetate by acetoclastic methanogens (Gujer & Zehnder, 1983). *Methanosarcina* and *Methanosaeta* (previously known as *Methanothrix*) are the two major genera of acetoclastic methanogens. *Methanosarcina* uses several methanogenic substrates such as methanol, methylamines, and sometimes $H_2/CO_2$. *Methanosaeta* can only grow on acetate (Khanal, 2008). As it is shown in Table 4.2, both of these generas were detected in the seed sludge.

The members of unclassified archaea, euryarchaeota and Methanomicrobiales were distributed as 58%, 8% and 7% respectively (Figure 4.4).

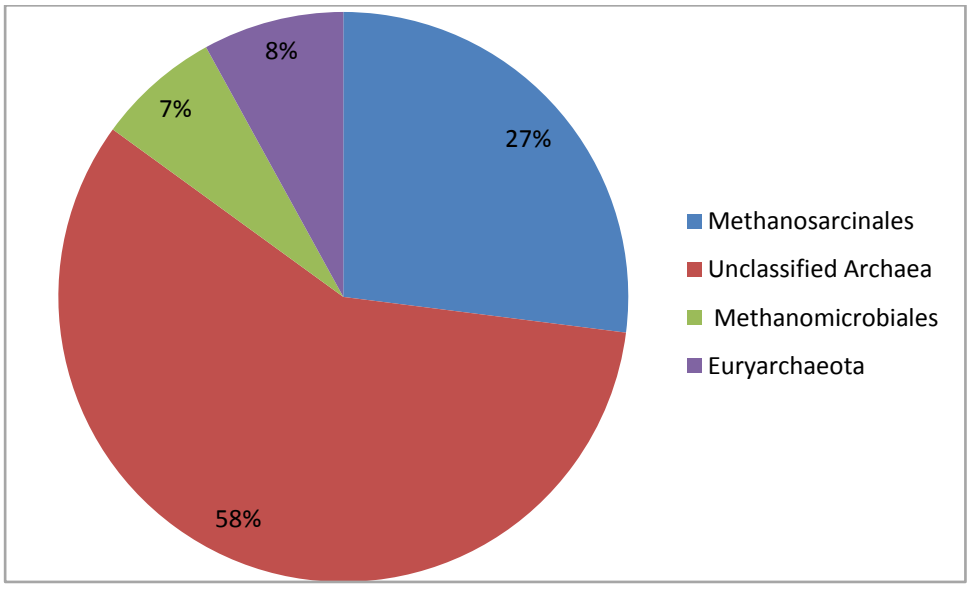



**Figure 4.4 :** Distribution of phylogenetic phylums from clone sequence of archaeal 16S rDNA clone library.

The dominant Methanosarcinales OTU, 02a (JQ684643), related cluster, represented by 7,23% of all archaeal clones. It was mostly closed to an uncultured Methanosaeta sp. clone JM-ASBR-Arch-117 (Ma *et al.*, 2012). Other OTUs of this phylum were isolated from anaerobic sludge samples and tidal flat sediments. The OTU, 01b (X51423) was most closely related (95%) to Methanothrix soehngeni, which utilizes acetate for production of methane (Eggen *et al.*,1991).

The dominant Methanomicrobiales OTU, 12a (M59143), related cluster, represented by 4,81% of all archaeal clones was most closely related (100%) to Methanoplanus limicola (Rouviere *et al.*, 1992).

OTUs including the members of euryarchaeota and unclassified archaea were isolated from lake sediment, mesophilic anaerobic digester which treats municipal wastewater sludge, rich minerotrophic fen, and compost (Fan & Wu, 2011; Riviere, *et al.*, 2009; Cadillo-Quiroz *et al.*,2008; Lee *et al.*, 2006). The phylogenetic tree constructed by archaeal clones was given in Figure 4.5.

**Table 4.2 :** Affiliation of archaeal clones and their abundance in the seed sludges.

| Clone Name | Clone Frequency (%) | Accession No | Environmental Database | Similarity |
|---|---|---|---|---|
| 01a | 0,0120 | JF947134 | Uncultured Methanosaeta sp. | 97 |
| 01b | 0,0120 | X51423 | Methanothrix soehngenii | 95 |
| 02a | 0,0723 | JQ684643 | Uncultured Methanosaeta sp. | 89 |
| 03a | 0,0120 | FR823806 | Uncultured archaeon clone arch.R1-49 | 92 |
| 04a | 0,0241 | JQ684642 | Uncultured Methanosaeta sp. clone JM-ASBR-Arch-116 | 92 |
| 04b | 0,0120 | JQ684638 | Uncultured Methanosaeta sp. clone JM-ASBR-Arch-112 | 92 |
| 05a | 0,0361 | JQ684588 | Uncultured Methanosaeta sp. clone JM-ASBR-Arch-60 | 96 |
| 06a | 0,0241 | JN617394 | Uncultured archaeon clone A2-66 | 93 |
| 06b | 0,0361 | JQ684600 | Uncultured Methanosaeta sp. clone JM-ASBR-Arch-73 | 95 |
| 06c | 0,0241 | JQ684605 | Uncultured Methanosaeta sp. clone JM-ASBR-Arch-78 | 92 |
| 07a | 0,0241 | JQ684643 | Uncultured Methanosaeta sp. clone JM-ASBR-Arch-117 | 91 |
| 07b | 0,0843 | JN397898 | Uncultured archaeon clone SSA0402-03 | 93 |
| 08a | 0,0241 | CU917427 | Uncultured Methanosarcinales archaeon clone QEEG1AC031 | 92 |
| 08c | 0,0120 | JN617394 | Uncultured archaeon clone A2-66 | 97 |
| 09a | 0,0241 | CU916696 | Uncultured Methanosarcinales archaeon clone QEEG1AC031 | 92 |
| 10a | 0,0241 | HM061282 | Uncultured archaeon clone Arch.a24.46 | 97 |
| 10b | 0,0120 | EU156000 | Methanosphaerula palustris | 95 |
| 10c | 0,0120 | EU156000 | Methanosphaerula palustris | 94 |
| 11a | 0,0602 | CU915960 | Uncultured Methanomicrobiales archaeon clone QEEL1CB101 | 81 |
| 12a | 0,0482 | M59143 | Methanoplanus limicola | 100 |
| 12b | 0,0120 | M59144 | Methanoplanus limicola | 100 |
| 13a | 0,0120 | JN173203 | Uncultured Methanogenium sp. clone A673 | 100 |
| 14a | 0,0241 | M59134 | Methanogenium marisnigri | 97 |
| 15a | 0,0482 | M59147 | Methanocorpusculum parvum | 97 |
| 16a | 0,0241 | JQ179819 | Uncultured archaeon clone C4TRA06 | 98 |
| 17a | 0,0241 | JQ179798 | Uncultured archaeon clone C4ALB04 | 98 |
| 18a | 0,0241 | DQ365140 | Uncultured compost archaeon clone 4A01 | 95 |
| 19a | 0,1807 | CU917453 | Uncultured Methanomicrobiales archaeon clone QEEC1DA051 | 97 |
| 20a | 0,0482 | CU917453 | Uncultured Methanomicrobiales archaeon clone QEEC1DA051 | 100 |
| 21a | 0,0241 | CU915998 | Uncultured Methanomicrobiales archaeon clone QEEK1BA061 | 97 |
| 22a | 0,0120 | CU917453 | Uncultured Methanomicrobiales archaeon clone QEEC1DA051 | 92 |

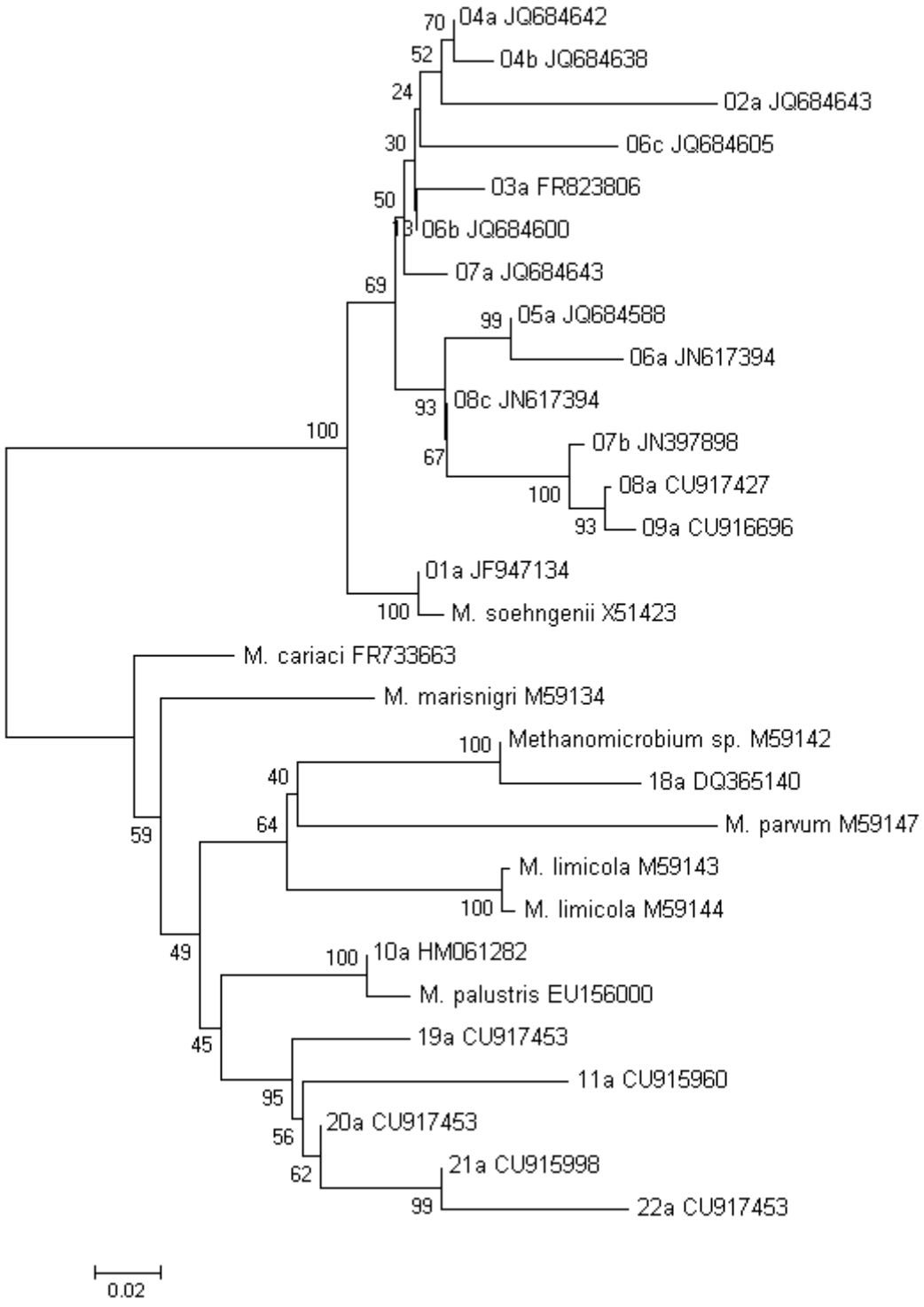



**Figure 4.5 :** Neighbor-joining full-length 16S rDNA trees showing the genetic distances among individual clones from archaeal 16S rDNA clone library. Tha *bar scale* represents 2 nucleotide substitutions per 100 nucleotides.

### 4.3 Acute Tests

Acute tests were designed to evaluate short-term inhibitory effect of the multi-component mixture of selected antibiotics, tetracycline (TET), sulfamethoxazole (SMX), and erythromycin (ERY) on the metabolic activity of both *homoacetogens and methanogens*, which are specific trophic groups in the acclimated sludge. The experiments were designed as three sets which were differ according to the substrate composition (acetate, butyrate and VFA mixtures). Antibiotic concentrations were between 1-250 mg/L at different mixture composition.

The test bottles were monitored by the parameters in terms of biogas/methane production and VFAs removal. Biogas generation was monitored daily throughout the experiment period. VFA concentrations were measured at the end of the test.

A preliminary test was conducted before the inhibition experiments, mainly to determine the optimum initial dose of acetate, butyrate, and VFA mixture. For this purpose, initial substrate concentrations in the range of 500–2000 mg/L were tested in the control reactor without antibiotic addition and 1500 mg/L of each substrate was determined as the optimum initial dose.

#### 4.3.1 Acetate as a sole carbon source

Acetate is a key intermediate in the anaerobic food chain. It is estimated that nearly 70% methane is produced from acetate other than hydrogenotrophic pathway (Jones, 1991). Two genera of archaea, *Methanosarcina* and *Methanosaeta*, are known to use acetate as a substrate for methanogenesis. *Methanosaeta* uses only acetate for methane production, whereas *Methanosarcina*, utilizes both methylated compounds such as methanol and methylamines and acetate (Smith & Ingram-Smith, 2007).

It was determined that Methanosarcinales (27%) is the most abundant classified phylum of archaeal clone library of the seed sludge which includes *Methanosarcina* and *Methanosaeta*. Acetate tets bottles were set up to identify the inhibition effect of the antibiotic mixtures on acetoclastic methanogens.

#### 4.3.1.1 Methane production

Average of total methane production in the control bottles was 64 mL at the end of the test period which was 95% of total biogas production. Figures 4.6 - 4.10 indicate total and maximum methane productions of the test bottles with acetate and different antibiotic combinations. The complete inhibition was observed in none of the test bottles.

Figure 4.6a reports the total methane production in the test bottles with the addition of three-component mixture (ETS) of antibiotics compared with the control bottles. 1 mg/L antibiotic mixture added test bottles showed nearly the same pattern with control bottles. The significant inhibition effect induced when ETS dosage was increased up to 50 mg/L where the total methane production decreased to 32 mL. The maximum inhibition effect was observed at 100 mg/L concentration. The methane production was 17 mL at 100 mg/L ETS concentration.

Figure 4.6b indicates the maximum methane production profile. The maximum methane production was 220 mL CH4/gVSS.day in control bottles. A slight decrease was observed at 1 mg/L concentration, maximum methane production was decreased to 130 mL $CH_4$/gVSS.day at this concentration. 25 – 50 mg/L concentrations showed nearly the same pattern with each other. At 100 mg/L concentration, the maximum methane production was 34 mL $CH_4$/gVSS.day, which was 85% lower than control bottles.

There was a significant correlation between the total methane production and antibiotic concentration according to Pearson's correlation ($p<0,01$).

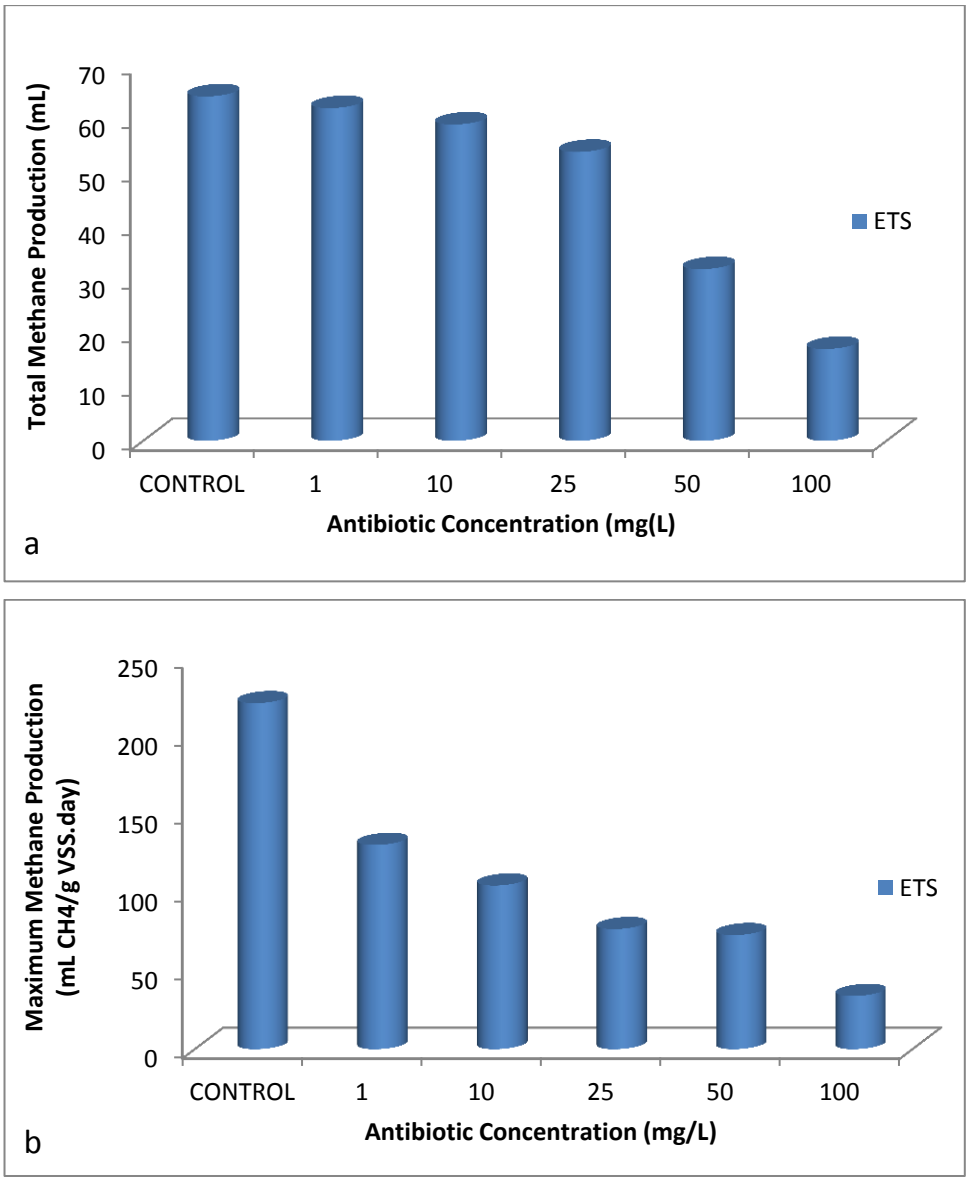



**Figure 4.6 :** Inhibition effect of ETS dosage on (a) total methane production, (b) maximum methane production when acetate as a sole carbon source.

Figure 4.7 shows the effects of ET mixture on total and maximum methane production. ET mixture indicated quite similar methane profile to control bottles at 1 mg/L. The profiles associated with 10 – 25 mg/L indicated similar pattern with each other. Significant decreases were observed at concentrations between 100 – 250 mg/L. The maximum methane production of sludge was decreased significantly at 100 mg/L ET concentration (Figure 4.7b). At 250 mg/L, maximum methane production was decreased to 31 $CH_4$/gVSS.day.

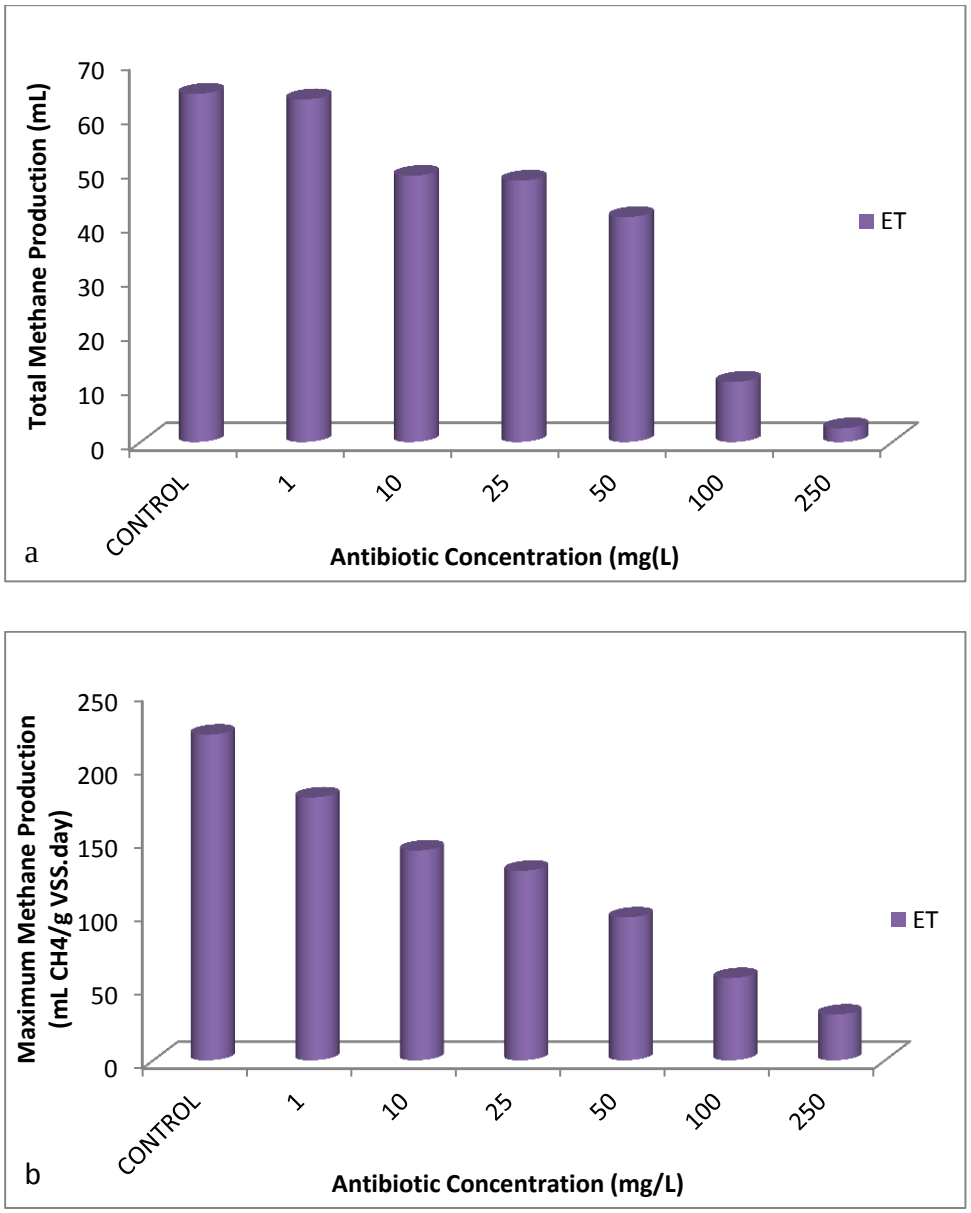



**Figure 4.7 :** Inhibition effect of ET dosage on (a) total methane production, (b) maximum methane production when acetate as a sole carbon source.

The effects of ST mixture on total methane production were shown in Figure 4.8a. Significant inhibition effect was induced at 250 mg/L. The methane content of biogas composition was decreased (92% – 75%) with increasing antibiotic concentration (1 – 250 mg/L). There were no significant difference between the concentration 1-25 mg/L on maximum methane production of sludge (Figure 4.8b). A slight decrease on maximum methane production was observed at 250 mg/L ST concentration. There was nearly no methane production at this concentration.

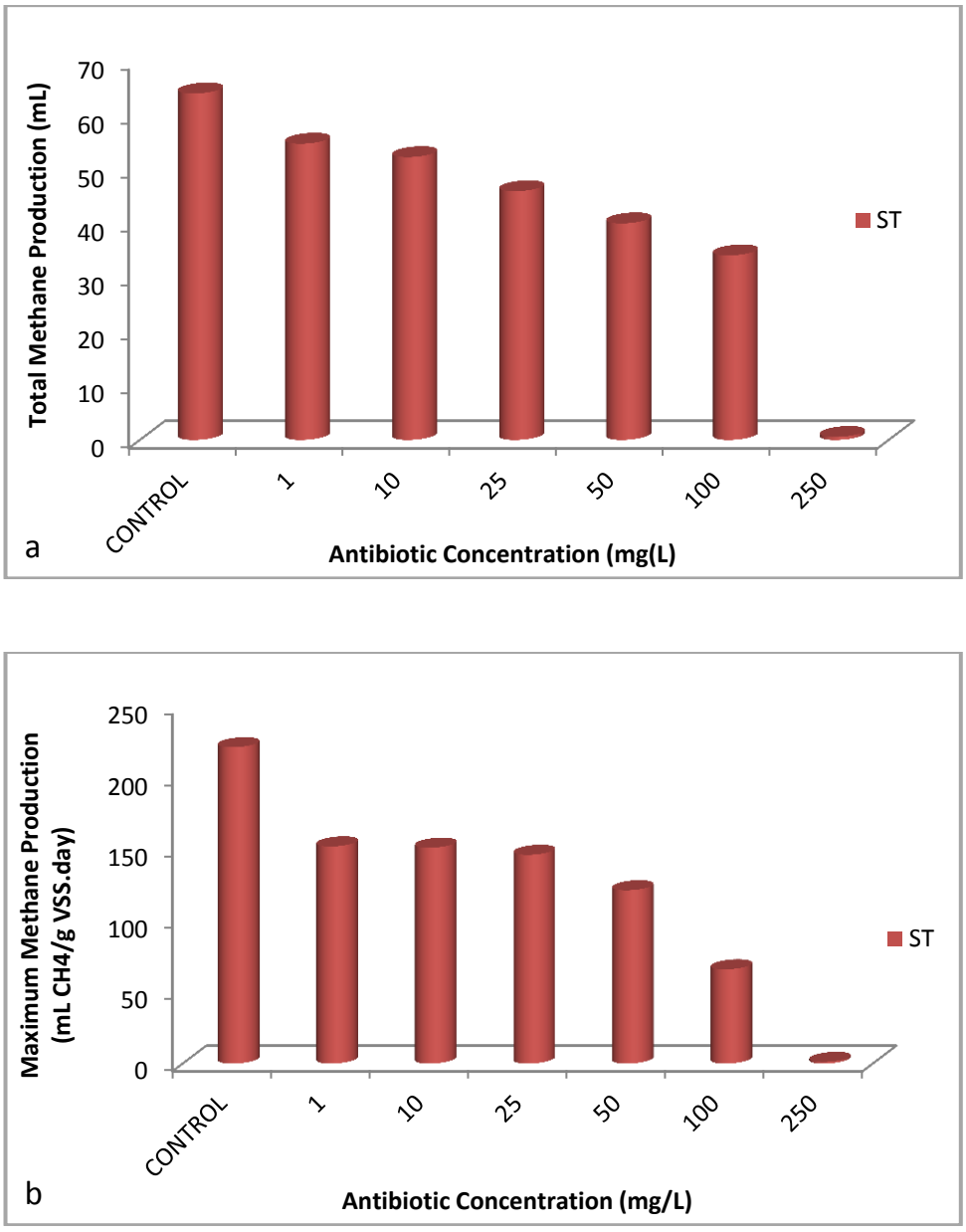



**Figure 4.8 :** Inhibition effect of ST dosage on (a) total methane production, (b) maximum methane production when acetate as a sole carbon source.

As shown in Figure 4.9a ES mixture indicated nearly similar pattern at 1 – 50 mg/L concentrations. The inhibition effect was seen clearly at 100 mg/L. Maximum inhibition effect was seen at 250 mg/L ES concentration. Figure 4.9b shows the changes on maximum methane production of sludge with ES addition. The same pattern was seen at 1 – 100 mg/L concentrations on maximum methane production of sludge.

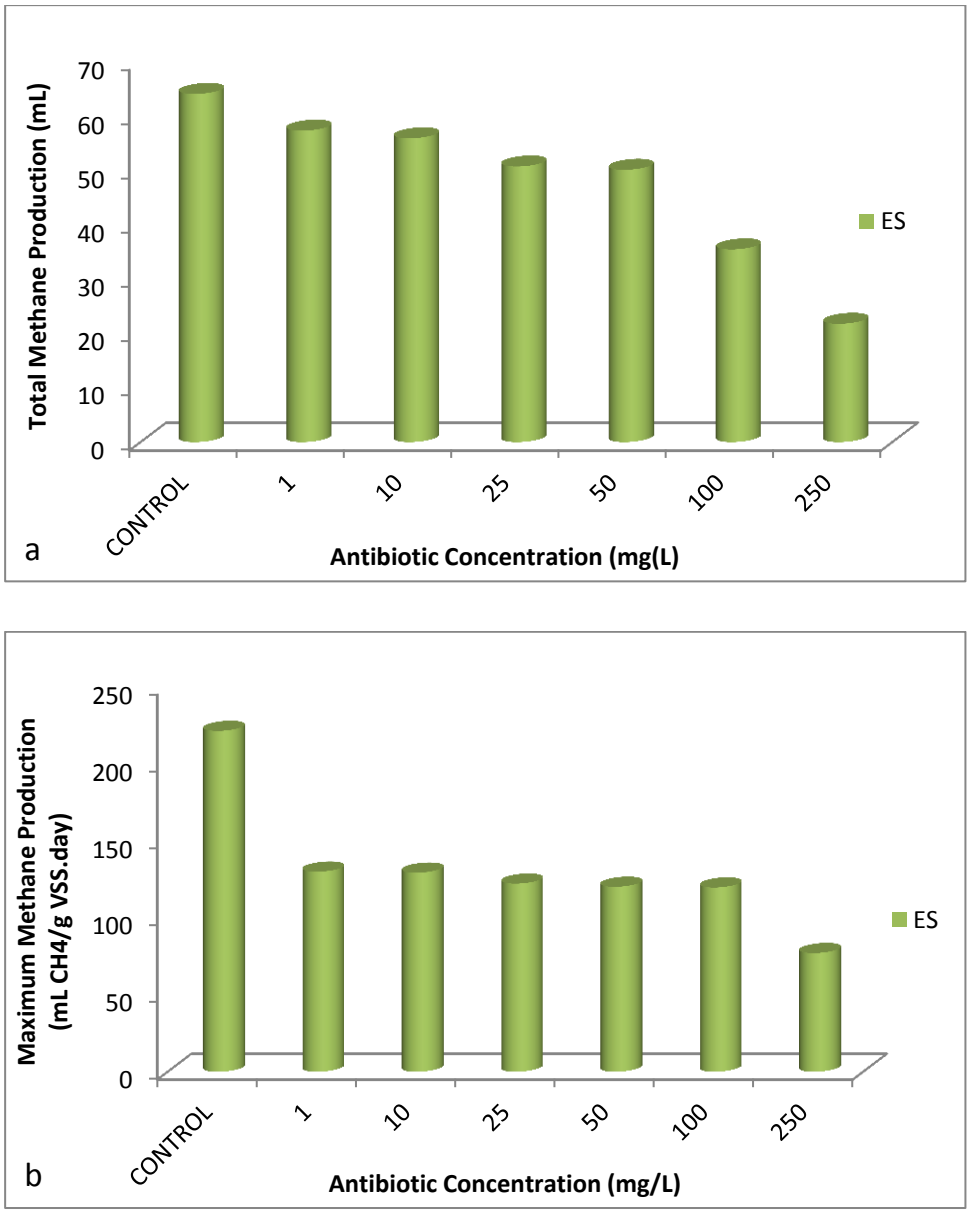



**Figure 4.9 :** Inhibition effect of ES dosage on (a) total methane production, (b) maximum methane production when acetate as a sole carbon source.

Figure 4.10 shows the total and maximum methane production for all combinations of antibiotic mixtures. The highest inhibition effect of antibiotics on total methane production was observed at 250 mg/L ST mixture. In the study carried out by Gartiser *et al.* (2006) was indicated that, erythromycin and sulfamethoxazole had no inhibition effect on biogas production. Also Fountulakis and his colleaugues stated that, sulfamethoxazole had not affected methanogenesis even at high concentrations (400 mg/L). While ET combinations constituted the lowest effect at 1 mg/L, it caused the highest inhibition effect at 10 mg/L and 100 mg/L. At 25 mg/L, ST

mixture caused the highest inhibition effect in all combinations of antibiotics. However, at 50 mg/L the highest decrease on the total methane production was caused by ETS mixture. Figure 4.10b indicates the effects of antibiotic mixtures on the maximum methane production rate. ETS mixture had the highest inhibition effect for all concentrations.

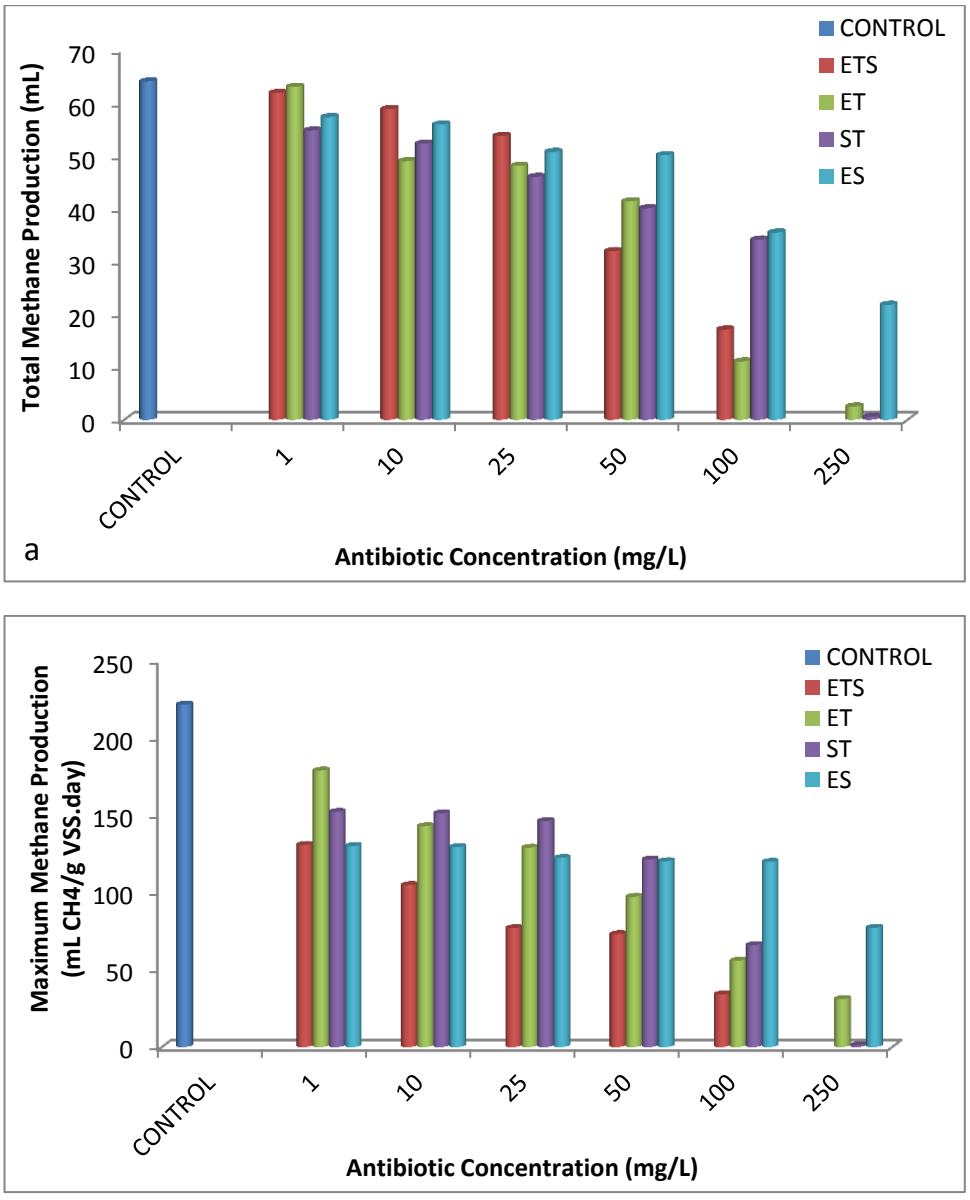



**Figure 4.10 :** Inhibition effect of antibiotic mixtures on (a) total methane production, (b) maximum methane production when acetate as a sole carbon source.

The $EC_{50}$ level is a commonly used parameter for inhibitory impact which defined as the inhibitor concentration, induces 50% inhibition on measured response of the microbial community (Ruiz *et al.*, 2009). The corresponding $EC_{50}$ values were

calculated as 50 mg/L for ETS, 65 mg/L for ET, 102 mg/L for ST, and 138 mg/L for ES. In a study carried out by Cetecioglu and her colleagues (2012), reported that $EC_{50}$ levels of single compounds were 155.4 mg/L for ERY, 204.4 mg/L for TET, and 198.5 mg/L for SMX. It was determined that, antibiotic mixtures had the lower EC50 values than single compounds.

### 4.3.1.2 Utilization of volatile fatty acids

Initial acetate concentrations in all serum bottles were 1500 mg/L. Acetate accumulation was observed in all antibiotic added reactors (Figure 4.11). Amin *et al.* (2006) evaluated the effects of erythromycin on the removal of acetic acid. It was stated that, even in the presence of high concentrations of erythromycin was not inhibit the acetic acid removal. However, antibiotic mixtures including erythromycin caused the acetic acid accumulation after 50 mg/L. At 50 mg/L concentration, ETS mixture showed the highest inhibitory effect on acetate removal. At 100 mg/L concentration ET mixture had the highest acetic acid concentrations at the end of the test period. On the other hand, at 250 mg/L ST containing bottles caused the highest acetic acid accumulation.

Acetic acid was negatively correlated with total methane production ($p<0,01$) but, it was positively correlated with antibiotic concentration ($r=0,889$; $p<0,01$).

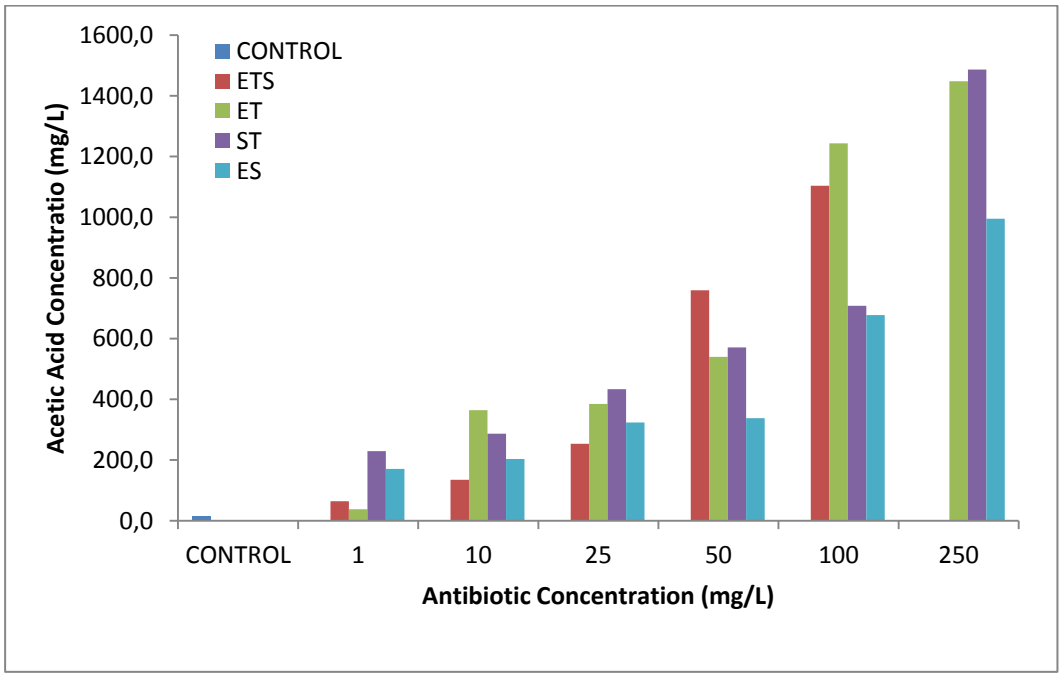



**Figure 4.11 :** VFA concentration in effluents of acetate set.

### 4.3.2 Butyrate as a sole carbon source

Volatile fatty acids (butyrate, propionate etc.) are not directly used by methanogens. They serve as substrates for proton reducing syntrophic bacteria. Different groups of syntrophic bacteria use specific VFAs and accumulation of these VFAs give information about sensitivity of relavant syntrophic bacteria to antibiotics (Amin *et al.*, 2006). In this direction, butyrate was selected as a sole carbon source to examine the inhibition effects of selected antibiotic mixtures on different microbial populations.

Degradation of butyrate is carried out by Gram positive beta-oxidizing syntrophic bateria (e.g., *Syntrophomonas* spp. And *Syntrophospora* spp.) (Shimada et al., 2008). *Syntrophomonas* is belong to the Firmicutes phylum which was comprised 21% of seed sludge's bacterial 16S rDNA clone library.

#### 4.3.2.1 Methane production

Figures 4.12 - 4.16 indicate the total and maximum methane production for different combination of antibiotic mixtures, which contain butyrate as a sole carbon source. At the end of the test period, average total methane production in the control bottles was 102,5 mL which was the 93% of total biogas production. None of the tested antibiotic mixtures completely inhibited biogas production.

As shown in Figure 4.12a ETS mixture indicated nearly a similar pattern at 1-10 mg/L and 25 – 100 mg/L concentrations. The methane content of biogas decreased from 88% to 80% with increasing antibiotic concentration from 1 mg/L to 100 mg/L. On the other hand, there was a significant decrease occurred on maximum methane production by ETS mixture addition (Figure 4.12b). Significant decrease was observed after 10 mg/L concentration. While, maximum methane production was 167 mL $CH_4$/g VSS.day, this value was decreased to 84 mL $CH_4$/g VSS.day at 10 mg/L ETS concentration. At 100 mg/L, maximum methane production was 65 mL $CH_4$/g VSS.day.

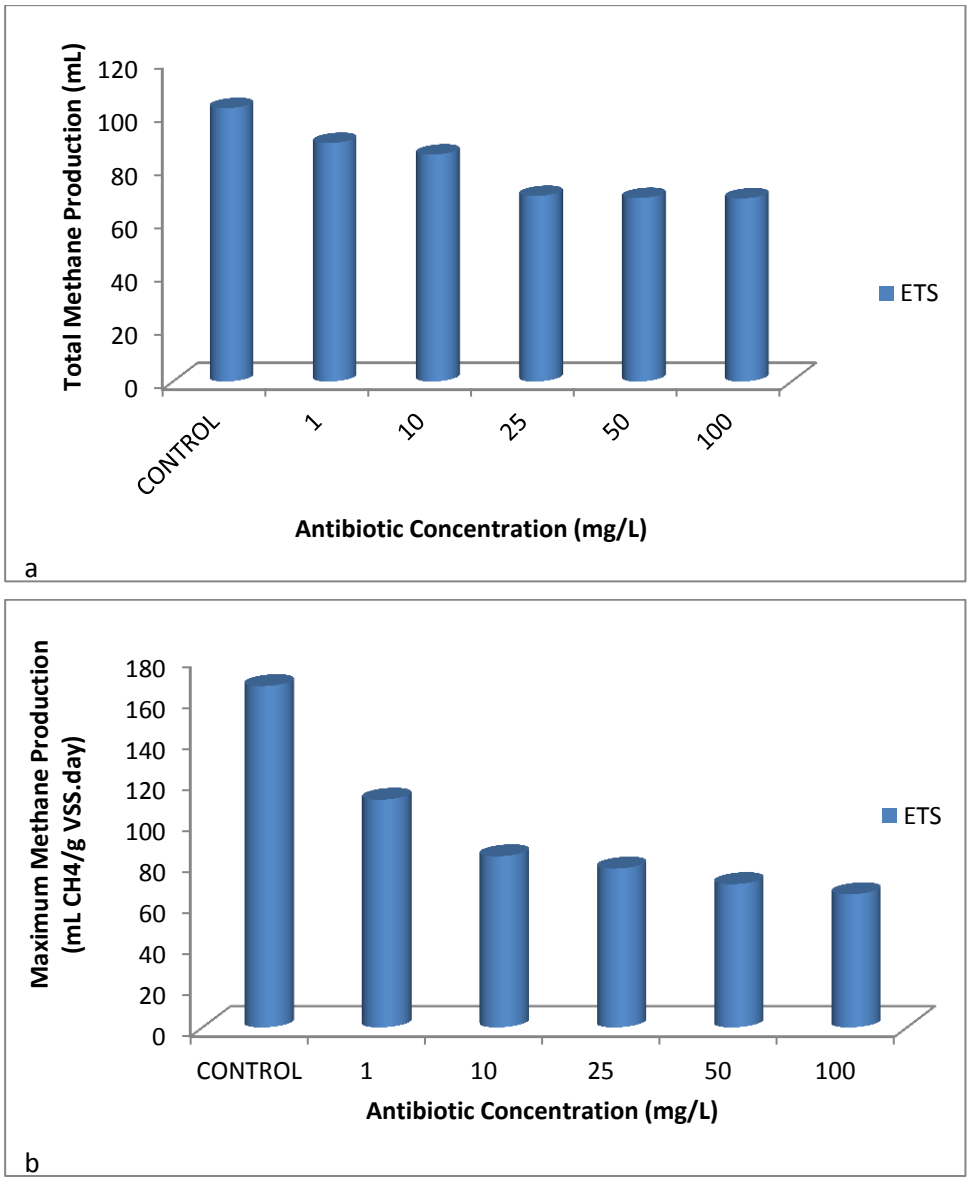



**Figure 4.12 :** Inhibition effect of ETS mixture dosage on (a) total methane production (b) potential methane production when butyrate as a sole carbon source.

In ET mixture, there was no significant inhibitory effect like in ETS mixture for tested concentrations (Figure 4.13a). Increasing in the antibiotic concentration caused to a decrease in the total methane production, but decrease level was not remarkable. According to Amin and his colleagues (2006), ERY had a substantial effect on butyrate utilization as well as methane production rate. However, in our study significant inhibition effect was not detected in all tests that included ERY. It is suggested that, antagonistic effects were occurred in tested antibiotic mixtures. Christensen *et al.* (2006) reported that, synergistic effects were seen in the mixture

oxytetracycline and erythromycin, while antagonistic effects were observed in the tests that included flumequine - erythromycin and flumequine - oxytetracycline mixtures.The methane ratio in the biogas decreased from 93% to 86% with increasing the ET concentration. However, as in the ETS mixture, there was a slight decrease on the maximum methane production with antibiotic mixture addition (Figure 4.13b). While the maximum methane production was 166,4 mL $CH_4$/g VSS.day in the control bottles, this value was decreased to 68 mL $CH_4$/g VSS.day at 250 mg/L ET concentration.

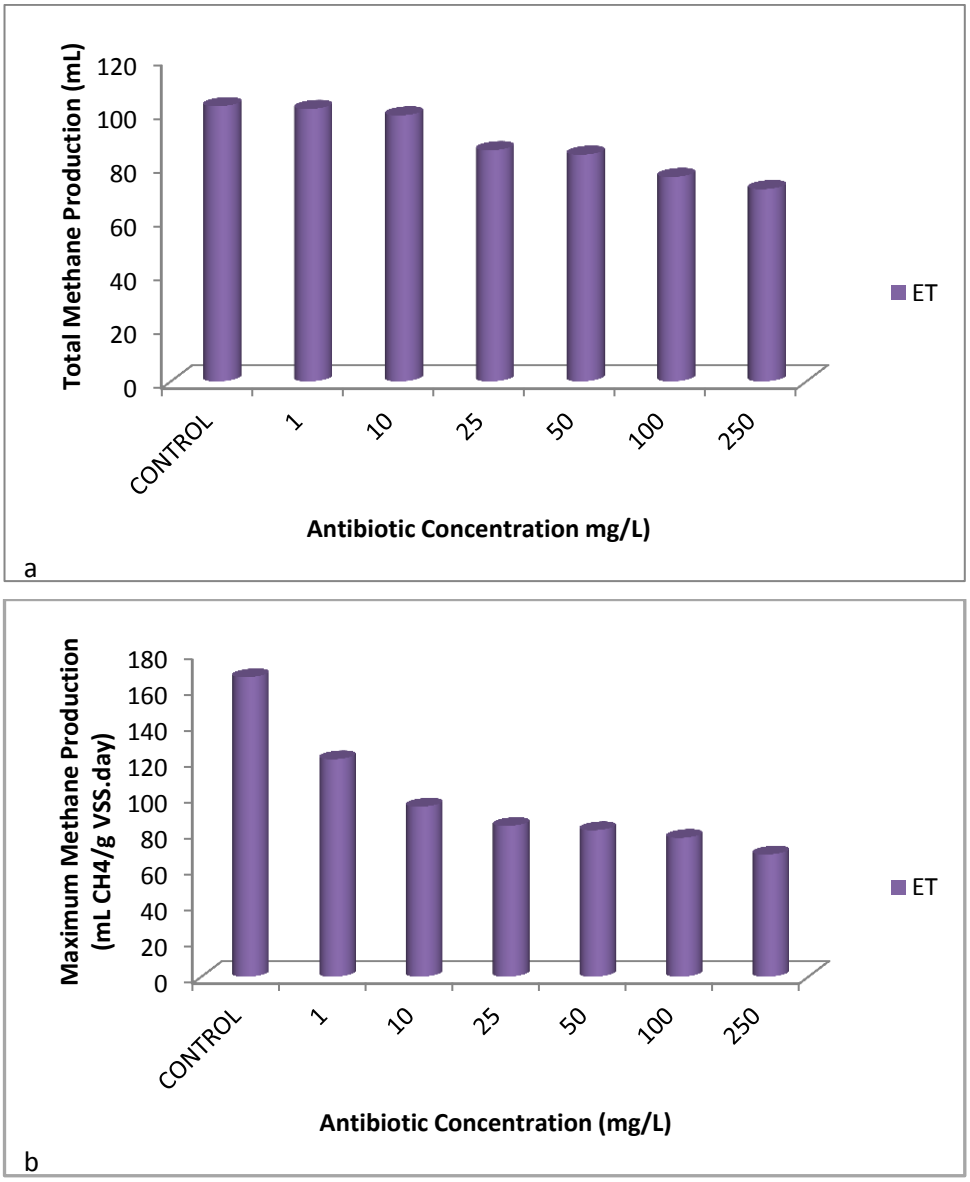



**Figure 4.13 :** Inhibition effect of ET mixture dosage on (a) total methane production (b) potential methane production when butyrate as a sole carbon source.

For ST mixture, 1 mg/L concentration showed nearly the similar pattern with control bottle (Figure 4.14a). 10 – 50 mg/L concentrations indicated a similar trend with each other. The significant decrease on the total methane production was induced after 100 mg/L. Methane percentage in the biogas decreased from 93% to 88%. The maximum methane production profile indicated similar pattern with ET mixture (Figure 4.14b). The significant inhibitory effect was started at 1 mg/L concentration. At 250 mg/L, the maximum methane production was 49,5 mL $CH_4$/g VSS.day.

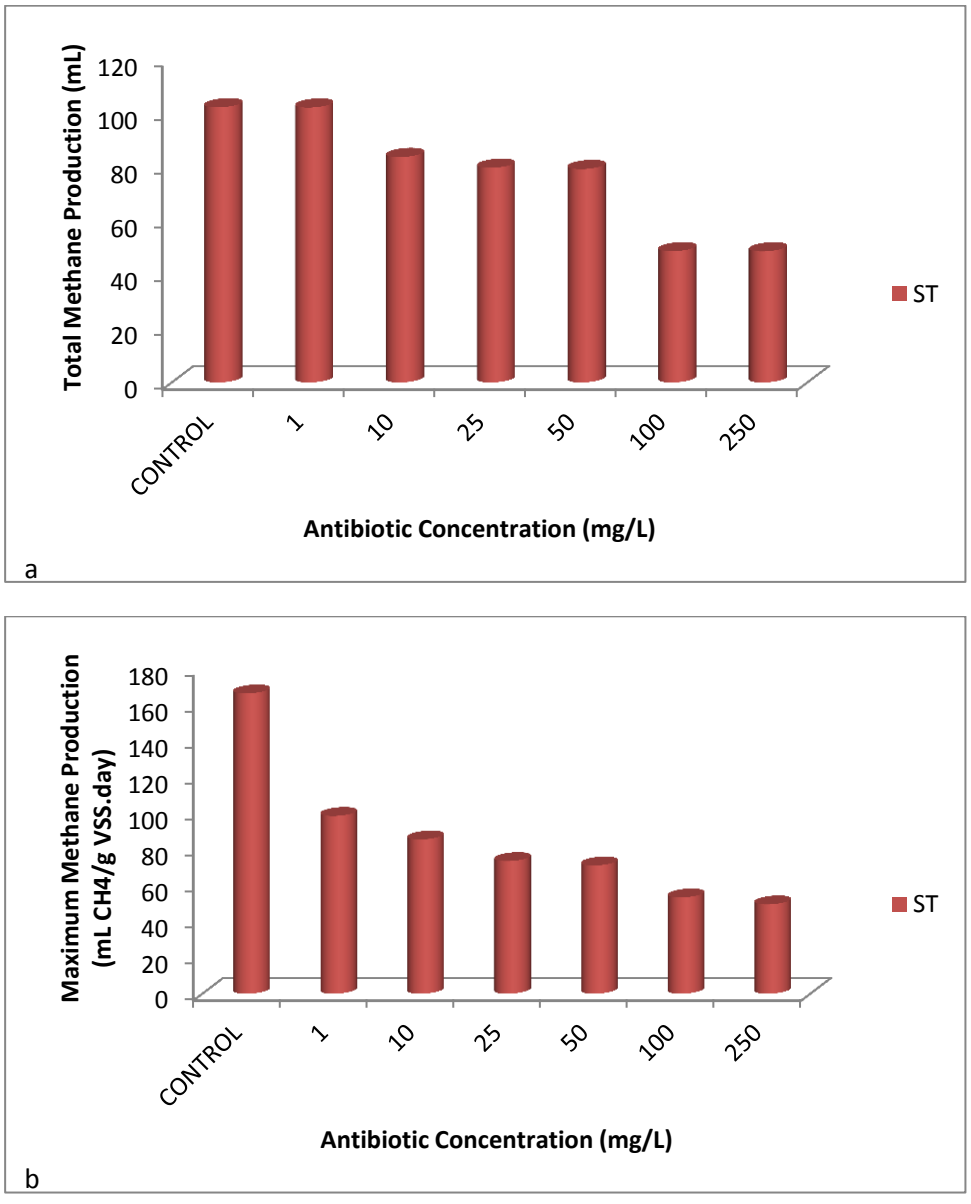



**Figure 4.14 :** Inhibition effect of ST mixture dosage on (a) total methane production (b) potential methane production when butyrate as a sole carbon source.

Figure 4.15 shows the inhibition effect of ES mixture on the total and maximum methane production. Total methane production of the test bottles with 1 mg/L of ES mixture was close to the control bottles like in ST mixture. There was no slight decrease with increasing the antibiotic concentration. However after the mixture dosage increased to 100 – 250 mg/, the inhibitory effect can be seen clearly. The maximum methane production was decreased in stepwise manner with increasing antibiotic concentration.

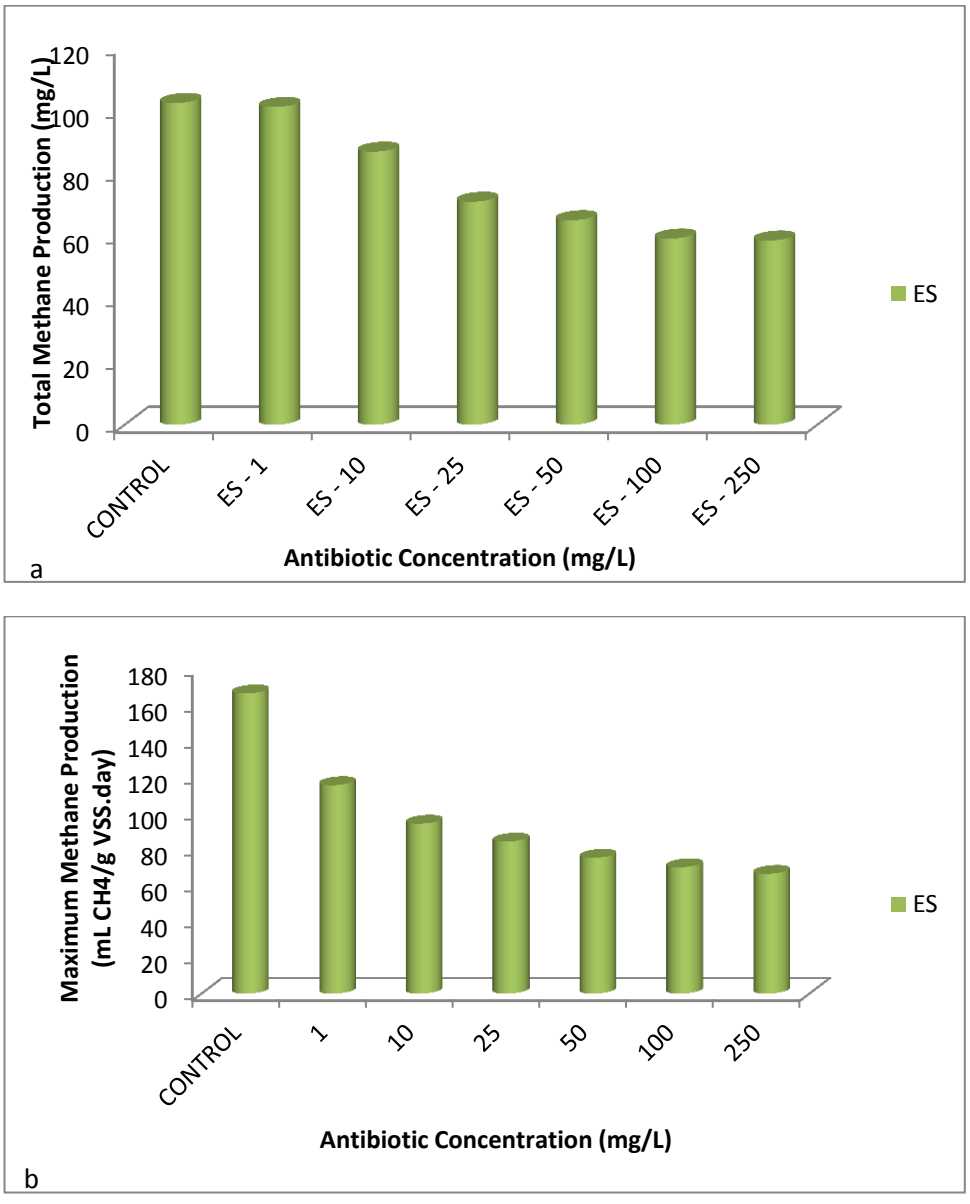



**Figure 4.15 :** Inhibition effect of ES mixture dosage on (a) total methane production (b) maximum methane production when butyrate as a sole carbon source.

Inhibition effects of all antibiotic mixtures on the total and maximum methane production was shown in Figure 4.16. At the 1 – 25 mg/L antibiotic concentrations, ETS mixture caused the highest inhibitory effect on the total methane production. At 50 mg/L the highest decrease on the total methane production caused by ES mixture. At 100 – 250 mg/L ST mixture indicated the highest inhibitory effect on the total methane production.

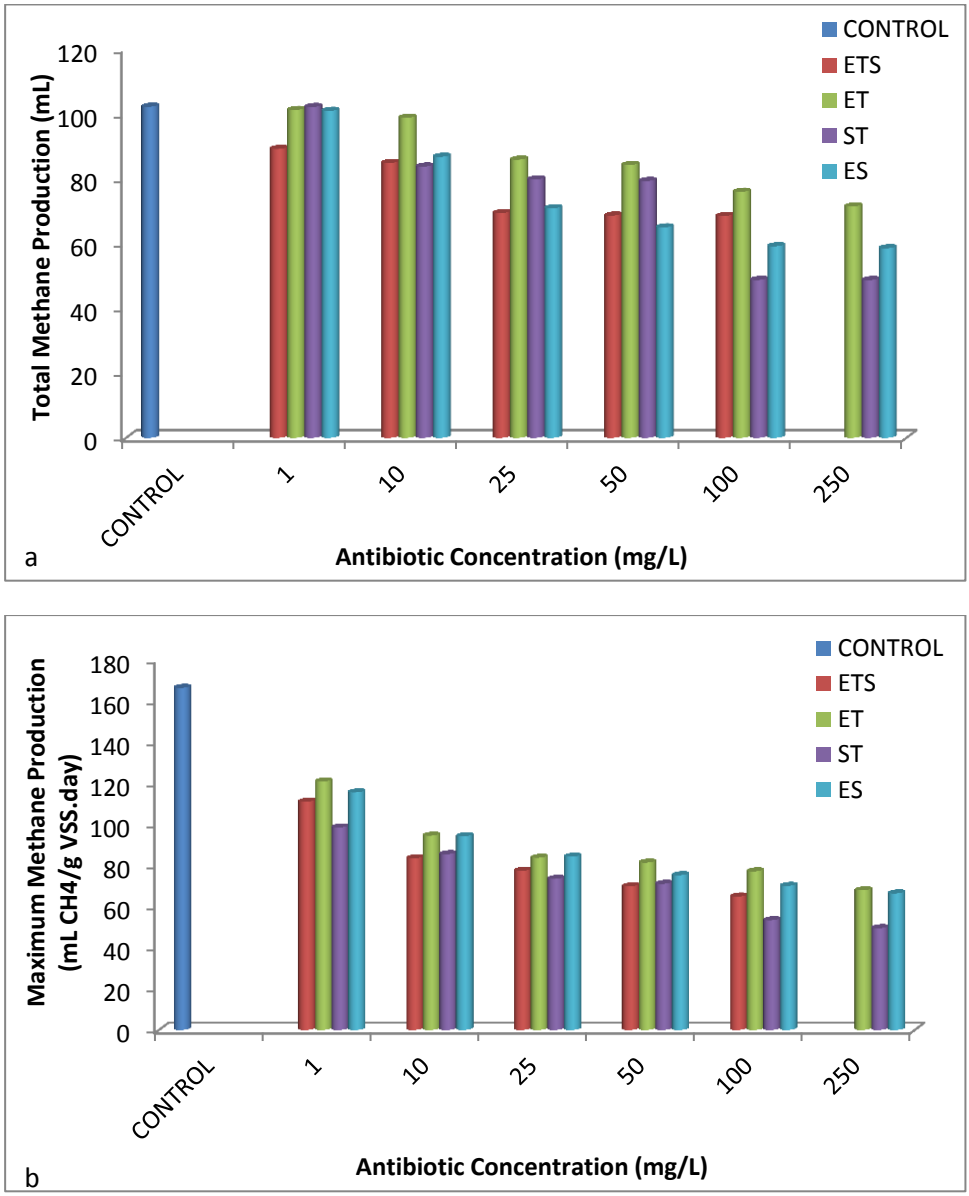



**Figure 4.16 :** Inhibition effect of all antibiotic mixtures (a) on total methane production, (b) maximum methane production while butyrate as a sole carbon source.

The $EC_{50}$ levels were determined just for ST mixture (96 mg/L). For other mixtures, $EC_{50}$ values were predicted above the tested antibiotic concentrations.

There was a significant correlation between the total methane production and antibiotic concentration according to Pearson's correlation ($p<0,01$; $r=-0,703$).

### 4.3.2.2 Utilization of volatile fatty acids

Initial butyrate concentrations in all serum bottles were 1500 mg/L. As seen in the Figures 4.17 – 4.20, VFA accumulation was occurred with increasing antibiotic concentration. There was a significant correlation between total methane production and butyric acid concentration ($p<0,01$; $r=-0,99$). According to Pearson's correlation, propionic acid, isobutyric acid, and isovaleric acid were also significantly correlated with total methane production ($p<0,01$). Amin and his colleagues (2006) stated that, the erythromycin addition had a substantial effect on the removal of butyric acid even at lower concentrations. They pointed out that, butyrate is used by bacteria, and erythromycin is more effective against Gram-positive bacteria.

Figure 4.17 indicates VFA concentrations for ETS mixture. A slight increase in VFA concentration was observed at 25 mg/L ETS included test bottles where butyric acid accumulation was higher than other bottles. Cetecioglu (2011) found that, VFA accumulation started after 250 mg/L for SMX and ERY whereas it observed in TET bottles after 25 mg/L. After 100 mg/L, acetic acid, isobutyric acid and propionic acid accumulations were increased.

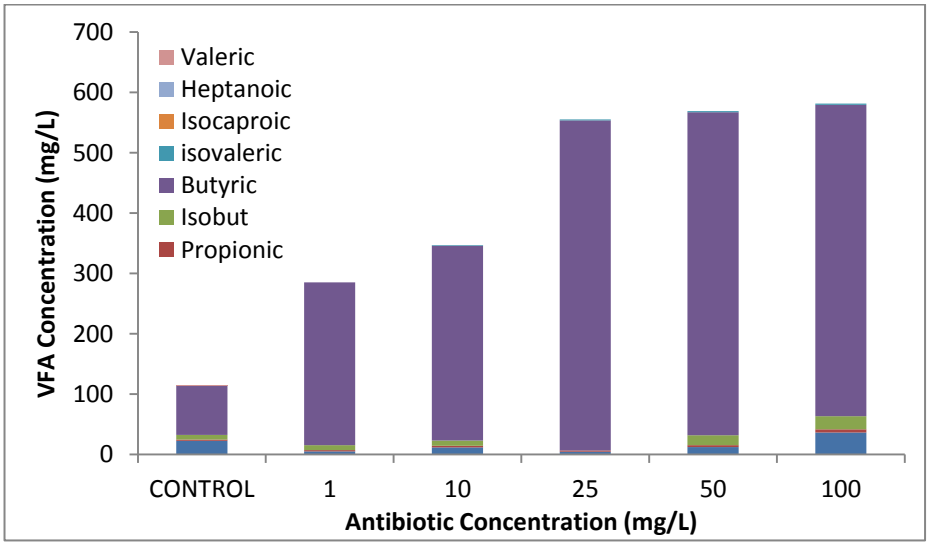



**Figure 4.17 :** VFA concentration in effluents of ETS mixture while butyrate as the sole carbon source.

In ET mixture, VFA accumulation was increased significantly at 25 mg/L concentration like in ETS mixture (Figure 4.18). Butyric acid concentration was increased from 113 mg/L to 509 mg/L with increasing the antibiotic concentration 1 – 250 mg/L.

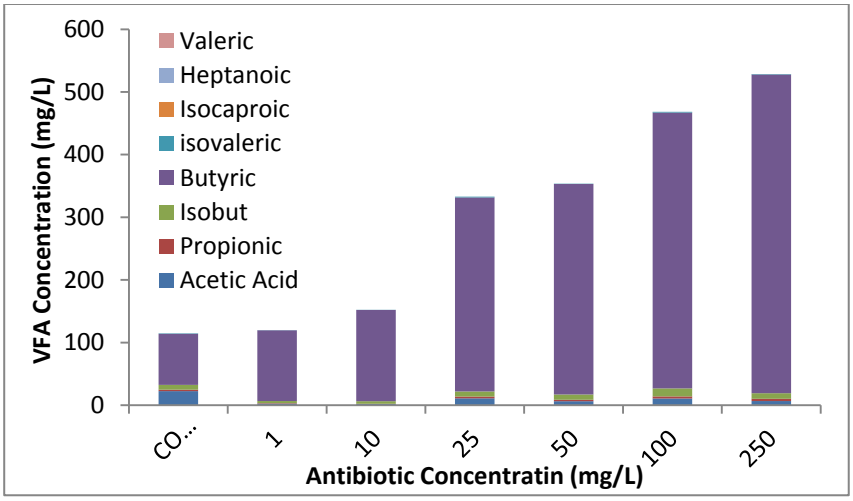



**Figure 4.18 :** VFA concentration in effluents of ET mixture while butyrate as the sole carbon source.

Figures 4.19 – 4.20 indicate VFA concentrations for ST and ES mixtures, respectively. Significant VFA accumulation was started at 10 mg/L antibiotic mixture concentration both in ST and ES included test bottles. While there was a sudden increase in VFA concentration between 50 and 100 mg/L ST included test bottles, VFA accumulation was occurred in stepwise manner in ES set.

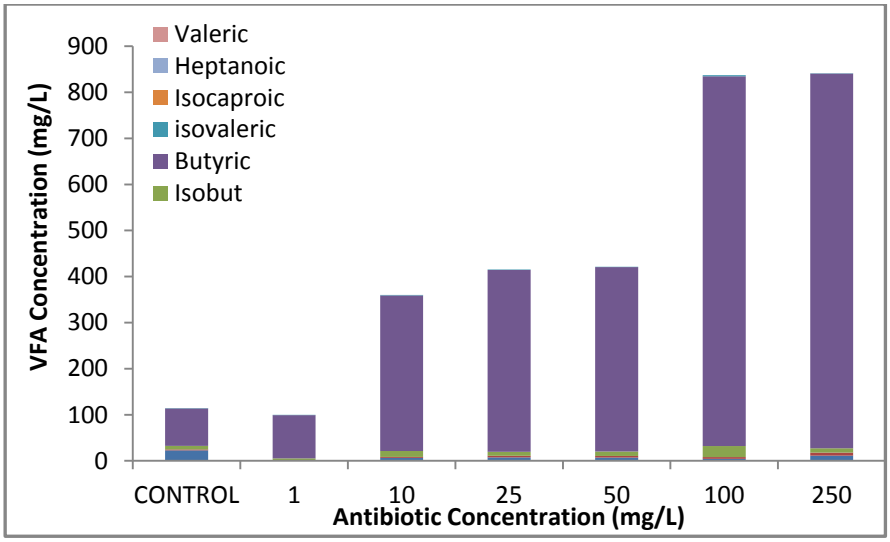



**Figure 4.19 :** VFA concentration in effluents of ST mixture while butyrate as the carbon source.

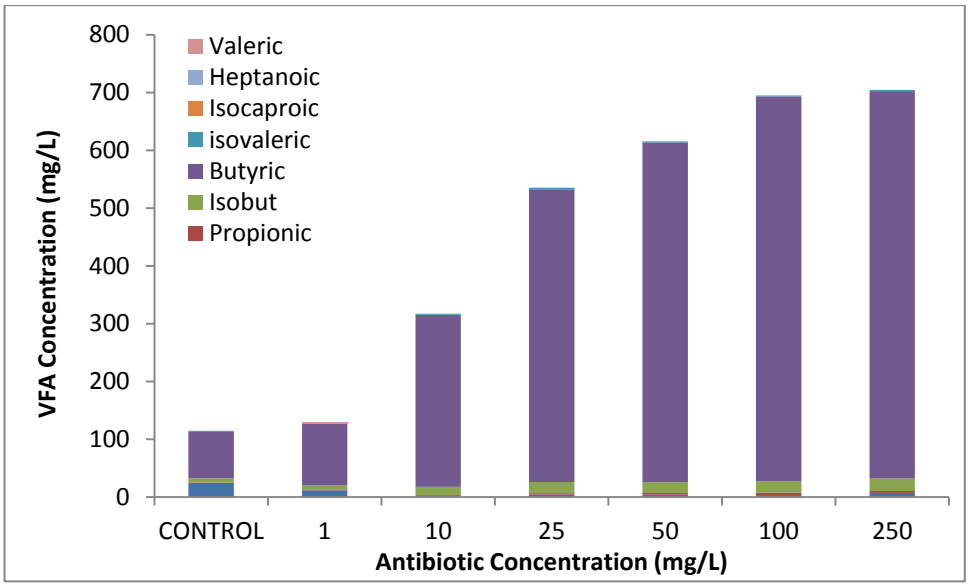



**Figure 4.20 :** VFA concentration in effluents of ES mixture while butyrate as the carbon source.

### 4.3.3 VFA mixture as a sole carbon source

VFA mixture was used as substrate for evaluation both homoacetogens and all methanogens. *Clostridium aceticum* and *Acetobacterium woodii* are the two mesophilic homoacetogenic bacteria isolated from sewage sludge which are belong to the Firmicutes phylum (Novaes, 1986). Firmicutes phylum was comprised 21% of seed sludge's bacterial 16S rDNA clone library.

Acetoclastic methanogens are responsible nearly 70% of total methane generation. They are belong to the Methanosarcinales phylum, which is the most abundant classified phylum (27%) of the seed sludge's archaeal clone library.

Hydrogenotrophic pathway contributes up to 28% of the methane generation in anaerobic systems. Euryarchaeota and Methanomicrobiales were comprised 8% and 7% of seed sludge's archaeal clone library, respectively.

#### 4.3.3.1 Methane production

There was a significant correlation between total methane production and antibiotic concentrations ($p<0,01$; $r=-0,712$). Figures 4.21 - 4.25 show the effects of antibiotic mixtures on the total and maximum methane production for the test bottles, which contain VFA mixtures as the sole carbon source. At the end of the test period, average of total methane production in the control bottles was 80,45 mL which was

the 93% of total biogas production. Complete inhibition on biogas production was not seen for the tested antibiotic concentrations.

As seen in Figure 4.21a, test bottles with 1 mg/L concentration ETS mixture showed nearly the similar pattern with control bottles. The total methane production was decreased in stepwise manner with increasing the ETS dosage. The significant inhibition on total methane production was detected at 100 mg/L ETS concentration. On the other hand, significant decrease on maximum methane production was observed at 25 mg/L (Figure 4.21b).

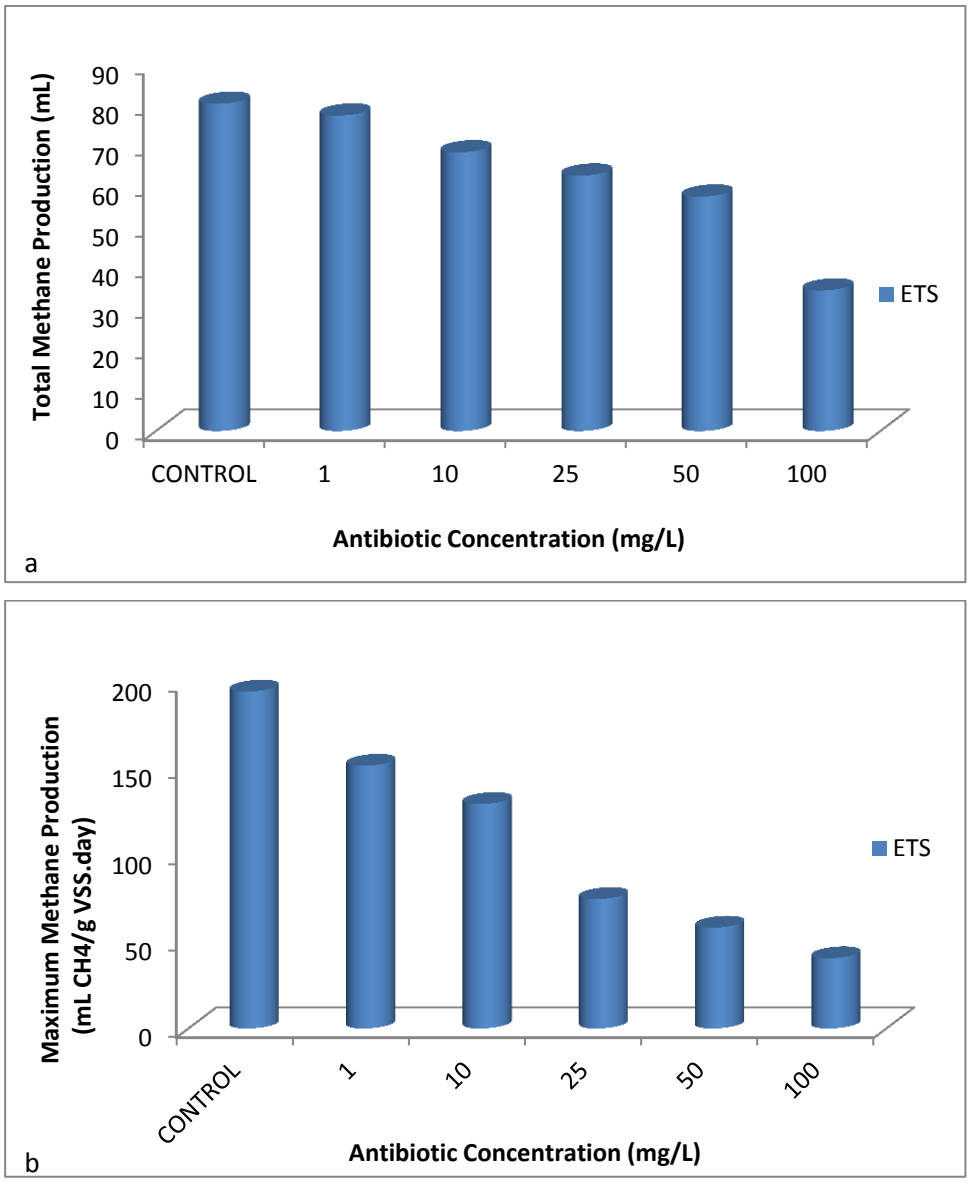
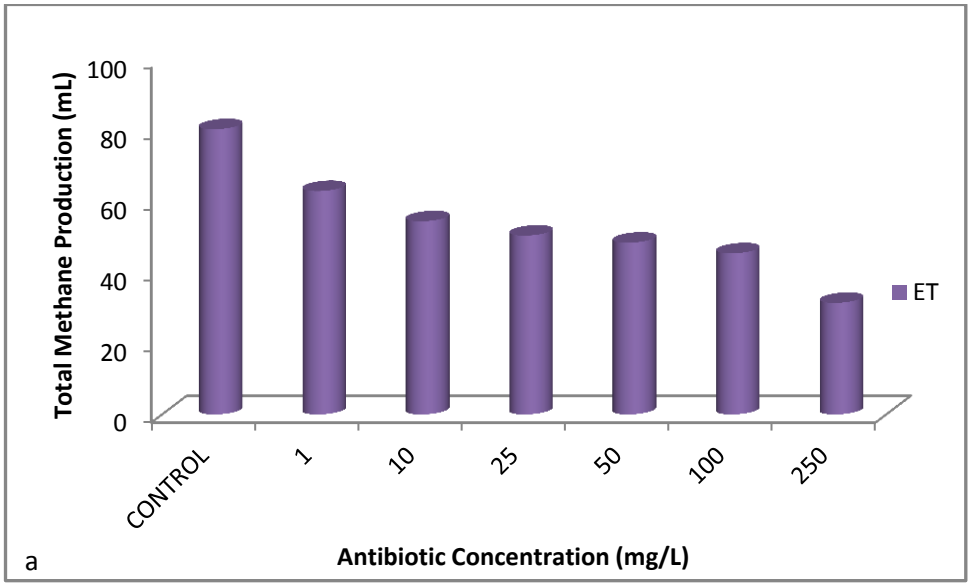



**Figure 4.21 :** Inhibition effect of ETS mixture dosage on (a) total methane production (b) maximum methane production when VFA mixture as a sole carbon source.

The effects of 10 – 100 mg/L ET mixture on total methane production was indicated nearly similar trend with each other (Figure 4.22a). Total methane production was decreased to 31,5 mL at 250 mg/L ET concentration. The methane content of biogas decreased from 87% to 69% with increasing ET concentration from 1 mg/L to 250 mg/L. Significant decrease on maximum methane production was observed at 50 mg/L concentration (Figure 4.22b).



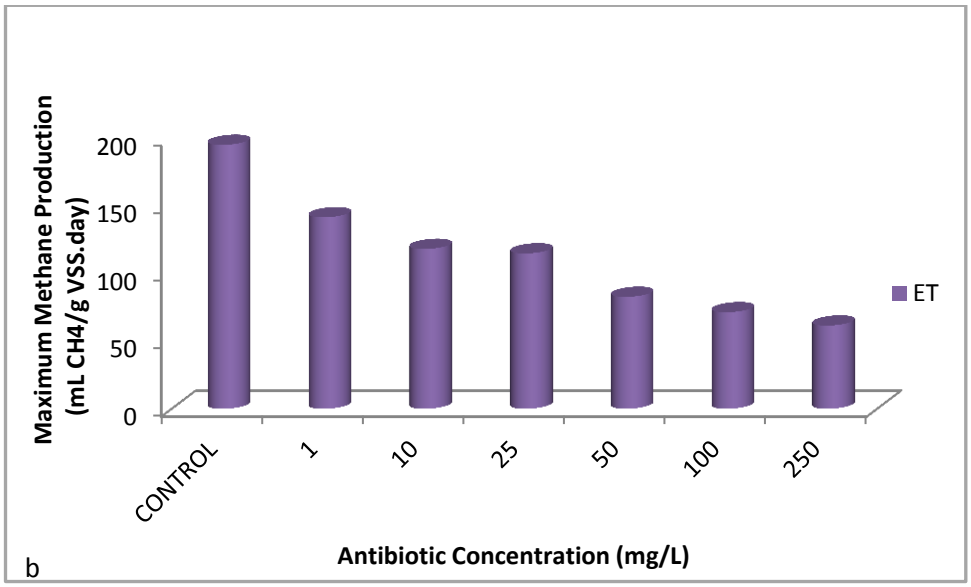




**Figure 4.22 :** Inhibition effect of ET mixture dosage on (a) total methane production (b) maximum methane production when VFA mixture as a solecarbon source.

For ST mixture, 1 – 10 mg/L concentrations indicated a similar trend with each other (Figure 4.23a). The significant decrease on total methane production was induced after 50 mg/L ST dosage. Methane percentage in the biogas decreased from 93% to 85%. The maxium methane production was decreased in stepwise manner with decreasing the antibiotic concentration (Figure 4.23b). The significant inhibitory effect on maximum methane production was observed after 100 mg/L.

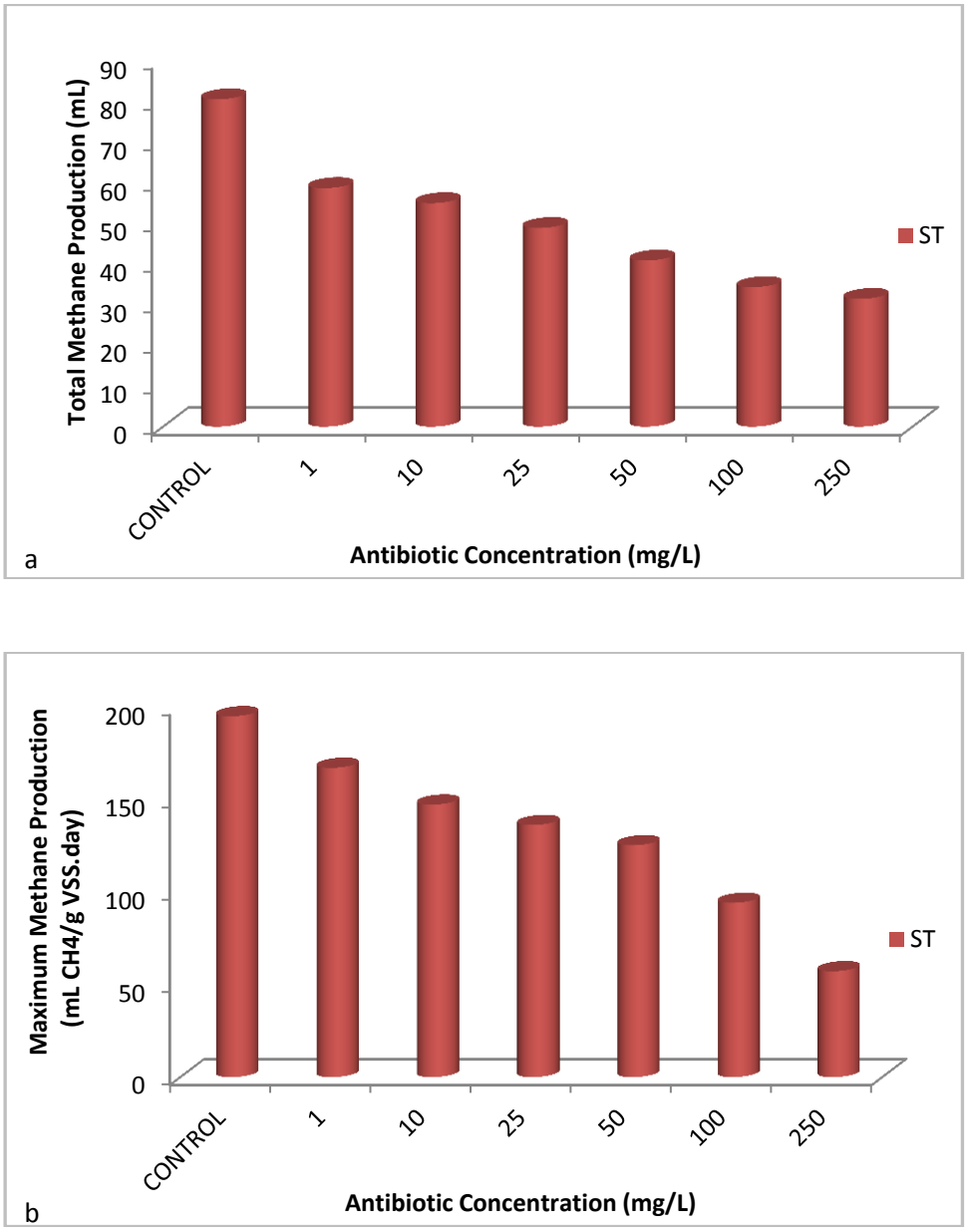
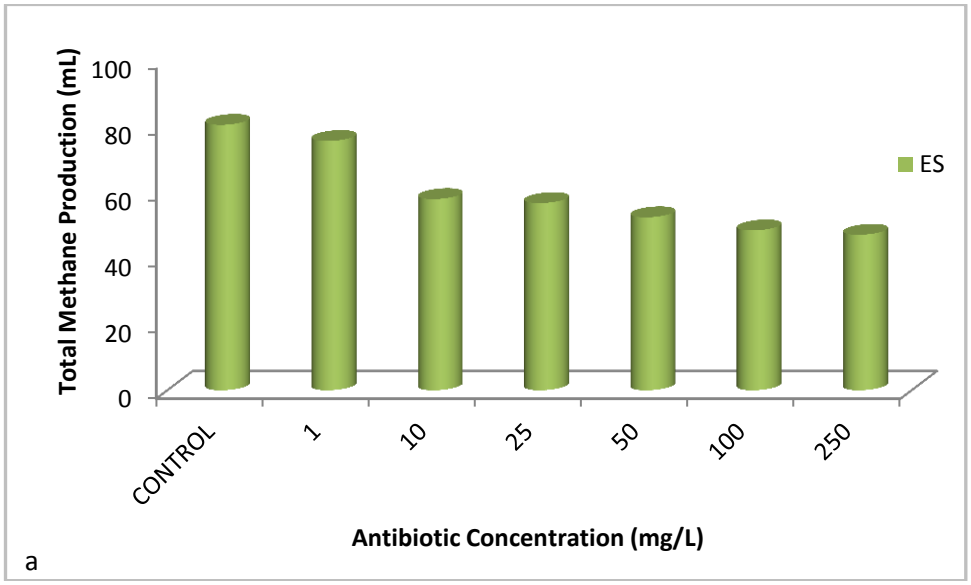



**Figure 4.23 :** Inhibition effect of ST mixture dosage on (a) total methane production (b) maximum methane production when VFA mixture as a sole carbon source.

Figure 4.24a shows the inhibition effect of ES mixture on total methane production. Total methane production of the test bottles with 1 mg/L ES mixture was close to the control bottles. Inhibitory effect can be seen clearly at 10 mg/L. The test bottles, included between 10 – 250 mg/L ES, showed nearly the similar pattern with each other. The significant decrease on maximum methane production was detected at 250 mg/L ES concentration.



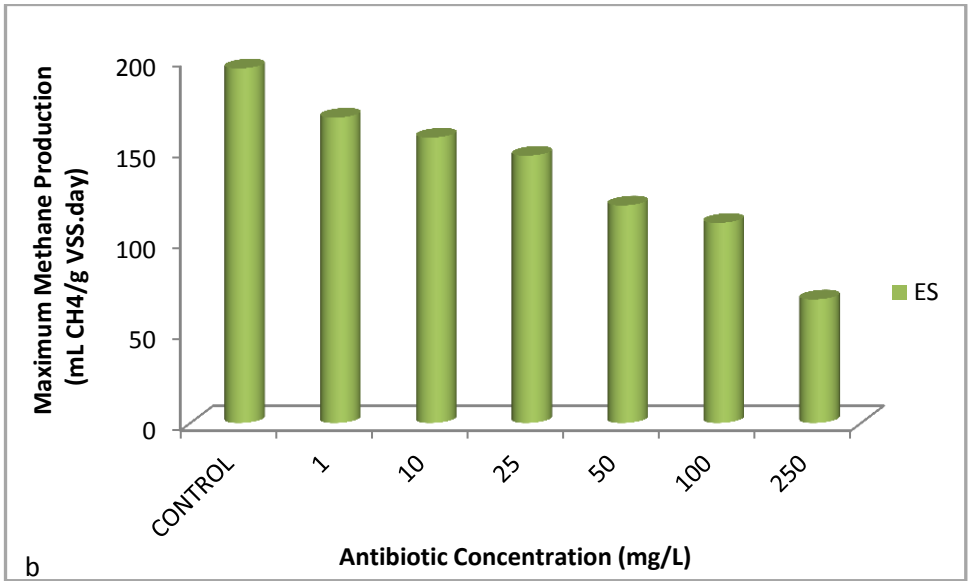




**Figure 4.24 :** Inhibition effect of ES mixture dosage on (a) total methane production (b) maximum methane production when VFA mixture as a sole carbon source.

Figure 4.25a shows the total methane production for all combinations of antibiotic mixtures. While ST mixture was induced the highest inhibition effect on total methane production at 1 – 25 – 50 mg/L concentrations, ETS mixture was caused the lowest effect. At 100 mg/L, ETS and ST mixtures induced nearly the same inhibitory effect on total methane production. At 250 mg/L, ET and ST mixtures indicated nearly the same inhibitory effect. It was expected that, three component mixture would have the highest inhibitory effect, but results did not agree with that prediction. This result was agree with the study carried out by Al-Ahmad (2009). According to the study, the eleven component antibiotic mix, which included ERY and SMX, did not affect biodegradation performance of activated sludge. Figure 4.25b shows the maximum methane production for all combination of antibiotic mixtures. At 1 – 10 mg/L, ET mixture caused the highest inhibition effect whereas ETS mixture induced the highest decrease between 25 – 100 mg/L concentrations.

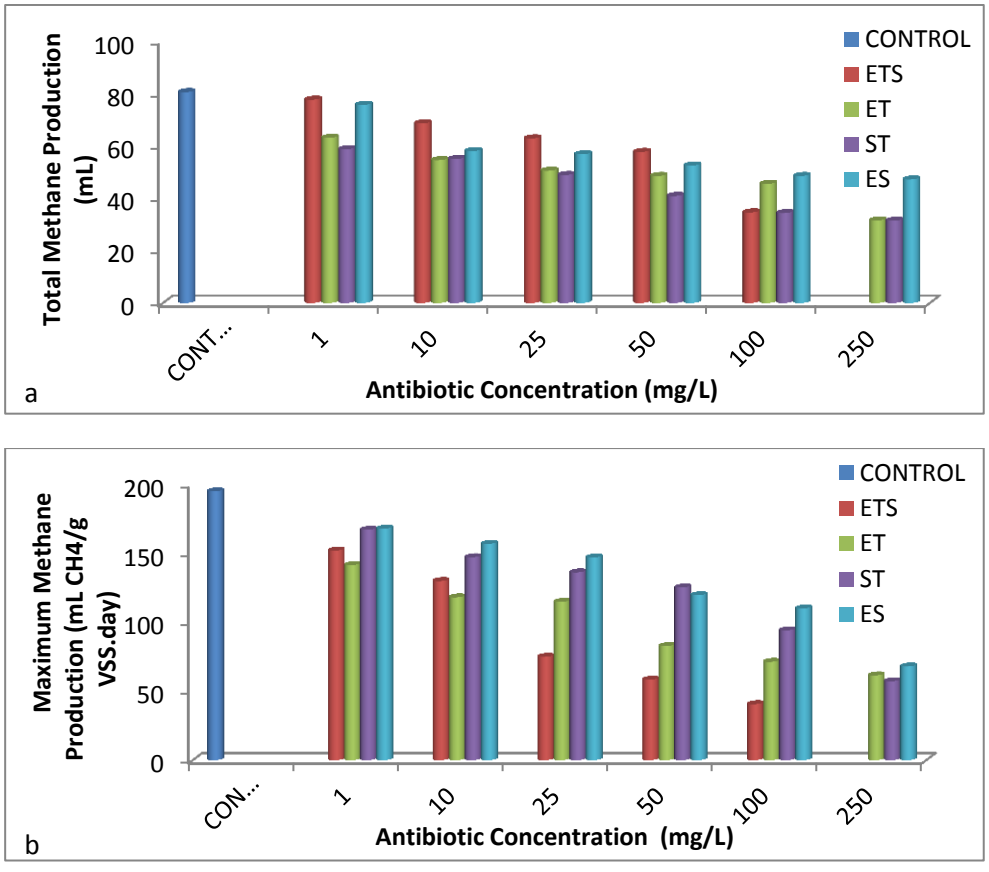



**Figure 4.25 :** Inhibition effect of all antibiotic mixtures (a) on total methane production, (b) maximum methane production while VFA mixture as a sole carbon source.

The $EC_{50}$ level is a commonly used parameter for inhibitory impact which defined as the inhibitor concentration, induces 50% inhibition on measured response of the microbial community (Ruiz *et al.*, 2009). The corresponding $EC_{50}$ values were calculated as 88 mg/L for ETS, 106 mg/L for ET, 52 mg/L for ST. $EC_{50}$ value of ES mixture was predicted above the tested antibiotic concentrations.

In a study carried out by Cetecioglu and her colleagues (2012), reported that $EC_{50}$ levels of single compounds were 85.8 mg/L for ERY, 46.2 mg/L for TET, and 55.4 mg/L for SMX. $EC_{50}$ values of single compounds were lower than all mixtures except ST mixture. $EC_{50}$ value of ST mixture was lower than single SMX.

### 4.3.3.2 Utilization of volatile fatty acids

Initial VFA mixture (acetate, propionate, and butyrate) concentrations in all serum bottles were 1500 mg/L. VFA concentrations were shown in the Figures 4.26 – 4.29. According to Pearson's correlation, acetic acid (r=-0,822), propionic acid (0,905), and butyric acid (0,946) were significantly correlated with total methane production (p<0,01).

VFA concentration was increased in stepwise manner with increasing ETS concentration (Figure 4.26). At 250 mg/L ETS concentration, the highest accumulation of all VFAs was observed.

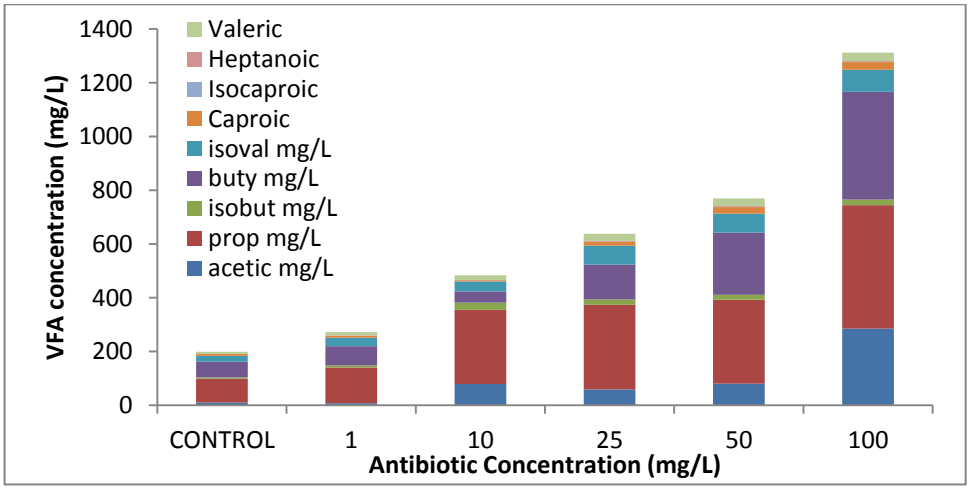



**Figure 4.26 :** VFA concentration in effluents of ETS mixture while VFA mixture as the sole carbon source.

Figure 4.27 indicates the effect of ET mixture on VFAs concentrations. VFAs were started to accumulate after 1 mg/L ET addition. In the study carried out by

Cetecioglu (2011), VFA accumulation started after 250 mg/L for SMX and ERY. But in this study, it was clearly seen that VFA accumulation started even in the presence of low concentrations. Cetecioglu pointed out that, removal of acetic acid was not affected. The datas obtained from this study confirmed with this finding.

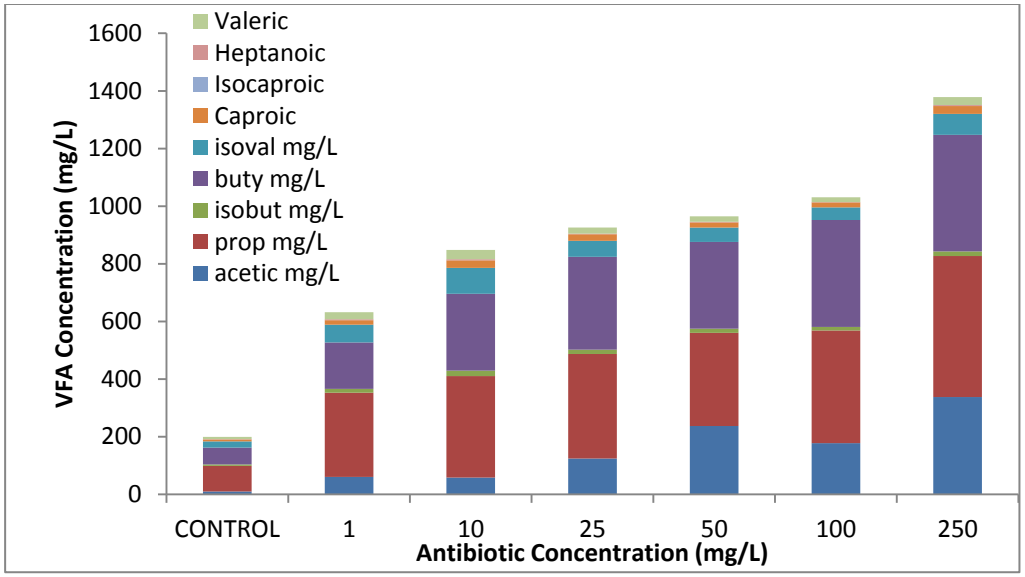



**Figure 4.27 :** VFA concentration in effluents of ET mixture while VFA mixture as the carbon source.

A slight increase in VFA concentrations was observed at 1 mg/L ST included test bottles (Figure 4.28). In ES mixture, VFAs were started to accumulate after 10 mg/L. 10 – 25 mg/L concentrations were indicated nearly similar trend with each other.

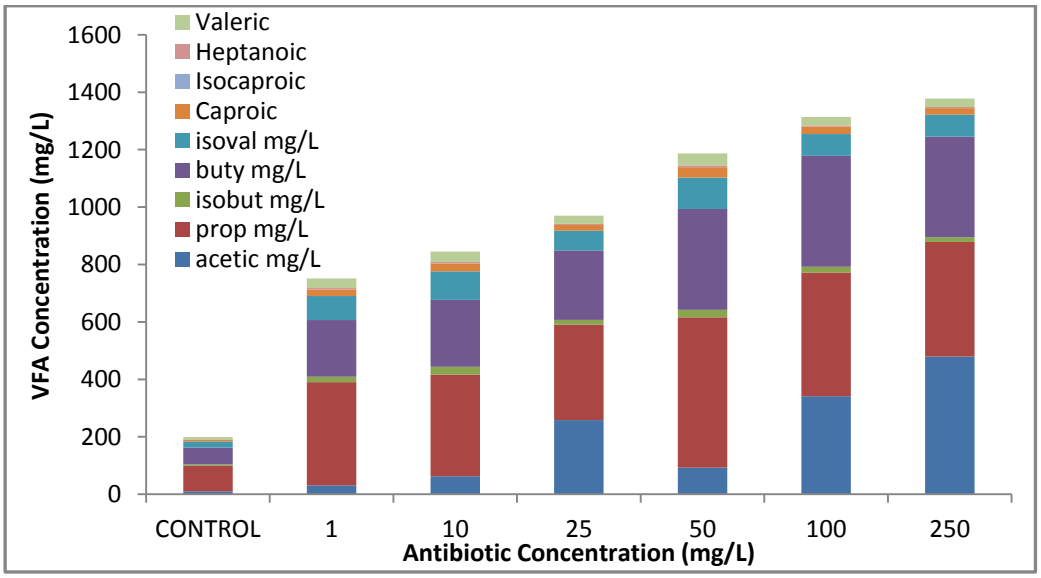



**Figure 4.28 :** VFA concentration in effluents of ST mixture while VFA mixture as the carbon source.

Figure 4.29 indicates the effect of ES mixture on VFAs concentrations. VFAs were started to accumulate after 1 mg/L ES addition. 10 – 25 mg/L concentrations were indicated nearly similar trend with each other as in the ST tests.

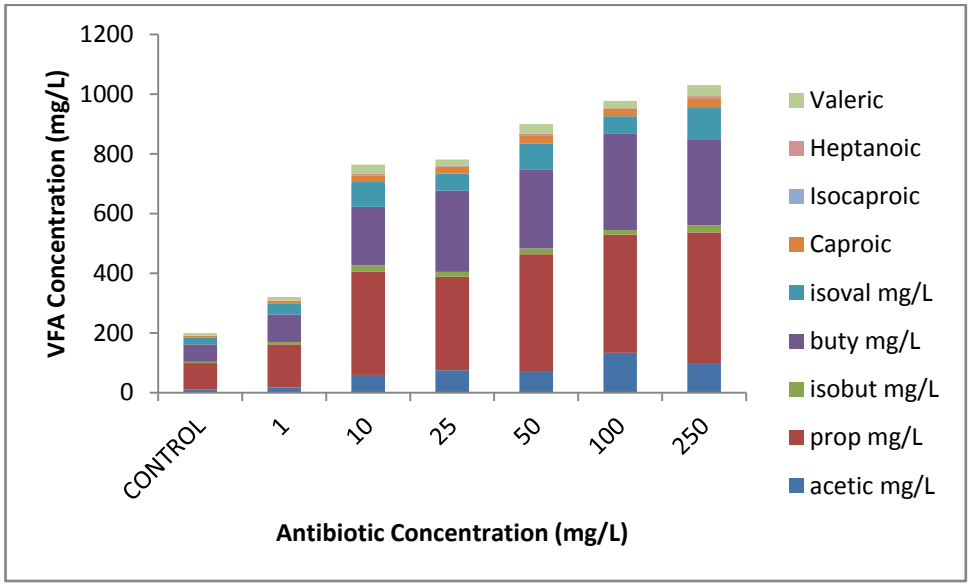



**Figure 4.29 :** VFA concentration in effluents of ES mixture while VFA mixture as the carbon source.

## 5. CONCLUSION

In this study, the short-term effects of TET, ERY, and SMX mixtures, which are widely used antibiotics, on methanogenic activity were examined. Acetate, butyrate, and VFA mixture were used as the sole carbon sources to evaluate the inhibition effect on specific trophic groups of anaerobic process. Antibiotic dosages were in the range of 1.0–250 mg/L. Biogas generation/composition and substrate removal were monitored during the test period.

Statistical analyses showed that, there is a strong correlation between antibiotic mixture concentration and methane generation. For all substrate conditions, 250 mg/L ST mixture had the highest inhibitory effect on total methane production. After 100 mg/L, short-term inhibitory effect of antibiotic mixtures was seen clearly. In the acetate fed test bottles, $EC_{50}$ values of mixtures were calculated lower than single compounds. Synergistic effects were observed in the mixtures. Acetoclastic methanogens are the most sensitive group of microorganisms participating in the anaerobic process. The results showed that, mixtures had higher inhibition effect on total methane production of acetoclastic methanogens than single compounds. In butyrate set, $EC_{50}$ values were predicted higher than tested concentrations. Two possible explanation can be made for these results. The antagonistic effect might be occurred between antibiotics in the mixture or the microorganisms, which responsible of butyrate degradation are resistant to the tested antibiotics. In VFA mixture fed test bottles, $EC_{50}$ values were calculated higher than single compounds for ETS, ET and ES mixtures surprisingly. The antagonistic effect was seen in these test bottles. $EC_{50}$ value of ST mixture was lower than single SMX, synergistic effect was seen in this mixture.

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