ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EVALUATION OF ANAEROBIC BIODEGRADABILITY CHARACTERISTICS OF ANTIBIOTICS AND TOXIC/INHIBITORY EFFECT ON MIXED MICROBIAL CULTURE

Ph.D. Thesis by
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Department : Environmental Engineering

Programme : Environmental Biotechnology

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ANTİBİYOTİKLERİN ANAEROBİK BİYOLOJİK AYRIŞABİLİRLİK ÖZELLİKLERİNİN VE KARIŞIK MİKROBİYAL KÜLTÜR ÜZERİNE TOKSİSİTE/İNHİBİSYON ETKİLERİNİN DEĞERLENDİRİLMESİ

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To the memory of my father, Cahit Çetecioğlu...

FOREWORD

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July 2011

Zeynep Çetecioğlu

Environmental Engineer and
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ABBREVIATIONS

ACP **:** Anaerobic Contact Reactor
AnMBR **:** Anaerobic Membrane Bioreactor
API : Active Pharmaceutical Ingredients
ASBR : Anaerobic Sequencing Batch Reactor
BOD : Biochemical Oxygen Demand
CBP : Cumulative Biogas Production
CMP : Cumulative Methane Production
COD **:** Chemical Oxygen Demand
CSTR **:** Continuous Stirred Tank Reactor
DGGE **:** Denaturing Gradient Gel Electrophoresis
EGSB **:** Expanded Granular Sludge Bed Reactor
ERY **:** Erytromycin
FISH **:** Fluoresence *in situ* Hybridization
HRT **:** Hydraulic Retention Time
MOA **:** Mode of Action
OHPA **:** Obligate Hydrogen Producing Acetogens
PCR **:** Polymerase Chain Reaction
PMP **:** Potential Methane Production Rate
Q-PCR **:** Quantitative Real-Time PCR
RFLP **:** Restriction Fragment Length Polymorphism
RT-PCR **:** Reverse Transcription PCR
SCFA **:** Short Chain Fatty Acid
SMA **:** Specific Methanogenic Activity
SMX **:** Sulfamethoxazole
SRT **:** Solid Retention Time
SS **:** Suspended Solid
SSCP **:** Single-Strand Conformation Polymorphism
TET **:** Tetracycline
TGGE **:** Temperature Gradient Gel Electrophoresis
TOC **:** Total Organic Compound
TS **:** Total Solid
TVS **:** Total Volatile Solid
UASB **:** Upflow Anaerobic Sludge Blanket Reactor
VFA **:** Volatile Fatty Acid
VSS **:** Volatile Suspended Solid

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EVALUATION OF ANAEROBIC BIODEGRADABILITY CHARACTERISTICS OF ANTIBIOTICS AND TOXIC/INHIBITORY EFFECT ON MIXED MICROBIAL CULTURE

SUMMARY

In last decades, pharmaceuticals production and consumption have been increased. Occurrence and fate of these compounds are one of the main issues because of their unknown potential risks and their effects on the environment. Antibiotics are one of these compounds and approximately 500 tonnes of them are produced and consumed every year in the worldwide. Antibiotics are resistant to conventional biological treatment process and the wastewaters including these compounds are directly discharged to the receiving waterbodies without efficient treatment. Also pharmaceutical industries need to use physico-chemical treatment approaches to remove these compounds from their high-strength wastewater. The occurrence of the antibiotics in the ecosystem increases from day to day and it causes to spread antibiotic-resistant pathogens which are important threat aganst to public health.

Anaerobic treatment is one alternative for high-strength wastewater including inhibitory compounds such as pharmaceutical industry wastewater. In the literature, there are a few studies reported information about the anaerobic treatment of antibiotic containing wastewater. Also these reported studies have presented limited information about anaerobic systems. A comprehensive study including inhibitory effects and biodegradability characteristics of these compounds with microbial community definition is a lack in the literature.

The focusing items in this dissertation are acute and chronic effects of the three selected antibiotics; *Sulfamethoxazole*, *Erythromycin*, *Tetracycline*; on anaerobic systems were examined. Also the microbial community in terms of bacterial and archaeal species were defined in the system under different operation conditions. The presence and active microbial diversity changes were monitored and quantified. Addition of them, chronic effects of these selected antibiotics on homoacetogenic and methanogenic pathways were investigated on mRNA level studies.

In the scope of this dissertation, significant chronic effects of the selected antibiotics were started from lower levels compared to short-terms tests. While the significant acute effects were observed after 50 mg/L for the three compounds, in the chronic tests, the reactors collapsed at 45 mg/L of SMX addition and 3 mg/L of ERY and TET additions. Also long-term effects of these compounds on the microbial diversity reflected that Clostridium species were generally abundant within the systems, they could be got the resistance against the antibiotics. While acetoclastic and hydrogenotrophic species were abundant in the ERY added system, hydrogenotrophic methanogens were dominant in the SMX and TET reactors. Also the effects on these compounds on the metabolic pathways were determined. According to expression level of the selected enzymes on mRNA based approache, the inhibition was also observed on methanogenic step within all reactors, however

specific methanogenic activity of SMX fed sludge increased even performance of the reactor ceased. At the ERY and TET fed reactors, after the reactors collapsed, antibiotic addition was stopped and the reactors were operated for a while to recover the performance again. While the performances of the reactors couldn't be recovered, the specific methanogenic activity of ERY fed sludge and the expression level of the selected enzymes from methanogenic pathway increased.

ANTİBİYOTİKLERİN ANAEROBİK BİYOLOJİK AYRIŞABİLİRLİK ÖZELLİKLERİNİN VE KARIŞIK MİKROBİYAL KÜLTÜR ÜZERİNE TOKSİSİTE/İNHİBİSYON ETKİLERİNİN DEĞERLENDİRİLMESİ

ÖZET

İlaç etken maddelerinin üretimi ve tüketimi son yıllarda oldukça artmıştır. Henüz çevre üzerindeki riskleri ve etkileri tam olarak bilinemediği için, bu maddelerin varlıkları ve giderimleri en önemli araştırma konularından biri olmuştur. Antibiyotikler bu maddelerden biridir ve yıllık üretim ve tüketimleri tüm dünyada yaklaşık 500 tondur. Antibiyotikler konvansiyonel biyolojik arıtma proseslerine dirençli oldukları için alıcı su kaynaklarına tam olarak arıtılamadan deşarj edilmektedirler. Bu maddeleri içeren ilaç endüstrisi atıksuların arıtımı için fiziko-kimyasal arıtıma ihtiyaç duyulmaktadır. Miktarları her geçen gün ekosistemde artan bu ilaç etken maddeleri, doğada antibiyotiğe dirençli patojen organizamların artışına sebep olmakta ve bu durum halk sağlığı için büyük bir tehdit oluşturmaktadır.

Anaerobik arıtım ilaç endüstrisi gibi organik ve inhibitör madde içeriği yüksek olan atıksuların arıtımı için alternatif oluşturmaktadırlar. Literatürde antibiyotik içeren atıksuların anaerobik arıtımı ile ilgili az sayıda çalışma bulunmaktadır. Bu çalışmalar da anaerobik sistemlerle ilgili oldukça sınırlı bir bilgi sunmaktadır. Bu tip maddelerin, inhibisyon etkisi, biyolojik olarak arıtılabilirliği ve bu proseste rol alan mikrobiyal komünitenin tanımlanması ile ilgili kapsamlı bir çalışma henüz literatürde rastlanmaıştır.

Bu tezde seçilen 3 farklı antibiyotiğin (*Sülfometaksazol, Eritromisin ve Tetrasiklin*) anaerobik sistemler üzerindeki akut ve kronik etkileri çalışılmıştır. Ayrıca farklı işletme koşullarında bakteriyel ve arkeyal kömünite tanımlanmıştır. Mikrobiyal çeşitlilik ve aktif kömünitedeki değişimler izlenmiş ve kantifikasyonu yapılmıştır. Bunlara ilaveten seçilen antibiyotiklerin homoasetojenik ve metanojenik yolizleri üzerindeki kronik etkisi mRNA bazlı çalışmalar ile tespit edilmiştir.

Bu kapsamda elde edilen sonuçlar, seçilmiş antibiyotiklerin anaerobik sistemlerde kronik inhibisyon etkilerinin kısa dönem testlerine göre daha düşük konsantrasyonlarda başladığını göstermiştir. Akut testlerde her 3 antibiyotik için biyolojik aktvitedeki belirgin düşüş 50 mg/L'den sonra başlarken, kronik testlerde bu etki SMX reaktörü için 45 mg/L, ERY ve TET reaktörleri için 3 mg/L antibiyotik konsantrasyonunda gözlemlenmiştir. Uzun dönemli işletmede bütün reaktörlerde baskın bakteriyel grup *Clostridium* olarak tespit edilmiştir, bu gruptaki bakteriler her 3 antibiyotiğe karşı direnç kazanmış olabilirler. ERY reaktöründe hem asetoklastik hem de hidrogenotrofik metanojenler baskın durumdayken, SMX ve TET reaktörlerinde hidrogenotrofik türler baskındır. Ayrıca bu maddelerin metabolik yolizleri üzerindeki etkileri de belirlenmiştir. Seçilmiş enzimlerin mRNA düzeyinde incelenmiş olan ekspresyon seviyeleri, inhibisyonun metanojenik yolizleri üzerindeki etkisini ortaya koyarken, SMX reaktör çamurunun aktivitesi, reaktör performansının tersine antibiyotik konsantrasyonu ile orantılı olarak artmıştır. ERY ve TET reaktörleri çöktükten sonra bir süre antibiyotik beslemesi kesilerek sistemin kendini

toparlaması için işletilmeye devam edilmiştir. Reaktörlerin performansında herhangi bir düzelme olmazken, ERY çamurunun aktivitesinde ve seçilen enzimlerin ekspresyonunda artış tespit edilmiştir.

1. INTRODUCTION

Xenobiotics called as unfamiliar to existing enzyme systems and persistent in the environment and resistant biodegradation like heavy metals, metalloids and man-made organic compounds are of rising concern in the urban water cycle. The major reason is that these chemicals persist in the environment, bioaccumulate through food chain, and pose a risk of adverse effects to human health and the environment (van der Meer, 1992). There are numerous sources of xenobiotics in urban water systems because of the excess usage of these compounds with the increasing life standarts. Increased focus on use of rainwater and reuse of wastewater for industrial as well as domestic non-potable purposes further increase the exposure to xenobiotics, but the conventional urban water cycle approaches are not designed to deal with xenobiotics. The main problems for environmental engineers are that these compounds cannot be detected by conventional parameters like chemical oxygen demand (COD), biochemcial oxygen demand (BOD) or total organic carbon (TOC) etc. and treated by a classical sewage treatment plants. The presence of these micro pollutants and persistent organics that come out of various industrial processes has increased the necessity in the removal and the determination of the effect on the treatment systems of these compounds. Therefore, detection of existing and fate of these compounds and developing innovation technologies to treat them are the main purpose of the scientist and engineers.

The medicines that are accepted as one of these xenobiotics are produced approximately 100,000 tons every year in worldwide. While the worldwide average yearly consumption of medicine per person is 15 g (Kummerer, 2004), this value is accepted to be 50-150 g a year in advanced countries (Sedlak *et al.*, 2005; Stockholm County Council, 2005). The yearly consumption of antibiotics, which are aimed to use in this study, is 500 tons throughout the world according to the data of 2001. Approximately 90% of the consumed antibiotics after being partially metabolized or not being metabolized are excreted by the help of urea or feces from the body and transferred to the domestic sewage plants. These antibiotics are discharged into the

receiving environment with no or low elimination after being treated in conventionally operated domestic sewage plants. While the concentration of these materials in domestic wastewaters and surface waters are in µg/l level, in pharmaceutical wastewater they are in 100-1000 mg/L level (Daughton and Ternes, 1999; Stuer-Lauridsen, 2000; Chelliapan *et al.*, 2006; Amin *et al.*, 2006). As this low concentration in the surface wastewaters cause important problems in the ecosystem, it necessitates the removal of high antibiotic amount that are found in the pharmaceutical wastewaters. However, because the chemical removals of these materials are costly, biological treatment is essential. Antibiotics are the one of these compounds and the most often discussed pharmaceuticals because of their potential role in the spread and maintenance of (multi)resistance of bacterial pathogens. There are lots of studies that have been done in Europe and North America on the detection and removal of antibiotics in the receiving environment and the treatment plant (Hartig *et al.*, 1999; Hirsch *et al.*, 1999; Alder *et al.*, 2001; Golet *et al.*, 2001; Lindsey *et al.*, 2001; Golet *et al.*, 2002; Kolpin *et al.*, 2002; Golet *et al.*, 2003; McArdell *et al.*, 2003; Miao *et al.*, 2004; Göbel *et al.*, 2005). However, the studies on the treatability of these antibiotics biologically are quite few (Drilla *et al.*, 2005; Kim *et al.*, 2005). Also the scope of the studies done on the biodegradability potential of these materials is limited (Gartiser *et al.*, 2007a and b; Alexy *et al.*, 2004; Matamaros *et al.*, 2008). In these studies, the biodegradability of these chosen antibiotics under aerobic and anaerobic conditions has been tried to determine by only chemical analysis. Studies on the microbial groups and species that are responsible for degradation have not been done yet.

1.1 Aim and Scope

Xenobiotics, including both inorganic elements like the heavy metals, metalloids and organic compounds such as pesticides, surfactants, preservatives, solvents, fragrances, flavours, endocrine disrupters and pharmaceuticals are of rising concern in the urban water cycle. There are more than 100,000 xenobiotic compounds on the market in the European Union and approximately 30,000 of these are “everyday” chemicals i.e. estimated to be used in volumes over 1 ton each year. It has been estimated that 70,000 xenobiotics may potentially be hazardous for humans and/or ecosystems. In order to assess the role of the xenobiotics information is needed with respect to the

sources, flow paths, fate (transport, treatment, natural attenuation) and impact on humans, livestock and the environment. Furthermore, it is necessary to have suitable tools like chemical analytical methods or eco-toxicological test methods for collecting the information that is needed and determining the potential risk.

Antibiotics are among these compounds and every year approximately 500 ton of them are produced and consumed. Nearly the 90% of the consumed antibiotics are excreted out of the body with no or little metabolization via the urea or the fecal matter and end up in domestic sewage plants. These antibiotics are discharged into receiving environment with no removal or low removal rate from the conventionally operated treatment plants.

Even though there are studies present in the literature considering the detection and the behavior in the receiving environment about these antibiotics that are resistant to degrade biologically, the literature information about the biodegradation and the active microbial groups in the degradation of this type of xenobiotics is very limited.

In this scope, determination of biodegradation characteristics of the refractory compounds and their toxic/inhibition effects on microbial community is substantial for environmental engineering. Four sub-topics will be investigated during the study.

These sub-topics are:

- Acute and chronic effect of compound on microbial community
- Anaerobic biodegradability characteristics of the chosen xenobiotic compound
- Profile of microbial community having role on biodegradation
- Syntrophic relations and metabolisms of microbial community which responsible to fate of these compounds

1.2 Problem Definition

Antibiotic including wastewater generated from houses, hospitals, pharmaceutical industries, stock farms are one of the current environmental problems. This kind of the wastewaters cannot be treated in the conventional treatment plants and discharge directly to the receiving water bodies such as rivers, lakes, and seas. The concentration of these active compounds and their metabolites in the water bodies

increase gradually and it causes to gain the antibiotic resistance of the pathogen organisms. Also these water bodies are mostly used as drinking water sources. Up to this study, there are some studies in the literature focused on the biological treatability of this compound, especially under aerobic conditions. Most of the studies examined the fate of the antibiotics in full-scale sewage treatment plants and receiving water bodies. Different treatment strategies based on the lab-scale studies including the influent and effluent antibiotic concentration together with conventional parameter to analyze specific fate of these compounds and microbial community analysis not only to identify the species which have a role in degradation but also to monitor changes the groups depending on time and antibiotic concentration are the most important lack of the literature. This study aims to bridge the gaps between bioreactor operation and manipulation/change of microbial community during to treat the selected pharmaceuticals.

In this scope, this thesis presented results obtained from:

- Acute tests to determine the short-term effect of the antibiotics on the acclimated anaerobic biomass.
- Chronic tests to observe the long-term effects and biodegradability characteristics of the selected compounds in the anaerobic systems.
- Methanogenic activity test to
- Molecular studies to identify bacterial and archaeal species in the systems and their behaviors during time and antibiotic concentration.

2. LITERATURE BACKGROUND

2.1 Pharmaceuticals

Pharmaceuticals used in medicine and veterinary applications have been one of the main concerns for the environment in last decade. A vast amount of literature has been published about their occurrence, fate, and risk and these subjects are still hot topics in the environmental science (Daughton and Ternes, 1999; Kolpin *et al.*, 2002; Boyd *et al.*, 2003; Bendz *et al.*, 2005; Clara *et al.*, 2005; Glassmeyer *et al.*, 2005; Kim and Aga, 2007; Avisar *et al.*, 2009; Benotti and Brownawell, 2009; Segura *et al.*, 2009; Rodríguez-Rodríguez *et al.*, 2011).

These active compounds called as pharmaceutical (sometimes called as active pharmaceutical ingredients, APIs) are complex molecules with different physicochemical and biological properties and functionalities (Kummerer, 2009). Their more or less specific biological activity is the main reason to use and develop as API. Approximately 3000 different pharmaceutical compounds including painkillers, antibiotics, antidiabetics, beta-blockers, contraceptives, lipid regulators, antidepressants, impotence drugs and cytostatic agents are used in Europe (Ternes *et al.*, 2006). Globally production and consumption of these compounds are considered around 100,000 ton/year (Kummerer, 2004). They are also most notably characterized by their ionic nature and classified as parent compounds and metabolites and /or transformation compounds:

Classification of small molecule APIs according to chemical structure is used mainly for the active substances within subgroups of medicines, e.g.,within the group of antibiotics or the subgroups within the antibiotics such as β-lactams, cephalosporins, penicillins or quinolones. In this case, the compounds can be treated as groups with respect to chemical behavior. Other classifications refer to the mode of action (MOA) e.g., anti-metabolites or alkylating agents within the group of cytotoxics/antineoplastics, the targets or the effects. In the case of classification according to MOA, chemical structures of molecules within the same group can be very different and therefore their environmental fate can vary, too. In this case,

compounds cannot be handled as a group with respect to environmental issues. Their fate and effects on humans or on other target organisms such as bacteria or parasites and on non-target organisms in the environment can be classified according to not only different pharmaceuticals of special interest of the compounds, but also because of the differences in their occurrence, This can be illustrated by comparing pharmacology and eco-pharmacology as seen in Table 2.1. Eco-pharmacology deals with all aspects of a pharmaceutical within the environment and emphasizes that pharmaceuticals in the environment are an issue for doctors and pharmacists. This concept is much broader than pharmacoenvironmentology, a concept, which has only recently been derived from it (Rhaman *et al.* 2007). Their environmental relevance is not yet clear in spite of high consumption of them.

Table 2.1: Pharmacology and eco-pharmacology (Kummerer, 2008).

	Pharmacology (humans)	**Ecopharmacology (environment)**
Numbers of compounds administered	One or only a few compounds at the same time	An unknown cocktails of different compounds
Desirable physicochemical properties	Stable	Readily (bio)degradable
Administration	Tarfeted on demand controlled	Diffuse, i.e. emissions from medical care units and the community
Wanted effects/side effects	Active wanted effects, side effects	Wanted effects in target organism are often most important, side effects in the environment
Metabolism/ biotransformation/ affected organism	One type of organism	Various type organisms of different trophic levels

2.1.1 Parent compounds, metabolites and transformation products

The studies which were carried out in the recent years, not only these parent compounds but also metabolites and transformation products of these compounds are important for the environment and have a different role in the ecosystem (Golan *et al.* 2007). The compounds can be changed due to biotic and abiotic processes in the environment. These structural changes start in the body of human and animals. It could be a microbial process occurred in the gut and/or enzymatic reaction such as cytochromes. The second structure of APIs derived from metabolism in the body is called as *metabolites*. After extraction of the body, these changes undergo in the effluent streams and also in the wastewater treatment plants by photochemically and

biologically (Qiting and Xiheng, 1988; Ravina *et al.*, 2002; Schroder, 2002; Ternes *et al.*, 2003; Zuhlke *et al.*, 2004; Lee *et al.*, 2007; Trautwein *et al.*, 2008; Mendez-Arriaga *et al.*, 2008). The chemicals from differentiation of APIs by biotic and abiotic processes in the environments after excretion such as biodegradation by microorganisms or fungi, chemical oxidation, photochemical reaction are called as *transformation products* (Burhenne *et al.*, 1997, Mendez-Arriaga *et al.*, 2008). Figure 2.1 shows the processes, which were explained above.

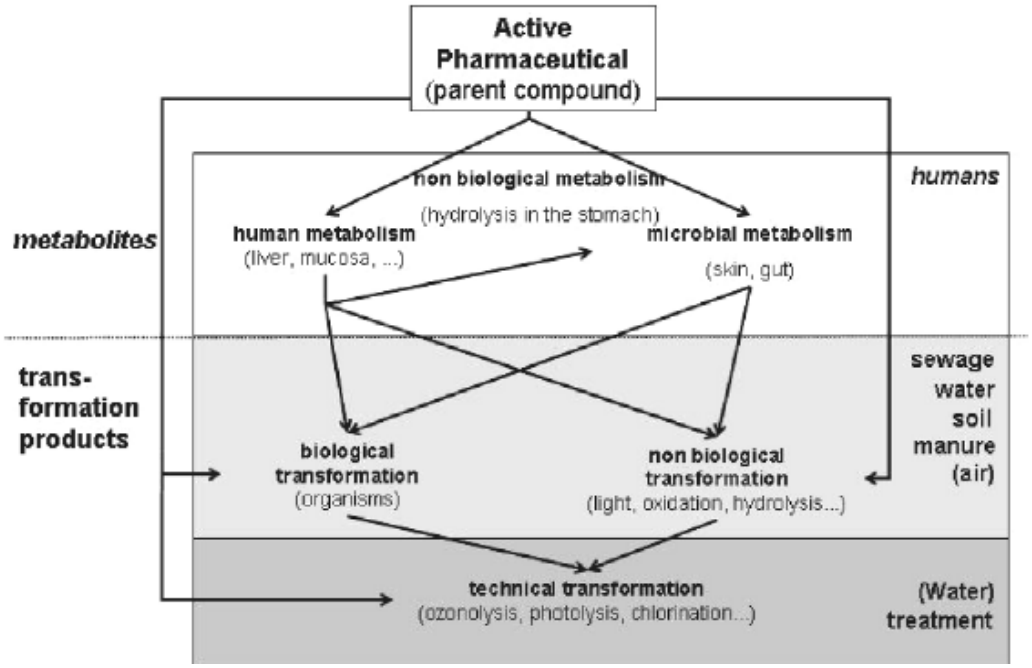

Figure 2.1: Metabolites and transformation products (Kummerer, 2008).

Both pharmacological and toxicological properties of the metabolites and transformation products differ from their parent drugs. Pharmaceuticals may be applied via oral or intravenous ways depending on the compound itself and the medical circumstances. This application way has an effect on their metabolism that is their structural change within the body of the target organism (e.g., within humans in the case of pharmaceuticals administered to humans). Metabolism may lower activity and/or enhance water solubility. However, metabolism is mostly incomplete. It is assumed that toxicity of APIs decreases after metabolized, however, sometimes metabolism causes to create more active compounds.

2.2 Antibiotics

Antibiotics are among the most important groups of pharmaceuticals and chemotherapeutic agents that inhibit or terminate the growth of microorganisms, such as bacteria, fungi, or protozoa without affecting host (Korolkovas, 1976; Foye *et al.*, 1995). The term antibiotic used for drugs that block any of these microorganisms. Other terms as chemotherapeutics or antimicrobials are not synonymous because of their scopes; the terms of antimicrobial is used for the medicine which is also effective against viruses and the expression ‘‘chemotherapeutical” refers to compounds used for the treatment of disease which kill cells, specifically microorganisms or cancer cells. The term ‘‘chemotherapeutical” may also refer to antibiotics (antibacterial chemotherapy).

The expression of antibiotic is originally used to describe any agent with biological activity against living organisms; however, ‘‘antibiotic” now refers to substances with antibacterial, anti-fungal, or anti-parasitical activity. During the years, this definition has been changed and now it includes also synthetic and semi-synthetic products. There are approximately 250 different compounds registered for use in medicine and veterinary application (Kummerer and Henninger, 2003).

In this thesis the term “antibiotic” refers only to drugs that kill or inhibit bacteria. Antibiotics that are sufficiently nontoxic to the host are used as chemotherapeutic agents in the treatment of infectious diseases of humans, animals and plants. They are extensively used for prevention and treatment of diseases caused by microorganisms in human and veterinary medicine as well as in aquaculture nowadays. Also, they are being still used as growth factor in livestock farming. Some compounds may be used for different purposes such as in growing fruit and in bee keeping other than human or veterinary medicine. The application purposes may vary from country to country.

Antibiotics are classified as their chemical structures and the mechanism of inhibition of microorganisms and they can be divided into subgroups such as β-lactams, quinolones, tetracyclines, macrolides, sulfonamides and others. The active compounds of antibiotics are often complex molecules, which may have different functionalities. In the environment, these molecules could be found as neutral, cationic, anionic, or zwitterionic forms. Because of the different functionalities within one molecule, their physicochemical and biological properties may change with pH

levels (Cunningham, 2008).

In this study, sulfamethoxazole (SMX), erytromycin (ERY) and tetracycline (TET) were selected as model compounds.

2.2.1 Sulfamethoxazole

The systematic name of this compound is 4-amino-*N*-(5-methylisoxazol-3-yl)-benzenesulfonamide as seen in Figure 2.2.

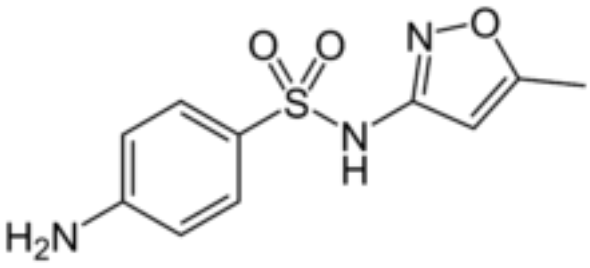

Figure 2.2: Chemical structure of Sulfamethoxazole (Url-1, 2011).

Sulfamethoxazole and other sulfonamides have a similar structure to *p*-aminobenzoic acid and inhibit to the synthesis of nucleic acids in sensitive microorganisms by blocking the conversion of *p*-aminobenzoic acid to the coenzyme dihydrofolic acid, a reduced form of folic acid; dihydrofolic acid is obtained from dietary folic acid so sulfanomides do not have any influence on human cells. Their action is primarily bacteriostatic, although they may be bactericidal where concentrations of thymine are low in surrounding medium. The sulfonamides have a broad spectrum of action, but the development of widespread resistance has greatly reduced their usefulness, and susceptibility often varies widely even among nominally sensitive pathogens like Gram-positive and Gram-negative cocci.

There are several mechanisms of resistance including alteration of dyhydropteroate synthetase, the enzyme inhibited by sulfonamides, to a less sensitive form, or an alteration in folate biosynthesis to an alternative pathway; increased production of *p*-aminobenzoic acid; or decreased uptake or enhanced metabolism of sulfonamides.

Resistance may result from chromosomal alteration, or may be plasmid-mediated and transferable, as in many resistant strains of enterobacteria. High-level resistance is usually permanent and irreversible. There is complete cross-resistance between the different sulfonamides (Sweetman, 2009).

2.2.2 Erythromycin

The systematic name of this antibiotic is (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-6-{[(2*S*,3*R*,4*S*,6*R*)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-14-ethyl-7,12,13-trihydroxy-4-{[(2*R*,4*R*,5*S*,6*S*)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy}-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione as seen in Figure 2.3. It is a macrolide groıup compound with a broad and essentially bacteriostatic action against many Gram-positive and to a lesser extent some Gram-negative bacteria, as well as other organisms including some Mycoplasma spp., Chlamydiaceae, Rickettsia spp., and spirochaetes.

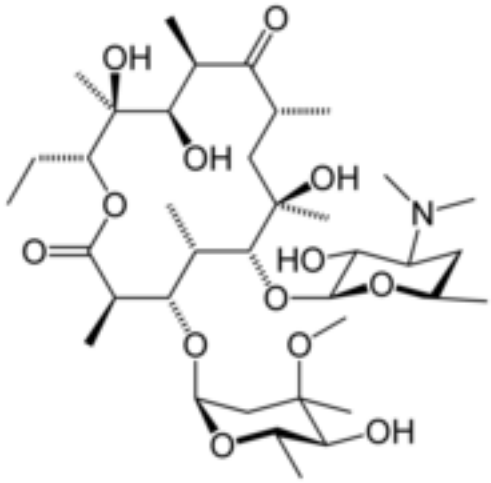

Figure 2.3: Chemical structure of Erythromycin (Url-2, 2011).

Erythromycin and other macrolide group antibiotics bind reversibly 50S subunit of the ribosome, resulting the blockage of the transpeptidation or translocation reactions, restriction of protein synthesis, and hence inhibition of cell growth. Its action is mainly bacteriostatic, but high concentrations are slowly bactericidal against the more sensitive strains. Because these compouns penetrate readily into white blood cells and macrophages there has been some interest in their potential synergy with the host defence mechanism *in vivo*. The actions of erythromycin are increased moderately alkaline pH (up to about 8.5), particularly in Gram-negative species, probably because of improved cellular penetration of the nonionised form of the drug.

Several resistance mechanisms of it have been reported of which the most common is a plasmid-mediated ability to methylate ribosomal RNA, resulting in decreasing binding of the antimicrobial drug. This can result in cross-resistance between erythromycin and other macrolides, lincosamides, and streptogramin B, because they

share a common binding site on the ribosome and this pattern of resistance is referred to as the MLS_B phenotype.

Decreased binding of antimicrobial to the ribosome may also occur as a result of a chromosomal mutation, resulting in an alteration of the ribosomal proteins in the 50S subunit, which conveys one-step high-level erythromycin resistance.

Other forms of erythromycin resistance may be due to the production of plasmid-determined erythromycin esterase that can inactivate the drug, or to decreased drug penetration. The latter may be partly responsible for the intrinsic resistance of Gram-negative bacteria like the Enterobacteriaceae, but has also been shown to be acquired as a plasmid-mediated determinant in some organisms; production of a protein which increase drug efflux from the cell is thought to explain the M phenotype resistance, in which organisms are resistant to 14-and 15-carbon ring macrolides, but retain sensitivity to 16-carbon ring macrolides, lincosamides, and streotogramins (Sweetman, 2009).

2.2.3 Tetracycline

The systematic names of this compound are 42-(amino-hydroxy-methylidene)-4-dimethylamino-6,10,11,12a-tetrahydroxy-6-methyl-4,4a,5,5a-tetrahydrotetracene-1,3,12-trione or 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro- 3,6,10,12,12a-pentahydroxy-1,11-dioxo-naphthacene-2-carboxamide or (4*S*,6*S*,12a*S*)-4-(dimethylamino)- 3,6,10,12,12a-pentahydroxy- 6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a- octahydrotetracene-2-carboxamide as seen in Figure 2.4.

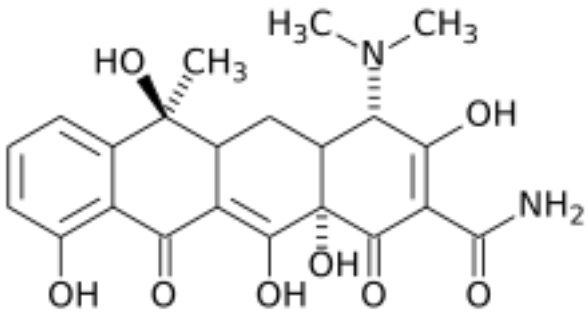

Figure 2.4: Chemical structure of Tetracycline (Url-3, 2011).

Tetracyclines are mainly bacteriostatic, with a broad spectrum of antimicrobial activity including *Chlamydiaceae*, *Mycoplasma spp.*, *Rickettsia spp.*, spirochaetes, many aerobic and anaerobic Gram-positive and Gram-negative pathogenic bacteria, and some protozoa.

This compound is transferred into sensitive bacterial cells by an active transport process. Once within the cell they bind reversibly to 30S subunit of ribosome, preventing the binding of aminoacyl transfer RNA and restricting protein synthesis, and hence cell growth. Although they also inhibit protein synthesis in mammalian cells they are not actively taken up, permitting selective activity against the infecting organisms.

It affects mostly Gram-positive and Gram-negative cocci, other Gram-positive bacteria, Gram-negative anaerobes *Bacteriodes fragilis* may sometimes susceptible, although wild strains are often resistant, and *Fusobacterium* may also be sensitive.

Resistance to the tetracyclines is usually plasmid-mediated and transferable. It is often inducible, and appears to be associated with the ability to prevent accumulation of antibacterial within the bacterial cells, both by decreasing active transport of the drug into the cell or by increasing tetracycline efflux.

Unsurprisingly, given the widespread use of tetracyclines (including as components of animal feeds, although this is now banned in some countries), resistant strains of the majority of sensitive species have now been reported (Sweetman, 2009).

2.2.4 Consumption and occurrence

The yearly consumption of antibiotics worldwide is estimated between 500 tons (Wise, 2002). Approximately 90% of the consumed antibiotics are excreted via urinary or fecal pathways from the human body after partial or no metabolism and they are transferred to the domestic sewage plants or directly to the environment. Conventional biological treatment of domestic sewage provides very low or no reduction for these compounds, which usually by-pass treatment and accumulate in the receiving waters.

Antibiotic consumption changes depending on the country and/or region however the situation is scarce and heterogenous. Country specific consumption for groups of antibiotics in DDDs can be found for Europe on the ESAC homepage

(http://www.esac.ua.ac.be/main.aspx?c=*ESAC2&n=1066l). Using patterns of different regions and countries are given Table 2.2. The relative importance of the different use patterns in different countries is still not known.

An increasing number of studies have been done to determine the source, occurrence, fate, and effects on the ecosystem of antibiotics. However, there is still a lack of understanding and knowledge of these compounds. So studies maybe focus on the strategies about stream segregation and at-source treatment of the concentrated streams appears.

Table 2.2: Country specific antibiotics consumption and occurrence data.

Region/ Country	Total volume used in human medicine (ton/year)	Volume used in human medicine (gram per capita)	Thereof in hospitals (%)	Unuse medicaments	Measured in sewage up to (µg/L)	Measured in surface water up to (µg/L)	Reference
World wide	100000-200000	N.D.	N.D.	N.D.	N.D.	N.D.	Wise, 2002
EU + Switzerland	8367	22.4	N.D.	N.D.	N.D.	N.D.	FEDESA, 2001
USA	4860	17	70	N.D.	1.9	0.73	Union of Concerned Scientists, 2001; Kolpin *et al.*, 2002
Canada	N.D.	N.D.	N.D.	N.D.	N.D.	0.87	Miao *et al.*, 2004
Switzerland	34.2	4.75	20-40	N.D.	0.57	0.2	Kummerer, 2004
Germany	411	4.95	25	20-40	6	1.7	Kummerer and Henninger, 2003; Rönnefahrt, 2005
Denmark	40	7.4	N.D.b	N.D.	5N.D.	N.D.	Kummerer, 2008
Austria	38	4.7	N.D.	20-30	N.D.	N.D.	Sattelberger, 1999
Netherlands	40.9	3.9	20	N.D.	4.4	0.11-0.85	Verbrugh and de Neeling, 2003
Italy	283	4.88	N.D.	N.D.	0.85-	0.25	Calamar *Et al.*, 2003;
Turkey	N.D.	31.4	N.D.	N.D.	N.D.	N.D.	Karabay, 2009

N.D.: not defined

2.2.5 Production and manufacturing

Pharmaceutical industries have minor importance on the sewage treatment plants. Only in some Asian countries, wastewaters from this industry contributes to the sewage and cause an increase in the concentration of single compound up to mg/L level (Larsson *et al.*, 2007; Li *et al.*, 2008a,b). Also in developed countries, manufacturing plants increases the total antibiotic concentrations in the domestical wastewater (Thomas, 2008).

The main problem for this industry, they still use the physicochemical treatment technologies in the plant to remove the compounds from their wastewater. However, this approach is expensive.

2.2.6 Elimination and treatment

In the literature, there are lots of studies focused on the fate of these compounds in conventional domestical wastewater treatment plants and also lab-scale applications in the innovative treatment methods. Elimination and/or treatment of these organic compounds are the results of biotic and abiotic processes. While biotic process is the biodegradation by microorganisms, abiotic processes are sorption, hydrolysis, oxidation-reduction, and photolysis.

2.2.6.1 Sorption

Before to assess the sorption characteristics of antibiotics, it is necessary to consider their physical and chemical parameters. Tolls (2002) investigated the sorption behavior of these compounds in soil and the results showed that sorption mechanism of antibiotics could be very complex and difficult.

Additionally, binding to particles or the formation of complexes may prevent their detection. For example, tetracyclines are able to form complexes with double cations such as magnesiumor calcium (Christian *et al.*, 2003). Also humic substances cause the change in the surface properties and sites available for sorption and reactions. Gu and Karthikeyan (2008) reported that there is a strong interaction between humic acids, hydrous Al oxide and tetracycline. Some studies showed that antibiotics used in medicine such as fluoroquinolones and macrolides can reach the terresrial environment by sewage sludge (Trivedi and Vasuden, 2007; Gu and Karthikeyan,

2008). Also Golet *et al.* (2002) and Giger *et al.* (2003) also confirmed that fluoroquinolone concentration is high in the sewage sludges.

Also sorption mechanism is a significant process for sulphonamides (Tolls, 2001; Kreuzig and Holtge, 2005; Heise *et al.*, 2006; Schmidt *et al.*, 2008). However, knowledge about the interaction of antibiotics with sludge and of sediments with sludge in activated sludge plants as well as the subsequent potential for their release back into the environment is still too sparse.

2.2.6.2 Photolysis

Photochemical process can be important in the surface waters and treatment plant effluents as another elimination process (Viola *et al.*, 2004; Edhlund *et al.*, 2006; Paul *et al.*, 2007; Werner *et al.*, 2007; Hu and Coats, 2007; Hu *et al.*, 2008; Lorenzo *et al.*, 2008). In the environment, photolysis process is not effective in turbid water or river and lakes, which are shadowed. So, the in the lab-scale experiments cannot reflect the photochemical process in the nature. Also, effectiveness of depletion process can differ under different environmental conditions such as pH, temperature, water hardness (Werner *et al.*, 2006) and depends on type of matrix, location, season, latitude (Kallenborn *et al.*, 2008).

One of the problems about this type of process is that incomplete photo-transformation and photo-degradation can cause to more or less stable or toxic compounds although this does not necessarily have to happen (Cokgor *et al.*, 2006; Arslan-Alaton and Caglayan, 2006; Gonzalez *et al.*, 2007; Iskender *et al.*, 2007; Paul *et al.*, 2007).

The significance and extent of direct and indirect photolysis of antibiotics in the aquatic environment are different for each compound because some of them are light sensitive (e.g. quinolones, tetracyclines, sulphonamides, tylosin, nitrofuran antibiotics). However, not all compounds are photo-degradable (Turiel *et al.*, 2005). Tetracyclines are senstive to photo-degradation. Samuelsen (1989) investigated the sensitivity of oxytetracycline towards light in seawater as well as in sediments. The antibiotics proved to be stable in sediments rather than in seawater. As no mechanism of decomposition other than photolysis is known for them (Oka *et al.*, 1989), the substance remains in the sediment for a long period, as shown by Lunestad and Goksøyr (1990). Boree *et al.* (2004) showed that sulphanilic acid was found as a degradation product common to most of the sulpha drugs.

2.2.6.3 Hydrolysis and thermolysis

Another important pathway for the non-biotic decomposition of organic substances in the environment is hydrolysis. Some instability in water could be demonstrated for some tetracycylines (Halling-Sørensen, 2000). In general, the hydrolysis rates for oxytetracycline increase with reascept to temperature at pH 7. The half-lives of oxytetracycline under investigation changed by differences in temperature, light intensity and flow rate from one test tank to another. However sulphonamides and quinolones are known as resistant antibiotic to hydrolysis.

2.2.6.4 Oxidation

Pharmaceutical industry wastewaters including antibiotic are well known for the difficulty of their elimination by conventional biological treatment methods and their important contribution to environmental pollution is due to their fluctuating and recalcitrant nature. For this reason, oxidation processes are usually applied.

The presence of carbon–carbon double bonds, aromatic bonds or nitrogen is a necessary essential for this application. However, the presence of these structural elements does not provided the fast and full degradation or even the complete degradation.

The effect of ozonation on the degradation of oxytetracycline in aqueous solution at different pH values (3, 7 and 11) was reported by Li *et al.* (2008c). The study was designed that ozonation as a partial step in a combined treatment concept is a potential technique for biodegradability enhancement. It has been shown that COD removal rates increase with increasing pH as a consequence of enhanced ozone decomposition rates at elevated pH values. The results of bioluminescence data indicate that the initial by-products after partial ozonation (5–30 min) of oxytetracycline were more toxic than the parent compound (Li *et al.*, 2008c).

Sulfamethoxazole was also efficiently degraded by ozonation (Dantas *et al.*, 2007). An improvement in biodegradability by the increasing of BOD5/COD ratio from 0 to 0.28 was observed by the authors after 60 min of ozonation. The acute toxicity of the intermediates was checked and a slight acute toxicity increment in the first stage of ozonation was found. pH variation was found as important parameter on TOC and COD removal efficiencies. The complete sulfamethoxazole removal was achieved for an in photo-Fenton process (Gonzalez *et al.*, 2007). Toxicity and inhibition tests

pointed in the same direction: no toxic effect of oxidized intermediates was determined and also no inhibition was detected on activated sludge activity.

2.2.6.5 Biodegradation

Biodegradability of most antibiotics has been checked and it was found that they are not biodegradable under aerobic conditions until today. (Richardson and Bowron, 1985; Al-Ahmad *et al.*, 1999; Wiethan *et al.*, 2000; Kummerer *et al.*, 2000; Ingerslev *et al.*, 2001; Ingerslev and Halling-Sørensen, 2001; Thiele-Bruhn, 2003; Alexy *et al.*, 2003, 2004; Gartiser *et al.*, 2007a; Li *et al.*, 2008c). Biodegradability characteristics have been weak for most of the compounds investigated in laboratory tests such as the OECD test series (301–303, 308) – even for some of the ß-lactams (Alexy *et al.*, 2004). Out of 16 antibiotics tested, only benzyl penicillin (penicillin G) was completely mineralized in a combination test (combination of the OECD 302 B and OECD 301 B tests; Gartiser *et al.*, 2007a).

Biodegradation for tetracycline was not observed during a biodegradability test (sequence batch reactor), and sorption was found to be the principal removal mechanism for tetracycline in activated sludge (Kim *et al.*, 2005).

Some antibiotics occurring in soil and sediment proved to be quite persistent in laboratory testing as well as in field studies. Some of them were not biodegradable also under anaerobic conditions (Gartiser *et al.*, 2007b) others did (Maki *et al.*, 2006). Substances extensively applied in fish farming had long half-lives in soil and sediment, as reported in several investigations (Jacobsen and Berglind, 1988; Hansen *et al.*, 1992; Samuelsen *et al.*, 1992, 1994; Hektoen *et al.*, 1995; Capone *et al.*, 1996; Marengo *et al.*, 1997; Lai *et al.*, 2008). However, some substances were at least partly degradable (Donoho, 1984; Gilbertson *et al.*, 1990; Samuelsen *et al.*, 1991, 1994; Capone *et al.*, 1996; Thiele-Bruhn, 2003). Maki *et al.* (2006) found that ampicillin, doxycycline, oxytetracycline, and thiamphenicol were significantly degraded, while josamycin remained at initial levels. Tylosin was biodegraded (Hu and Coats, 2007).

2.3 Inhibition and Toxicity

Inhibitors which cause slowing and/or halting enzymatic reactions are molecular agents. They are most important pharmaceutical agents nowadays (Lehninger *et al.*, 2005).

Toxicity is is the degree to which a substance can damage an organism. Toxicity can mention to the effect on a whole organism, such as an animal, bacterium, or plant, as well as the effect on a substructure of the organism.

2.3.1. Types of inhibition

Inhibition types are divided into two main classes as reversible and irreversible inhibition.

Inhibition effect can be recovered in the reversible inhibition type. One common type of reversible inhibition is called *competitive*. Inhibitors competes with the substrate for the active site of enzyme in the competitive one. While the inhibitor fills up the active site it prevents binding of the substrate to the enzyme. Many competitive inhibitors are compounds that resemble the substrate and combine with the enzyme to form an enzyme-inhibitor complex, but without leading to catalysis. Even fleeting combinations of this type will reduce the efficiency of the enzyme.

The other types of reversible inhibition is uncompetitive. An uncompetitive inhibitor binds at a site distinct from the substrate active site and, unlike a competitive inhibitor, binds only to the enzyme-substrate complex.

The last type of reversible inhibition is mixed inhibition also binds at a site distinct from the substrate active site, but it binds to either enzyme or enzyme-substrate complex.

Also there is a special case of mixed inhibition which is defined as noncompetitive inhibition. In this inhibition, the inhibitor reduces the activity of the enzyme. In practice, uncompetitive and mixed inhibition are observed only for enzymes with two or more substrates and are very important in the experimental analysis of such enzymes.

In the irreversible inhibition, the inhibitory effect cannot be recovered. The irreversible inhibitors are those that bind covalently with or destroy a functional group

on an enzyme that is essential for the enzyme's activity, or those that form a particularly stable noncovalent association. Formation of a covalent link between an irreversible inhibitor and an enzyme is common. Irreversible inhibitors are another useful tool for studying reaction mechanisms. Amino acids with key catalytic functions in the active site can sometimes be identified by determining which residue is covalently linked to an inhibitor after the enzyme is inactivated.

A special class of irreversible inhibitors is the suicide inactivators. These compounds are relatively unreactive until they bind to the active site of a specific enzyme. A suicide inactivator undergoes the first few chemical steps of the normal enzymatic reaction, but instead of being transformed into the normal product, the inactivator is converted to a very reactive compound that combines irreversibly with the enzyme. These compounds are also called mechanism-based inactivators, because they hijack the normal enzyme reaction mechanism to inactivate the enzyme. Suicide inactivators play a significant role in rational drug design, a modern approach to obtaining new pharmaceutical agents in which chemists synthesize novel substrates based on knowledge of substrates and reaction mechanisms. A well-designed suicide inactivator is specific for a single enzyme and is unreactive until within that enzyme's active site, so drugs based on this approach can offer the important advantage of few side effects (Lehninger *et al.*, 2005).

2.4 Fundamentals of Anaerobic Treatment

Anaerobic degradation is a complex process that the organic compounds are mineralized to CH_4 and CO_2 in the absence of oxygen. Different microbial groups have role on this process. Anaerobic degradation of the organic matters is occurred with a multi step and parallel reactions which different microbial groups have role. These steps are generally classified, namely:

- hydrolysis
- acidogenesis
- acetogenesis
- methanogenesis

Various models have been suggested to explain these biochemical stages such as nine-stage model (Harper and Pohland, 1986), six-stage model (Lester *et al.*, 1986)

and three-stage model (Gerardi, 2003). Biochemical reactions in the nine-stage model of anaerobic digestion are listed below (Harper and Pohland, 1986) and shown diagrammatically in Figure 2.5. The biochemistry and microbiology of anaerobic digestion is a complex biogenic process involving a number of microbial populations, linked by their individual substrate and product specifities (Hutnan *et al.*, 1999).

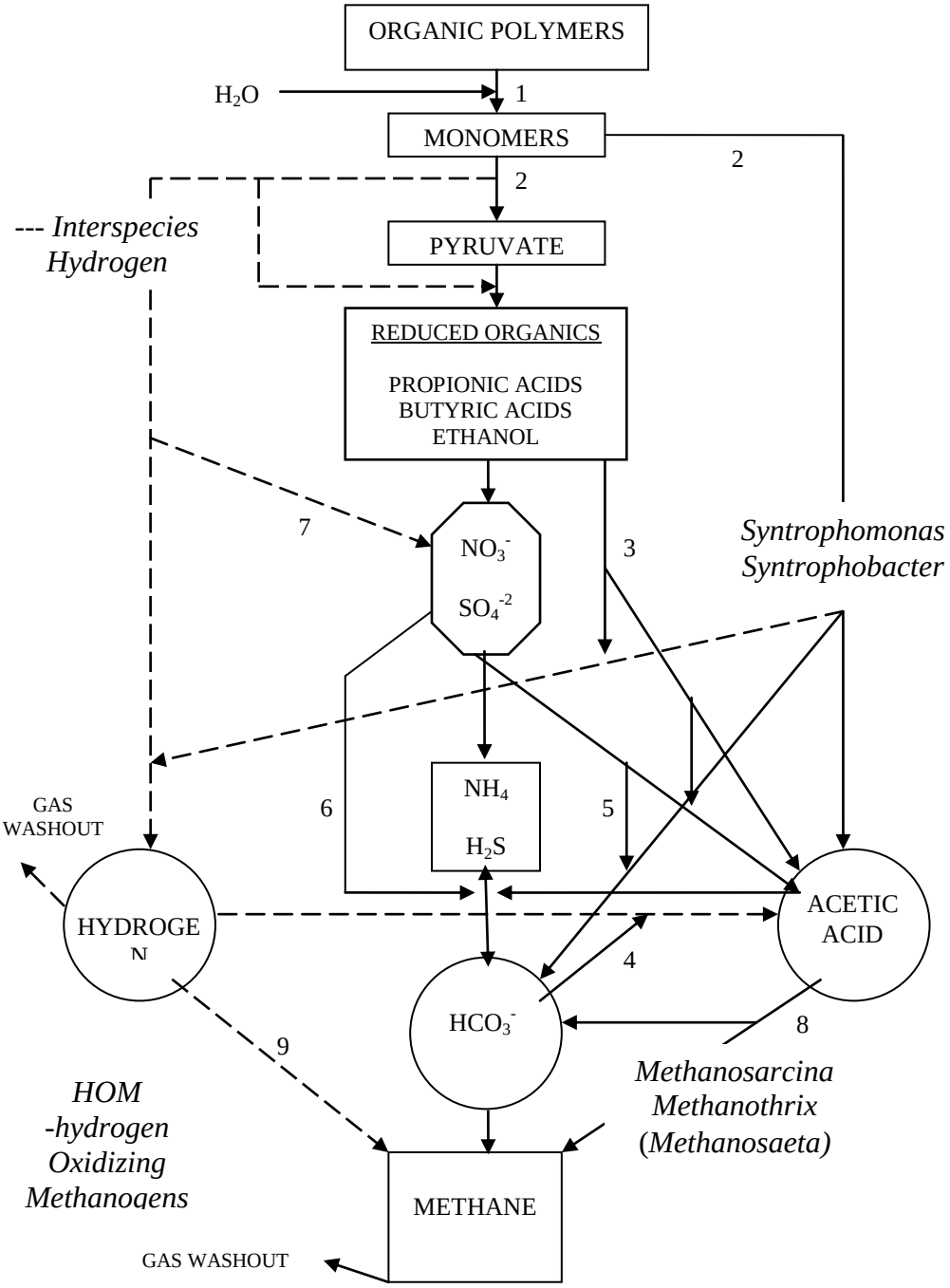

Figure 2.5: Substrate conversion patterns associated with the anaerobic digestion (Harper and Pohland, 1986).

2.4.1 Hydrolysis

Hydrolysis, which is the first step of the anaerobic degradation, consists the extracellular enzymatic reactions to serve the particulate organic matters for bacteria. This process is only surface phenomena where the complex polymeric particles such as carbohydrates, proteins, and lipids are degraded through the action of exo-enzymes, which are produced in the cell but are released through the "slime" coating the cell to the insoluble substrate attached to the slime (Gerardi, 2003), to monomers, which can transport to the cell barriers. While all bacteria produce endoenzymes, it is not the same for exoenzymes. Also there is no bacterium can prıduce all type of exoenzymes that are needed to degrade the large variety of particulate and colloidal substrates that are found in sludges and wastewaters. The substrate types and the spesific exoenzymes are given in Table 2.3. Each enzyme has role on degradation of only a specific substrate or group of substrates. Therefore, a large and diverse community of bacteria is needed to ensure that the proper types of exoenzymes and endoenzymes are available for degradation of the substrates present.

Table 2.3: Exoenzymens and substrates (Gerardi, 2003).

Substrate	Exoenzyme	Example	Bacterium	Product
Polysaccharides	Saccharolytic	Cellulase	Cellulomonas	Monosaccharides
Proteins	Proteolytic	Protease	Bacillus	Amino acids
Lipids	Lipolytic	Lipase	Mycobacterium	Fatty acids

Hydrolysis can be a rate limiting steps for all treatment of the waste and/or wastewater because the hydrolytic bacteria, their enzymes, the availability of free accessible surface area of the organic compounds and the overall structure of the solid substrate are of paramount importance for the succeeding steps in the anaerobic degradation sequence (Stronach *et al.*, 1986, Pavlostathis and Giraldo-Gomez, 1991, Zeeman *et al.*, 1996). Furthermore, this step is very sensitive to temperature. Also pH, cell residence time and the waste constituents in the reactor directly influence to the reaction rate.

In the hydrolysis step, a wide range of bacteria takes the role. Bacillus play role in the degradation of proteins and fats, while The strictly anaerobic genus *Clostridium* includes many species that are highly active in the production of extracellular hydrolytic enzymes and is responsible for degradation of compounds containing

cellulose and starch. The types of hydrolytic microorganisms are classified namely as, the cellulytic (*Clostridium thermocellum*), proteoytic (*Clostridium bifermentas, Peptococcus*), lipolytic (genera of clostridia and micrococci) and aminolytic (*Clostridium butyricum, Bacillus subtilis*) bacteria (Hungate, 1982; Payton and Haddock, 1986). The hydrolytic bacteria may also break down the some intermediate products to simple volatile fatty acids, carbon dioxide, hydrogen, ethanol (Eastman and Ferguson, 1981).

As seen in Table 2.3, the spesific exoenzymes carries out the hydrolysis. Lipases convert lipids to long-chain fatty acids. The produced long-chain fatty acids are degraded by β-oxidation to produce acetyl-CoA. Proteins are generally hydrolyzed to amino acids by proteases, secreted by *Bacteroides, Butyrivibrio, Clostridium, Fusobacterium, Selenomonas,* and *Streptococcus.* The amino acids produced are then degraded to fatty acids such as acetate, propionate, and butyrate, and to ammonia as found in *Clostridium, Peptococcus, Selenomonas, Campylobacter,* and *Bacteroides.* Polysaccharides such as cellulose, starch, and pectin are hydrolyzed by cellulases, amylases, and pectinases.

2.4.2 Acidogenesis

Acidogenesis is the second step of the anaerobic degradation in which the hydrolysis products are transferred to bacterial cells and subsequently fermented or anaerobically oxidized. In this phase, ammonia acids, simple sugars and long chain fatty acids are converted to carbon dioxide, hydrogen, alcohols, ammonia and short-chain fatty acids such as acetic, butyric, propionic, valeric acid. Acidogenesis is carried out by a large group of hydrolytc and non-hydrolytic microorganisms. The products of this step differ with not only type of bacteria but also temperature, pH, and the composition of the influent feed. The catabolism of these organic compounds is carried out by a large number of both obligatory and facultatively anaerobic microorganisms. While single amino acids are degraded under anaerobic conditions by *Clostridia, Mycoplasmas* and *Streptococci*, butanol, butyric acid, acetone and iso-propanol are generally converted by the bacteria of the genera *Clostridium* and *Butyribacterium*, for example *Clostridium butyricum, Clostridium acetobutylicum,* and *Clostridium butylicum* produce butyrate, acetone and butanol and only butanol in addition to hydrogen, carbondioxide and iso-proponol. The genera *Clostridium* and *Butyribacterium* are

involved in the production of butyrate, buthanol, butyric acid, acetone and iso-propanol.

2.4.3 Acetogenesis

The third stage of the anaerobic degradation is acetogenesis in which the short chain fatty acids (SCFA), other than acetate, are further converted to acetate, hydrogen and carbon dioxide by two groups of bacteria, which have a sytrophic relationship between methanogenic archaea. The process in which a molecule is removed from fatty acids having more than two carbons at each reaction step until all fatty acids are converted to acetate molecules is generally called as β-oxidation. The activity of the organisms playing role in the step is of paramount importance, since methanogens cannot utilize the fatty acids other than acetic acid.

The first group bacteria taking the role in acetogenesis, the obligate hydrogen producing acetogens (OHPA), also called proton-reducing acetogens, produce acetic acid, carbon dioxide and hydrogen from the major fatty acid intermediates (propionate and butyrate), alcohols and other higher fatty acids (valerate, isovalerate stearate, palmitate and myristate via β-oxidation). These species are capable of growth only in environments that maintain a low concentration of the metabolic product hydrogen. Only a limited number of OHPA species have been isolated and identified, namely Syntrophomonas wolfei and Syntrophobacter wolinii, which oxidize butyrate and propionate, respectively (Malina and Pohland, 1992; Gujer *et al.*, 1983).

The second group in acetogenesis is homoacetogens which are strictly anaerobic microorganisms catalyzing the formation of acetate from hydrogen and carbon dioxide. Homoacetogens are known in the genera Acetobacterium, Acetoanaerobium, Acetogenium, Butribacterium, Clostridium and Pelobacter. Homoacetogenic bacteria are also syntrophs like OHPA because they participate in the interspecies hydrogen transfer process, which maintains the low hydrogen concentrations required by the OHPA. Acetogenic bacteria reproduce very slowly. Generation time for these organisms is usually greater than 3 days (Anderson *et al.*, 2003).

2.4.4 Methanogenesis

In the final step of the anaerobic degradation, the products of the previous step are mineralized to methane and carbon dioxide by methanogens via two conversion mechanisms including decarboxylation of acetic acid and reduction of carbon dioxide in the absence of other electron acceptors such as oxygen, nitrate, and sulfate except bicarbonate and protons as terminal electron acceptors (Garcia *et al.*, 2000; De Bok *et al.*, 2004; Stams *et al.*, 2006). This stage is considered as the rate-limiting step in the anaerobic process because of the slow growth rate of the methanogens (Malina *et al.*, 1992). Methanogens is a phylogenetic member of Archaea domain that are distinguished from Eubacteria by a number of characteristics, including the possesxion of membrane lipids composed of isoprenoids ether-linked to glycerol or other carbohydrates, a lack of peptidoglycan containing muramic acid, a distinctive ribosomal RNA sequences (Woese, 1987). Some extreme halophiles and some extremely thermophilic, sulfur-dependent microbes (Woese, 1987) are also included in this grup and they are phylogenetically distinct from eukaryotes and bacteria.

Methanogens, which have orders and sub-groups, are strictly anaerobic organisms and gain their energy by producing CH_4 and CO_2 from simple substrates such as H_2, CO, acetate, formate, and a few alcohols (Figure 2.6). Alternatively, methanogens produce CH_4 by the reduction of the methyl groups in acetate, methanol, trimethylamine, and dimethylsulfide, part of which are oxidized to CO_2 to generate the electrons necessary for reduction of the methyl group to CH_4. Some methanogens are able to use H_2 as second substrate to reduce the methyl, for example in methanol. The substrates including CO_2-type, methyl substrates and acetate converted to methane by various methanogenic *Archaea* are listed in Table 2.4. All reactions are thermodynamically exergonic at standard conditions allowing occur in nature, if substrate concentrations are sufficiently high.

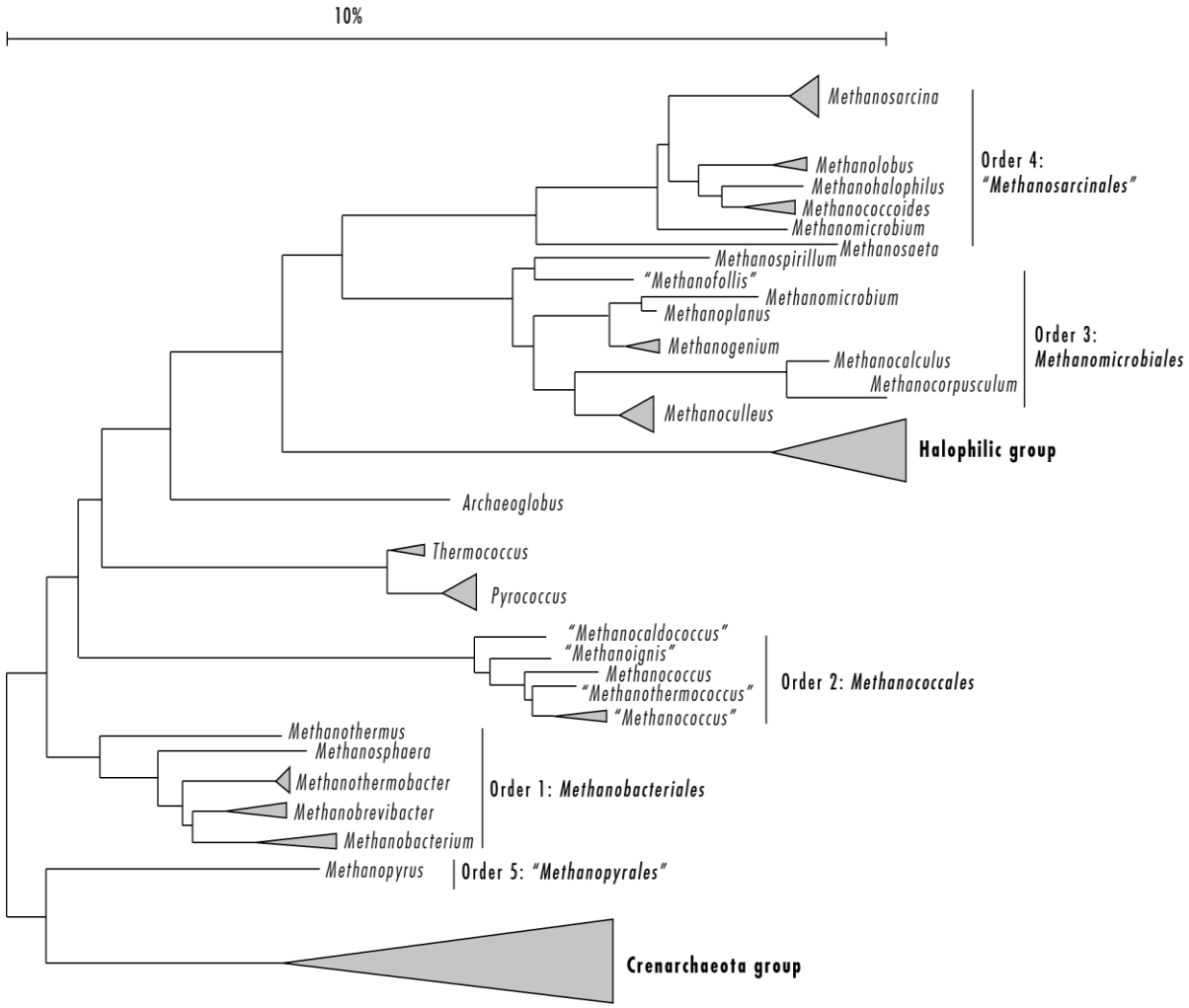

Figure 2.6: Phylogeny of methanogens, domain Archaea. (Non-methanogens are indicated by their group names, large triangles) (Garcia *et al.*, 2000).

Table 2.4: Substrates converted to methane by various methanogenic *Archaea* (Madigan *et al.*, 2009).

Substrates and Reactions	**Organisms**
I. CO_2-type substrates (Carbon dioxide with electrons derived from H_2, certain alcohols, or pyruvate; Formate, Carbon monoxide)	
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	Most methanogens
$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	Many hydrogenotrophic methanogens
CO_2+4isopropanol$\rightarrow CH_4$ + 4acetone + $2H_2O$	Some hydrogenotrophic methanogens
$4\ CO + 2H_2O \rightarrow CH_4 + 3\ CO_2$	*Methanothermobacter* and *Methanosarcina*
II. Methylated C1 compounds (Methanol, Methylamine, Dimethylamine, Trimethylamine, Methylmercaptan, Dimethylsulfide)	
$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	*Methanosarcina* and other methylotrophic methanogens
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	*Methanomicrococcus blatticola* and *Methanosphaera*
$2\ (CH_3)_2\text{-}S + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ H_2S$	Some methylotrophic methanogens
$4\ CH_3\text{-}NH_2 + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_3$	Some methylotrophic methanogens
$2(CH_3)_2\text{-}NH + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_3$	Some methylotrophic methanogens
$4\ (CH_3)_3\text{-}N + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_3$	Some methylotrophic methanogens
$4CH_3NH_3Cl + 2H_2O \rightarrow 3CH_4 + CO_2 + 4\ NH_4Cl$	Some methylotrophic methanogens
III. Acetate	
$CH_3COOH \rightarrow CH_4 + CO_2$	*Methanosarcina* and *Methanosaeta*

As seen in Table 2.4, CO_2, formate and CO are called as CO2-type substrates and reduced to methane in accordance with Eq 3.1. Hydrogenotrohic methanogens such as *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales* and *Methanosarcinaceae* are responsible in this process. Most hydrogenotrophic methanogens that can reduce CO_2 to methane use H_2 as the primary electron donor. Many hydrogenotrophic methanogens are also able to use formate and secondary alcohols, such as 2-propanol, 2-butanol, and cyclopentanol as electron donors. A small number of methanogens can also use ethanol.

$$CO_2+4H_2 \rightarrow CH_4+2H_2O \qquad \Delta G°=-131kJ/mol \text{ (Batstone } et\ al.\text{, 2002)} \qquad \textbf{(2.1)}$$

The methyl groups, which are the second class substrate of methanogenesis, are converted to methane via two conversion mechanisms. First of them is the formation of methane by reducing methyl group substances using an external electron donor such as H_2. In the conversion equations methanol (CH_3OH) is used as a model methyl

substrate, as given in Eq. 3.2. In the second mechanism, the methane is produced via reducing methyl group substances using an external electron donor such as H_2. In the conversion equations, methanol (CH_3OH) is used as a model methyl substrate, as given in Eq. 3.3. Methanogens that are able to use methylated compounds, or methylotrophicmethanogens, are limited to the order *Methanosarcinales*, except for *Methanosphaera* species, which belong to the order *Methanobacteriales*.

$CH_3OH+H_2 \rightarrow CH_4+H_2O$ $\Delta G°=-113kJ/mol$ (Batstone *et al.*, 2002) **(2.2)**

$4CH_3OH \rightarrow 3CH_4+CO_2+2H_2$ $\Delta G°=-319kJ/mol$(Batstone *et al.*, 2002) **(2.3)**

The last type of substrate that is used by methanogens is acetate. Acetate is a major product of the fermentation and accounts for 70% of the methane production in anaerobic bioreactors. Acetate is degraded by cleavage, with the carboxyl group being oxidized to CO_2 and the methyl group being reduced to CH_4. The CH_4-producing reaction is generated by the activity of methyl-CoM reductase, which converts methyl-CoM (methyl-coenzymeM) and HS-HTP (N-7-mercaptoheptanoyl-O-phospho-L-threonine) to CH_4 and a heterodisulfide consisting of HS-HTP and CoM-SH. This reaction is occurred by all methanogens, independent of primary substrate. The subsequent reduction of the heterodisulfide to CoM-SH and HS-HTP is coupled to the generation of a proton motive force. This reaction is the most important one for energy conservation and is universal for all methanogens. In the first step, acetate has to be converted to acetylcoenzyme A (acetyl-CoA), which requires the expenditure of energy. Formation of acetyl-CoA occurs by two different reactions (Zinder, 1993). In *Methanosarcina* spp., acetate is first phosphorylated with ATP by an acetate kinase producing acetyl-P and ADP. Subsequently, the acetyl-P is converted by a phosphotransacetylase with CoA-SH to acetyl-CoA and phosphate. In summary, conversion of acetate to acetyl-CoA requires one energy-rich phosphate bond of ATP in *Methanosarcina* spp. In *Methanosaeta* spp., on the other hand, acetate is activated using an acetyl-CoA synthetase, which converts acetate, CoA-SH, and ATP to acetyl-CoA, AMP, and pyrophosphate. In summary, this reaction requires two energy-rich phosphate bonds of ATP. This means that *Methanosaeta spp.* use more energy for acetate activation than *Methanosarcina spp*. Acetate is catabolized by the members of only two genera of methanogens, that is *Methanosarcina* and *Methanosaeta*, which

belong to the families of *Methanosarcinaceae* and *Methanosaetaceae*, respectively. *Methanosarcina* has a low affinity for acetate and dominate when acetate concentrations are high. On the other hand, *Methanosaeta* has a high affinity for acetate and dominate when acetate concentrations are as low. *Methanosaeta*, which uses only acetate as substrate, is a superior acetate utilizer in that it can use acetate at concentrations as low as 5–20 μM, while *Methanosarcina* requires a minimum concentration of about 1 mM. Members of the family *Methanosarcinaceae*, including *Methanosarcina* spp. are also able to utilize H_2/CO_2, methanol, metyl amines and pyruvate besides acetate as energy substrate for CH_4 production. The difference between the organisms in terms of acetate affinity depends probably on the differences in the first step of acetate metabolism. *Methanosarcina* uses the low-affinity acetate kinase (AK)-phosphotransacetylase (PTA) system to activate acetate to acetyl-CoA. On the other hand, *Methanosaeta* uses the high-affinity adenosine monophosphate (AMP)–forming acetyl-CoA synthetase.

2.4.5 Characterization of microbial communities in anaerobic reactors

Anaerobic bioreactors are used for waste(water) treatment and much is known about the pathways occurred in the system. However, only little part of the microbial ecology within these systems has been revealed, yet. This small fraction cannot reflect the composition and diversity of the ecosystems. So, in the full-scale application, the black-box model is still acceptable for design and operation. Despite, culture dependent techniques are being developed day to day, a few percent of Bacteria and Archaea have been isolated. Also their dynamic in the system and relations between each other are still unknown.

To overcome this unpredictable and unexplainable lack, molecular tools have been used in last decades (Amann *et al.*, 1995; Muyzer *et al.*, 1996; Head *et al.*, 1998). Using the molecular tools in the anaerobic treatment systems, we can find the answers of the questions such as ‘which species do exist?’, ‘which species are active?’, ‘how many microorganisms are there?’, which species do utilize the specific compounds?’.

Microbial ecology studies need identification of species based on a comprehensive classification system that perfectly reflect the evolutionary relations between the microorganisms (Pace, 1996). Numerous authors pointed out that traditional classification systems based on phenotypic characteristics such as morphology,

physiology, and structure of cell components) offer a few information on evolutionary relations and the cultivation was necessary for identification (Pace, 1996 and 1997).

Zuckerkandl and Pauling (1965) indicated that nucleic acids could document evolutionary history. Due to the pioneering studies, nucleic acids, especially 16S rRNA, are the ultimate biomarkers and hereditary molecules probably because of their essential role in protein synthesis, making them one of the earliest evolutionary functions in all cellular life-forms (Olsen *et al.*, 1986; Pace *et al.*, 1986; Woese, 1987; Stahl *et al.*, 1988). In particular, 16S rRNA and 16S rDNA have been used in phylogenetic analysis and accepted as ideal evolutionary chronometers because:

i) They are a key element for all prokaryotic organisms, and evolutionary homologous,

ii) They are very conserved against to mutations.

iii) Their conserved and variable regions reflect the differences between species and provided to analyze phylogenetically as seen in Figure 2.7 (Woese, 1987, Amann *et al.*, 1995; Head *et al.*, 1998). Therefore to design primers and probes are possible.

iv) They are sufficiently large molecules (≈1500 nucleotides) to provide the information species and strain level (Woese, 1987).

v) They have enough copy in most of the cells (10^3 to 10^5 copies), which provide easy detection (Amann *et al.*, 1995).

vi) There is no evidence about transfer of this gene between the organisms.

Using 16S rRNA, Woese and his colleagues developed the first phylogenetic trees, which show the evolutionary relations of all organisms as seen Figure 2.8.

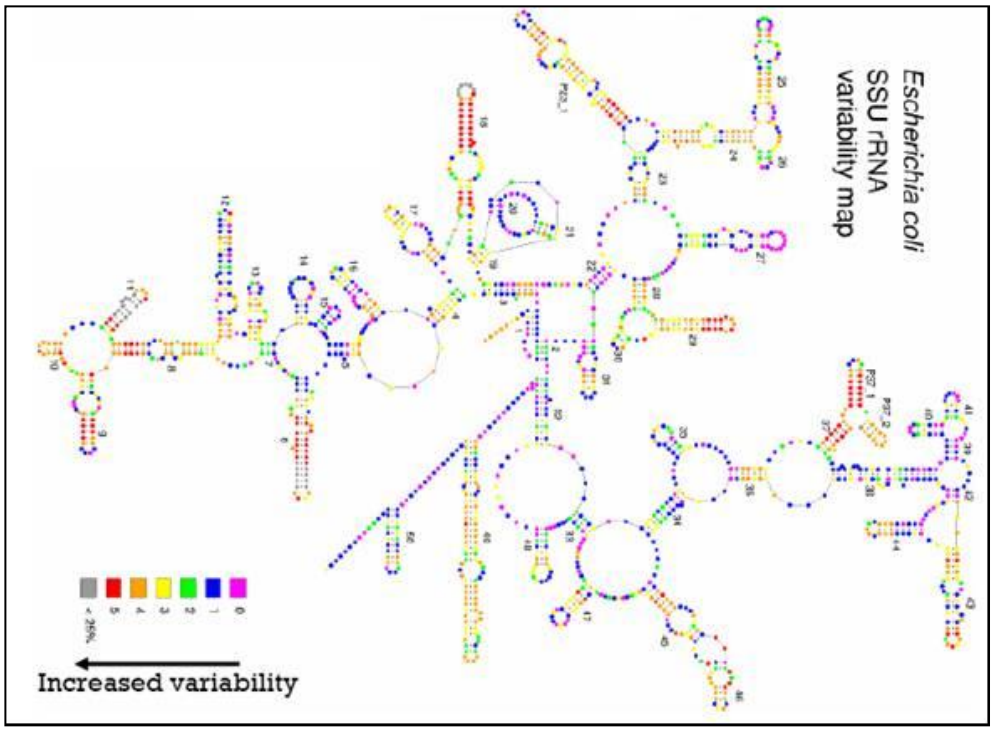

Figure 2.7: Secondary structure of the 16S rRNA of *E. coli*, showing conserved and variable regions (van de Peer *et al.*, 1996).

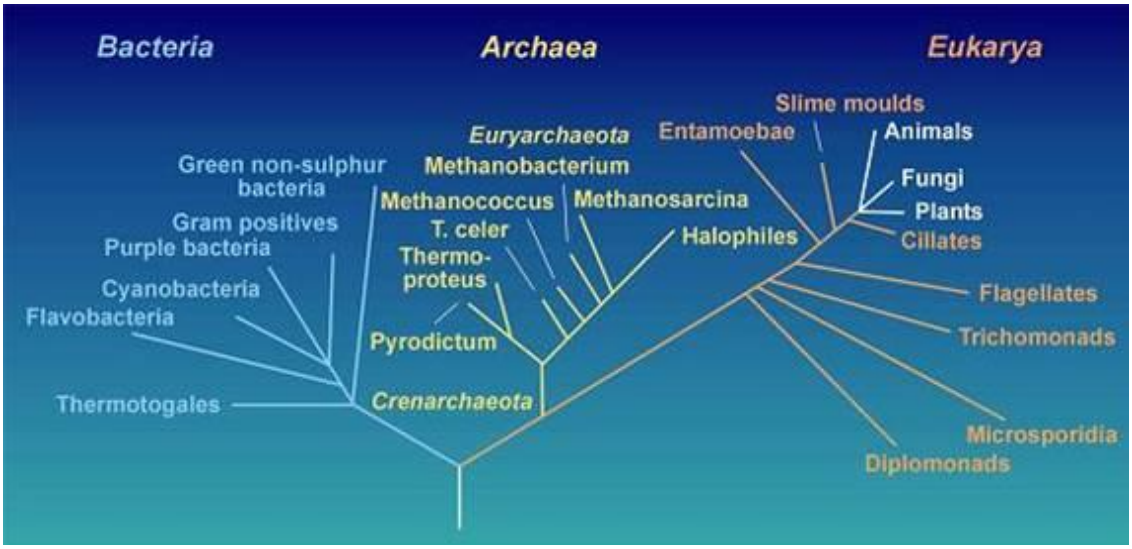

Figure 2.8: The rRNA tree of life (Madigan *et al.*, 2009).

In the last decade, metabolically functional genes such as methyl coenzyme M reductase subunit A gene (mcrA) in methanogenesis, nitrite reductases (nirK), nitrate reductase (narG), nitrous oxide reductase (nosZ) genes in nitrification process, ammonia monooxygenase genes in nitrification process have been used to investigate diversities in the ecosystems (Gray and Head, 1999; Kowalchuk *et al.*, 1999; Nogales *et al.*, 2002; Nunoura *et al.*, 2008).

2.4.5.1 Molecular tools

The microbiological analysis based on 16S rRNA and rDNA sequences for characterization are given in Figure 2.9.

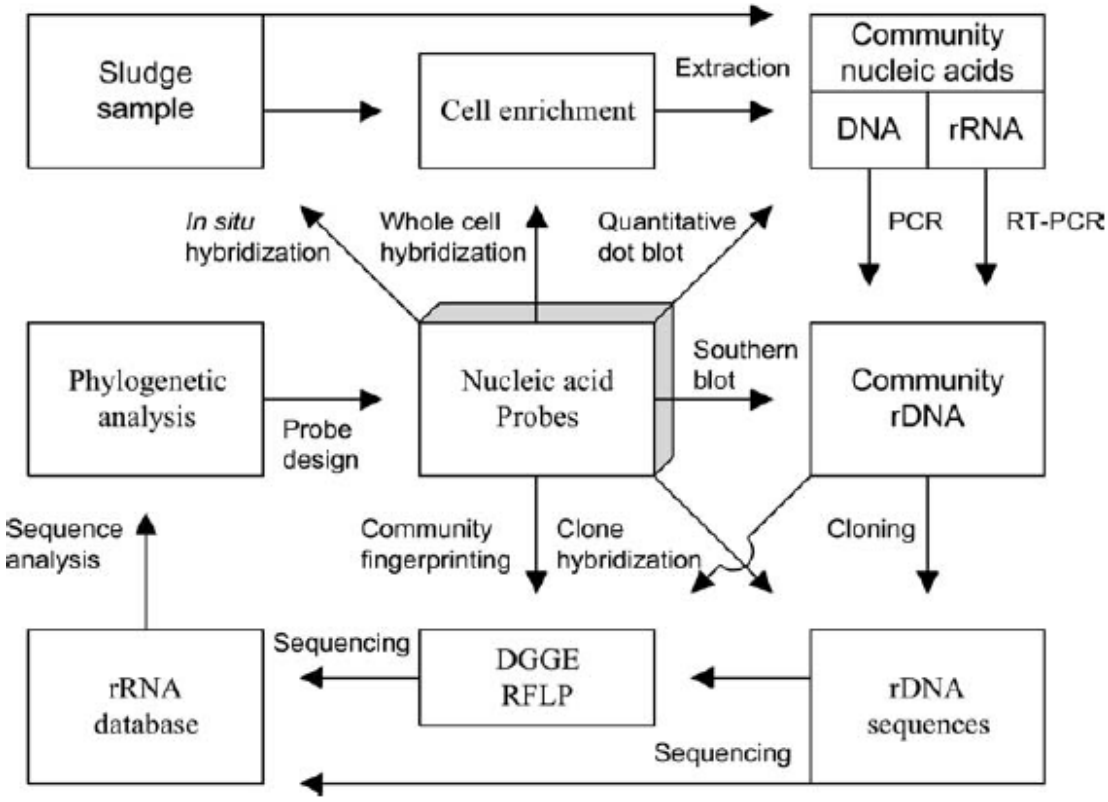

Figure 2.9: Strategies based on rRNA sequence analysis for characterization of microbial communities without cultivation (arrows indicate the interconnected use of methods, experimental materials, and information in the study of microbial ecosystems. RT-PCR: reverse transcription to produce DNA from RNA, followed by PCR. DGGE: denaturing gradient gel electrophoresis. RFLP: restriction fragment length polymorphism (Hofman-Bang *et al.*, 2003).

Nucleic Acid Isolation

DNA and RNA isolation are the first step of PCR-based methods. The important point in this step is to obtain representative and bias-free samples. Also adequate nucleic acid should be retrieved. After the sampling, DNA samples can be stored at -20 ºC until extraction. However, RNAs should be isolated quickly and under appropriate conditions.

Several methods have been used for the isolation from different kind of samples such as sludge, water, manure, soil, and sediment (Moran *et al.*, 1993; Ibrahim and Ahring, 1999; Frischer *et al.*, 2000; Cetecioglu *et al.*, 2009). Obtaining RNA or DNA

quantitatively from all cells in a complex community without bias can be difficult. In general, mechanical lysis methods have shown less bias than enzymatic lysis methods, leading to the recovery of intact high molecular weight nucleic acids (Yu and Mohr, 2001).

Polymerase Chain Reaction (PCR)

PCR are used to amplify a selective, small part of DNA, which is extracted from environmental samples. Nowadays, it is generally the first step of the phylogenetic analysis. The products of the PCR are analyzed by further techniques such as cloning-sequencing, DGGE or TGGE which have the potential to separate the PCR products originating from different DNA sequences representing populations in the original samples (Hofman-Bang, 2003).

Reverse-Transcription PCR (RT-PCR)

RT-PCR is used to synthesis complementary DNAs (cDNAs) from RNA (rRNA or mRNA) to analyze active community based on 16S rRNA or expression level of enzymes via mRNAs (Felske *et al.*, 1998; Miskin *et al.*, 1999). In 16S rRNA approach, positive relationship between growth rate and cellular rRNA content. So, it seems to allow a better analysis of active communities. Still, this approach needs to be improved because it is known that inactive but viable microorganisms will contain ribosomes, in order to be able to react to changing environmental conditions.

The amount of ribosomes in starved microorganisms can be considerable, when compared to the ribosome content during growth at maximal growth rates, and high levels can be maintained over long periods of time (Fukui *et al.*, 1996) and it has been shown that autotropic ammonia-oxidizing bacteria maintain very high levels of rRNA even in the absence of detectable ammonia oxidation activity (Wagner *et al.*, 1995).

Quantitative Real-Time PCR (Q-PCR)

Q-PCR is applied for exact quantification of the microbial groups as kingdom, family or genus level (Conklin *et al.*, 2006; Siggins *et al.*, 2011) and for determination of expression level of specific enzymes on mRNA level (Lopez-Gutierrez *et al.*, 2004; Nunoura *et al.*, 2008; Ince *et al.*, 2011). It is a technique based on the continuous monitoring of changes of fluorescence in the PCR tube during amplification. In contrast to traditional PCR, in which end-point detection is occurred, quantification is done based on the exponential phase of amplification in Q-PCR (Malinen *et al.* 2003).

Thus, the bias often observed in the PCR template-to-product ratios can be largely avoided (Suzuki & Giovannoni 1996).

Cloning and Sequencing

Construction of clone libraries is the most common molecular analysis for environmental samples. This technique allows identifying the species on strain level. After amplicons obtained from PCR, the single sequences are retrieved for sequencing via cloning (Madigan *et al.*, 2009). Analysis of 16S rRNA clone library to assess microbial diversity and populations in ecosystems is an important approach (Giovanni *et al.*, 1990). The steps of this technique are given in Figure 2.10: (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).

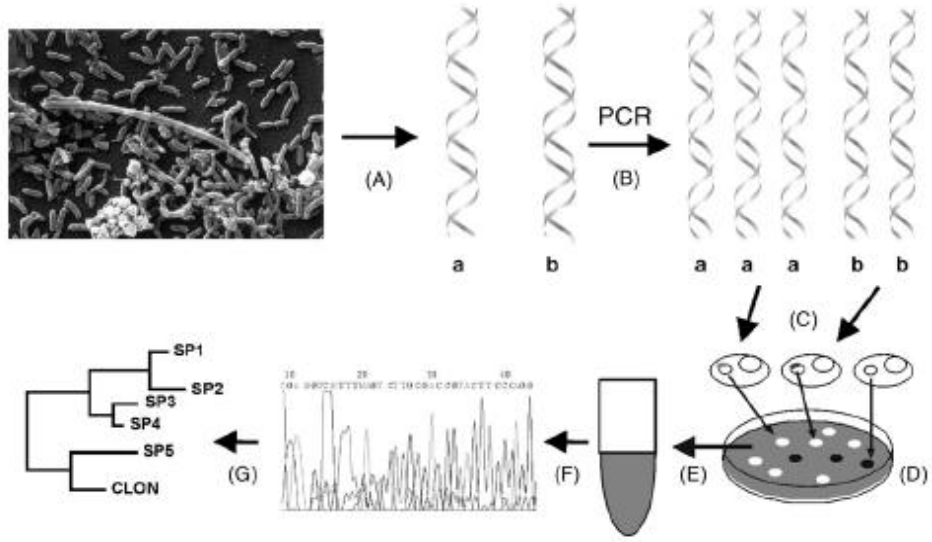

Figure 2.10: Outline of the cloning procedure for studying a microbial community (Sanz and Kochling, 2007).

Fingerprinting Techniques

A lot of fingerprinting techniques such as DGGE, TGGE, RFLP, T-RFLP, and SSCP, have been developed for estimation of diversity in ecosystems, screening clone libraries, following the diversity changes with respect to time and location (Hofman-Bang, 2003).

The DGGE (denaturing gradient gel electrophoresis) technique has been used to characterize the microbial diversity in different environments such as activated sludge (Curtis and Craine, 1998), sediments (Muyzer and De Wall, 1993), lake water (Ovreas *et al.*, 1997), hot springs (Santegoeds *et al.*, 1996), biofilm (Santegoeds *et al.*, 1998). It provides a fast, less labor-intensive approach to comparing community composition in many different samples than sequencing of clone libraries but little direct information on population identity (Muyzer and Smalla, 1998). The method can be used to as both qualitative and semi-quantitative approaches for biodiversity estimations.

TGGE (temperature gradient gel electrophoresis) is also similar technique. The only differences between DGGE and TGGE, while chemical denaturing polyacrylamide gel is used to separate equal-length DNA fragments in DGGE, temperature gradient is applied for the same target in TGGE.

Another fingerprinting method is RFLP (restriction fragment length polymorphism) in which rDNA can be digested with restriction enzymes and the banding pattern obtained from previous step has been used to identify different genotypes of microorganisms (Ingianni *et al.*, 1997) and to monitor population changes in environmental samples (Smit *et al.*, 1997; Vachee *et al.*, 1997).

SSCP (single-strand conformation polymorphism) is used for separation of DNAs based on their secondary structures product by NaOH prior to non-denaturing polyacrylamide gel electrophoresis. The advantage of the technique is that even point mutations can be detected as a change of conformation in the secondary structure of the single stranded DNA. In this method, each SSCP peak reflects to a distinct microbial sequence, indicating the presence of a microbial strain or species retrieved from the sample (Leclerc *et al.*, 2001; 2004).

Fluoresence *in situ* Hybridization (FISH)

This technique is commonly applied investigate the overall taxonomic composition of bacterial communities or assemblages in environmental samples. Fluoresence probes can be designed to be complementary to species-, group-, or kingdom-specific target sites. Cells are fixed to make them permeable to the probe, which then hybridizes its specific target site. There are a lot of studies which FISH was used to explore microbail communities in activated sludge, anaerobic reactors, marine and freshwater environments, and in both pristine and contaminated aquifers (Wagner *et al.*, 1993; Borneman *et al.*, 1996; Snaidr *et al.*, 1997; Shi *et al.*, 1999; Cottrell and Kirchman, 2000; Ince *et al.*, 2007 and 2009).

2.5 Environmental and Operational Factors Effecting Anaerobic Treatment

Methanogenesis is assumed that the rate limiting step of the anaerobic degradation. However, all metabolic products play a regulative role in maintaining the overall process because of syntrophic relation. Low growth rate of methanogens can make the anaerobic system sensitive to environmental changes (Xing *et al.*, 1997) and disturbances in populations from one trophic level may affect the entire community (Raskin *et al.*, 1996). The most important environmental and operational factors in the anaerobic systems are listed below.

2.5.1 Temperature

Oscillation in temperature of even a few degrees affects almost all biological activities as the inhibition of some anaerobic microorganisms, especially methanogens. The main reason of this, ionization equilibrium, solubility of substrates, substrate removal rate and other constants such as specific growth rate, decay biomass yield, and half- saturation constant are depends on the temperature. Three different temperature conditions are used for the operation of the anaerobic systems; psycrophilic, mesophilic and thermophilic. However the most common application is mesophilic conditions t temperatures from 25°C to up to 40°C with the optimum at approximately 35°C. Psycrophilic bacteria function between 0-20°C. Thermophilic digestion operates at temperature ranges of 50-65°C. Temperature fluctuations become more important in high loading rates. Also methane production in the thermophilic range is usually performed at industrial wastewater treatment plants that

are able to heat wastewaters or sludges (Gerardi, 2003). Because of the impact of temperature on enzymatic activity, SRT in the system should increase with decreasing temperatures. Although anaerobic microorgansism can be acclimated to operating temperatures out of their optimum range, biomass activity and reactor performance may be adversely affected. Because methanogens grow slowly and are very sensitive to small changes in temperature, acclimation must proceed very slowly.

2.5.2 Alkalinity and pH

Sufficient alkalinity is essential for proper pH control. Alkalinity serves as a buffer that prevents oscillation in pH. pH influences on enzymatic activity and reactor performance. Acceptable enzymatic activity of bacteria occurs above pH 5.0, but acceptable range for methanogens are changed between pH 6.2. and 8.0. However, ph range of 6.8 to 7.2 is optimum for the single-stage reactors (Pohland, 1987; Malina *et al.*, 1992). The pH in the anaerobic reactors initially decreases with the production of volatile acids if the adequate alkalinity is not provided. A decrease in alkalinity can be caused by:

1) an accumulation of organic acids due to the failure of methanogens to convert the organic acids to methane,

2) a slug discharge of organic acids to the anaerobic reactor, or

3) the presence of wastes that inhibit the activity of methanogens.

2.5.3 Nutrients

All microorganisms need to macro and micro-nutrient for basal metabolism and growing under both aerobic and anaerobic conditions. Macro nutrient requirement of anaerobic systems is lower than aerobic systems. Methanogens need trace amounts of elements called as micronutrients besides nitrogen and phosphorus for their fundamental requirements of microbial metabolism (Speece, 1996). Methanogens are needed iron, nickel, magnesium, calcium, sodium, barium, tungstate, molybdate, selenium and cobalt (Henze *et al.*, 1983). Some of the elements such as selenium, tungsten and nickel are important in the enzyme systems of acetogenic and methanogenic microorganisms (Stronach *et al.*, 1986).

2.5.4 Mixing

Anaerobic reactors should be mixed for appropriate conditions. Mixing enhances the process efficiency by distributing microorganisms, substrate, and nutrients throughout the reactor as well as equalizing temperature. Mixing could be energy consuming process but it is applied most of treatment systems.

2.5.5 Retention Time

According to wastewater characteristics, the adequate hydraulic retention time (HRT) should be provided to allow the mineralization of organic compound by microorganisms.

2.5.6 Toxicants

Anaerobic digestion is known as a favourable process for inhibitory or toxic materials that cause disturbances in biological systems. There are many compounds, both organic and inorganic, which may be toxic or inhibitory to the anaerobic process, although the general effect which results from the addition of most substances may vary from stimulation to toxicity. The effective concentration of an inhibitory or toxic substance may vary.

Inhibition of methanogenesis is generally indicated by reduced methane production and increased concentration of volatile acids. Methanogenesis is generally the most sensitive step to inhibitory or toxic material although all groups involved in process can be affected. Bacteria are affected by increasing undesirable environmental conditions. However; methanogens can be acclimatized to these compounds (Speece and Parkin, 1983).

A wide range of toxicants is responsible for the occasional failure of anaerobic reactors such as volatile acids, alkali and alkaline earth salts, ammonia nitrogen, sulfide and heavy metals.

2.5.6.1 Volatile Fatty Acids (VFA) inhibition

The most common inhibition, which restricts the stability of an anaerobic reactor, is known as the accumulation of volatile fatty acids produced by the acidogenic culture. Acetic acid is identified as the least toxic volatile acid in literature whereas propionic acid has often been defined as the major source of system failure in anaerobic

digesters. The maintenance of a balance between the rate of volatile acids production and their conversion to methane is essential factor on the effectiveness of anaerobic digestion.

2.5.6.2 Alkali and alkaline salts inhibition

Salt toxicity is normally associated with the cation rather than the anion of the salt. Excess concentrations of alkali and alkaline earth-metal salts such as Na, K, Ca, or Mg particularly in industrial wastewaters can cause instabilities in the system and are defined as inhibitory.

2.5.6.3 Ammonia-Nitrogen inhibition

Normally, the presence of ammonium bicarbonate resulting from digestion of heavily proteinaceous or urea wastes is beneficial to the digester as a source of nitrogen and as a buffer to changing values of pH.

However, both the ammonium ion (NH_4^+) and dissolved ammonia gas (NH_3) may become inhibitory at high concentrations. These two forms are in equilibrium with each other, the relative concentration of each depending upon the pH as indicated in the Eq. 3.4 below:

$$NH_4^+ \longleftrightarrow NH_3 + H^+ \qquad \textbf{(2.4)}$$

At high hydrogen ion concentration (pH 7.2 or lower), the equilibrium is shifted to the left so the inhibition is related to the ammonium ion concentration. At higher pH levels, the equilibrium shifts to the right and the ammonia gas may become inhibitory.

2.5.6.4 Sulfide inhibition

Sulfide inhibition is a potential problem in anaerobic treatment primarily due to biological production of sulfates and other sulfur-containing inorganic compounds, as well as from anaerobic protein degradation.

Sulfate in the influent of an anaerobic digester particularly inhibits methanogenesis due to both the competition for acetate and production of sulfide from sulfate reduction. As sulfate reduction to produce hydrogen sulfide (H_2S) is energetically favored over methane production, excessive amounts of sulfide are produced by

anaerobic digestion of industrial wastewaters containing high concentrations of sulfates.

Sulfides in anaerobic digesters may distribute between a soluble and insoluble form, and also as gaseous hydrogen sulfide depending upon the digester pH and the quantity of the gas produced per volume of waste. The higher the gas production per unit volume of waste, the higher will be the amount of sulfides driven from solution as a gas.

Heavy metal sulfides are insoluble and precipitate from solution, so they are not harmful to the microorganisms.

2.5.6.5 Heavy metal inhibition

Heavy metals are known as the most common source of inhibition or toxicity in anaerobic systems. They have been a great deal of interest on the inhibition of anaerobic biodegradation since their accumulation in the sludge or solids fraction during the treatment may be detrimental on the enzyme systems of biomass.

The most important feature, which distinguishes them from other toxicants is that they are not biodegradable.

Toxic elements and compounds such as chromium, chromates, nickel, zinc copper, arsenic and cyanides are classified as highly toxic inorganic toxins.

Particularly, the presence of low but soluble concentrations of copper, zinc and nickel is known to be quite toxic and these salts are associated with most of the problems of heavy metal toxicity in anaerobic treatment. The inhibitory levels of these metals have been reports as being 170 mg/L (copper), 163 mg/L (zinc) and 180 mg/L (cadmium). However, trace stimulatory quantities of most of these heavy metals are required for enzymatic activation.

Concentration of more toxic heavy metals which can be tolerated are related to the concentrations of sulfides available to combine with the heavy metals to form the very insoluble sulfide salt.

2.6 Anaerobic Bioreactor Configurations

One of the most critical point in design of the anaerobic systems is selection of the appropriate bioreactor type and configuration to maximize metabolic, nonoxidative bioenergy production. Anaerobic reactors can be classified as low rate or high rate as shown in Figure 2.11.

Low-rate anaerobic reactors are unmixed and temperature, SRT, and other environmental conditions are not regulated. The organic loading rate is low ranges varied between 1 and 2 kg COD/m^3day. These reactor configurations are not suitable for methane production. However, to enhance biogas production some of the anaerobic ponds and lagoons are covered.

High-rate anaerobic systems maintain a very high biomass level in the reactor. Environmental conditions are well regulated to optimize performance of the reactor. The organic loading rates change from 5 to 30 kg COD/m^3day or even higher. High-rate anaerobic reactors are more appropriate for methane production.

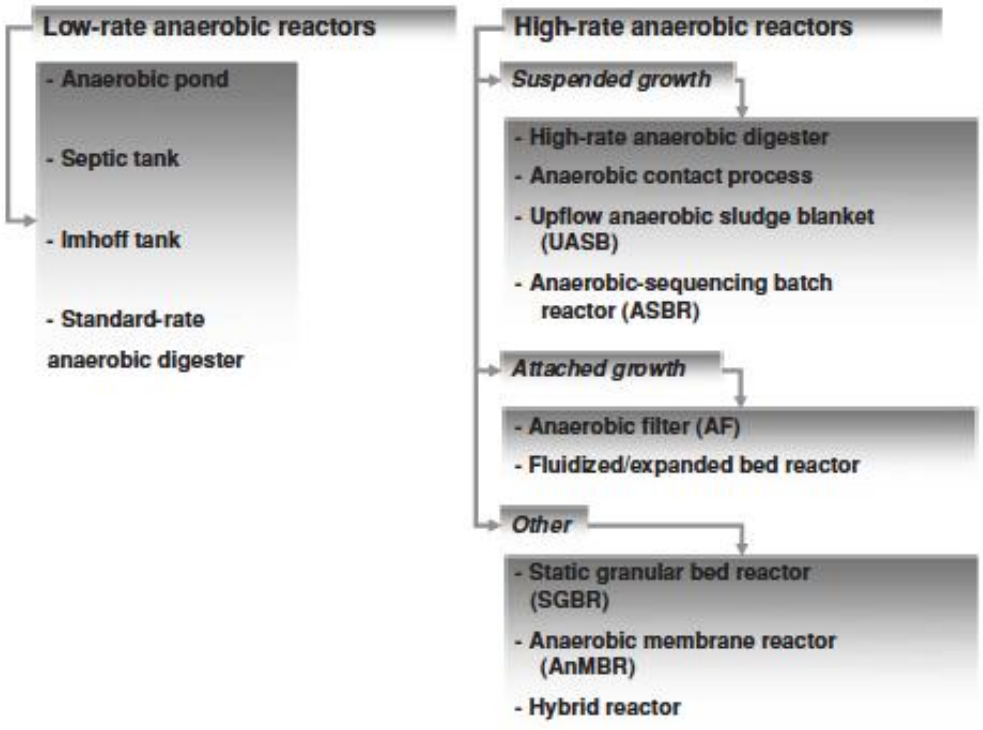

Figure 2.11: Classification of anaerobic reactors. (Khanal 2008).

2.6.1 Anaerobic contact reactors

The anaerobic contact process (ACP), which is essentially a CSTR with an external settling tank to settle biomass with recycling to provide long SRT, is improved to

overcome the disadvantage of the completely mixed digesters, which are among the earliest configurations of anaerobic digestion. Waste streams with high concentrations of particulates and very high concentrations of soluble biodegradable organic materials are suitable for these systems.

Typical reactor biomass concentrations change between 4 and 6 g/L, with maximum concentrations as high as 25–30 g/L, depending on the settleability of sludge. Organic loading rates of 0.5 to 10 kg COD/m^3.day can be applied to the reactor with HRTs of range between 0.5 and 5 days. The required SRT can be maintained by controlling the recycle rate similarly to an activated sludge process.

2.6.2 Upflow anaerobic sludge blanket (UASB) reactors

The upflow anaerobic sludge blanket reactor (UASB) reactor developed to treat sugar-rich soluble wastewater (Lettinga *et al.,* 1991) is characterized by an anaerobic granular sludge with a notably high metabolic activity and good biosolids settleability. This reactor is essentially a suspended growth system in which proper hydraulic and organic loading conditions are maintained

UASB reactors are operated at a superficial upflow velocity less than 2 m/h. Under this condition, an extremely long SRT of 200 days can be achieved at HRT as low as 6 h (Hulshoff Pol *et al.*, 2004). Due to its ability to absorb extremely high VOLR (up to kg COD/m^3.day), UASB is ideal for methane production applications from high-strength wastewaters including soluble compounds (Hulshoff Pol *et al.*, 2004).

In this system, the influent wastewater is uniformly distributed through a specially designed distributor at the bottom of the reactor. The active anaerobic granules in the reaction zone mineralize the organic matter to biogas. Large and dense granules remain suspended within the sludge bed due to superficial upflow velocity and rising biogas bubbles. A gas collection system is used for produced biogas. The effluent is collected in a series of weirs placed at the top of the reactor.

2.6.3 Anaerobic sequencing batch reactors (ASBR)

The anaerobic sequential batch reactor (ASBR) was developed by Dague and coworkers in the early 1990s (Dague *et al.,* 1992). The ASBR system was developed for treatment of high-strength and medium solids content feeds (TS: 1–4%) with high loading rate. Because of sequential operation, a single reactor can be used as a

reaction vessel and as a settling tank, achieving high biomass levels in the reactor regardless of HRT. The ASBR process retains biomass due to bioflocculation followed by biogranulation similar to a UASB reactor.

ASBRs are run by four operational steps: settle, decant, feed, and react as seen in Figure 2.12. The sequencing frequency and the feed volume processed with each sequence determine the hydraulic loading (HRT) and the strength of the wastewater. One of the most variable is also biomass concentration and it directly affects biomass settleability. Initial food-to-microorganism (F/M) ratio has an impact on bioflocculation and biochemical reactions. The optimization and well-operation provide a decrease in the cycle time. Reaction time depends on the intrinsic kinetics of substrate consumption by the biomass and on the mass-transfer rates that must be enhanced by mixing (Bosma *et al.*, 1997). Factors affecting performance of ASBRs have been reported as geometric reactor characteristics (Sung and Dague, 1995), mixing (Brito *et al.*, 1997), F/M ratio (Sung and Dague, 1995; Timur and Ozturk, 1999), and wastewater loading strategy (Ratusznei *et al.*, 2003). Mixing is one of the most important factors affecting ASBR performance and is applied during the reaction step in order to improve the mass transfer rates, increasing the overall organic matter uptake rates. It can be supplied by recirculating the biogas produced during the anaerobic digestion (Sung and Dague, 1995) and liquid recycle or mechanical agitation (Brito *et al.*, 1997). Wastewater loading strategy has also been reported as an important factor in ASBRs. Longer fill cycles has more advantages because of rapidly acidified substrates (Bagley and Brodkorb, 1999). However, the approach should be improved for the treatment of complex wastewaters in the reactors. The variable substrate concentration in the reactor during the sequencing operation results in a variable food-to-microorganism (F/M) ratio in the reactor: high substrate concentrations during and immediately after feeding (high F/M ratio) and low substrate concentrations at the end of the react step (low F/M ratio). The feast and famine conditions can be considered as an advantage for obtaining high removal efficiency. High F/M ratio verifies high substrate utilization rate providing high driving forces for metabolic activity, in accordance with Monod kinetics (Dague *et al.*, 1998). The low F/M ratio occurring at the end of the react step through the end of the decant step is known to stimulate bioflocculation, granulation, efficient biomass settling (Sung and Dague, 1995) and a long SRT.

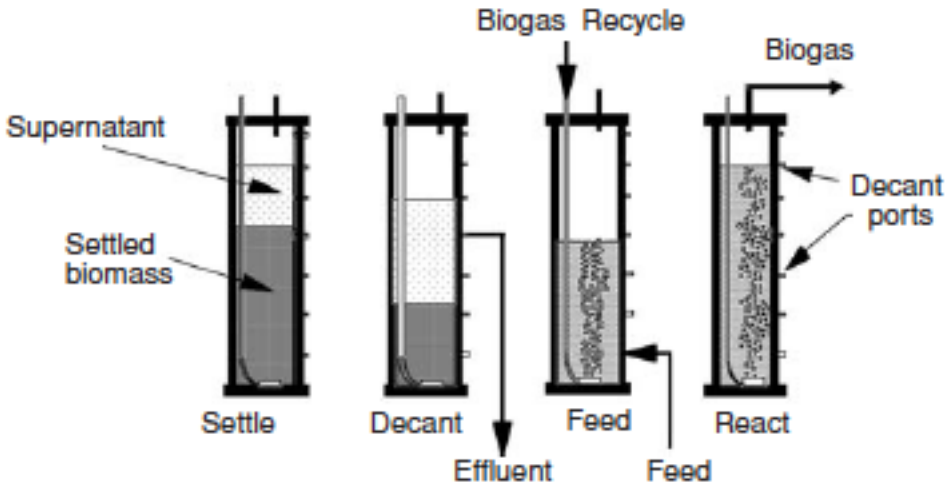

Figure 2.12: Operating steps for the anaerobic-sequencing batch reactor (Dague *et al.*, 1992).

2.6.4 Fixed bed processes

Fixed bed processes include a flooded bed of inert filter medium, which is used for the increase of biomass concentrations required for efficient anaerobic treatment of wastewaters. While wastewater is passing through the medium, soluble organic compounds diffuse in surfaces of the attached biomass where the organics are mineralized to methane and carbondioxide. Fixed bed processes can be used for almost all types of industrial wastewaters with low (COD<1000 mg/L) to intermediate (COD>20000 mg/L) concentrations.

2.6.5 Anaerobic expanded/fluidized bed processes

An expanded granular sludge bed (EGSB) reactor is derived from UASB concept (Kato, 1994). It was designed according to a faster rate of upward-flow velocity for the wastewater passing through the sludge bed. The use of effluent recirculation in a UASB (or a high height/diameter ratio) results in the EGSB reactor (Seghezzo *et al.*, 1998). The higher upflow liquid velocity keeps in the granular sludge bed under an expanded condition (Zoutberg and Frankin, 1996). The increased flux permits partial expansion (fluidization) of the granular sludge bed to enhance wastewater-microorganism contact as well as improving segregation of small inactive suspended particle from the sludge bed. The increased flow velocity is either accomplished by utilizing tall reactors, or by incorporating an effluent recycle (or both). The EGSB

design is appropriate for low strength soluble wastewaters (less than 1 to 2 g soluble COD/L) or for wastewaters that contain inert or poorly biodegradable suspended particles, which should not be allowed to accumulate in the sludge bed.

2.6.6 Anaerobic membrane bioreactor

The anaerobic membrane bioreactor (AnMBR) includes a membrane unit integrated to the reactor or in an external loop to aid in solid–liquid separation. An AnMBR is capable of keeping biomass and thus can be operated at an extremely long SRT regardless of HRT. The most noticeable advantage of this technology is the reductions in costs in recent years (Visvanathan *et al.* 2000), membranes have great potential in anaerobic biotechnology for renewable energy generation.

This system provides an advantage to treatment of the wastewater with high particulate matter (such as stillage from biofuel plants, sludge, and food waste), which traditionally has been digested in a CSTR. Integration of a membrane may overcome the drawbacks of CSTR such as increasing SRT.

3. MATERIALS AND METHODS

3.1 Experimental Approach

This thesis involved acute and chronic anaerobic tests with microbiological analysis to evaluate the inhibitory effect of antibiotics in terms of conventional parameters and cellular activities. For this aim, firstly a stock anaerobic sequencing batch (ASBR) reactor was operated to acclimate the biomass.

In the second step, a series of batch reactors seeded with acclimated microbial culture were set-up to determine the short-term effect of the antibiotics. Each batch reactor was also inoculated with a different concentration of the selected antibiotics in the range of 1 – 1000 mg/L tested in the experiments. Batch reactors were kept running for an observation period of six days for assessing short-term inhibitory impact. COD removal was monitored using both soluble COD and VFA measurements at the beginning and at the end of the observation period. Biogas production and methane generation were monitored daily throughout the experiment.

In the third step, four different ASBRs were set-up and operated to evaluate the long-term effects of the antibiotics. The reactors were operated at the 50-day SRT and 2.8-day HRT. Influent and effluent COD, VFA, biogas production and composition, pH, temperature, SS and VSS were monitored daily. Biomass was taken in different antibiotic feeding phases to determine the methanogenic activity of the sludge.

Additionally microbial characterization of the seed by cloning-sequencing was done and the bacterial and archaeal clone libraries were compared to the sludges which were collected from different antibiotic feeding regimes. All samples including seed sludge were quantified by Q-PCR. Also methanogenic sub-groups was determined by FISH. DNA and RNA based approaches were applied to monitor the microbial community changes during long-term inhibition experiment by DGGE.

Finally, mRNA level expression of selected enzymes was determined to evaluate the long-term effects of selected antibiotic within the cells.

3.2 Bioreactor Operations

In the scope of this study, a variety of reactor was operated. The characteristics of the reactors and related information are explained in detail below.

3.2.1 Laboratory scale anaerobic sequencing batch reactor (stock reactor)

An anaerobic sequencing batch reactor (ASBR) that has 8 L total volume was set-up and operated at 35 ^{0}C. The reactor was operated with a 24-hour cycle consisting of fill (10 min), react (23 h), settle (45 min) and decant (5 min). The rector was mixed using a magnetic stirrer at 90 rpm. The system was inoculated by an anaerobic sludge taken from a mesophilic anaerobic contact reactor treating the wastewater of raki (alcohol) and fresh grape alcohol. The MLVSS concentration of the reactor was 7000 mg/L. The reactor was fed by 4500 mg/L of COD equivalent substrate mixture. The organic compounds and concentration of model wastewater was given in Table 3.1. Trace element solution as mg/L ($FeCl_2.4H_2O$, 2; $CoCl_2.6H_2O$, 2; $MnCl_2$, 0.32; $CuCl_2$, 0.024; $ZnCl_2$, 0.05; H_3BO_3, 0.05; $(NH_4)Mo_7O_{24}.4H_2O$, 0.09; Na_2SeO_3, 0.068; $NiCl_2.6H_2O$, 0.05; EDTA, 1; resazurine, 0.5; HCl (36%) 0.001 mL), vitamin as mg/L (4-aminobenzoic acid, 0.04; D(+)-biotin, 0.01; nicotinic acid, 0.1; calcium D(+)-pantothenate 0.05; pyroxidine dihydrochloride, 0.15; thiamine, 0.1 in NaP buffer (10 mM, pH 7.1) and 0.05 mg/L B12 solution were added to the wastewater. The pH of the model wastewater was adjusted 7 and the pH in the reactor varied from 6.8 to 7.2.

The reactor was operated with an organic loading rate (OLR) 1400 mg/L*d for the first 10 days of operation and then increased to 4500 mg/L*d in a stepwise manner. The solid retention time was approximately 15 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 2.8 days.

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

Table 3.1: Organic components and concentrations of model wastewater.

Components	COD (mg/L)
Starch	2090
Glucose	1350
NaAcetate	240
NaButyrate	330
NaPropionate	490
Total COD	4500

3.2.2 Laboratory scale anaerobic batch reactors (acute tests)

Acute tests were constructed as batch studies based on specific methanogenic activity (SMA) principles in anaerobic cabinet. Laboratory bottles (total volume 120 mL) sealed with butyl rubber septa were used as reactors. The liquid volume as well as the headspace volume was 60 mL. Biomass was taken from acclimated anaerobic sludge of lab scale ASBR. 2000 mg/L VSS were added to the serum bottles. Acute effects of antimicrobials on the biomass were tested in two different substrate sets as acetate and volatile fatty acid (VFA) mixture. Acetate and VFA concentrations in a range (range: 1000-5000 mg/L) were initially tested in order to reach maximum potential methane production (PMP) rate during the batch tests. Among those 4000 mg/L of acetate concentration and 3000 mg/L of each VFA concentration (acetate, butyrate and propionate) were found to be optimum. Antibiotic concentrations (Sulfamethoxazole-SMX, Tetracycline-TET, Erythromycin-ERY; range: 1, 10, 25, 50, 100, 250, 500, 750 and 1000 mg/L) were maintained within the batch reactors for acute studies in duplicate bottles. In addition to these test tubes, two control bottles, which only included acetate or VFA mixture, were prepared. During 6 days (test duration), the bottles were stored at 35±2 ^{0}C and shaken daily by hand. Headspace pressure was measured every day by hand-held pressure transducer (Lutron PM-9107, U.S.A.). The percentage inhibition of biogas production was calculated from the amount produced in the respective test and control bottles.

3.2.3 Laboratory scale anaerobic sequencing batch reactors (chronic tests)

An anaerobic sequencing batch reactor (ASBR) that has 1 L total volume was set-up and operated at 35 ^{0}C. The reactor was operated with a 24-hour cycle consisting of fill (10 min), react (23 h), settle (45 min) and decant (5 min). The rector was mixed using a magnetic stirrer at 90 rpm. The system was inoculated by an anaerobic sludge taken from the stock reactor treating model wastewater given the components in Table 3.2.

The MLVSS concentration of the reactor was 5000 mg/L. The reactor was fed by 2250 mg/L COD equivalent substrate mixture. The organic compounds and concentration of model wastewater was given in Table 3.2. Trace element solution which is adapted from a previous study (Amin *et al.*, 2006) as mg/L ($FeCl_2.4H_2O$, 2; $CoCl_2.6H_2O$, 2; $MnCl_2$, 0.32; $CuCl_2$, 0.024; $ZnCl_2$, 0.05; H_3BO_3, 0.05; $(NH_4)Mo_7O_{24}.4H_2O$, 0.09; Na_2SeO_3, 0.068; $NiCl_2.6H_2O$, 0.05; EDTA, 1; resazurine, 0.5; HCl (36%) 0.001 mL), vitamin as mg/L (4-aminobenzoic acid, 0.04; D(+)-biotin, 0.01; nicotinic acid, 0.1; calcium D(+)-pantothenate 0.05; pyroxidine dihydrochloride, 0.15; thiamine, 0.1 in NaP buffer (10 mM, pH 7.1) and 0.05 mg/L B12 solution were added to the wastewater. The pH of the model wastewater was adjusted 7 and the pH in the reactor varied from 6.8 to 7.2.

The reactor was operated with an organic loading rate (OLR) 1.4 g/L*d for the first 10 days of operation and then increased to 2.25 g/L*d in a stepwise manner. After 78^{th} day, 1 mg/L of antibiotic addition was started. While SMX concnetration was increased up to 45 mg/L in a stepwisemanner, ERY and TET was fed up to 3 mg/L during the operation. The solid retention time was approximately 50 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 of the reactors was 2.8 days.

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

Table 3.2: Organic components and concentrations of model wastewater.

Components	COD (mg/L)
Starch	1045
Glucose	675
NaAcetate	120
NaButyrate	165
NaPropionate	245
Total COD	2250

3.3 Seed Sludge

Seed was obtained from a full-scale UASB reactor (with a volume of 490 m^3) being the first stage of a two-stage anaerobic-aerobic biological treatment plant at 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 58000 mg/L and 44000 mg/L, respectively.

3.4 Analytical Methods

Biogas production by the ASBRs was monitored using Milligas Counter (Ritter Digital Counter, U.S.A.). The methane content of the in the biogas and VFA concentrations were measured using gas chromatograph (Perichrom, France and Agilent Technologies 6890N, USA, respectively). Suspended solids (SS), volatile suspended solids (VSS), total suspended solids (TS), total volatile suspended solids (TVS) and soluble COD were determined according to Standard Methods (APHA, 2005).

3.5 Specific Activity Test

Methanogenic activity tests were performed using the pressure transducer technique (Colleran *et al.*, 1992). The pressure increase in sealed vials fed with non-gaseous substrates such as acetate, proponate, butyrate was monitored. The hand-held pressure transducer (Lutron PM-9107, U.S.A.) was capable of measuring a pressure in a range of 5 to 7000 mbar, corresponding to 0.01 mol biogas in 60 mL headspace. The liquid volume as well as the headspace volume was 60 mL. 2000 mg/L VSS were added to the serum bottles. Acetate and VFA mixture (acetate, butyrate, propionate) concentrations in a range of 1000-5000 mg/L were initially tested in order to reach maximum potential methane production (PMP) rate during the batch tests. Among those 4000 mg/L of acetate concentration and 3000 mg/L of VFA concentration were found to be optimum. The basal medium in the batch experiments was prepared based on OECD311 protocol under strict anaerobic conditions (2006). During the 6-day test duration, the bottles were stored at 35±2 °C and shaken daily by hand. Headspace pressure was measured every day by hand-held pressure transducer.

3.6 Antibiotic Measurement

3.6.1 Sludge

3.6.1.1 Method optimization

The extraction procedure of the sludge samples consists of two steps:

1- extraction of the antibiotics by sonication

2- clean-up of the extracts by SPE.

For the method development, three different methods were examined to obtain the highest recovery as given in Table 3.3.

Table 3.3: Extraction protocoles and recoveries.

				Recoveries %		
Method No	**Extraction**	**SPE**	**Reference**	**SMX**	**ERY**	**TET**
1	5% (w/v) sodium acetate, 100 mM EDTA in a 50:50 solution with methanol, adjusted to pH 8 with sodium hydroxide	OASIS HLB, 6mL, 200 mg cartridge was used.	O'Connor *et al.*, 2007	107.8±25.7	133.7±40.0	188.1±63.8
2	5% (w/v) sodium acetate, 100 mM EDTA in a 50:50 solution with methanol, adjusted to pH 8 with sodium hydroxide	Prior to SPE, all extracts were acidified by HCl. OASIS HLB, 6mL, 200 mg cartridge was used.	O'Connor *et al.*, 2007 and this study	63.8±29.2	N.D.	117.2±29.0
3	0.2 M citric acid buffer pH4:acetonitrile	Prior to SPE, 0.2 g of Na2EDTA was added into each sample. OASIS HLB, 6mL, 200 mg cartridge was used.	Yang *et al.*, 2010	89.5±11.3	89.0±20.4	202.8±45.9

According to the recovery results, Method 1 was selected for SMX and ERY samples and Method 2 was selected for TET samples.

3.6.1.2 Extraction

20 mg of freeze-dried sludge samples were weighted in 15 mL centrifuge tubes and 10 mL of the extraction buffer was added to each tube. The tubes were sonicated for 15 min and then they were centrifuged at 1370Xg at 25ºC during 10 min. The supernatants were transffered to 60 mL glass tubes. The extraction protocol was performed 3 times in each sample and obtained supernatants were evaporated at 25ºC under nitrogen stream to remove the organic solvent before clean-up step.

3.6.1.3 Clean-up

Before clean-up step, evaporated samples were diluted by MilliQ water to 500 mL and filtered. For the clean-up, OASIS HLB cartridges (6mL, 200 mg, Waters, USA) were used. The cartridges were conditioned by 5 mL methanol followed by 5 mL HPLC grade water. The samples were loaded to cartridges at a rate of approximately 1 mL/min. The cartridges were washed by 10 mL HPLC grade water and then dried by vacuum during 30 min. The samples were eluted by 6 mL of methanol. The extracts were exaporated less than 50 μL under nitrogen streams and then reconstituted to 1 mL with 50:50 methanol:water mixture. Before analysis, 10 ppb of a internal standard mixture (sulfamethoxazole D6, roxithromycin and chlorotetracycline) was added. The concentratin of antibiotics in the samples was quantified by internal standard calibration curve, in order to correct for possible matrix effects.

3.6.2 Wastewater

Wastewater samples were filtered and diluted by 50:50 methanol:water mixture according to their concentration in the reactors. Antibiotics were quantified by external standard calibration.

3.6.3 Instrumental analysis

Chromatographic separation was achieved using a Waters Acquity Ultra-Performance™ liquid chromatograph system, equipped with two binary pumps system (Milford, MA, USA) using an Acquity BEH T_3 colum (50mm x 2.1mm i.d., 1.7 μm particle size). Compounds were analyzed under positiove ionization mode. The optimized separation conditions were as follows: solvent (A) acetonitrile, solvent (B) water with 0.1% formic acid at a flow rate of 0.5mL/min. The gradient elution

was: initial conditions 5% A; 0–3 min, 70%A; 3.0–3.5 min, 100% A; 3.5–5.0 min, 100% A; from 5.0 to 5.1 return to initial conditions; 5.1 to 6, equilibration of the column.

The sample volume injected was 5µL. The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Target antibiotics were quantified from the samples by MRM mode. MRM transitions for antibiotics and internal standards used in the quantification of sludge samples are indicated in Table 3.4.

Settings for source-dependent parameters were as follows: curtain gas (CUR), 30V; nitrogen collision gas (CAD) medium; source temperature (TEM) was 650ºC; ion spray voltage was 5500 V; ion source gases GS1 and GS2 were set 60 and 50V, respectively.

Table 3.4: Target Compounds and Their Optimized QqLIT-MS/MS Parameters by SRM Negative and Positive Ionization Mode.

Compound	SRM 1	DP-CE-CXP	SRM2	DP-CE-CXP
Sulfamethoxazole	253.992>156.100	81-23-12	253.992>92	81-37-12
Sulfamethoxazole D6 (IS)	257.943>160.000	101-23-18		
Erithromycin	734.299>158.100	116-39-14	734.299>576.400	116-27-22
Roxithromycin (IS)	837.377>679.4	91-31-26		
Tetracycline	445.000>410.000	101-27-14	445.000>154.000	76-37-18
Chlorotetracycline (IS)	479.043>444.100	51-31-18		

3.7 Microbial Characterization and Enzyme Expression Level

Triplicate samples were collected from seed and reactor sludges and then GDNA and total RNA isolation were applied for further molecular analysis.

3.7.1. Genomic DNA (GDNA) extraction

Genomic DNAs were extracted from 1-mL sludge samples using FastDNA Spin Kit for Soil (Qbiogene Inc., U.K.) following the manufacturer's instructions. Extracted

GDNA concentrations were determined Qubit 2.0 Flouremeter (Invitrogen, UK) and diluted to 25 ng/µl by DNase free water. The extracted GDNAs were stored at -20 ºC until the further analysis.

3.7.2 Total RNA isolation and cDNA synthesis

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

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

3.7.3 PCR amplification of 16S rDNA

Amplication of bacterial and archaeal 16S rDNA was performed by PCR using specific primers. The primers and annealing temperatures are given in Table 3.5.

Amplification was occurred in a 50-µl reaction volume including 50 ng of DNA, 10 pmol of each primer, 10 mM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 5 µl of 10×*Taq* buffer and 4 U of *Taq* DNA polymerase (iTaq, U.K.). Nested PCR approach was applied to enhance specifity in archaeal 16S rDNA amplification. For the second-round nested amplification 1 µl of the first-round product was used as template, with reaction composition being the same as previously. PCR amplification was performed in a Techne TC-5000 thermal cycler (Barloworld Scientific Ltd., U.K.) with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min and extension at 72°C for 2 min and a final extension at 72°C for 10 min. PCR products were visualized by electrophoresis (Thermo-Scientific Ltd., U.K.) on a 1% (w/v) agarose gel in 1× Tris–borate–EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 7 V cm^{-1} and gel images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after staining with ethidium bromide.

Table 3.5: Primers using in PCR amplification and their annealing temperatures.

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

[1]*Escherichia coli* numbering.
[2]5′-GC clamp on Arch344f and Bact341f (GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGG).

3.7.4 Cloning, sequencing and phylogenetic analysis

The bacterial and archaeal PCR products of the seed sludge of acute and chronic 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 PureLink PCR Purification Kit (Invitrogen, UK). To select the positive ones, 200 clones were collected from each clone library and colony PCR were applied using the vector-specific primers M13f and M13r. Bact341f_GC- Bact534r nad Arch344f_GC-Univ522r primers were used to re-amplify positive bacterial and archaeal inserts, respectively. The re-amplified PCR products were loaded to DGGE gels 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 FASTA (Pearson, 1988) 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 $1 - (n_1/N)$, where n_1 is the number of clone types that was encountered only once in the library and N is the total number of clones analyzed (Good 1953).

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

3.7.5 Denaturing gradient gel electrophoresis (DGGE)

Existing and active community profiles of the sludges were determined by DGGE analysis as described by Muyzer *et al.* (1993). The bacterial and archaeal PCR products obtained from GDNAs and cDNAs by Bact341f_GC-Bact534r and Arch344f_GC-Arch855, respectively.

PCR product (10 μl) was mixed in equal volumes with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll in water) and run on a 10% polyacrylamide gel (acrylamide–*N*,*N*'-methylenebisacrylamide ratio, 37.5:1) in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) over a chemical denaturing gradient of urea and formamide equivalent to 30–60% denaturant (100% denaturant is 7 M urea and 40% (v/v) formamide). To aid the conversion and normalization of gels, a marker consisting of 16S rDNA mix from archaeal and

bacterial clone libraries was added on the outside of the gel as well as after every four samples. Electrophoresis was performed using the D-Code system (Bio-Rad Laboratories, Ltd., UK) at 200 V constant current at 60°C, for 4.5 h. Gel images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after stained with SybrGold (1:10000 diluted; Molecular Probes Inc., UK) according to the supplier's instructions.

Images were converted, normalized and analyzed by using the Bionumerics 5.0 software (Applied Maths, Belgium), and data were exported to excel and used for numerical analysis with Minitab (Minitab Inc.) and Past (http://folk.uio.no/ohammer/past/) softwares. Similarities between tracks were calculated by Pearson product-moment correlation coefficients (*r*) and UPGMA clustering.

3.7.6 Quantitative real time polymerase chain reaction (Q-PCR)

3 primer sets targeting the bacteria, archaea and methanogens were used to quantify existing and active microbial community by using the template extracted GDNAs and synthesized cDNA, respectively. Furthermore 3 primer sets targeting metabolic genes encoding formylterahydrofolate synthetase (FTHFS), methyl-coenzyme M reductase (mcrA) and acetyl-coA synthetase (ACAS) were also used to determine the inhibition level on mRNA expression of the homoacetogenesis, methanogenesis and specifically acetoclastic methanogenesis, respectively. All primers using Q-PCR analysis were given in Table 3.6.

The procedure recommended by Roche was followed and a Light Cycler Master Kit (Roche, Applied Science, Switzerland) was used to set up the reaction (2.0 µl master mix, 1.6 µl MgCl2 1.0 µl Primer F and R, 13.4 µl H2O, 1 µl sample). Absolute quantification analysis of the GDNA was carried out with a LightCycler 480 Instrument (Roche Applied Science, Switzerland). The amplification protocol was as follows: initial denaturation for 10 min at 94 ºC followed by 45 cycles of 10 s at 94 ºC, 5 s at specific annealing temperature as given in Table 5.5, 16 s at 72 ºC. The standard curves for Q-PCR.

Table 3.6: Q-PCR primers

Primer Sets	Target	Annealing (°C)	Reference
Bac519f- Bac907r	16S rDNA	53	Lane, 1991
Arc 349f- Arc 806r	16S rDNA	60	Takai, 2000
Met 348f- Met 786r	16S rDNA	55	Sawayama, 2006
fhs_f fhs_r	FTHFS	53	Lovell and Leaphart, 2005
mcrA_f mcrA_r	mcrA	51	Luton *et al.*, 2002
MSaeta_Aco-A _f MSaeta_Aco-A _r	ACAS	56	Ince *et al.*, 2011

Standard curves for Q-PCR constructed from clones of PCR products. PCR products that are to be sequenced were purified by ethanol precipitation and then were sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA) using primer MSaeta_Aco-A _f. After the primer specificity was confirmed by the sequence analysis, these amplicons were used as standards. Dilution series of the purified PCR product were used as calibration standards for real time PCR quantification after their DNA concentrations were determined by the fluorometer (Qubit, Invitrogen, Carlsband, CA, USA). Standard curves were constructed in each PCR run and the copy numbers of the genes in each sample were interpolated using these standard curves.

For each PCR run with SYBR Green I detection, a melting curve analysis was performed to confirm the specificity in each reaction tube by the absence of primer dimers and other nonspecific products. Reactions for all samples were shown to have only one melting peak, which indicated a specific amplification making it suitable for accurate quantification.

3.7.7 Fluorescence in situ hybridization (FISH)

Relative quantification in terms of bacteria, archaea and methanogenic sub-groups were done by FISH. Sludge samples were transferred into sterile containers with the addition of absolute ethanol (1:1, v/v) on-site. Samples were transferred to the laboratory in cool-boxes maintained at 4°C or less. Upon arrival, 1mL aliquots of the sample were fixed in freshly prepared 4% paraformaldehyde (PFA) in PBS (130 mM NaCl, 10 mM sodium phosphate, pH 7.2) for at least 3 hours at 4°C. After fixation,

cells were washed once with PBS, resuspended in PBS-absolute ethanol (1:1, v/v) and stored at -20°C (Harmsen *et. al.*, 1996). 16S rRNA-targeted oligonucleotide probes were used for identification of microbial domain and groups were given in Table 3.7.

10μl of 100 times dilution of the fixed sample which resulted in between about 50 and 300 cells per field of view was spotted onto a gelatin-coated slide and air dried. Cells were dehydrated at room temperature in increasing concentrations of ethanol (50%, 80% and 98%). Dehydrated cells were prehybridized in hybridization buffer (0.9 mol l^{-1} NaCl, 2 mg ml^{-1} Ficoll, 2 mg ml^{-1} Bovine Serum Albumin, 2 mg ml^{-1} polyvinyl pyrrolidone, 5 mmol l^{-1} EDTA, pH 8.0, 25 mmol l^{-1} NaH_2PO_4, pH 7.0, 0.1% SDS, 5-35% deionised formamide) at the intended hybridisation temperature for 20 min (Amann *et al.*, 1990; Manz *et al.*, 1992). After prehybridisation, probe at a final concentration of 5 ng μl^{-1} was added into the hybridization buffer and incubated at the optimal hybridisation temperature for 3 hours. Following hybridization, 2 μl of 4',6-diamidino-2-phenylindole (DAPI) DNA stain (final concentration of 3.3 μg/ml) was added into the hybridization buffer and incubated at room temperature for 10 minutes. The cells were washed twice in wash buffer containing 20 mmol l^{-1} Tris-HCl (pH 7.2), 0.01% SDS, 5 mmol l^{-1} EDTA and between 0.9 mol l^{-1} and 56 mmol l^{-1} NaCl according to the formula of Lathe (1985) for 15 min at the optimal washing temperature before a final wash with deionized water. Slides were air dried and one drop of Citifluor antifadent (Citifluor Ltd., U.K.) was added to the sample. Slides were examined under Olympus BX 50 Epifluorescence Microscope equipped with a 100 W high-pressure mercury lamp and U-MWIB/U-MWG filter cubes. Images were captured using a Spot RT charged coupled device (CCD) camera using the software supplied by the camera manufacturer (Diagnostic Instruments Ltd., UK). The images were processed and analyzed using Image-Pro Plus version 5.1 image analysis software (Media Cybernetics, U.S.A.). For all times, triplicate samples were collected from the reactor. Counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated.

Table 3.7: Oligonucleotide probes.

Probe	Target Group	Probe sequence (5'-3')	Labelling (5')	Reference
EUBMIX	*Bacteria*	GCTGCCTCCCGTAGGAGT	Fluorescein	Amann *et al.*,1990
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	CY3	Stahl *et al.*, 1988
MC1109	*Methanocaccaceae*	GCAACATAGGGCACGGGTCT	CY3	Raskin *et al.*, 1994
MB310	*Methanobacteriaceae*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin *et al.*, 1994
MG1200	*Methanomicrobiaceae*	CGGATAATTCGGGGCATGCTG	CY3	Raskin *et al.*, 1994
MS821	*Methanosarcina*	CGCCATGCCTGACACCTAGGCCAGC	CY3	Raskin *et al.*, 1994
MX825	*Methanosaeta spp.*	TCGCACCGTGGCCGACACCTAGC	CY3	Raskin *et al.*, 1994
NON338	Non sense probe	ACTCCTACGGCAGGCAGC	TAMRA	Manz *et al.*, 1992

3.8 Statistical Analysis

To determine the significant inhibition of the acute and chronic tests, gas production rate of the batch tests and COD removal efficiencies of the ASBRs were compared using ONE WAY ANOVA test, which was followed by running a Post-hoc Dunnett's test and student's T-test, respectively. Graphpad Prism 4 software was used for all statistical analysis.

4. RESULTS AND DISCUSSION

In the first section of the results and discussion part (Section 4.1), stock anaerobic sequencing batch reactor operation data were represented in terms of conventional operation parameters. The seed sludge obtained from an alcohol distillery industry was acclimated to a defined synthetic wastewater, which was also used in the other steps of the study.

In the section 4.2, the data about the characterization of seed sludges were represented. Two different seed sludges were used in this study. One of them was taken from an alcohol distillery industry and used for the inoculation of stock ASBR, which was explained in the previous section. The second seed was obtained from the stock ASBR and used for inoculation of the following experiments; acute and chronic tests. The second seed sludge was investigated in terms of microbiological approaches and specific activity tests. The first one was only investigated by molecular tools to determine the microbial shifts during the operation of stock ASBR.

In the section 4.3, the acute effects of the selected antibiotics were discussed. The short-term effects of the compounds on only acetoclastic methanogens and homoacetogens with methanogens were investigated in terms of biogas production, biogas composition, COD and VFAs removal efficiencies.

In the last section (4.4), the chronic effects and/or biodegradability characteristics of the antibiotics were particularly discussed. To investigate the long-term effects, conventional engineering approaches were used together analytical measurement of antibiotics and DNA and RNA based molecular tools.

4.1 Stock Anaerobic Sequencing Batch Reactor

4.1.1 Performance of the reactor

The ASBR that served as the stock reactor for biomass seeding was operated at an organic loading rate (OLR) 4.5 g COD/L by a defined synthetic wastewater including starch, glucose, butyrate, propionate and acetate. The aim of using these substrates

was set to work in each stage of anaerobic treatment to investigate specific inhibition step. Starch, glucose, butyrate, propionate and acetate were used to run hydrolysis, fermentation, acetogenesis and methanogenesis steps (Speece, 1996, Batstone *et al.*, 2002).

The reactor was operated with 2.8-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 1400 mg/L COD to 4400 mg/L COD. At steady-state, it exhibited a stable performance, with an average effluent soluble COD of 285±75 mg/L corresponding to a COD removal of around 93% and an average biogas production of 1.79±0.4 L/d as seen in Figure 4.1 and 4.2. During this period, the methane content in the biogas averaged 75±1.4%. This corresponds to an average specific methane production of 0.32 L methane per g COD removed, which is consistent with previously reported values (Tchobanoglous *et al.*, 2003).

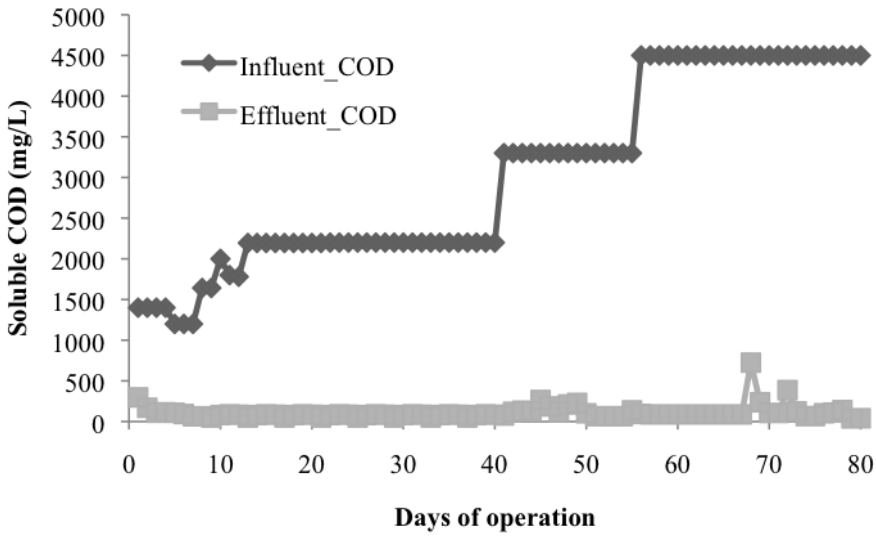

Figure 4.1: Stock ASBR performance in terms of influent and and effluent soluble COD concentration.

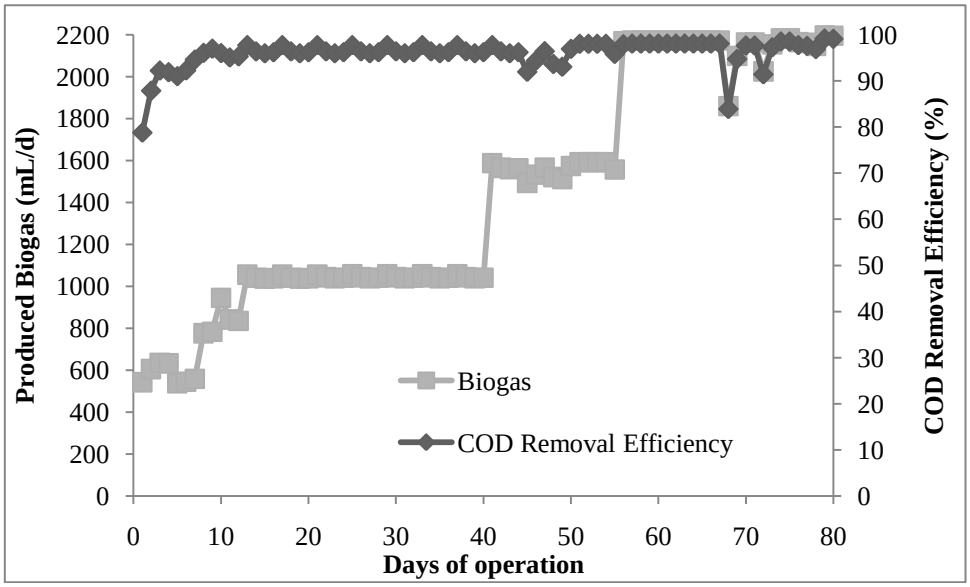

Figure 4.2: Stock ASBR performance in terms of biogas production and COD removal efficiency.

4.2 Characterization of Seed Sludge

Seed sludge which was used in acute and chronic tests were characterized in terms of poteantial methane production capacity and dominant microbial spacies. 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. DGGE analysis was applied to compare the detected microbial species by clone libraries to all OTUs in the sludge samples. Additionally, bacterial, archaeal and methanogenis groups were quantified by Q-PCR and FISH.

4.2.1 Specific methanogenic activity (SMA) test

To determine the potential acetoclastic methanogenic activity of the seed sludge, acetate concentrations between 1000-5000 mg/L were tested by SMA test. Also VFA mixtures (butyrate:propionate:acetate) which the concentrations varied between 1000 to 5000 mg/L were applied to the seed sludge to measure the maximum methane production capacity. 4000 mg/L of acetate and 3000 mg/L of VFA mixture were found as optimum concentrations for the next analysis.

4.2.2 Bacterial and archaeal 16S rDNA clone libraries

To identify the microbial community of seed sludge and to explore the microorganisms which associated with degradation and/or having resistance of the chosen antibiotics in the following steps, bacterial and archaeal 16S rDNA gene clone libraries were constructed from the seed sludge of acute and chronic tests and phylogenetic analysis performed according to sequencing results. Additionally, the clone libraries were compared with DGGE profiles of not only seed of acute and chronic test but also seed of the stock ASBR.

All clones were screened by DGGE and this analysis showed that 22 different OTUs were obtained from 120 bacterial clones and 25 different OTUs were found in 140 archaeal clones. Bacterial and archaeal coverages of the clone librariers were calculated as 91.6% and 97.1%, respectively. All different OTUs were sequenced and the closest relatives of the sequencing results of bacterial and archaeal were given in Table 4.1 and 4.4, respectively.

The majority of sequences obtained from the bacterial clone library represented Firmicutes (61%) as seen in Figure 4.3. The second most abundant phylum was Bacteroidetes (21%). Members of *Verrucomicrobia*, unclassified bacteria, *Chloroflexi*, *Actinobacter* and *Proteobacteria* were distrubeted as 6%, 6%, 4%, 1% and 1%, respectively. 57% of Firmicutes members belonged to *Clostridium sp.*, which are sulfide reducing bacteria and found in different places.

The dominant Firmicutes OTU, B9 (FR836443), related cluster, represented by 33.64% of all bacterial clones was most closely related to the *Clostridium sp. Kas107-1* which was isolated from gramineous plants' root as a member of anaerobic N_2 fixing consortium (Minamisawa *et al.*, 2004). Other close relatives of Firmicutes phylum were mosty isolated and/or identified from different anaerobic reactors in which domestical wastewater sludge, manure and slaughter house wastes, brewery industry wastewater and synthetical wastewaters were treated (Briones *et al.*, 2007; Sousa *et al.*, 2007; Riviere *et al.*, 2009; Bagge *et al.*, 2010). Other OTUs related to Firmicutes phylum mostly closed to isolates from soil, digesting system of mouse and cleaning room (Rawls *et al.*, 2006; Rui *et al.*, 2009; Probst *et al.*, 2010). The second abundant phylum was Bacteroidetes and this phylum was represented by three OTUs. The dominant OTU was B7 (FR836441) and it represented 14.95% of all bacterial

clones. It is most closely related (97%) to an unclassified *Porphyromonadaceae* sequnce obtained from an anaerobic bioreactor treating cassava pulp and pig manure (Panichnumsin *et al.*, 2009). Also second dominant OTU in Bacteroidetes phylum which has members using sugars or proteins for producing acetate ans succinate (Madigan *et al.*, 2009), B14 (FR836448) in this study was also most closely related (97%) to an uncultured *Paludibacter* clone obtained from same study. The third and last OTU, B19 (FR836453) in the Bacteroidetes clustal related (92%) to a culture species, *Paludibacter propionicigenes*, which was isolated from rice paddy soil (Akasaka *et al.*, 2003).

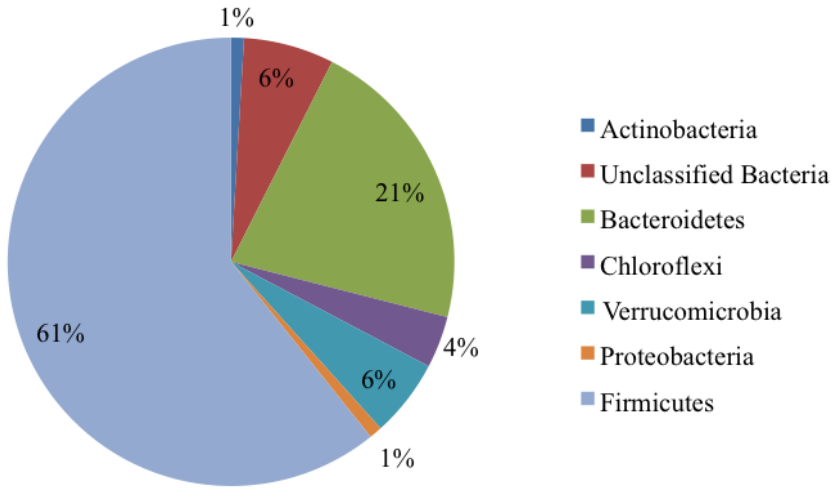

Figure 4.3: Distribution of phylogentic phylums from clone sequences of bacterial 16S rDNA clone library.

OTUs including the members of Actinobacteria which are responsible to produce of propionic acid, acetic acid and CO_2 (Madigan *et al.*, 2009); Verrumicrobia which are globally distributed and soil bacteria (Hedlund *et al.*, 1997; Hugenholtz *et al.*, 1998; Floyd, *et al.*; 2005*); Proteobacteria*; *Chloroflexi* which are formerly known as the green nonsulphur bacteria, are associated with extreme habitats, e.g. microbial mats in hot springs (Boomer *et al.*, 2002; Nubel *et al.*, 2002) and hypersaline environments (Nubel *et al.*, 2001), are known as (filamentous) anoxygenic photothophs (Hanada *et al.*, 2002; Hanada & Pierson, 2002; Nubel *et al.*, 2002); and unclassified Bacteria phylums constituted the small clusters. The closest cultured species of these OTUs are given in Table 1. The closest relatives of tho OTUs which are the members of these phylums were firstly isolated and/or obtained from different ecosystems such as anaerobic reactors, groundwater, sediment, and also fermented milk (Wu *et al.*, 2001;

Schwarz *et al.*, 2007; Ueki *et al.*, 2007; Jagevall *et al.*, 2008; Nayak and Kale, 2008; Bauer *et al.*, 2009; Cockell, 2010).

Also, the bacterial OTUs which match the predominant bands of two seed sludges (seed sludges of stock ASBR and acute and chronic tests) in the DGGE profiles were given in Table 4.1. 11 of the bacterial OTUs became practically undetectable in the DGGE profiles.

The phylogenetic tree constructed by bacterial clones was given in Figure 4.4.

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

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Seed Sludge_Stock Reactor	Seed Sludge_ Acute and Chronic Tests
B1	2.80	FR836435	*Clostridium sp. BG-C66* (FJ384378)	96		
B2	0.93	FR836436	*Clostridium sp. Kas301-1* (AB114242)	96		
B3	1.87	FR836437	*Clostridium intestinale* (AM158323)	95	•	
B4	4.67	FR836438	Uncultured Clostridium sp. clone AUCLO214 (AY648564)	96	•	•
B5	1.87	FR836439	Uncultured Firmicutes bacterium clone QEDS2BE07 (CU921543)	93	•	
B6	2.80	FR836440	Uncultured Firmicutes bacterium clone QEDN7DC08 (CU926749)	97		•
B7	14.95	FR836441	Uncultured bacterium clone BS7_65 (GQ458219)	98	•	•
B8	3.74	FR836442	*Syntrophomonas zehnderi strain OL-4* (DQ898277)	89		•
B9	33.64	FR836443	*Clostridium sp. Kas107-1* (AB114241)	96	•	•
B10	5.61	FR836444	Uncultured bacterium clone C3-25 (AM181973)	91	•	•
B11	4.67	FR836445	*Bacterium CBIC19I3* (HM240274)	95		
B12	3.74	FR836446	Uncultured bacterium UASB_TL84 (AF254390)	94	•	•
B13	0.93	FR836447	Uncultured Candidatus Cloacamonas sp. clone 8SN (EU887773)	92		
B14	5.61	FR836448	Uncultured bacterium clone BS1_25R (GQ458208)	97	•	
B15	2.80	FR836449	Uncultured bacterium clone aaa97f10 (DQ816701)	96		
B16	0.93	FR836450	*Acinetobacter sp. 1B3* (EU337120)	96		
B17	2.80	FR836451	Uncultured Sarcina sp. clone JPL-2_O10 (FJ957591)	89		
B18	0.93	FR836452	Uncultured bacterium clone 30c07 (EF515566)	87		
B19	0.93	FR836453	*Paludibacter propionicigenes* (AB078842)	92		
B20	0.93	FR836454	*Bacterium 061128-OL-KR37-AA 3-0 10000x -1A* (FJ037613)	92	•	•
B21	0.93	-	*Propionibacteriaceae bacterium FH044* (AB298766)	99		
B22	1.87	FR836458	Uncultured bacterium clone D14R15C96 (FM956760)	98		

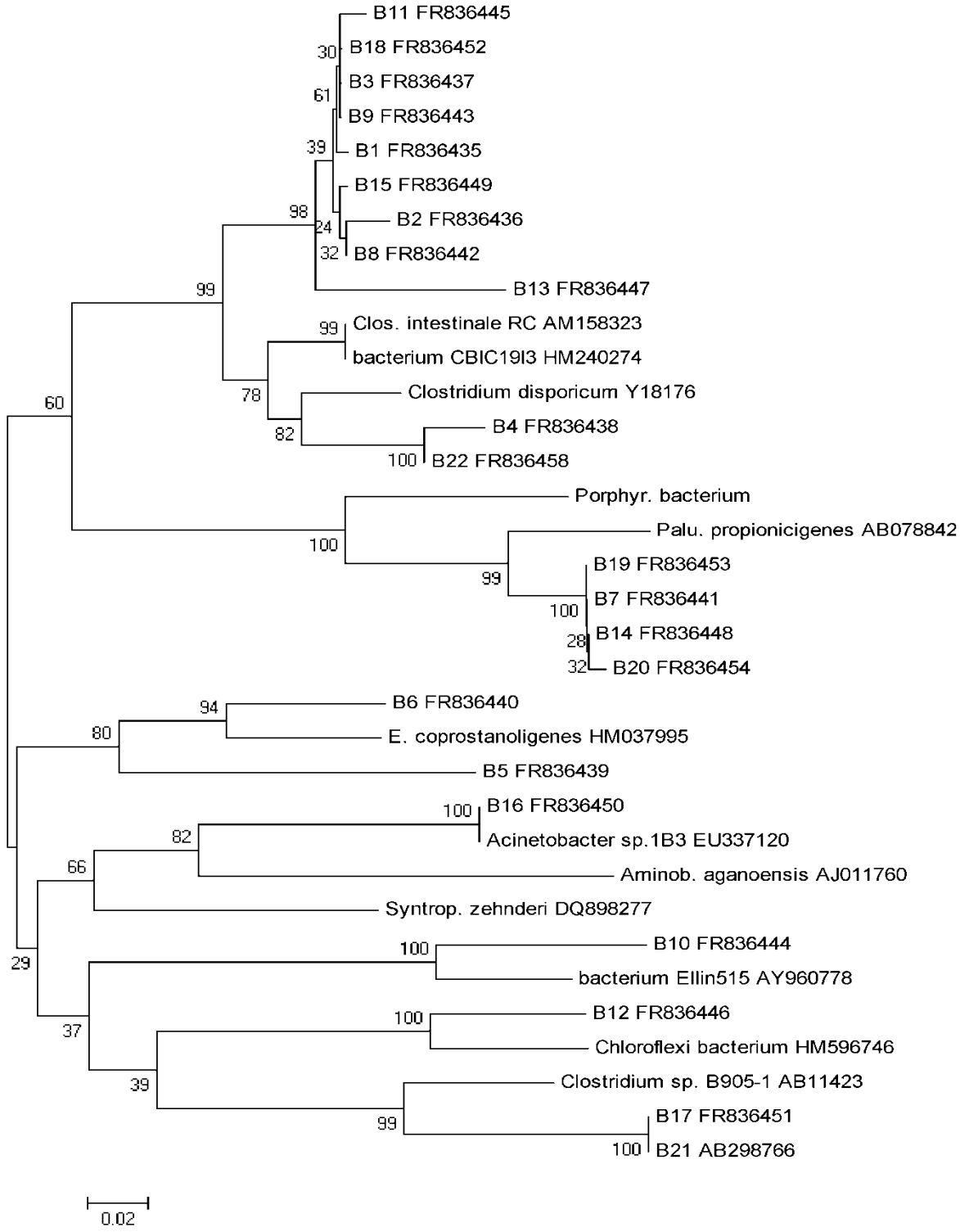

Figure 4.4: Neighbor-joining full-length 16S rDNA trees showing the genetic distances among individual clones from bacterial 16S rDNA clone library. Tha *bar scale* represents 2 nucleotide substitutions per 100 nucleotides.

The bacterial diversity indices of the seed sludges were represented in Table 4.2.

Table 4.2: Shannon-Weaver diversity indices, Margalef's species richness measure, and eveness measure estimates for bacterial DGGE profiles of the seed sludges.

	Stock Reactor	Chronic Reactors
Taxa	27	33
Shannon-Weaver diversity	3.152	3.449
Evenness	0.8662	0.9537
Margalef's richness	4.128	4.954

The most abundant of sequences obtained from the archaeal clone library represented in order of Methanomicrobiales (28%) which has members utilizing H_2+CO_2, alcohols and formate, Methanosarcinales (24%) which mostly acetoclastic methanogens belong to this genus, and Methanobacteriales (21%) which has members utilizing H_2+CO_2, formate and methanol+H_2 as seen Figure 6. The members of unlassified euryarchaeota, unclassified archaea and Methanococcales which has members utilizing H_2+CO_2, pyruvate+CO_2 and formate were distributes as 18%, 6% and 3%, respectively. 75.36% of OTUs were members of methanogens.

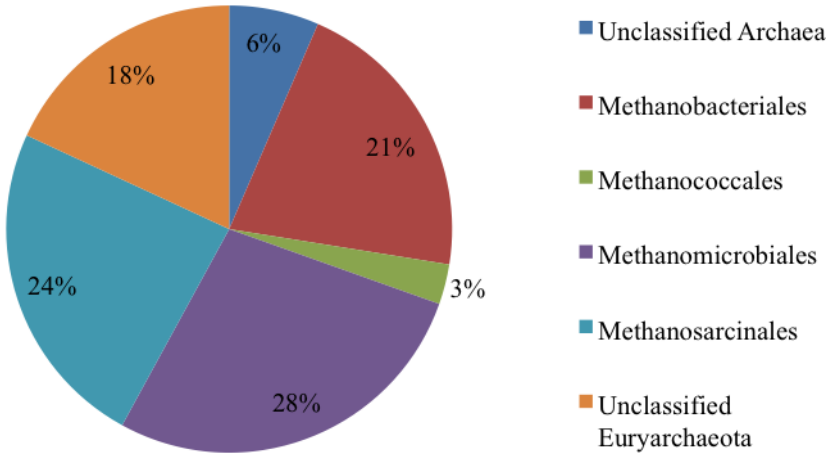

Figure 4.5: Distribution of phylogentic phylums from clone sequences of archaeal 16S rDNA clone library.

The dominant Methanomicrobiales OTU, A17 (FR836472), related cluster, represented by 9.42% of all archaeal clones was most closely related to an unclassified Methanomicrobiales specie, Methanogenic archaeon Prasan1 (Prasanna *et al.*, 2010). Other close relatives of Methanomicrobiales were mostly isolated and/or identified from anaerobic reactor treating waste(water) (Yang *et al.*, 1985; Klocke *et al.*, 2008; Cadillo-Quiroz *et al.*, 2008; Ueki *et al.*, 2009).

The most dominant OTU (12.32%), A7 (FR836463), in all clone library belongs to the second abundant phylum which was Methanosarcinales. This dominant OTU most closely related (97%) to a cultured specie, Methanothrix soehngenii, which utilizes acetate to produce CH_4 (Eggen *et al.*, 1990). Abundance of the other OTUs belonging to this phylum varied between 2.17 and 3.62% and their closest relatives were isolated from diifeent ecosystems such as activated sludges, anaerobic digester, groundwater and marine canyon sediments (Elberson *et al.*, 1997; Watanabe *et al.*, 2000; Fredriksson *et al.*, 2010; Zhang *et al.*, 2010).

The third abundant phylum was Methanobacteriales and this phylum was represented by nine OTUs. The dominant OTU was A18 (FR836473) and it represented 7.25% of all archaeal clones. It is most closely related (96%) to Methanobacterium beijingense strain 4-1 cultured from the granular sludge of a mesophilic UASB reactor treating beer-manufacture wastewater (Ma *et al.*, 2005). Clone frequencies of the other OTUs belonging Methanobacteriales phylum changed from 0.72% to 3.62 and they were firstly obtained from different anaerobic reactor, animal rumen and freshwater samples (Jarvis *et al.*, 2003; Ma *et al.*, 2005; Rastogi and Shouche, 2005; Briee *et al.*, 2007; Cheng and Zhu, 2008; Kelly *et al.*, 2009).

OTUs including the members of unlassified euryarchaeota, unclassified archaea and Methanococcales phylums constituted small clusters and they were represented by 3, 1 and 1 OTU(s), respectively. They were obtained from natural and engineering-designed ecosystems such as freshwater sediment, soil and wastewater treatment plants (Kemnitz *et al.*, 2005; Briee *et al.*, 2007; Green and Blank, 2007; Xing *et al.*, 2010).The closest cultured species of these OTUs are given in Table 2.

Also, the archaeal OTUs which match the predominant bands of two seed sludges (seed sludges of stock ASBR and acute and chronic tests) in the DGGE profiles were given in Table 2. 11 of the archaeal OTUs became practically undetectable in the DGGE profiles.

The archaeal diversity indices of the seed sludges were represented in Table 4.3.

Table 4.3: Shannon-Weaver diversity indices, Margalef's species richness measure, and eveness measure estimates for archaeal DGGE profiles of the seed sludges.

	Stock Reactor	Chronic Reactors
Taxa	15	13
Shannon-Weaver diversity	2.599	2.526
Evenness	0.8966	0.962
Margalef's richness	2.123	1.901

The phylogenetic tree constructed by the archaeal clone library was given in Figure 4.6.

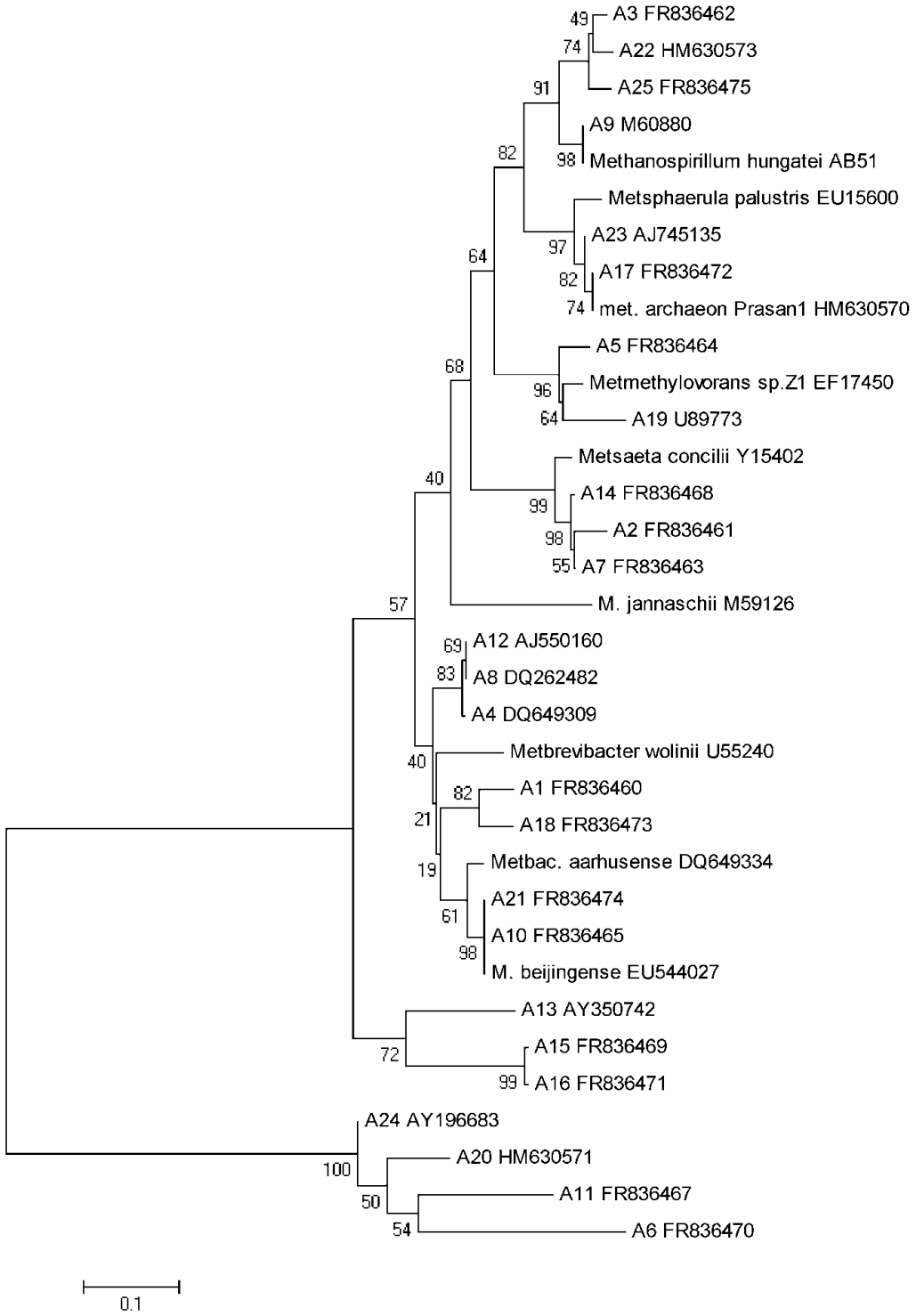

Figure 4.6: Neighbor-joining full-length 16S rDNA trees showing the genetic distances among individual clones from archaeal 16S rDNA clone library. Tha *bar scale* represents 10 nucleotide substitutions per 100 nucleotides.

Table 4.4: Affiliation of archaeal clones and their abundance within the seed sludges.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Seed Sludge_Stock Reactor	Seed Sludge_ Acute and Chronic Tests
A1	1.45	FR836460	Uncultured archaeon clone OK3 (GQ406364)	94		
A2	2.90	FR836461	Uncultured archaeon clone I17_Rya (HM639831)	98		•
A3	9.42	FR836462	*Methanogenic euryarchaeote Annu4* (FJ896263)	95	•	•
A4	2.90		*Methanobacterium formicicum strain S1* (DQ649309)	99		
A5	2.90	FR836464	Uncultured Methanosarcinales archaeon clone:KuA4 (AB077214)	98		
A6	4.35	FR836470	Uncultured euryarchaeote clone MVP-8A-21 (DQ676281)	98		
A7	12.32	FR836463	*Methanothrix soehngenii* (X51423)	97		
A8	0.72		Uncultured archaeon clone A6T20L208 (DQ262482)	99		
A9	7.25		*Methanospirillum hungatei* (M60880)	99	•	•
A10	0.72	FR836465	*Methanobacterium beijingense strain M4* (EU544027)	97	•	
A11	6.52	FR836467	*Methanosphaerula palustris strain E1-9c* (EU156000)	95		•
A12	0.72		*Methanobacterium sp. OM15* (AJ550160)	99		
A13	2.17		*Methanobacterium beijingense strain 8-2* (AY350742)	100	•	•
A14	3.62	FR836468	Uncultured Methanosarcinales archaeon clone S4 (GU475184)	95		•
A15	2.90	FR836469	Uncultured archaeon clone Sed-ARC-34 (EU255763)	98		
A16	3.62	FR836471	Uncultured euryarchaeote clone MVP-8A-26 (DQ676246)	96	•	
A17	9.42	FR836472	*Methanogenic archaeon Prasan1* (HM630570)	97	•	•
A18	7.25	FR836473	*Methanobacterium beijingense strain 4-1* (AY552778)	96		
A19	2.17		*Methanosarcina siciliae* (U89773)	99	•	•
A20	4.35		Uncultured archaeon clone EOTU21 (FJ896263)	99		
A21	1.45	FR836474	Uncultured archaeon clone OKA6 (GQ465437)	97		
A22	1.45		Uncultured archaeon clone ATB-KM-2942-A18 (EF680353)	99		•
A23	6.52		Archaeon LL25A3 (AJ745135)	99	•	•
A24	2.17		Uncultured archaeon clone WA10 (AB494239)	99		
A25	0.72	FR836475	*Methanogenic euryarchaeote Annu1* (HM630571)	95	•	

4.2.3 Quantification by Q-PCR and FISH

Quantitative changes in the 16S rDNA concentrations within two seed sludges using in this study were determined by quantitative real-time PCR (Q-PCR) (Figure 4.7). The concentrations showed that abundance of all microbial community increased during stock ASBR operation. While 16S rDNA gene concentration of bacterial community increased sharply from 3.65 X 10^8 copies/mL to 2.23 X 10^{10} copies/mL (~60 fold increasing), abundance of archaeal community changed from 1.00 X 10^7 copies/mL to 4.88 X 10^8 copies/mL (~50 fold increasing). Increase in the methanogen population was more than archaea. 16S rDNA gene concentration of methanogens ascended 4.05 X 10^6 copies/mL to 2.68 X 10^8 copies/mL (~66 fold increasing).

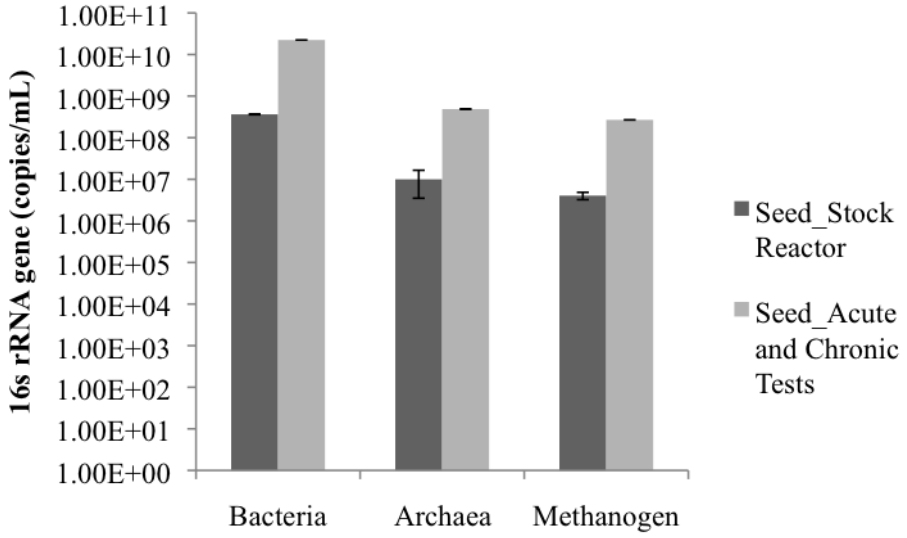

Figure 4.7: Quantitative changes in bacterial, archaeal, and methanogenic 16S rDNA concentrations determined by Q-PCR.

Also, relative amount of bacteria, archaea and important methanogenic subgroups was determined by FISH in the seed sludge of acute and chronic tests (Figure 4.8). Relative abundances of bacteria and archaea were 31.4% and 45.9%, relatively. The three hydrogen utilizing methanogenic groups, Methanobacteriaceae, Methanocaccaceae, Methanomicrobiaceae were distributed 8.6%, 6.1%, 5.4%. Acetoclastic methanogens were investigated based on Methanosarcina genus and Methanosaeta spp. specific FISH probes. Relative abundances of Methanosarcina genus and Methanosaeta spp. were found 18.9% and 10.3%, relatively.

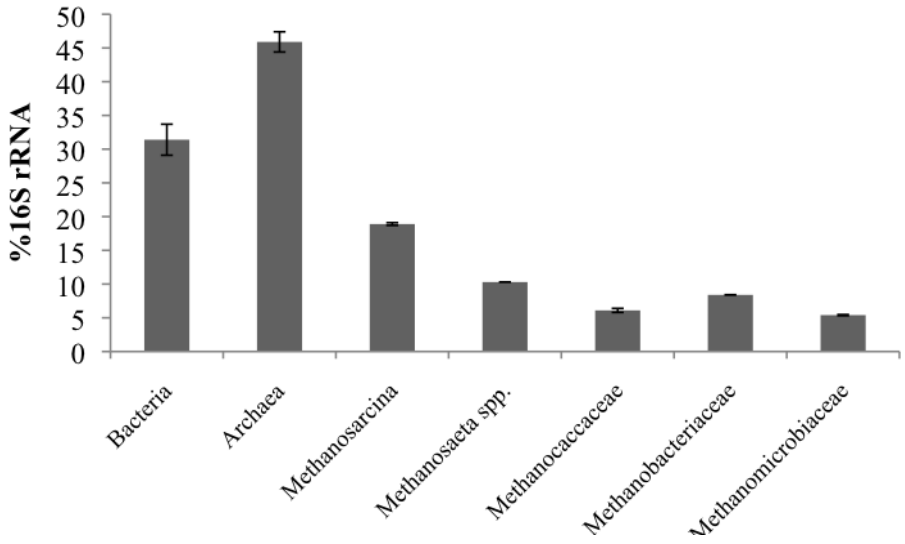

Figure 4.8: Relative abundance of methanogenic genus in seed sludge of acute and chronic tests.

4.3 Acute Tests

These experiments were designed to evaluate the short-term inhibitory impact of the selected antibiotics, *sulfamethoxazole* (SMX), *erythromycin* (ERY) *and tetracycline* (TET) on the metabolic activity of both *homoacetogens and methanogens*, which are specific trophic groups in the acclimated sludge. The experiments were designed as two sets;

1- acetate feeding to evaluate acetoclastic methanogens,

2- VFA mixture feeding to evaluate homoacetogens and all methanogens.

The experiments reflected the effect of the selected antibiotics in the range of 1 – 1000 mg/L at only one exposure. Batch reactors with an effective volume of 60 mL were run for six days; together with antibiotic dosage, each reactor was inoculated with 4000 mg/L of acetate corresponding to an initial acetate COD of around

4250 mg/L and 3000 mg/L of each VFA; acetate, butyrate and propionate corresponding to an initial COD of around 3180, 5400, and 4500 mg/L favoring acetoclastic methanogenic activity and homoacetogenic activity with all methanogens, respectively. Gas/methane production and COD/VFAs removal were selected as relevant parameters monitored. Cumulative biogas generation was monitored daily throughout the experiment period. COD/VFA concentrations were measured both at the beginning and at the end of the test.

4.3.1 Acute effects on acetoclastic methanogenic activity

4.3.1.1 Cumulative biogas production

The average cumulative biogas production (CBP) associated with the utilization of acetate in the control reactors was 97 mL at the end of six days, which is above the minimum required in ISO standards (ISO13641-1, 2003). The corresponding cumulative methane production (CMP) was 77 mL, which accounted for around 80% of biogas generated in control reactors. The CBM profiles together with the corresponding CMP values for the three selected antibiotics are plotted in Figures 6.9-6.11.

The CBP profile in the control reactor related to SMX dosage displayed the typical substrate utilization/microbial growth curve observed in similar studies (Lallai *et al.*, 2002; Ince *et al.*, 2009). The profile reached a plateau with no further biogas production after 120 hours indicating the depletion of all organic substrate supplied and therefore, confirming that the six-day period selected for the short-term tests was appropriate. As shown in Figure 4.9a, SMX dosages of 1 and 10 mg/L generated a CBP profile quite close to the control reactor with total biogas volumes close to 90 mL and methane volumes above 70 mL. The profiles associated with 25 and 50 mg/L of SMX were clearly different but they still yielded methane levels above 60 mL. Significant inhibition effect was induced when SMX dosage was increased to 100 – 250 mg/L. It is interesting to note that the shape of the CBP profile at SMX of 250 mg/L diverged from the rest showing the end of biogas production and substrate depletion much sooner – around 96 hours. Almost complete biogas and methane inhibition was observed for SMX dosages of 500 mg/L and higher concentrations. The same trend was observed in methane production as shown in Figure 4.9b, which displays the change in CMP levels and percent methane levels in the biogas as a function of increasing SMX dosages. The plot indicated the significant inhibition impact of SMX between 100 and 250 mg/L.

It should be noted that batch reactors were set as duplicates for each antibiotic dosage and they yielded almost identical CBP and CMP results. In fact, the data collected from the duplicates for each SMX concentration (1-1000 mg/L) were evaluated with one-way analysis of variance and individual p values were calculated via Dunnett's multiple comparison test (Conkle *et al.*, 2010 and Libralato *et al.*,

2010). The results indicated a highly significant decrease ($p < 0.01$) in CBP values for SMX concentrations equal to or higher than 25 mg/L (Figure 4.9a).

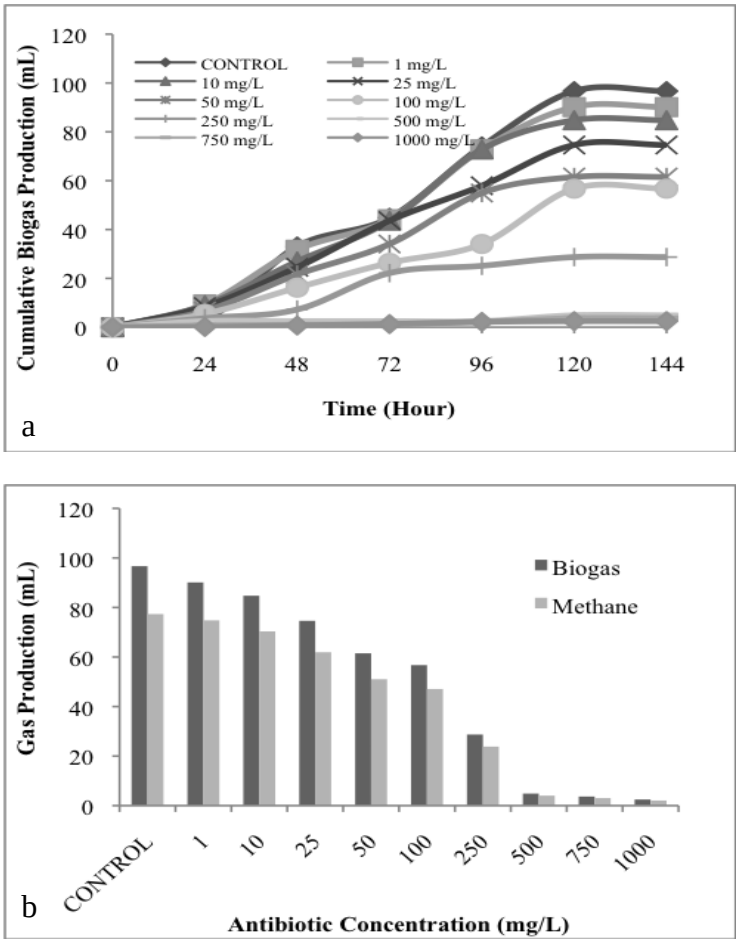

Figure 4.9: Inhibition effect of SMX dosage on (a) cumulative biogas production profiles (b) total biogas and methane production.

The experimental CBP profiles and CMP values obtained with the other antibiotics, ERY and TET, indicated a similar pattern as the those characterizing SMX (Figures 6.10 and 6.11), i.e. a significant inhibitory effect starting around 100 mg/L and almost total biogas and methane inhibition starting at 500 mg/L. The discrepancy in the shape of the CBP profile could be observed at 250 mg/L for both for ERY and TET. At this concentration, biogas generation stopped after 72 hours, half way through the experimental period. The statistical analysis of the results indicated a highly significant decrease in CBP values for all ERY concentrations including 1 mg/L, and a similar result except 1 mg/L for TET. The methane level of 83±0.5% observed in the control reactor persisted up to 100 mg/L TET fed test tubes but it

started to decrease and reached a low level of 68 % at the TET dosage of 1000 mg/L. However in ERY experiments, the methane percentage did not change with antibiotic concentration and it stayed at the level observed in the control reactor (83±0.1%) in all ERY fed sets.

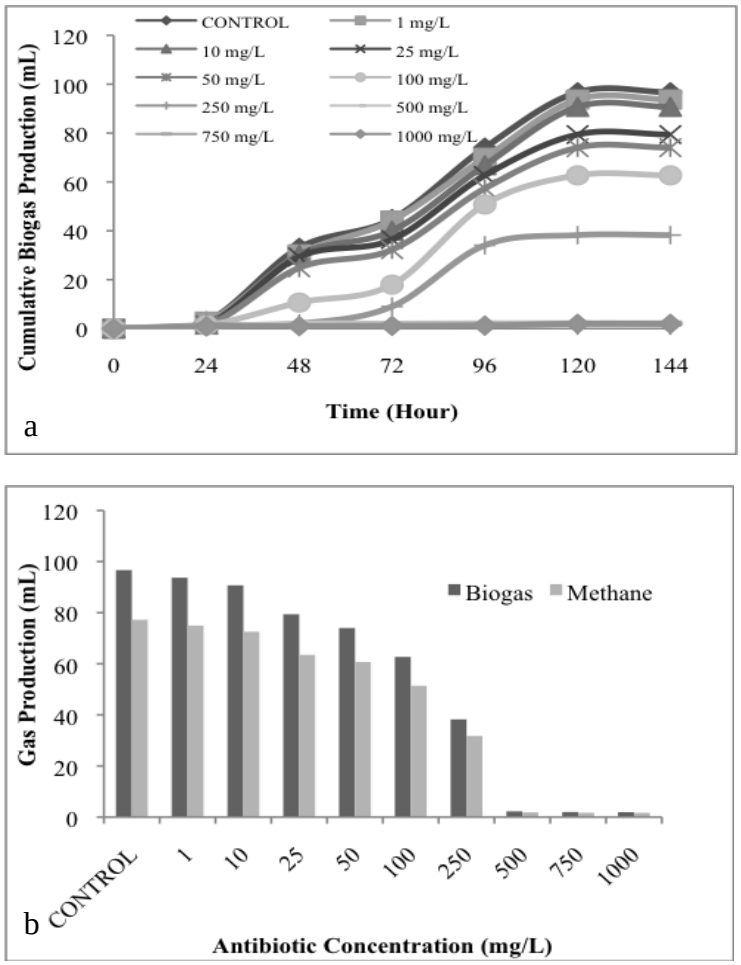

Figure 4.10: Inhibition effect of ERY dosage on (a) cumulative biogas production profiles (b) total biogas and methane production.

The EC50 level, i.e. the inhibitor concentration that induces 50% inhibition on measured response of the microbial community is a commonly used parameter for inhibitory impact (Speir *et al.*, 2007; Arienzo *et al.*, 2009; Ruiz *et al.*, 2009). The percent inhibition on CMP levels as a function of dosage, as plotted in Figure 4.12, showed that inhibition evaluated in terms of the EC50 values was also quite similar. The corresponding EC50 values were calculated as 198.5 mg/L for SMX, 155.4 mg/L for ERY and 204.4 mg/L for TET. These results do not confirm the findings of Gartiser and his colleques (2007b) reporting that 50% inhibition was observed at 37.3

mg/L of TET while no inhibition effect was detected for SMX and ERY up to 100 mg/L.

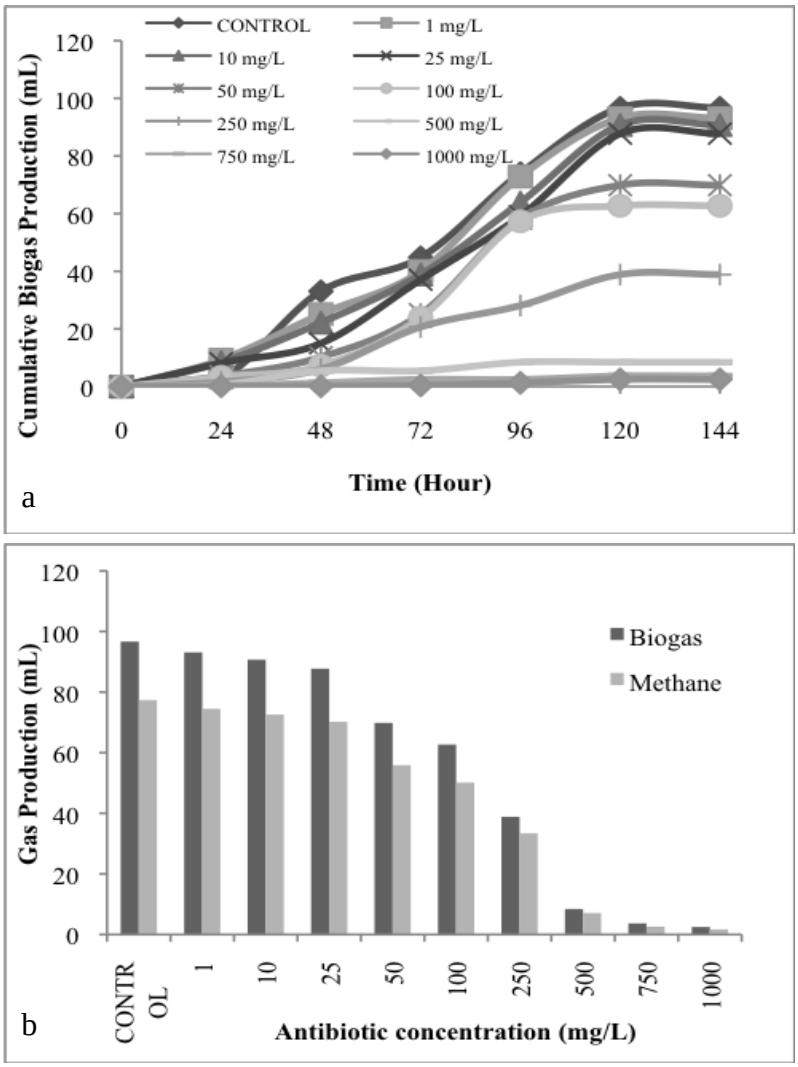

Figure 4.11: Inhibition effect of TET dosage on (a) cumulative biogas production profiles (b) total biogas and methane production.

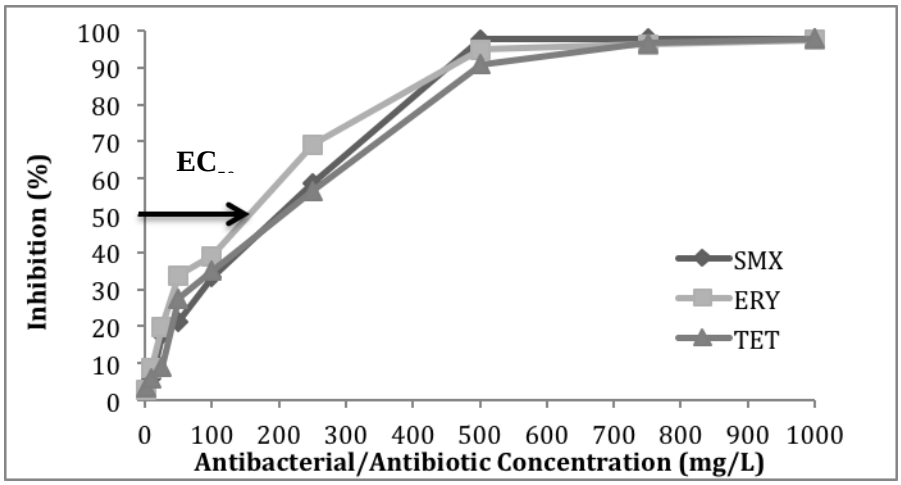

Figure 4.12: Inhibition of CMP levels and EC50 values associated with SMX, ERY and TET.

4.3.1.2 Chemical oxygen demand (COD) and acetate removal

Three parameters were considered in the evaluation of the COD removal pattern in the inhibition experiments: (a) the level of acetate COD remaining in the effluent; (b) the level of antibiotic COD in the effluent; (c) fraction of the antibiotic COD remaining or entrapped with biomass. Total COD and acetate were measured both at the beginning and at the end of each experimental run to set the grounds for differentiating the nature of remaining COD, i.e. acetate COD or antibiotic COD as shown in Figures 6.13-6.15. The results obtained may be summarized as follows: (i) Acetate that was initially inoculated as the sole organic carbon source was entirely removed in all the runs carried out with SMX and ERY; it was also entirely removed in the TET runs, except in reactors started with a TET dosage of above 250 mg/L, where less than 300 mg/L of acetate COD was measured to remain at the end of the observation period. Thus, while methane generation – i.e. the end product of methanogenic activity - was severely impaired, removal of acetate was not affected by the inhibitory action of antibiotics. (ii) Measurements always detected remaining COD at the end of the experiments, which mostly reflected the presence of antibiotics, together with residual soluble microbial products generated in the course of biochemical reactions (Germirli Babuna *et al.*, 1998), because parallel direct analyses revealed no acetate. (iii) The remaining part of the antibiotic represented only a fraction of the initially inoculated dosage; 0.19, 0.29 and 0.48 for SMX, ERY and TET, respectively, this fraction was relatively smaller for SMX, gradually increasing for ERY and TET; The results suggested that they mostly remained entrapped/adsorbed by the microbial community, based on the assumption that they

are practically non-biodegradable with short-term exposure to non-acclimated biomass (Alexy *et al.*, 2003; Alexy *et al.*, 2004; Gartiser *et al.*, 2007b).

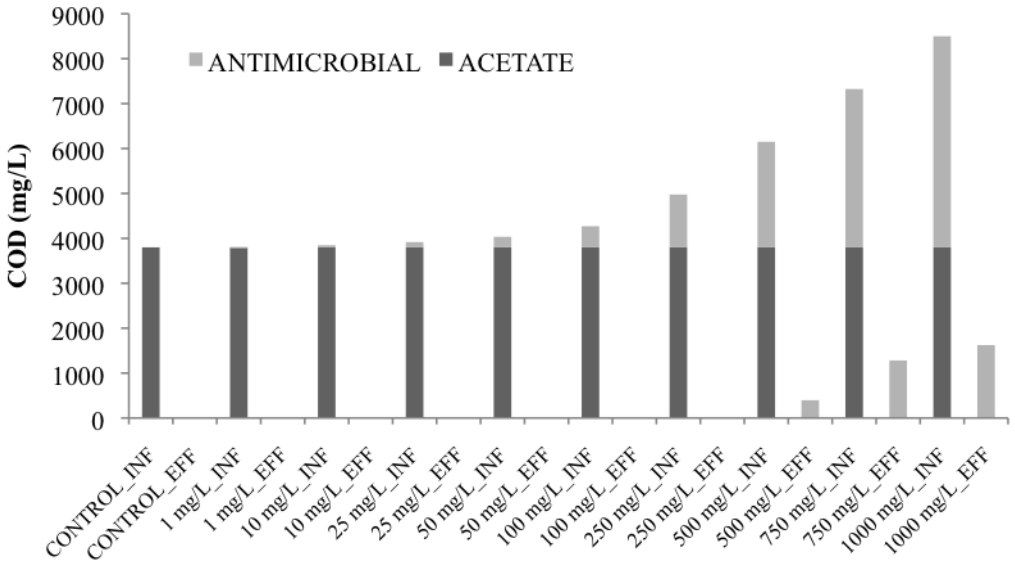

Figure 4.13: Initial and final COD concentrations in SMX experiments.

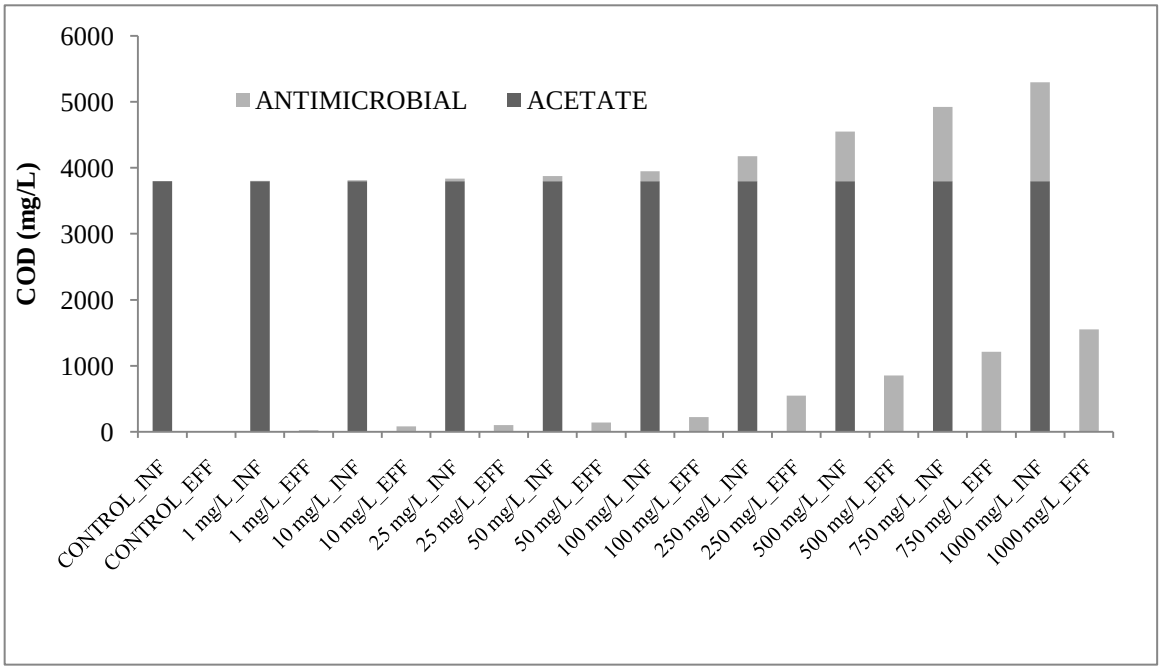

Figure 4.14: Initial and final COD concentrations in ERY experiments.

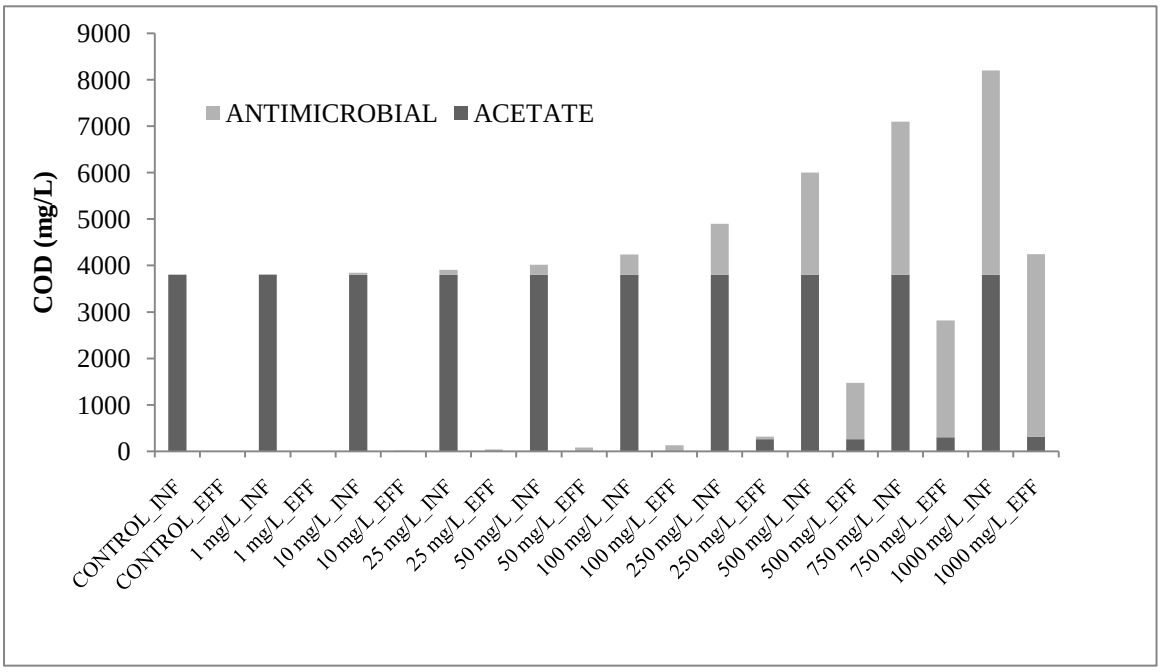

Figure 4.15: Initial and final COD concentrations in TET experiments.

The results indicated that the short-term inhibitory impact of antibiotics was observed as a significant reduction in methane production, while acetate removal was not impaired. The seemingly conflicting results may be better interpreted in terms of two different approaches: (i) the basic process stoichiometry that defines the relationship between methane generation and acetate utilization for metabolic activities, (ii) conceptual understanding of the inhibition mechanism.

The evaluation should also recognize the possibility of a metabolic shift – competitive growth of alternate species under inhibitory stress – likely to occur in the bottle reactors due to antibiotic dosage. In this scenario, high dosages of antibiotics would affect *acetoclastic methano*gens and would favor competitive utilization of acetate by *homoacetogenic bacteria*, which are capable of producing hydrogen and CO_2 from acetate (Girffin *et al.*, 1998). This process would produce the substrate for hydrogen utilizing methanogens; however, no H_2 was detected at the end of test. The main reason could be that hydrogenotrophic methanogens may have used H_2 and CO_2 on the instant, as they were produced. In view of the results obtained however, this scenario does not look as a viable option because, the methane percent in the biogas always remained in the range of 80–86%, although significantly reduced with increasing antibiotic additions, leaving substrate binding by uncompetitive inhibition as the only biochemical mechanism.

4.3.2 Acute effects on methanogenic activity

4.3.2.1 Cumulative biogas production

The CBP and CMP in control reactor at the end of the test duration were reached to 445 mL and 312 mL, respectively. CH_4 portion in the biogas was accounted for around 70%. When the total VFAs fed in the reactor were utilized completely, the theoretical CH_4 production should be 315 mL. The CBP and CMP profiles for all selected antibiotics are given in Figure 4.16-6.18.

CBP profiles in test tubes with respect to SMX dosage were similar to substrate utilization curve of other studies in the literature (Amin *et al.*, 2006 and Shimada *et al.*, 2008). The biogas production reached to maximum in 6 days and stopped. The result indicated that all organic substrate was completely utilized and the duration is appropriate for short-term inhibition tests.

While total biogas production of 1 and 10 mg/L of SMX added test bottles was quite close to control, the profiles of 25 and 50 mg/L of SMX added reactors were different as seen in Figure 4.16. However their methane productions were still higher than 150 mL. Significant inhibition effect of the SMX on the VFA utilization under anaerobic conditions was detected after 100 mg/L. Also 250 mg/ almost and higher concentration of SMX addition almost completely inhibited the metabolic activity. The plot indicated the significant inhibition impact of SMX at two different points, between 10 and 25 mg/L, and between 100 and 250 mg/L (Figure 4.16). The methane ratio in the biogas was detected almost same with control reactor as 70±1.5%.

Dunnett's multiple comparison test was used to evaluate the duplicate data with one-way analysis of variance and individual p values (Conkle *et al.*, 2010 and Libralato *et al.*, 2010). The results indicated a highly significant decrease ($p < 0.01$) in total biogas production values for SMX concentrations equal to or higher than 10 mg/L (Figure 4.16a).

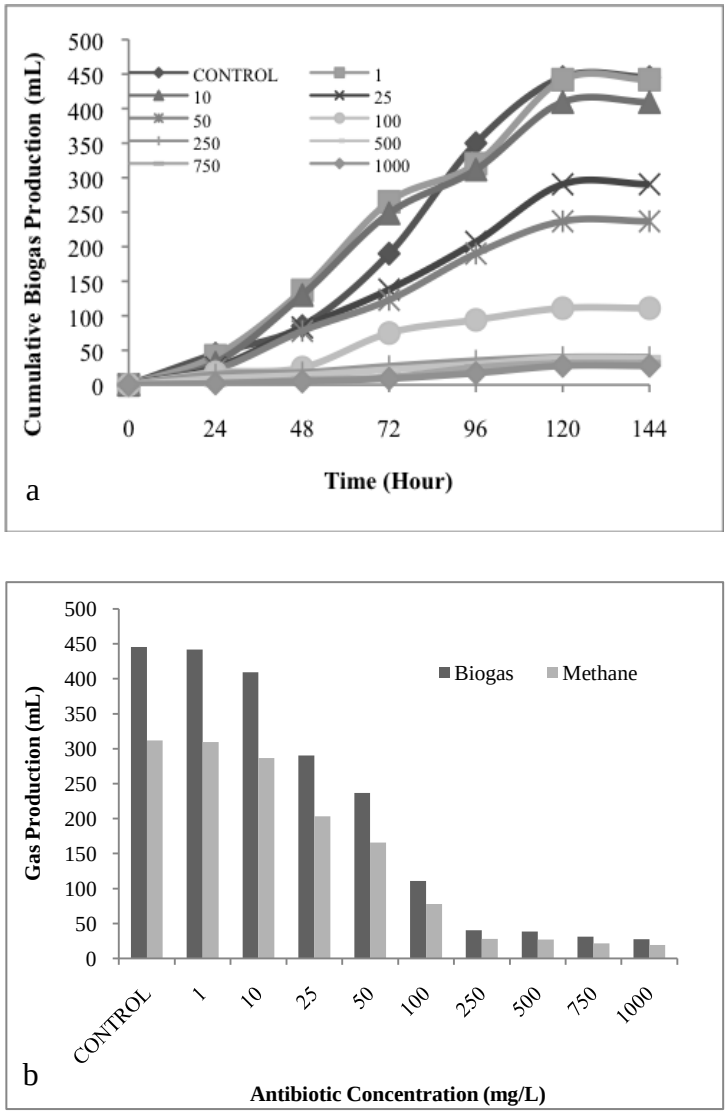

Figure 4.16: Inhibition effect of SMX dosage on (a) cumulative biogas production profiles (b) total biogas and methane production.

The experimental CBP profiles and CMP values obtained with the other antibiotics, ERY and TET, indicated different patterns as the characterizing SMX (Figures 6.17 and 6.18). In the ERY sets, a significant inhibitory effect was started 1 mg/L ($p < 0.01$) and complete biogas and methane inhibition were not observed, also in 1000 mg/L ERY fed reactors. The biogas and methane inhibition reached to 82.5% in 250 mg/L fed reactor and the inhibition effect was detected 83.5±0.9% up to 1000 mg/L.

Two discrepancies in the shape of the CBP profile could be observed at 250 mg/L and after 250 mg/L for ERY. The methane level in ERY fed reactor was almost stable as %62±1 while in the control reactor was 70%.

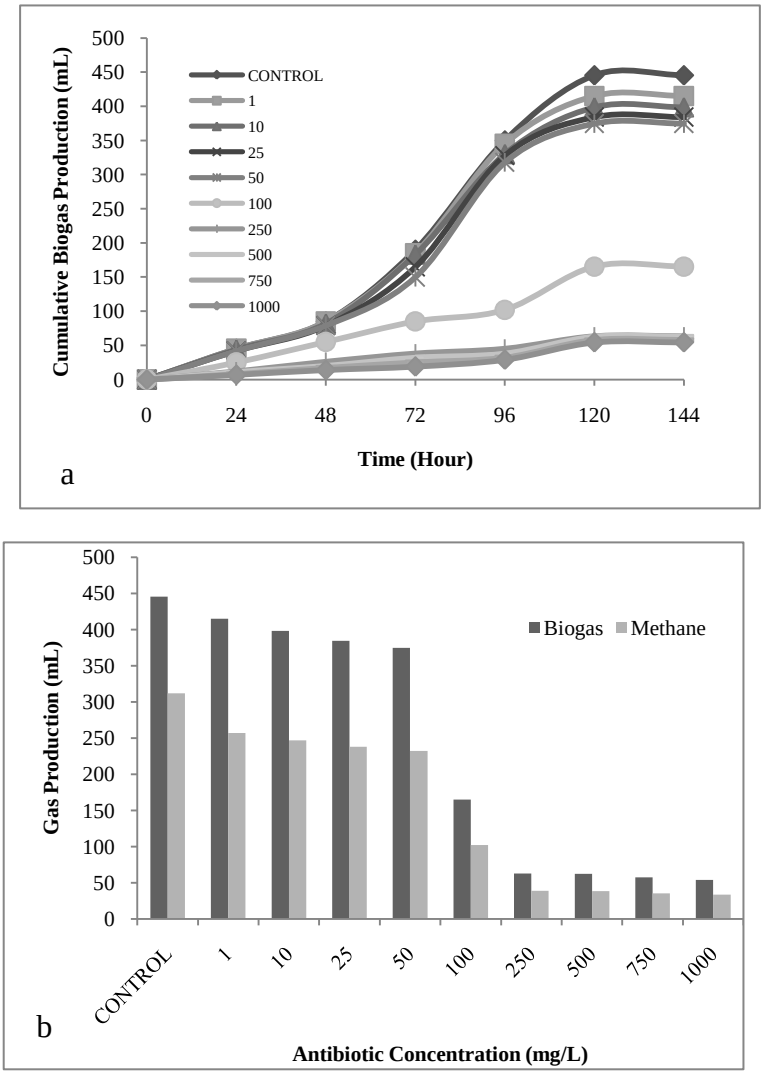

Figure 4.17: Inhibition effect of ERY dosage on (a) cumulative biogas production profiles (b) total biogas and methane production.

The biogas production profile of TET fed reactor were different from SMX and ERY because the inhibition effect of the antibiotic started from 1 mg/L and increased stepwise in the parallel of rising concentration as seen Figure 4.18. Also, 1000 mg/L concentration of TET was stopped the gas production distinctively. The significant decrease in the CBP was begun from 1 mg/L antibiotic addition like ERY fed reactors. While the methane percentage in the biogas was the same between 1-250 mg/L as 58%±1.7 and it started to decrease after 250 mg/L TET concentration until 43% at the TET dosage of 1000 mg/L.

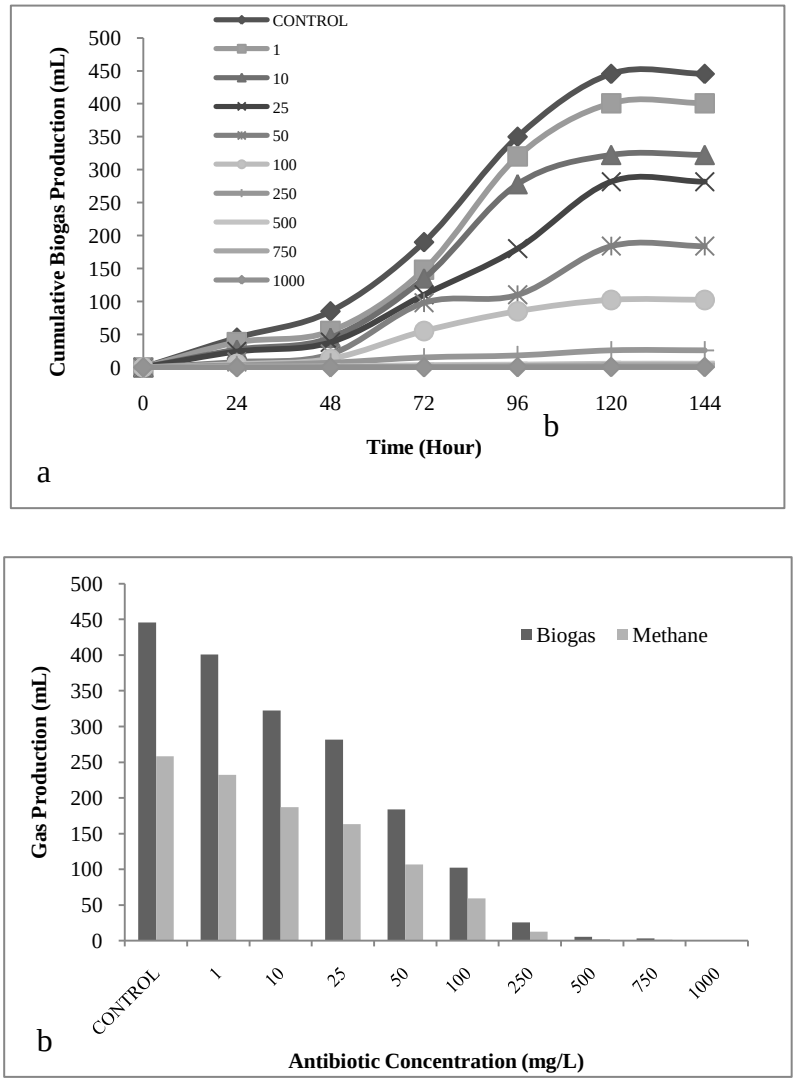

Figure 4.18: Inhibition effect of TET dosage on (a) cumulative biogas production profiles (b) total biogas and methane production.

Also EC50 values which show the 50% inhibition in the system was determined (Speir *et al.*, 2007). The inhibition profiles as percent were represented in Figure 4 and EC50 values of the compound are highlighted. EC50 values of the selected antibiotics were calculated as 55.4 mg/L for SMX, 85.8 mg/L for ERY and 46.2 mg/L for TET. Gartiser *et al.* (2007b) found the EC50 value of TET as 37.3 mg/L and this value is close to the result obtained from this study. However, they could not detect EC50 value for SMX and ERY, while Xu *et al.* (2011) showed that the EC50 value of SMX in natural river sediments 40.9 mg/L.

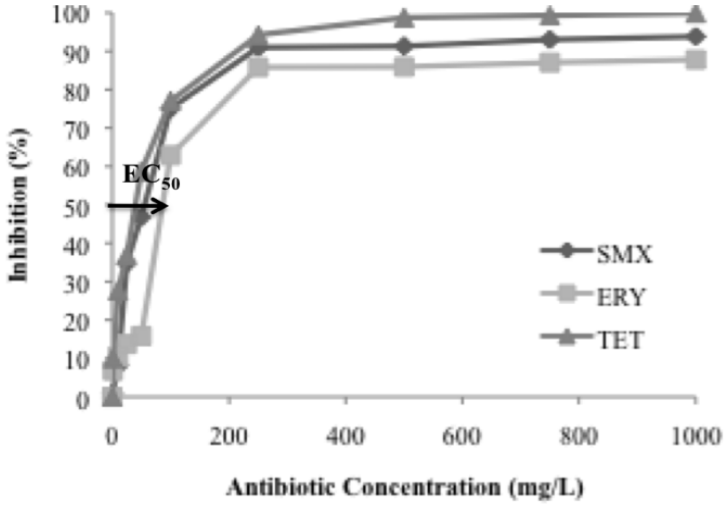

Figure 4.19: Inhibition of CMP levels and EC50 values associated with SMX, ERY and TET.

4.3.2.2 Chemical oxygen demand (COD) and volatile fatty acid (VFA) removal

Soluble COD and VFAs were measured both at the beginning and at the end of the experimental run sets as shown in Figure 4.20-6.25 for each compound. The results indicated that all VFAs were completely utilized in SMX and ERY runs until 250 mg/L of antibiotic dosage. 250 mg/L of SMX/ERY and above, it is clear that VFA removal mechanism was directly affected. Most of the propionic acid accumulation was detected. In 500 mg/L and higher concentrations, butyric acid, also iso-butyric acid, started to accumulate. The removal of acetic acid was not effected even in the presence of very high concentrations of the two compounds as seen Figure 4.21 and 6.23. Acetic acid results in this study are also similar to only acetic acid fed as sole carbon source runs (Cetecioglu *et al.*, submitted). In these runs, valeric acid, caproic acid and heptanoic acid, which are higher than four carbons, were determined despite they were not fed to the reactors. This result indicated that effluent COD included not only antibiotics and VFAs, which were used as carbon sources but also microbial metabolites (Aquino and Stuckey, 2004).

COD results showed that after 25 mg/L of SMX concentration and 10 mg/L of ERY concentration COD removal efficiency started to decrease. However, VFAs determined 250 mg/L and higher SMX and ERY dosages. The measured COD in the effluent may be antibiotics and/or residual soluble microbial products generated in the course of biochemical reactions (Germirli Babuna *et al.*, 1998 and Aquino and Stuckey, 2004).

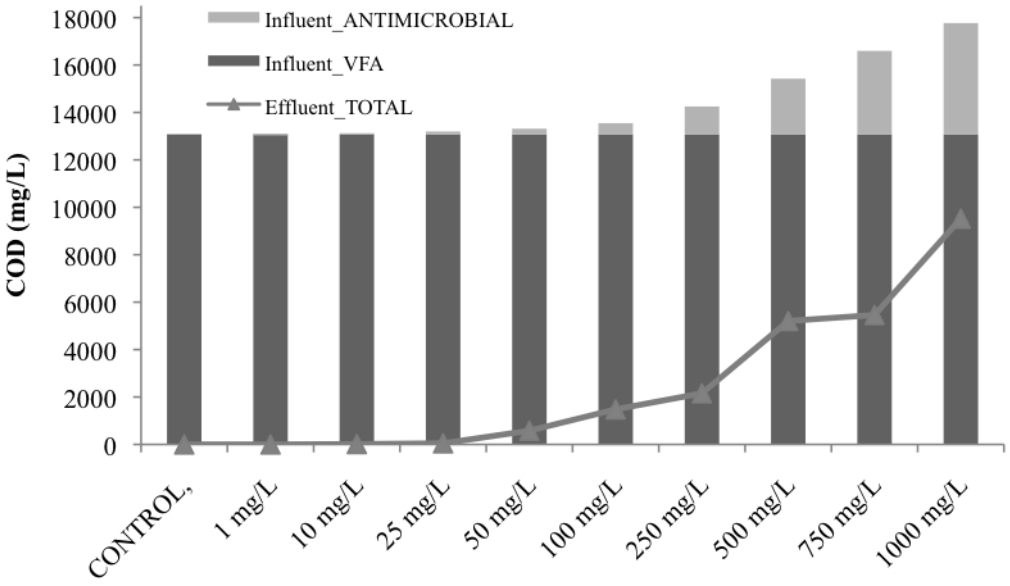

Figure 4.20: Initial and final COD concentrations in SMX experiments.

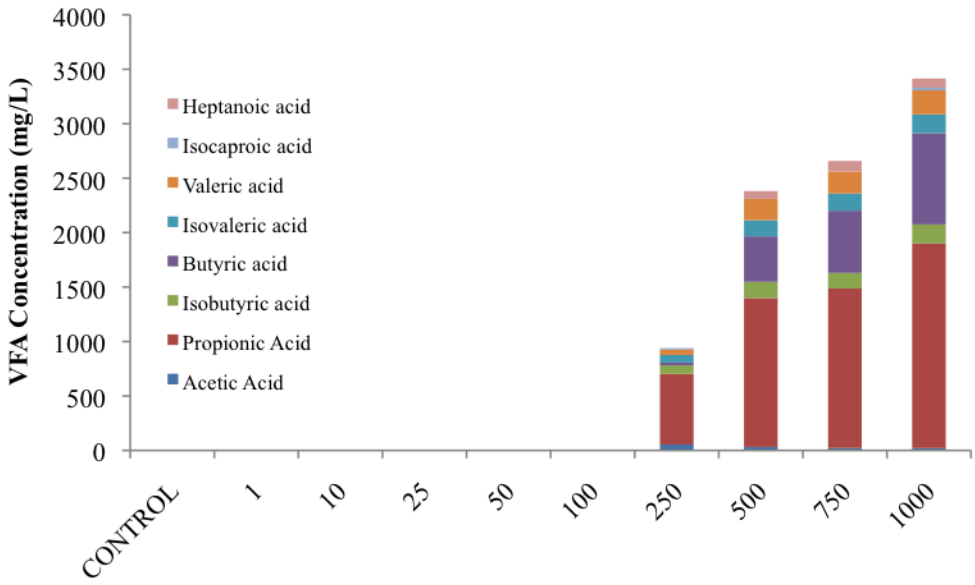

Figure 4.21: VFA concentration in effluents of SMX experiments.

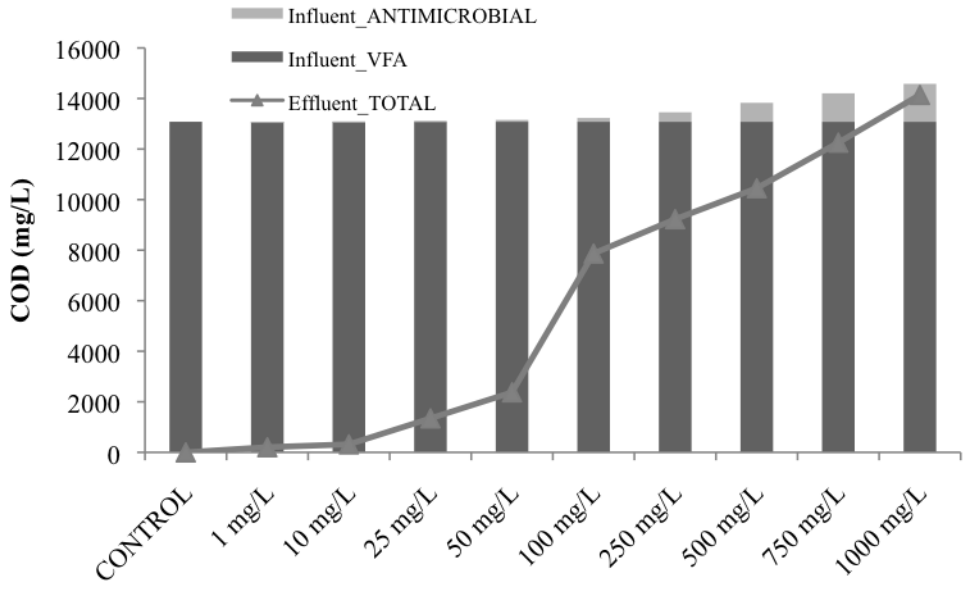

Figure 4.22: ERY initial and final COD concentrations in ERY experiments.

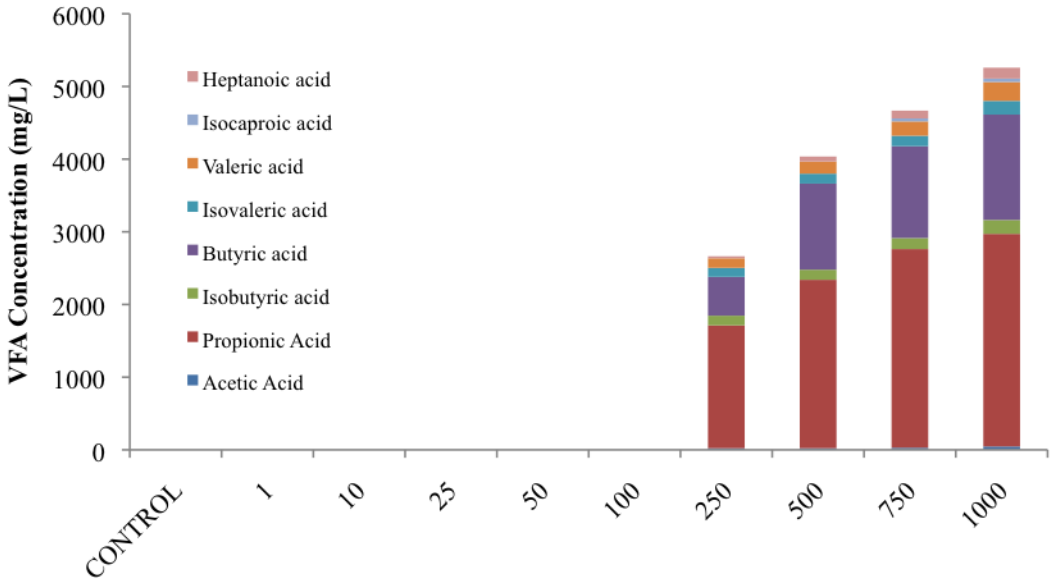

Figure 4.23: VFA concentration in effluents of ERY experiments**.**

COD and VFA concentrations of TET effluents were different from SMX and ERY. VFA accumulation started from 25 mg/L TET dosage and not only butyrate and propionate utilization pathways but also acetic acid utilization was affected as seen Figure 4.25. The results obtained from this study showed different profile about acetate utilization than only acetate fed batch tests (Cetecioglu et al, submitted). Cetecioglu and her colleagues (submitted) detected acetate accumulation in the system 250 mg/L and higher TET concentration. The reason of the detected acetate in the lower concentration can be the utilization of other VFAs by homoacetogens

(Speece, 1996 and Batstone, 2002). COD results obtained from TET fed test bottles were also parallel to VFA results (Figure 4.24). COD value in the effluents started to increase 25 mg/L and higher TET concentrations. 500 mg/L and higher concentrations COD results were measured higher than detected VFA concentrations. This difference could be as a result of the TET concentrations in the effluent. The lower concentration of TET probably was absorbed to the sludge (De Liguoro *et al.*, 2003 and Zhao *et al.*, 2010).

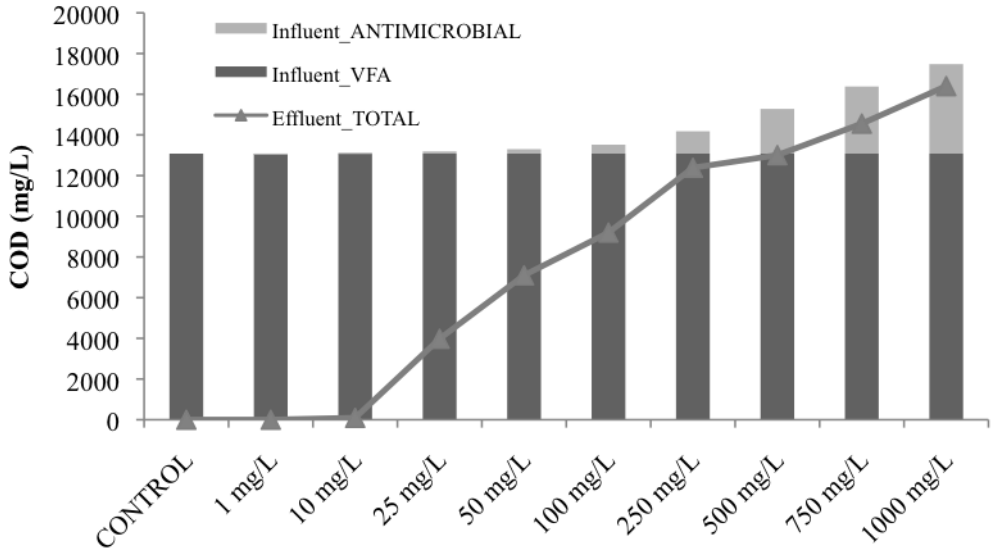

Figure 4.24: Initial and final COD concentrations in TET experiments.

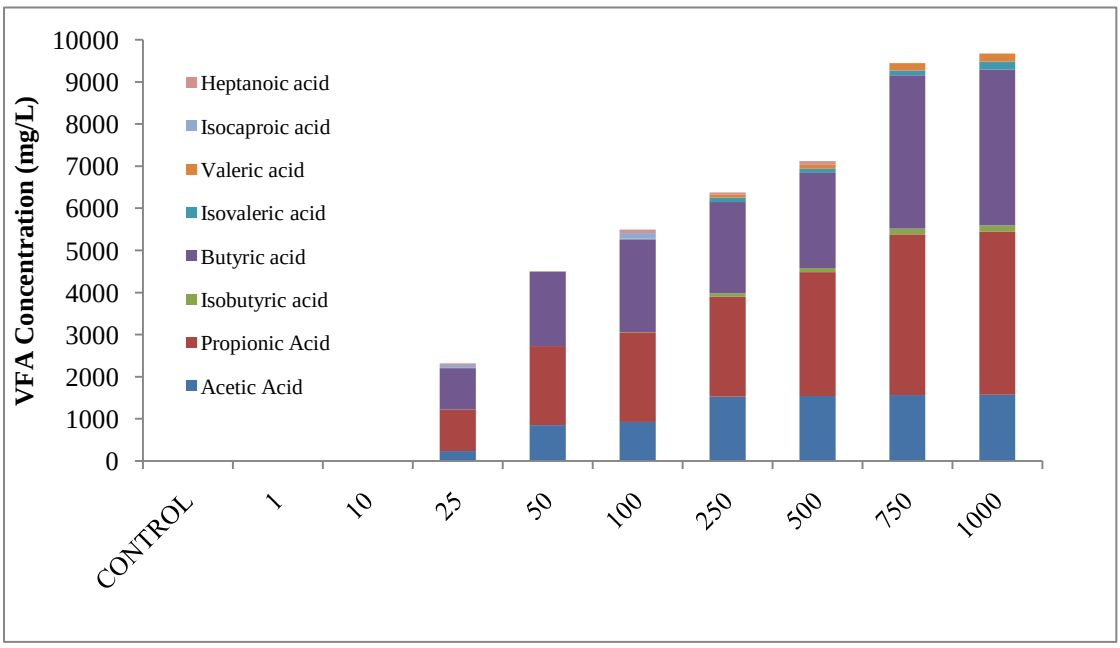

Figure 4.25: VFA concentration in effluents of TET experiments.

The quantification of methanogen subgroups in the seed sludge showed that the acetoclastic methanogens, Methanosarcina and Methanosaeta spp., were dominant. However, hydrogenotrophic methanogens were detected in the system while no hydrogen was measured in the biogas analysis. It indicated that hydrogen can be converted to CH_4 instantly by this group.

4.4 Chronic Tests

Four lab scale anaerobic sequencing batch reactors were inoculated with the seed sludge taken from lab scale stock ASBR. One of these reactors was operated as control reactor, which was only fed by synthetic wastewater. Other reactors were fed by synthetic wastewater with particular antibiotic; sulfamethoxazole, erythromycin, tetracycline. Performance of the antibiotic fed reactors with a comparison of the control reactor in terms of COD removal efficiency, biogas production, volatile fatty acids (VFAs) accumulation, antibiotic degradation were discussed in this section. Also long-term effects of antibiotics on the microbial community and specific metabolic pathways were presented.

4.4.1 Sulfamethoxazole

4.4.1.1 Performance of the Sulfamethoxazole (SMX) reactor

The performance of control and SMX reactors which were operated with 2.8-day HRT and 50-day SRT during 168 days was given in Figure 4.26 and 6.27. After inoculation, ASBRs were started with an initial organic loading rate (OLR) of 1.4 g COD/L*d. OLR was increased to 2.2 g COD/L*d in a stepwise manner during first 22 days and then stable reactor performance was observed for both of the reactors, with an average effluent soluble COD 75.9±27.2 and 81.2±33.8 mg/L corresponding to a removal efficiency of 96.5% and 96.1% and average biogas production of 1045±23 mL/d and 977±15 mL/d for control and SMX reactor, respectively. Chelliapan *et al.* (2006) showed that the OLRs up to 2.5 gCOD/L*d are proper for the treatment of pharmaceuticals wastewater containing antibiotics. High loading rate with low HRT is directly affects removal efficiency. The average methane content of biogases obtained from control and SMX reactors was 65.7%±3.3. This corresponds to an average specific methane production of 0.32 and 0.3 L methane per g COD removed at control and SMX reactors, respectively. These values are consistent with

reported in the literature (Tchobanoglous *et al.*, 2003). VSS concentration and pH were stable during all operation.

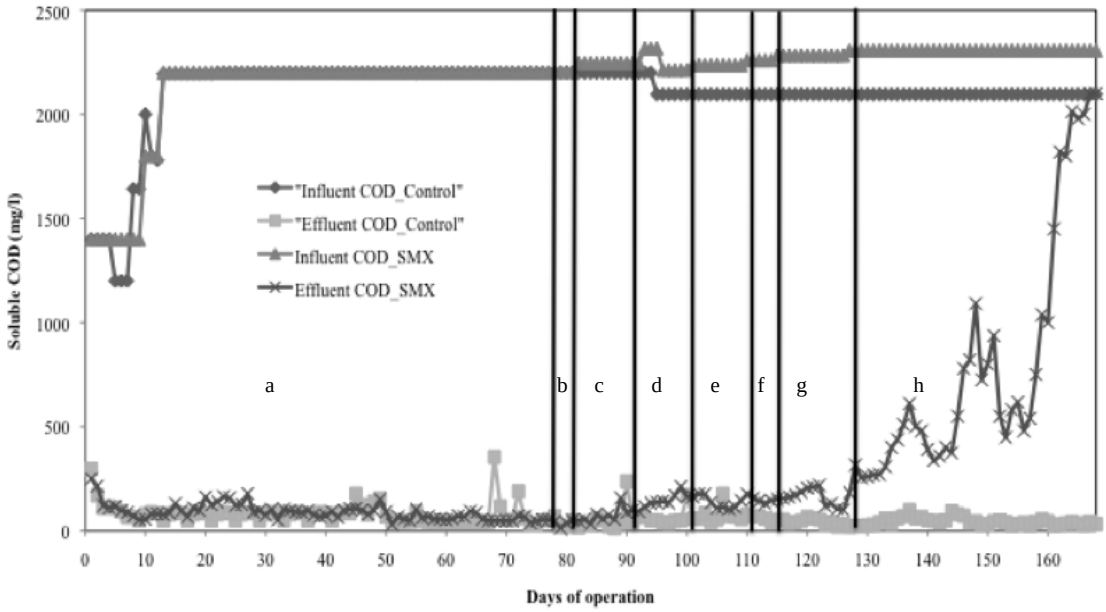

Figure 4.26: Performance of Control and SMX reactors in terms of influent and effluent soluble COD concentration (Phase a: 0 mg/L SMX, Phase b: 1 mg/L SMX, Phase c: 10 mg/L SMX, Phase d: 25 mg/L SMX, Phase e: 30 mg/L SMX, Phase f: 35 mg/L SMX, Phase g: 40 mg/L SMX, Phase h: 45 mg/L SMX).

Starting on day 78, 1 mg/L of SMX (Phase b) was added to the influent of one of the ASBR to evaluate the long-term influence and biodegradability characteristics during the acclimation period SMX on reactor performance, microbial ecology and anaerobic pathways. After 78th day, the SMX concentration in the influent was increased up to 45 mg/L (Phase h) in a stepwise manner. After first SMX addition to the reactor, the reactor was operated during 90 days. Soluble COD removal efficiency and biogas production were not affected significantly ($p>0.05$) by the addition of SMX from 1 to 25 mg/L to the ASBR influent (Phase b and c, days 78-92). The first remarkable effect of SMX on the reactor performance was detected after 25 mg/L of SMX addition on day 92. A mild increase in the effluent soluble COD concentration to 151.6±28.7 mg/L in SMX reactor was observed at Phase d, while effluent soluble COD concentration of control reactor was 62.4±41.2 mg/L ($p<0.001$). After obtained steability in the SMX reactor, antibiotic concentration was increased to 30 and 35 mg/L by gradually on day 101 and operated at this

concentration to day 115 (Phase e and f). Almost stable COD removal efficiency observed during these phases and no significant change was determined in terms of effluent soluble COD and biogas production in the SMX reactor ($p>0.05$). However significant effect of SMX was detected at these parameters in the comparison to control reactor ($p<0.01$). Subsequently reactor performance decreased substantially after 45 mg/L of SMX addition between 127th and 168th days. The average effluent soluble COD concentration of SMX reactor was 815.5±591.6 mg/L while effluent soluble COD concentration of control reactor was 44.6±18.6 mg/L.

In contrary of this result, Sponza and Demirden (2007) found that the COD removal efficiency decreased from 87% to 68% when the sulfamerazine concentration was increased from 10 mg/L to 90 mg/L. This contradiction could be originated from different anaerobic reactor types, operation conditions or different sulfonamid compound using in the studies.

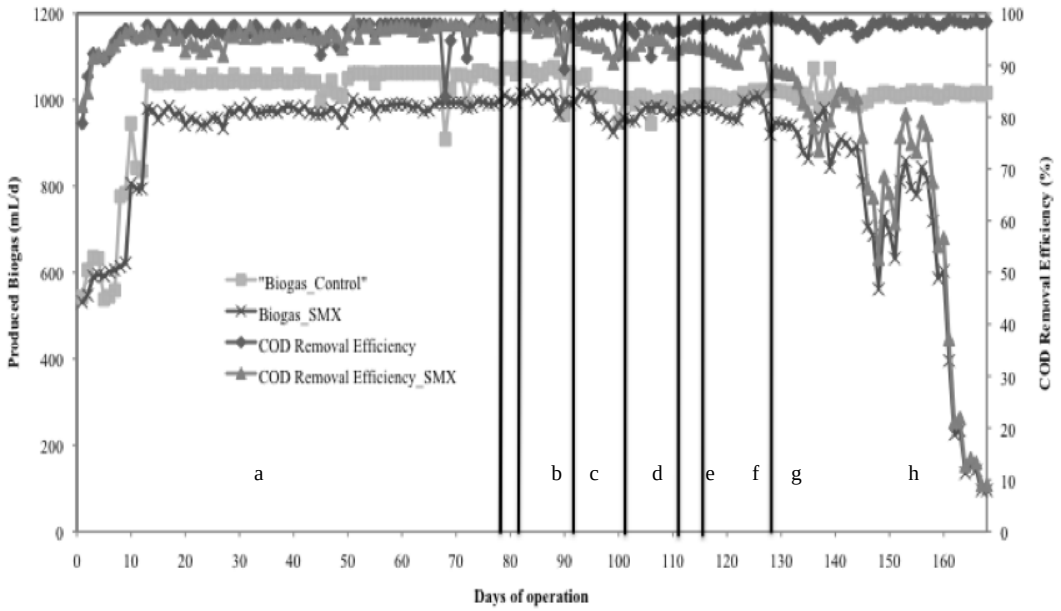

Figure 4.27: Performance of Control and SMX reactors in terms of biogas production and COD removal efficiency (Phase a: 0 mg/L SMX, Phase b: 1 mg/L SMX, Phase c: 10 mg/L SMX, Phase d: 25 mg/L SMX, Phase e: 30 mg/L SMX, Phase f: 35 mg/L SMX, Phase g: 40 mg/L SMX, Phase h: 45 mg/L SMX).

Biogas production was also parallel to effleunt soluble COD concentration and it was also dramaticaly dropped in the last phase. Also Shi *et al.* (2011) showed that methane production in the anaerobic digester, which treated manure from pig farms at 25 °C during 20 days, decreased approximately 33.3% at 25-50 mg/L of

sulfamethoxydiazine concentration, which is an antibiotic of sulfonamid group. In another study, different antibiotics effect including sulfonamids on the anaerobic lagoons was examined (Loftin *et al.*, 2005). According to this study, these compounds had the inhibitory effects changed between 20% and 45% on methane production under different concentrations varied 1 mg/L to 25 mg/L. The opposite of this represented study, the previous studies in the literature showed the inhibition in COD removal and biogas production even if low sulfonamid concentrations up to 25 mg/L.

During all operation days, methane content of biogas was stable (64.2%±4.7) in this study. However, Sponza and Demirden (2007) showed that the methane percentage in the system increased from 60% to 76% with respect to the sulfamerazine concentration.

The reactors had been inoculated with granular sludge. Throughout the entire period of rector operation, these granules remained stable and no significant dispersed growth and biofilm formation on the reactor walls was observed.

4.4.1.2 Volatile fatty acids

During the 168-day operation period, VFA could not detected in the effluent of control reactor. Also in the SMX reactor, after first SMX addition to the influent on day 78, no VFAs were detected in the effluent until 128th day. On this day, SMX concentration in the influent was increased to 45 mg/L as last phase. After that, the reactor performance in terms of not only VFA accumulation but also COD removal efficiency and biogas production started to decrease.

In the phase h, propionic acid accumulation was observed from the beginning. While propionic acid concentration in the effluent was observed in the beginning of the phase h_1 as 35 mg/L, it was increased during the operation stepwise and reached to 438 mg/L at the end of the operation period. After 146th day (Phase h_2), acetic acid accumulation started and the concentration reached to 342 mg/L at the end of the phase. Also this observed increase in VFA concentration represented approximately 50% of the effluent COD. The discrepancy between measured VFA and soluble COD in the effluent suggests that the increase of the effluent soluble COD could result from soluble microbial products (SMP) and residual antibiotic. The terms SMP is used for the organic compounds, which are the end products of substrate

metabolism and biomass decay excluding known intermediates such as VFAs (Barker and Stuckey, 1999). The composition of SMP is changed according to system and also is not characterized completely. Increase in the SMP concentration has been observed in the systems, which were exposed to toxic compounds (Aquino and Stuckey, 2004).

The results indicated that SMX affected the propionic acid degradation and acetic acid utilization pathways in the higher concentrations. Also valeric acid and butyric acid were measured in the effluent obtain from phase h, however their concentrations were less than 20 mg/L. Sponza and Demirden (2007) also found similar results while sulfamerazine, which is another antibiotic from sulfonamid group, was being fed to the anaerobic system, an increase in VFA accumulation was observed with respect to rising of antibiotic concentration. In that study, VFA accumulation around 420 mg/L was observed at10 mg/L of sulfamerazine addition and the value increased to 600 mg/L at higher than 65 mg/L of antibiotic concentration.

4.4.1.3 Antibiotic measurement

Antibiotic measurement was carried out for wastewater and sludge samples taken from different phases of SMX reactor. The results of the measurment is given in Figure 4.28.

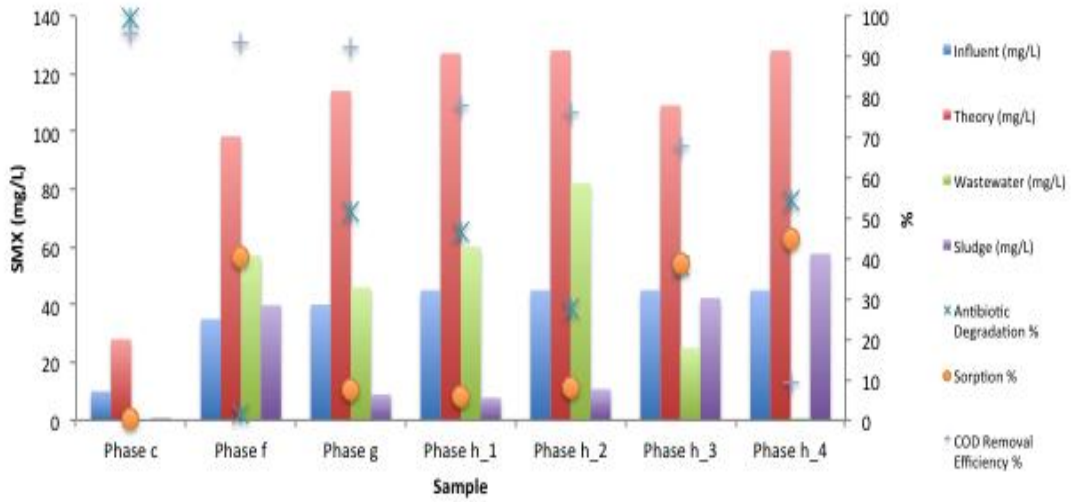

Figure 4.28: Antibiotic measurement results of SMX reactor.

As seen in Figure 4.28, in the first phases SMX was removed in the reactor in high efficiency. However, the sorption rate increased in the last two pahses, Phase h_3 and Phase h_4 suddenly. In the same time, COD removal effciiency of the reactor

decreased dramatically. It is clear that the long-term antibiotic feeding to the reactor caused an increase in accumulation of the antibiotic within the sludge and it directly affected reactor efficiency.

4.4.1.4 Specific methanogenic activity tests

Different microbial groups have different roles in the anaerobic degradation. It was anticipated that the sensitivity of these groups to SMX might vary. To further investigate this, the long-term effect of SMX on the utilization of particular substrate was investigated by specific methanogenic activity tests.

Utilization of Acetate:

Acetate utilization rates of anaerobic sludges taken from different operation days of the SMX reactor were observed during six days to identify long-term effect of SMX on the activity of acetoclastic methanogens. The cumulative biogas and methane productions were given in Figure 4.29. The acetoclastic activity of the seed sludge of chronic tests was found 77 mL of methane as given in section 4.2. After chronic study started, the methane production capacity of the sludge fed by 10 mg/L SMX decreased to 51 mL. The results show that low concentration of SMX had a clear adverse influence on acetoclastic methanogens. However, the acetate utilization capability increased with time and also surprisingly SMX concentration. As seen Figure 4.29, the cumulative biogas and methane productions were the lowest as 68 mL and 51 mL, respectively, in the phase c, in which 10 mg/L SMX was fed to the system. The values increased with the SMX concentration and reached the maximum at the end of the operation as 116 mL and 87 mL for biogas and methane production, respectively. The cumulative methane production obtained from the last samples was near to the theoretical produced methane. When 4000 mg/L of acetate utilized completely in the system, 97 mL of methane can be produced. The methane percentage in the biogas was the same in all sets, 70%.

As a result, the removal of acetic acid was not effected in higher SMX concentrations, presumably because it was accomplished by acetoclastic methanogens resistant to SMX.

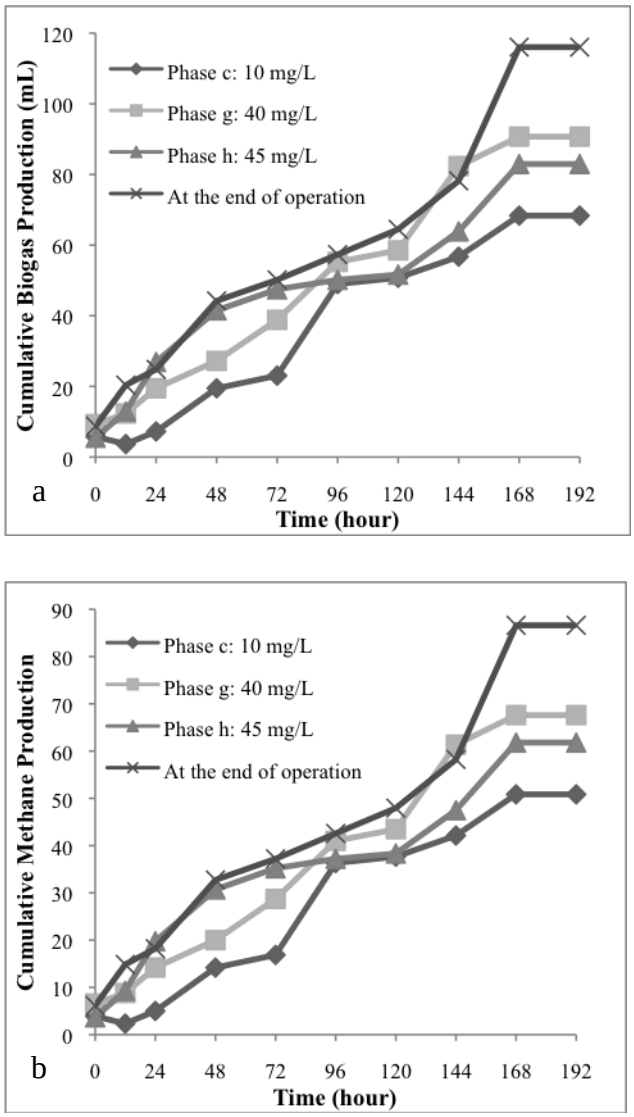

Figure 4.29: Specific methanogenic activity (SMA) test using 4000 mg/L of acetate (a) cumulative biogas production profiles (b) cumulative methane production.

Utilization of VFA mixture

VFA mixture utilization rates of anaerobic sludges taken from different operation days of the SMX reactor were also determined during six days to identify long-term effect of SMX on the homoacetogenic and methanogenic pathways. The cumulative biogas and methane productions were given in Figure 4.30. Theoretical methane production from this mixture is 315 mL. The results showed a clear inhibition effect of SMX of these metabolic pathways. Also the highest CMP value was also 40% of this theoretical value as seen in Figure 4.30b.

Even the inhibition effect of the SMX on the utilization of VFA mixture was more noticable compared to acetate utilization, the gas profiles of these two data sets were

similar. The lowest CBP and CMP were obtained from the sludge of phase c. The values increased with the SMX concentration and reached the highest values as 190 mL and 123 mL for CBP and CMP, respectively, at the end of the operation. Sponza and Demirden (2007) was found that specific methanogenic activity of the sulfamerazine, which is another antibacterial from sulfonamid group, fed anaerobic sludge increased approximately 2.5 fold 0 to 65 mg/L of sulfamerazine. However, the authors observed a decrease in SMA results after 65 mg/L of the compound.

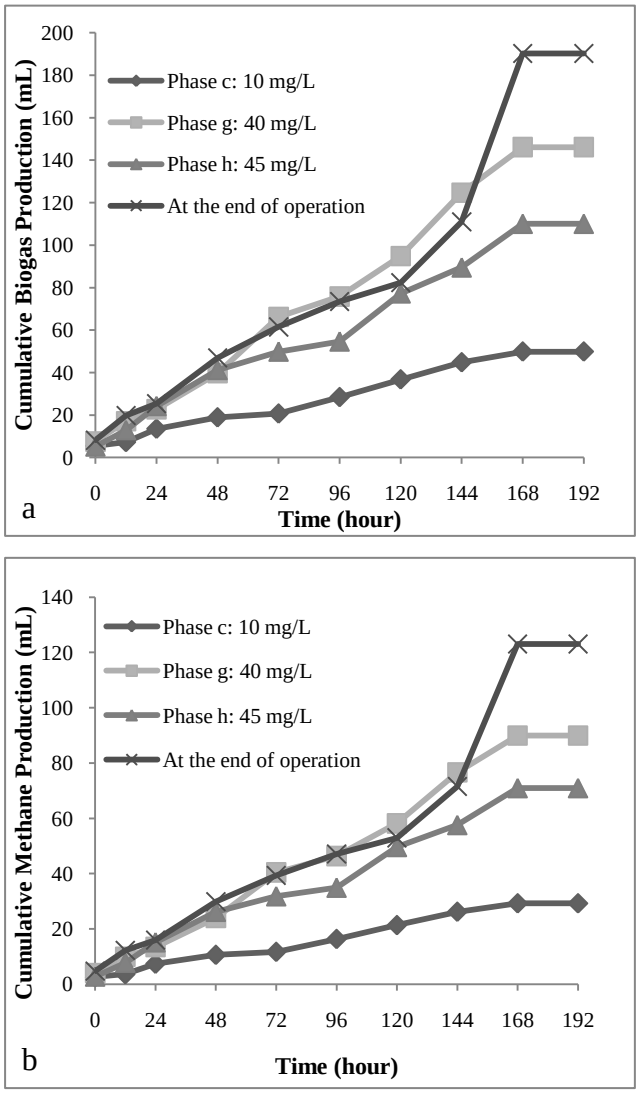

Figure 4.30: Specific methanogenic activity (SMA) test using 3000 mg/L of VFAs (a) cumulative biogas production profiles (b) cumulative methane production.

Also, the methane percentage in the biogas changed with the concentration of the SMX. While 60% of methane was detected in phase c, this ratio increased to 65% in the phase h. The results indicated that homoacetogens and hydrogenotrophic

methanogens could be more sensitive to SMX, however the system was recovered itself and acclimated to the compound even if high concentration.

Decreased utilization of butyrate and propionate is consistent with the fact that these substrates are used directly by bacteria, homoacetogens. SMX also has a bacteriostatic inhibition effect on folic acid production of especially gram positive and negative cocci (Sweetman. 2009). VFAs are not directly used by methanogens, however different groups of syntrophic bacteria use specific VFAs.

Biomass that had been exposed to SMX longer time exhibited a much higher methane production. Thus for the saturated fatty acid-beta acid oxidizing bacteria, long term exposure to SMX resulted in an adaptation which could be caused either by the development of the resistance in existing bacteria or by an increase in the abundance of resistant populations. However, this increased resistance to SMX was not universal for all populations, since the COD removal efficiency and biogas production in the ASBR did not recover for the duration of experiment.

4.4.1.5 Microbiological approach

16S rDNA and rRNA microbiological analysis were done to determine the effects of SMX on existing and active microorganisms, relatively. In this scope, genomic DNAs and total RNAs were extracted from the samples collected form different phases of the SMX reactor and RNAs were converted to cDNAs by reverse transcriptase PCR (RT-PCR). Amplicons were obtained using 16S rRNA gene spesific primers for further analysis on DGGE. Furthermore, GDNAs and cDNA were used to quantify total bacteria, archaea and methanoges by Q-PCR.

Predominant bands in the DGGE patterns

To assign the composition of the predominant community and its shift during the operation time in terms of existing and active species, 16S rDNA and rRNA DGGE profiles of the samples were compared to bacterial and archaeal clone libraries of the seed sludge, which were given in the section 6.2. The comparison results of bacterial and archaeal 16S rDNA DGGE band patterns were given in Table 4.5 and 4.6, respectively.

13 of the bacterial OTUs detected in the 16S rDNA DGGE profiles. According to these results, the matched Clostridum species (AB114241, AM158323, and

AY648564) almost detected during the operation and their intensities did not noticebly change with respect to SMX concentration and opertaion time. Clostridium species frequently presence in the anaerobic systems and they are resposible to fermentation and some species especielly produce the ethanol. Addition of them, Clostridium spesice have the role on the starch degradation by exo-enzymes (Hungate, 1982; Payton and Haddock, 1986). So, the results are not surprise for this system fed by a synthetic wastewater including starch. Other OTUs detected in the samples almost belong to the uncultured clones or unclassfied bacterial clutured species.

Differently from 16S rDNA bacterial DGGE pattern, the intensity of matched archaeal DGGE predominant band were higher as seen in Table 4.6. 14 of the archaeal OTUs detected in the 16S rDNA DGGE profiles. According to the results, acetoclastic methanogen species (U89773, FR836463, FR836464, and FR836468) disappered at the last phases. However, the abundance of hydrogenotrophic methanogens, especially Methanobacterium species, and methanogenic archeons (AY350742, AY552778, HM630570, FR836462, and FR836467) were higher and they seemed almost in every steps. When compared to the results obtained from rector operation and SMA tests, it was expected that acetoclastic methanogens were dominant in the system. Especially, the SMA test results indicated that acetoclastic methanogenic activity increased with respect to SMX concentration.

Table 4.5: Affiliation of bacterial clones and their abundance at the different phases of the SMX reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase d	Phase g	Phase h_1	Phase h_2	Phase h_3	Phase h_4
B1	2.80	FR836435	*Clostridium sp. BG-C66* (FJ384378)	96							
B2	0.93	FR836436	*Clostridium sp. Kas301-1* (AB114242)	96							
B3	1.87	FR836437	*Clostridium intestinale* (AM158323)	95	•	•	•	•	•	•	•
B4	4.67	FR836438	Uncultured Clostridium sp. clone AUCLO214 (AY648564)	96	•	••	••	•	•	•	
B5	1.87	FR836439	Uncultured Firmicutes bacterium clone QEDS2BE07 (CU921543)	93			•		•	•	•
B6	2.80	FR836440	Uncultured Firmicutes bacterium clone QEDN7DC08 (CU926749)	97			•				•
B7	14.95	FR836441	Uncultured bacterium clone BS7_65 (GQ458219)	98							
B8	3.74	FR836442	*Syntrophomonas zehnderi strain OL-4* (DQ898277)	89		•	••	•			
B9	33.64	FR836443	*Clostridium sp. Kas107-1* (AB114241)	96	•	••	••		•	•	•
B10	5.61	FR836444	Uncultured bacterium clone C3-25 (AM181973)	91	•	•	•	•			
B11	4.67	FR836445	*Bacterium CBIC19I3* (HM240274)	95							
B12	3.74	FR836446	Uncultured bacterium UASB_TL84 (AF254390)	94	•		•			•	

Table 4.5 (continued): Affiliation of bacterial clones and their abundance at the different phases of the SMX reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b: 82nd day	Phase d: 93rd day	Phase g: 128th day	Phase h: 136th day	Phase h: 145th day	Phase h: 158th day	Phase h: 166th day
B13	0.93	FR836447	Uncultured Candidatus Cloacamonas sp. clone 8SN (EU887773)	92							
B14	5.61	FR836448	Uncultured bacterium clone BS1_25R (GQ458208)	97		•	•	•			
B15	2.80	FR836449	Uncultured bacterium clone aaa97f10 (DQ816701)	96							
B16	0.93	FR836450	Acinetobacter sp. 1B3 (EU337120)	96							
B17	2.80	FR836451	Uncultured Sarcina sp. clone JPL-2_O10 (FJ957591)	89			•		•	••	
B18	0.93	FR836452	Uncultured bacterium clone 30c07 (EF515566)	87							
B19	0.93	FR836453	Paludibacter propionicigenes (AB078842)	92							
B20	0.93	FR836454	Bacterium 061128-OL-KR37-AA 3-0 10000x - 1A (FJ037613)	92						•	•
B21	0.93	-	Propionibacteriaceae bacterium FH044 (AB298766)	99							
B22	1.87	FR836458	Uncultured bacterium clone D14R15C96 (FM956760)	98						•	

Table 4.6: Affiliation of archaeal clones and their abundance at the different phases of the SMX reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase d	Phase g	Phase h_1	Phase h_2	Phase h_3	Phase h_4
A1	1.45	FR836460	Uncultured archaeon clone OK3 (GQ406364)	94							
A2	2.90	FR836461	Uncultured archaeon clone I17_Rya (HM639831)	98	••						
A3	9.42	FR836462	*Methanogenic euryarchaeote Annu4* (FJ896263)	95	••••	•••	•••	••	••	••••••	••
A4	2.90		*Methanobacterium formicicum strain S1* (DQ649309)	99							
A5	2.90	FR836464	Uncultured Methanosarcinales archaeon clone:KuA4 (AB077214)	98					••		
A6	4.35	FR836470	Uncultured euryarchaeote clone MVP-8A-21 (DQ676281)	98		••					
A7	12.32	FR836463	*Methanothrix soehngenii* (X51423)	97	••						
A8	0.72		Uncultured archaeon clone A6T20L208 (DQ262482)	99							
A9	7.25		*Methanospirillum hungatei* (M60880)	99							
A10	0.72	FR836465	*Methanobacterium beijingense strain M4* (EU544027)	97							
A11	6.52	FR836467	*Methanosphaerula palustris strain E1-9c* (EU156000)	95		••	••	••	•••		••
A12	0.72		*Methanobacterium sp. OM15* (AJ550160)	99							
A13	2.17		*Methanobacterium beijingense strain 8-2* (AY350742)	100	••		•••	•••	••••	•••	•••

Table 4.6 (continued): Affiliation of archaeal clones and their abundance at the different phases of the SMX reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase d	Phase g	Phase h_1	Phase h_2	Phase h_3	Phase h_4
A14	3.62	FR836468	Uncultured Methanosarcinales archaeon clone S4 (GU475184)	95	••						
A15	2.9	FR836469	Uncultured archaeon clone Sed-ARC-34 (EU255763)	98					•••		
A16	3.62	FR836471	Uncultured euryarchaeote clone MVP-8A-26 (DQ676246)	96	••	•••					
A17	9.42	FR836472	*Methanogenic archaeon Prasan1* (HM630570)	97	•••			••	•••	••	••
A18	7.25	FR836473	*Methanobacterium beijingense strain 4-1* (AY552778)	96	••		••	•••	•••	••	•••
A19	2.17		*Methanosarcina siciliae* (U89773)	99			••	••		••	
A20	4.35		Uncultured archaeon clone EOTU21 (FJ896263)	99							
A21	1.45	FR836474	Uncultured archaeon clone OKA6 (GQ465437)	97							
A22	1.45		Uncultured archaeon clone ATB-KM-2942-A18 (EF680353)	99							
A23	6.52		Archaeon LL25A3 (AJ745135)	99	•••	•••	•••	•••	•••	••	••
A24	2.17		Uncultured archaeon clone WA10 (AB494239)	99							
A25	0.72	FR836475	*Methanogenic euryarchaeote Annu1* (HM630571)	95							

Also to determine the active species and their shift and/or changes in the SMX reactor with respect to the SMX concentration and operation time, the amplicons obtained from 16S rRNAs were compared with the bacterial and archaeal clone libraries. The comparison results were given in Table 4.7 and 6.8, respectively.

13 of the bacterial OTUs detected in the 16S rRNA DGGE profiles. However, 3 of these OTUs were not the same with 16S rDNA DGGE comparison which is given in Table 4.5.

According to 16S rRNA DGGE results, the uncultured Clostridum species, which were detected in 16S rDNA results (AM158323 and AY 648564), also existed in every phase, however their abundance were decreasing with respect to the SMX concentration and operation time. This result also confirm the results obtianed from reactor performance and SMA tests. While the COD removal efficiency decreased, methanogenic activity increased. Also the bacterial 16S rRNA results showed that the inhibition of SMX firstly strated in the fermentation step of the anerobic degradation pathway. Clostridium species are the important group responsible in the fermentation in the anerobic systems (Hungate, 1982; Payton and Haddock, 1986). While Acinetobacter sp. 1B3 (EU337120) was deetcted in the seed sludge, it coulndn't be matched in DGGE band patterns of the samples taken from the SMX reactor. However, Acinetobacter spp. were found in the pharmaceutical industry and hospital effluents and it has been known that they are adapted to high concentration of antibiotics because of the resistance (Guardabassi *et al.*, 1998). Other predominant bacterial clones were mostly uncultured species. These uncultured bacterial clones may probably be species responsible to fermentation and homoacetogenesis, especieally VFA-oxidizing syntrophs (Speece, 1996; Madigan *et al.*, 2009).

In the archaeal 16S rRNA samples, only one acetoclastic methanogen (U897731) was detected in only one sample, phase h_2 surprisingly. Hydrogenotrophic methanogens represented different patterns. While Methanobacterium beijingense (AY350742) were highly dominant, abundance of other hydrogenotrophic methanogens decreased. Other detected species were mostly uncultured archaeal clones as seen in Table 4.8. In anaerobic digestion, degradation of organic compounds to CO_2 and CH_4 relies greatly on methanogenic archaea for ultimate stage (Madigan *et al.*, 2009). So, the results was expected excluding not detected acetoclastic methanogens. Generally, 70% of CH_4 is produced from acetate in the

anaerobic reactors (Whiticar *et al.*, 1986), and also in biogas composition of the SMX reactor during the operation, H_2 was not observed. Additionally, VFA concentration in the effluent indicated that acetate accumulation in the reactor started in Phase h_2 and SMA test results also indicated that acetoclatic activity increaaed especially in the last phases. This concept put forward to another pathway; homoacetogenesis with hydrogenotrophic methanogenesis. Acetate could be converted to H_2 +CO_2 by homoacetogens; and H_2 might be used to produce methane in also early phase of the operation. Hydrogenotrophic methanogens have been also detected during the anaerobic treatment of pharmaceutical industry wastewater and manure digestion especially including antibiotics (Gomez-Silvan *et al.*, 2010; Stone *et al.*, 2010).

Table 4.7: Affiliation of bacterial clones and their abundance within the active community at the different phases of the SMX reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase d	Phase g	Phase h_1	Phase h_2	Phase h_3	Phase h_4
B1	2.80	FR836435	*Clostridium sp. BG-C66* (FJ384378)	96						
B2	0.93	FR836436	*Clostridium sp. Kas301-1* (AB114242)	96						
B3	1.87	FR836437	*Clostridium intestinale* (AM158323)	95	••••	••••	•••	••	•	••
B4	4.67	FR836438	Uncultured Clostridium sp. clone AUCLO214 (AY648564)	96	••••	••••	••••	•••		•
B5	1.87	FR836439	Uncultured Firmicutes bacterium clone QEDS2BE07 (CU921543)	93	•••			•••	••	•
B6	2.80	FR836440	Uncultured Firmicutes bacterium clone QEDN7DC08 (CU926749)	97	•••	•••	•••		••	
B7	14.95	FR836441	Uncultured bacterium clone BS7_65 (GQ458219)	98	••••••••••	•••••••••	•••••••••	••••••	•••	•••
B8	3.74	FR836442	*Syntrophomonas zehnderi strain OL-4* (DQ898277)	89	••	••	•••			
B9	33.64	FR836443	*Clostridium sp. Kas107-1* (AB114241)	96						
B10	5.61	FR836444	Uncultured bacterium clone C3-25 (AM181973)	91	••					
B11	4.67	FR836445	*Bacterium CBIC19I3* (HM240274)	95						

Table 4.7 (continued): Affiliation of bacterial clones and their abundance within the active community at the different phases of the SMX reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase d	Phase g	Phase h_1	Phase h_2	Phase h_3	Phase h_4
B12	3.74	FR836446	Uncultured bacterium UASB_TL84 (AF254390)	94	••				••	•
B13	0.93	FR836447	Uncultured Candidatus *Cloacamonas sp. clone 8SN* (EU887773)	92						
B14	5.61	FR836448	Uncultured bacterium clone BS1_25R (GQ458208)	97	•••	•••	•••			
B15	2.8	FR836449	Uncultured bacterium clone aaa97f10 (DQ816701)	96						
B16	0.93	FR836450	*Acinetobacter sp. 1B3* (EU337120)	96						
B17	2.8	FR836451	Uncultured Sarcina sp. clone JPL-2_O10 (FJ957591)	89	•••					
B18	0.93	FR836452	Uncultured bacterium clone 30c07 (EF515566)	87						
B19	0.93	FR836453	*Paludibacter propionicigenes* (AB078842)	92			•••			
B20	0.93	FR836454	*Bacterium 061128-OL-KR37-AA 3-0 10000x -1A* (FJ037613)	92					•	
B21	0.93	-	*Propionibacteriaceae bacterium FH044* (AB298766)	99						
B22	1.87	FR836458	Uncultured bacterium clone D14R15C96 (FM956760)	98			•••			

Table 4.8: Affiliation of archaeal clones and their abundance within the active community at the different phases of the SMX reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase d	Phase g	Phase h_1	Phase h_2	Phase h_3	Phase h_4
A1	1.45	FR836460	Uncultured archaeon clone OK3 (GQ406364)	94						
A2	2.90	FR836461	Uncultured archaeon clone I17_Rya (HM639831)	98				•••		
A3	9.42	FR836462	*Methanogenic euryarchaeote Annu4* (FJ896263)	95			••••		••••	••••
A4	2.90		*Methanobacterium formicicum strain S1* (DQ649309)	99						
A5	2.90	FR836464	Uncultured Methanosarcinales archaeon clone:KuA4 (AB077214)	98						
A6	4.35	FR836470	Uncultured euryarchaeote clone MVP-8A-21 (DQ676281)	98						
A7	12.32	FR836463	*Methanothrix soehngenii* (X51423)	97						
A8	0.72		Uncultured archaeon clone A6T20L208 (DQ262482)	99						
A9	7.25		*Methanospirillum hungatei* (M60880)	99						
A10	0.72	FR836465	*Methanobacterium beijingense strain M4* (EU544027)	97	••••••	•••		•••		
A11	6.52	FR836467	*Methanosphaerula palustris strain E1-9c* (EU156000)	95	•••••		••••••			
A12	0.72		*Methanobacterium sp. OM15* (AJ550160)	99			••••			
A13	2.17		*Methanobacterium beijingense strain 8-2* (AY350742)	100	••••••	••••••••••	••••••••	••••••••••	••••••••••	••••••••••

Table 4.8 (continued): Affiliation of archaeal clones and their abundance within the active community at the different phases of the SMX reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase d	Phase e g	Phase h_1	Phase h_2	Phase h_3	Phase h_4
A14	3.62	FR836468	Uncultured Methanosarcinales archaeon clone S4 (GU475184)	95						
A15	2.9	FR836469	Uncultured archaeon clone Sed-ARC-34 (EU255763)	98	••	•••	•••	•••	•••	
A16	3.62	FR836471	Uncultured euryarchaeote clone MVP-8A-26 (DQ676246)	96						
A17	9.42	FR836472	*Methanogenic archaeon Prasan1* (HM630570)	97	•••	••••	••••	•••••	•••••	••••
A18	7.25	FR836473	*Methanobacterium beijingense strain 4-1* (AY552778)	96	••••	••••••	•••••••	••••••••	••••••	•••••
A19	2.17		*Methanosarcina siciliae* (U89773)	99				••••		
A20	4.35		Uncultured archaeon clone EOTU21 (FJ896263)	99						
A21	1.45	FR836474	Uncultured archaeon clone OKA6 (GQ465437)	97						
A22	1.45		Uncultured archaeon clone ATB-KM-2942-A18 (EF680353)	99						
A23	6.52		*Archaeon LL25A3* (AJ745135)	99	••••••	•••••	•••••	••••	•••••	•••••
A24	2.17		Uncultured archaeon clone WA10 (AB494239)	99						
A25	0.72	FR836475	*Methanogenic euryarchaeote Annu1* (HM630571)	95						

Cluster analysis of DGGE banding pattern

Microbial diversity and shifts in bacterial and archaeal communities, which presented in the SMX reactor during the operation time, were estimated based on the DGGE patterns of the partial 16S rDNA amplicons. A total of eight sludge samples were compared: The seed sludge of the chronic reactors and the sludges of SMX reactors. Result of DGGE analysis suggested that bacterial community was respectively, more diverse than archaeal diversity in the reactor; 71 bacterial and 51 archaeal bands were remarked in the SMX reactor samples.

Bacterial and archaeal diversities and species richness in seed sludge and sludges of SMX reactors were measured by the Shannon-Weaver diversity index and Margalef's species richness, respectively. The values were given in Table 4.9 and 4.10.

In seed sludge, while 33 of different bacterial species were detected, this number decreased to 7 at the end of operation as seen in Table 4.9. Also archaeal species number changed during the operation time; in the phase be, it increased to 21 from 13 (in seed sludge). However the archaeal spesices number dramatically decreased to in the last period of the operation. However the change in both bacterial and archaeal OTUs was not steady. After the addition of SMX to the reactor, bacterial and archaeal species were generally stable until the 45 mg/L of SMX concentration (phase h). In the last phase (phase h), both of them firstly increased and then dramatically decreased.

The results indicated that SMX addition to the acclimated sludge affected to species, especially bacterial ones, only in the highest concentration, 45 mg/L. In the previous phases, the diversity was almostly held steady. The sharp decrease in bacterial OTUs was observed at the beginning of the phase h. The diversity was recovered in a while, but it was not prevented decrease in bacterial OTUs again at the end of the operation.

Table 4.9: Shannon-Weaver diversity indices, Margalef's species richness measure, and eveness measure estimates for bacterial DGGE profiles of sludges taken from different phases of the SMX reactor.

	Seed_Chronic Reactors	Phase b	Phase d	Phase g	Phase h_1	Phase h_2	Phase h_3	Phase h_4
Taxa	33	27	18	34	13	25	26	7
Shannon-Weaver diversity	3.449	3.093	2.734	3.406	2.329	3.166	3.225	1.928
Evenness	0.9537	0.8166	0.8556	0.8866	0.7895	0.9482	0.9677	0.9823
Margalef's richness	4.954	4.08	2.797	5.064	2.265	3.967	4.274	1.33

Table 4.10: Shannon-Weaver diversity indices, Margalef's species richness measure, and eveness measure estimates for archaeal DGGE profiles of sludges taken from different phases of the SMX reactor.

	Seed_Chronic Reactors	Phase b	Phase d	Phase g	Phase h_1	Phase h_2	Phase h_3	Phase h_4
Taxa	13	21	13	13	10	19	10	10
Shannon-Weaver diversity	2.526	3.009	2.525	2.501	2.256	2.907	2.272	2.159
Evenness	0.962	0.9655	0.9605	0.9379	0.9544	0.963	0.9698	0.8666
Margalef's richness	1.901	2.933	1.825	1.826	1.478	2.568	1.43	1.354

The DGGE analyses were done by Pearson product-moment correlation coefficients and UPGMA clustering. The bacterial and archaeal 16S rDNA phylogenetic trees obtained from SMX reactor were given in Figure 4.31 and 4.32. The analysis of DGGE band patterns from the SMX reactor sludge samples revealed that the samples differentiated not only by SMX addition but also by operation time.

The changes in bacterial community during the operation reflect the clusters obtained from DGGE band patterns, which were divided into two clusters. The clusters were defined by less than 70% pattern similarity. One of them included Phase d, g and h_1. This cluster also divided into two sub-clusters based on their population origin, which were defined by a similarity pattern varied between 87.4% and 94.2. The other cluster including seed sludge and Phase b, h_2, h_3 and h_4 samples divided two

sub-clusters. In the first sub-cluster, seed sludge and Phase h_3 samples were defined by 81% similarity. In the second sub-cluster, Phase b, h_2 and h_4 samples were defined by the similarities changed 85.4% to 94.8%. As seen in dendogram (Figure 4.31), the pattern similarity of the samples did not change very distinctly.

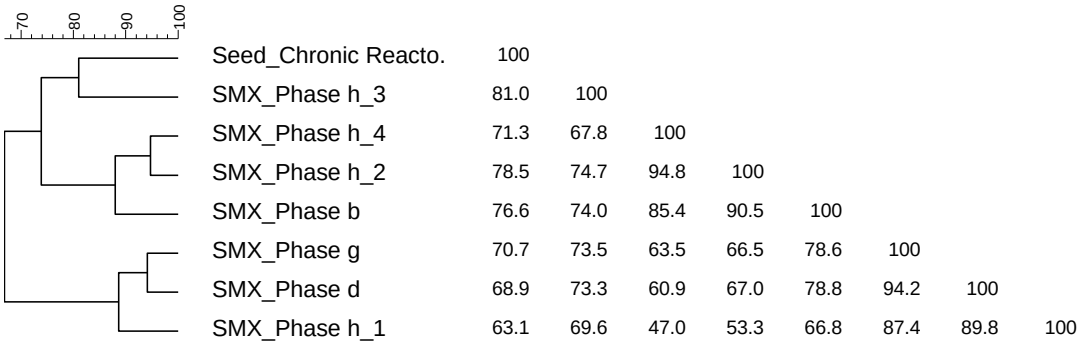

Figure 4.31: Cluster analysis and similarity matrix of bacterial 16S rDNA gene DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

As for the Bacteria, clustering of 16S rDNA gene DGGE banding pattern revealed two main clusters defined by less than 60% similarity for Archaea (Figure 4.32). In the first cluster, seed sludge and Phase_3 sample were defined by a similarity 56.2% These samples gave different patterns compared to others. The second cluster also divided into two sub-clusters. One of them included Phased, g and h_1 samples and the samples defined by the similarities varied between 77.1% and 83.8%. The second sub-cluster also gave similar profile and the samples in this subcluster, Phase b, h_2 and h_4, defined the similarities varied 85.6% to 89.4%. Differently from the bacterial dendogram, the archaeal pattern similarity of the samples changed more distinctly.

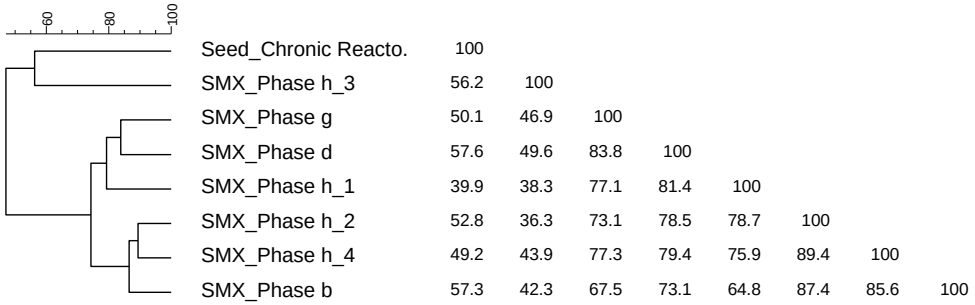

Figure 4.32: Cluster analysis and similarity matrix of archaeal 16S rDNA DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

DGGE analysis was also performed to find out the active community changes during the operation of the SMX reactor. The bacterial and archaeal phylogeneticdendograms obtained from DGGE band patterns were represented in Figure 4.33 and 4.34, respectively.

The bacterial band patterns obtained from 16S rRNA gave the similar result to 16S rDNA band patterns. Phase h_3 sample differentiated from other samples obviously. As seen in Figure 4.33, Phase h_3 samples was defined by a similarity as 65.1% to the closest sample, Phase h_4. The closest phases in terms of active bacterial community were Phase g and h_1, which were defined by a similarity 78.3%.

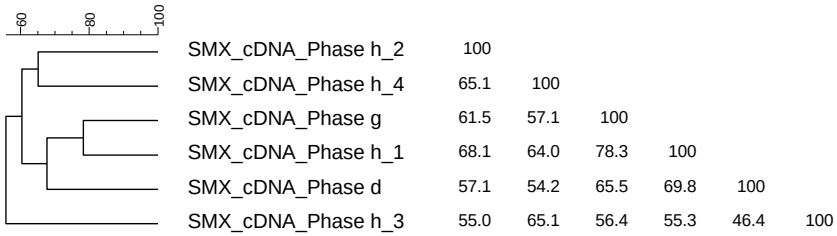

Figure 4.33: Cluster analysis and similarity matrix of bacterial 16S rRNA DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

In the archaeal phylogenetic tree of 16S rRNA, the dendogram structures and similarities were different from the dendogram of archaeal 16S rDNA. As seen in Figure 4.34, the distribution of the active archaeal community reflected the clusters, which were divided into two. One of this cluster included Phase h_3 and h_4 samples, which defined by a similarity 93.6%. The second cluster also divided into two sub-clusters. While Phase h_2 sample formed one sub-cluster defined by a similarity less than 90% to other sub-cluster. The second sub-cluster was constituted by two other clusters including Phase d, g and h_1 samples and these clusters were defined by the similarities varied between 92.3% and 97.8%.

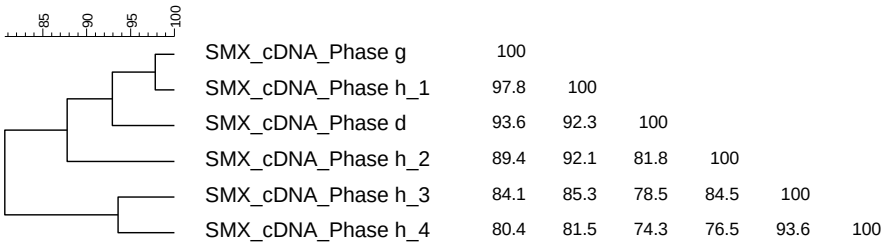

Figure 4.34: Cluster analysis and similarity matrix of archaeal 16S rRNA gene DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

No significant changes in 16S rDNA and 16S rRNA profile of bacterial and archaeal communities were detected. However, the microbial shift especially in the acitve community was expected, and thus this result is surprising.

Quantification by Q-PCR

Quantitative changes of 16S rDNA and 16S rRNA concentrations in terms of bacteria, archaea and methanogens were also determined by Q-PCR. The concentration profiles showed the temporal variations in the microbial community and the active population at different phases of the SMX reactor. As seen in Figure 4.35-4.37, the bacterial, archaeal and methanogenic populations increased after the reactor operation started. Also there is a decrease for all of them in Phase h_1. In the same Phase, COD removal efficiencey started to decrease in the reactor. The reason of the increase in the microbial community in Phase b could be adaptation of the new conditions. However, this adaptation was not observed in the reactor efficiency. The bacterial, archaeal and methanogenic community varied from 3.03 X 10^8 to 1.23 X 10^{10}, from 1.00 X 10^7 and to 2.53 X 10^9, and from 4.05 X 10^6 to 1.12 X 10^9, respectively. As seen in Figure 4.37, the highest methanogenic population was determined in Phase b. When compared to seed sludge, the amount of methanogens was increased approximately 275 times in Phase b. Also more oscillation in the microbial groups was observed in methanogens.

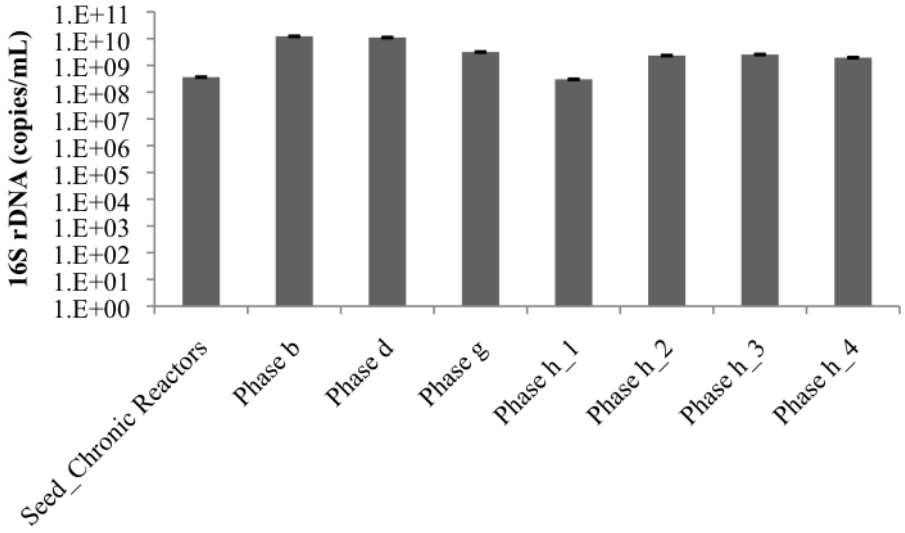

Figure 4.35: Quantitative changes in bacterial 16S rDNA concentrations at different phases of the SMX reactor.

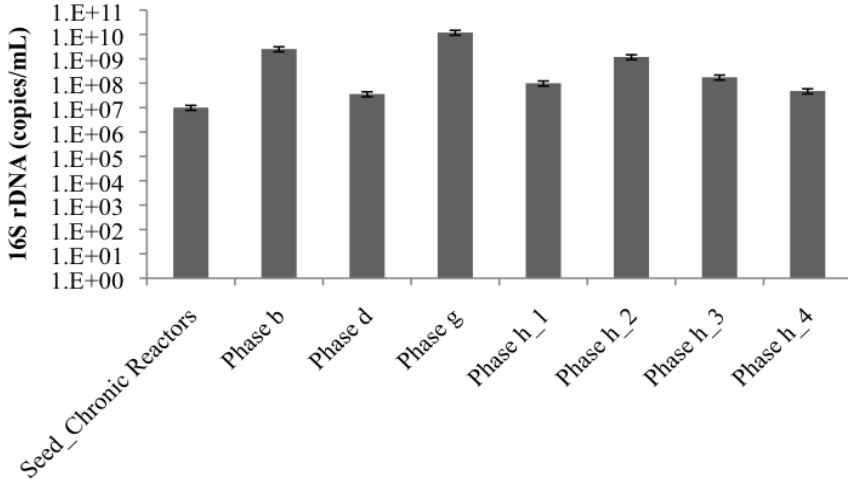

Figure 4.36: Quantitative changes in archaeal 16S rDNA concentrations at different phases of the SMX reactor.

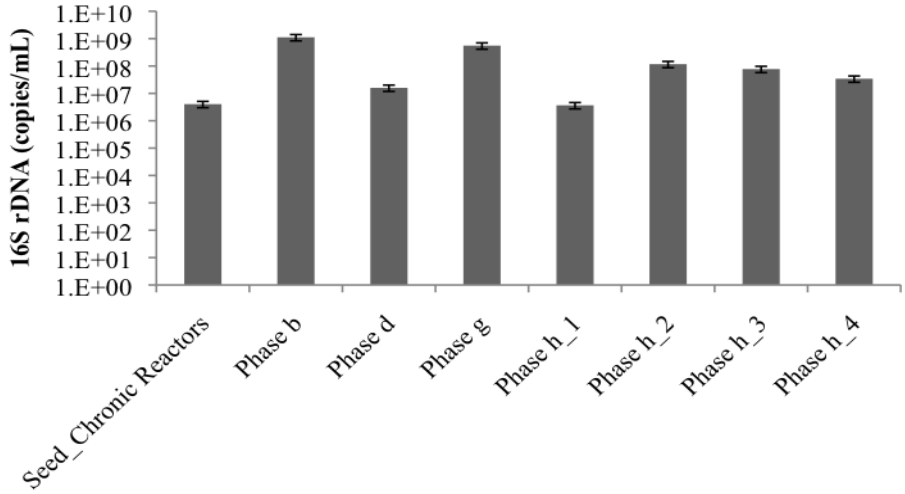

Figure 4.37: Quantitative changes in methanogenic 16S rDNA concentrations at different phases of the SMX reactor.

Besides quantification of 16S rDNA concentration, 16S rRNAs were quantified to determine the effects of SMX on the specific microbial groups. Quantification results of bacteria, archaea and methanogens were given in Figure 4.38-4.40, respectively. While the reactor operation data and SMA test results changed during the operation time, 16S rRNA expression level in the system was not changed noticeably.

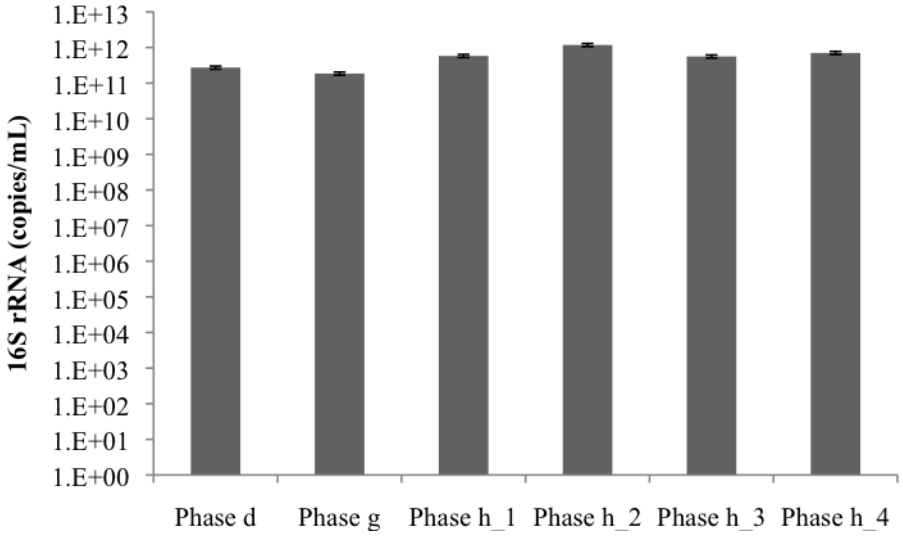

Figure 4.38: Quantitative changes in bacterial 16S rRNA concentrations at different phases of the SMX reactor.

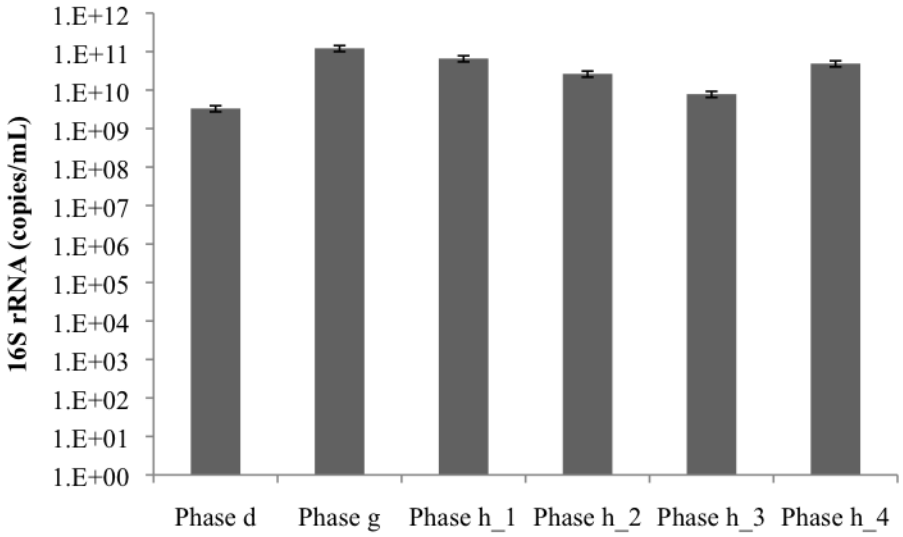

Figure 4.39: Quantitative changes in archaeal 16S rRNA concentrations at different phases of the SMX reactor.

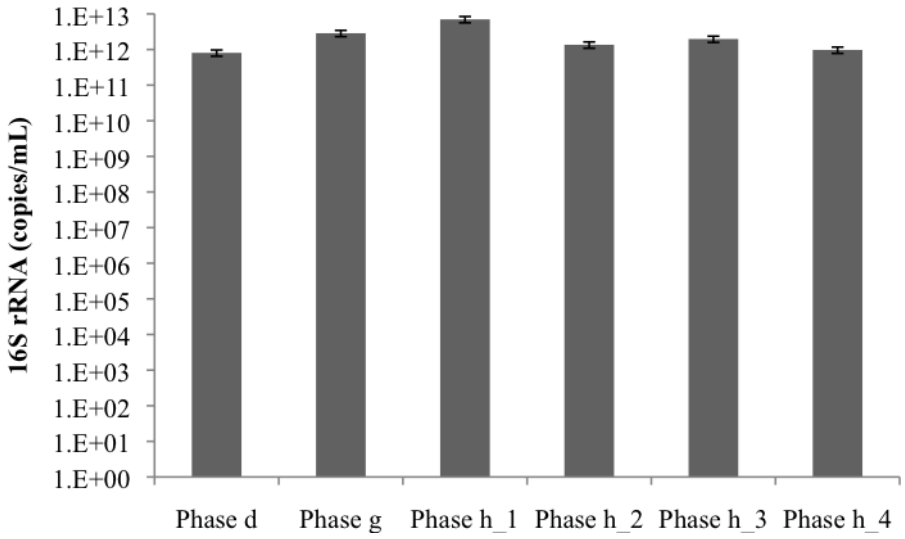

Figure 4.40: Quantitative changes in methanogenic 16S rRNA concentrations at different phases of the SMX reactor.

Enzyme expression

To detect the inhibition effect of the SMX on the anaerobic pathways three enzymes were selected based on mRNA level from the different metabolic pathways of the anaerobic degradation. Formylterahydrofolate synthetase (FTHFS), methyl-coenzyme M reductase (mcrA) and acetyl-coA synthetase (ACAS) were selected to determine the inhibition on homoacetogenesis, methanogenesis and specifically acetoclastic methanogenesis, respectively.

Despite acetate and other VFAs were not observed in effluent and active methanogenic species were detected in the system, FTHFS expression was surprisingly not observed in the mRNA level.

Expression of mcrA gene was detected in every phase of the SMX reactor. While biogas production was really low at the end of the reactor operation, expression level of mcrA gene was also very low (1.03×10^2 copies/mL) as seen in Figure 4.41. A stepwise decrease was observed in the mcrA gene expression with respect to SMX concentration. This result was supported by reactor operation data in terms of biogas production.

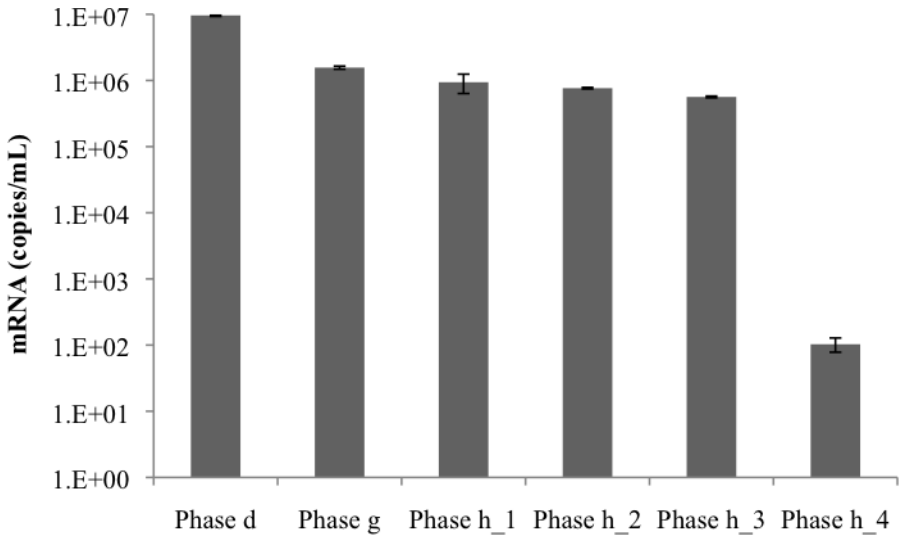

Figure 4.41: Quantitative changes in methyl-coenzyme M reductase expression level at different phases of the SMX reactor.

ACAS gene expression results were given in Figure 4.42. Differently from mcrA, ACAS couldn't be detected at the end of the reactor operation. Surprisingly, the expression level of ACAS was higher than mcrA at Phase d, g, and h_1. However, in DGGE analysis of archaeal 16S rRNA, cultured Methanosaeta couldn't be detected.

ACAS gene expression results were given in Figure 4.42. Differently from mcrA, ACAS couldn't be detected at the end of the reactor operation. Surprisingly, the expression level of ACAS was higher than mcrA at Phase d, g, and h_1. However, in DGGE analysis of archaeal 16S rRNA, cultured Methanosaeta couldn't be detected. The main reason of this discrepancy between DGGE analysis and Q-PCR results could be the potential bias of the molecular tools with differences in DNA extraction efficiency, gene copy number or PCR amplification and primer biases, must be considered. However, cultivation-independent, molecular techniques currently provide the best approach for analysis of complex natural microbial communities and in this study, sludge samples investigated were processed using identical techniques and data analyses, and biases are likely to have been similar, thus differences detected would be significant and would arise from structural differences in the examined samples.

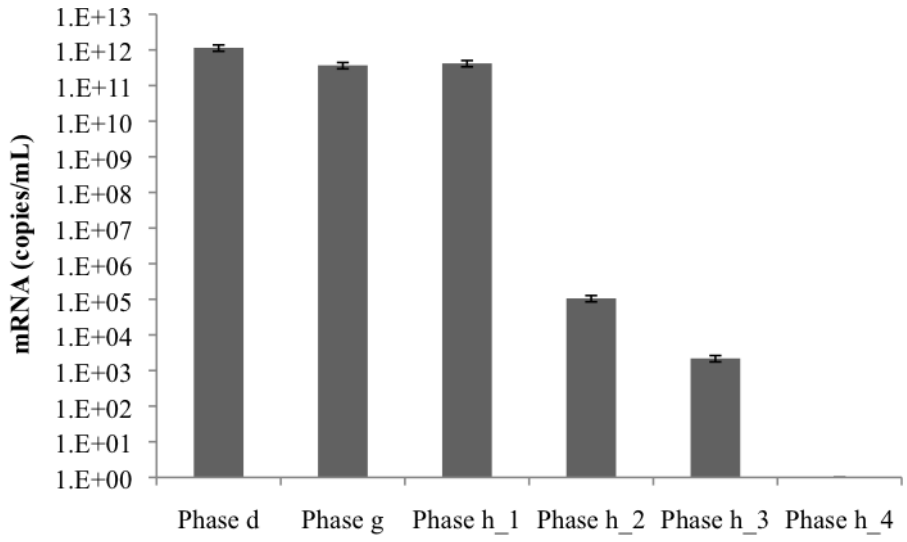

Figure 4.42: Quantitative changes in acetyl-coA synthetase expression level at different phases of the SMX reactor.

The acetyl-coA pathway has been known to be operational in acetate assimilation, and thereby, the FTHS expression can reflect the outgrowth of such bacteria. (Drake, 1994). However the temporal expression of this gene rules out the possibility that the expression was accounted for by assimilative metabolism (Akuzawa *et al.*, 2010).

4.4.2 Erythromycin

4.4.2.1 Performance of the Erythromycin (ERY) reactor

To evaluate the long-term effect of ERY on anaerobic systems, an ERY fed reactor was operated during 154 days with a control reactor. The performances of both reactors in terms of COD and biogas are given in Figure 4.43 and 4.44. After the start-up, the OLR was increased gradually from 1.4 g COD/L*d. OLR was increased to 2.25 g COD/L*d in first 22 days. In a previous study focusing on the influence of ERY to the anaerobic systems, OLR was selected as 2.9 g COD/L*d, which was close to this study (Amin *et al.*, 2006). Also in other studies evaluated anaerobic treatment of pharmaceutical industry wastewaters, the anaerobic reactors were operated by OLRs in a range between 0.43-3.73 g COD/L*d (Chelliapan *et al.*, 2006; Sponza and Demirden, 2007; Shimada *et al.*, 2008) and Chelliapan *et al.* (2006) found that high OLR with low HRT is positively affects COD removal efficiency in the anaerobic systems fed by pharmaceutical industry wastewater. Then, both of the reactors were operated at the steady-state until 78th day with an average effluent soluble COD 75.9±27.2 and 84.4±24.6 mg/L corresponding to a removal efficiency

of 96.5% and 96.5% and average biogas production of 1045±23 mL/d and 1041±12 mL/d for control and ERY reactor, respectively. Also methane percentage of the biogases produced in the Control and ERY reactors were 64.8%±2.6. This corresponds to an average specific methane production of 0.32 L CH_4/g COD_removed at control and ERY reactors before the feeding of antibiotic to the system. These values are consistent with reported in the literature (Tchobanoglous *et al.*, 2003). VSS concentration and pH were stable during all operation.

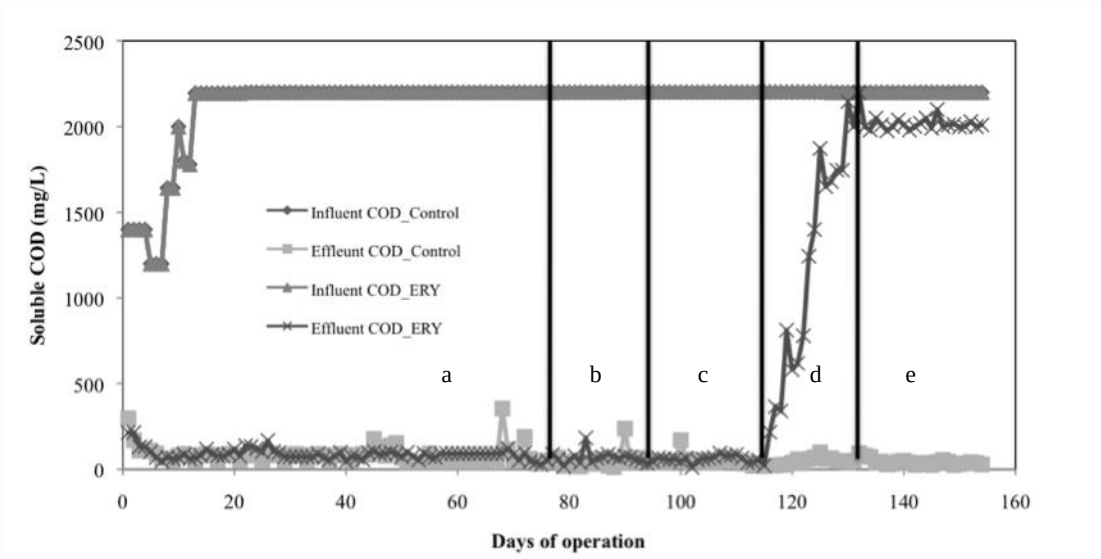

Figure 4.43: Performance of Control and ERY reactors in terms of influent and effluent soluble COD concentration (Phase a: 0 mg/L ERY, Phase b: 1 mg/L ERY, Phase c: 2 mg/L ERY, Phase d: 3 mg/L ERY, Phase e: 0 mg/L ERY).

On 78th day, ERY addition was started to ERY reactor. After first ERY addition, the reactor was operated during 76 days. Until 90th day, 1 mg/L of ERY was fed to the system (Phase b). No significant change was observed in COD removal efficiency and biogas production at Phase b. While effluent COD concentrations and COD removal efficiencies of control and ERY reactors were 50.1±18.3 and 70.5±38.9 mg/L, 97.7% and 96.8%, respectively; biogas production of the reactors during phase b was measured as 1058±9 mL and 1049±20 mL for control and ERY reactor, respectively. Methane content of the biogas produced in ERY reactor was the same with control reactor as 64%.

After 90th day, ERY concentration was increased to 2 mg/L and the ERY reactor was operated with this condition during 25 days (Phase c). Also no significant inhibition in terms of COD removal efficiency and biogas production was detected at this

phase. However, COD conversion to methane rate decreases from 0.32 to 0.3 L CH_4/g COD_removed.

The remarkable effect of ERY was detected at Phase d in which ERY concentration was increased to 3 mg/L. As seen in Figure 4.43 and 4.44 biogas production, effluent COD in connection with COD removal efficiency of ERY reactor was decreased dramatically and the highly significant effect was observed when compared to control reactor ($p<0.0001$). During phase d, COD removal efficiency and biogas production decreased day to day and reached to 23.6% and 204 mL at the end of the Phase d, respectively. During this period, performance of control reactor was stable. COD removal efficiency and biogas production of the control reactor were 97.8±1.1% and 1063±12 mL, respectively. While the methane content of biogas was 64±1.6% in the control reactor, this value was decreased to 52±8.2% in the ERY reactor.

After the COD removal efficiency decreased in phase d, the ERY addition was stopped and this period was showed as Phase e in Figure 4.43 and 4.44. Ass seen in the figures, the reactor performance could not be increase again even ERY was not added.

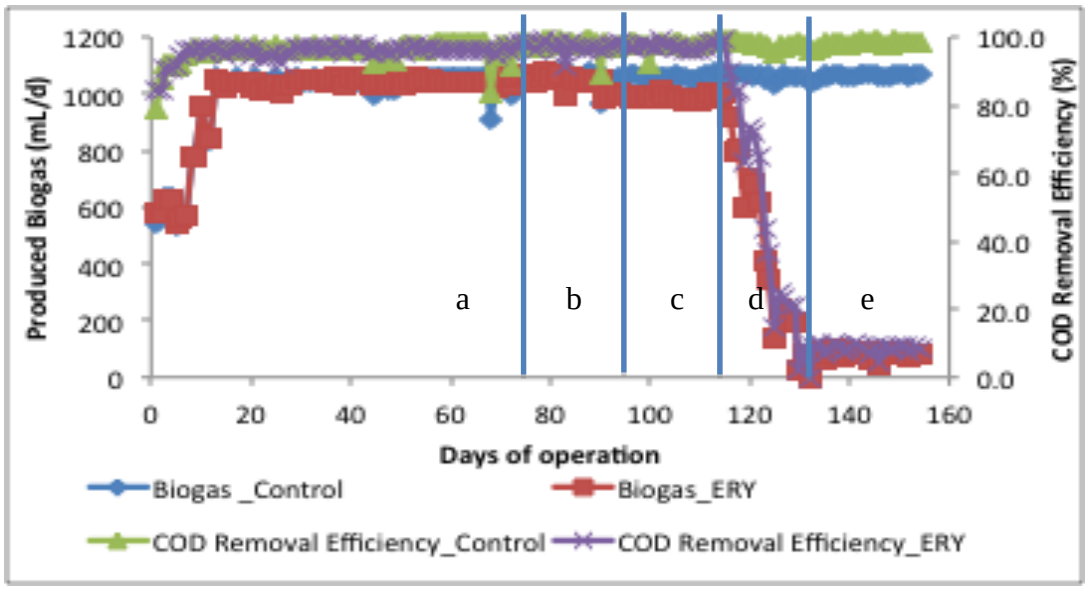

Figure 4.44: Performance of Control and ERY reactors in terms of biogas production and COD removal efficiency (Phase a: 0 mg/L ERY, Phase b: 1 mg/L ERY, Phase c: 2 mg/L ERY, Phase d: 3 mg/L ERY, Phase e: 0 mg/L ERY).

Amin and his colleagues (2006) examined the chronic inhibition effect of ERY on ASBR. However, the authors increased ERY concentration in the system suddenly from 2 g/L to 200 mg/L. The system responded to this shock effect by increased COD concentration in the effluent. The authors mentioned that the biogas production was stable like in the previous step in which 1 mg/L ERY addition. However in this study, the anaerobic system was dramatically collapsed even 3 mg/L of ERY was added and could not recovered the performance again.

In another study, chronic effect of tylosin, which is an antibiotic belonging to macrolide group, was determined (Shimada *et al.*, 2008). The experimental approach in this study was also similar to Amine *et al.* (2006). Tylosin concentration in the system was suddenly increased 100 times and the reactor performance decreased in terms of COD removal efficiency and VFA accumulation. Chelliapan *et al.* (2006) also focused on performance of up-flow anaerobic stage reactor (UASR) treating pharmaceutical industry wastewater containing macrolide antibiotics. They showed that COD removal efficiency decreased when OLR rate increased 1.86 g COD/L*d to 2.48 g COD/L*d under the operation conditions with low HRT (4 –day). Additionally, LaPara *et al.* (2002) mentioned that pharmaceutical wastewater especially containing high amount of fermentation by-products requires long HRT for efficient treatment. As a result, ERY reactor was operated high OLR with low HRT and this operation conditions could cause this dramatically collapsed. Loftine *et al.* (2005) also studied effect of tylosin tartrate on methane production in the anaerobic lagoons. According their results, this macrolide antibiotic inhibited the methane production as 23-30% with changing the concentration from 1 mg/L to 25 mg/L.

4.4.2.2 Volatile fatty acids

During the Phase a with OLR of 2.25 g COD/L*d without ERY addition, no VFA was detected in the effluent of ERY reactor. The first VFA in the effluent of ERY reactor was measured on 116th, the second day of Phase d, as 61 mg/L of propionic acid. In the 120th day, while the propionic acid concentration increased to 412 mg/L, butyric and valeric acids were determined in first time as 6 and 14 mg/L, respectively. During the Phase d, propionic acid concentration increased day to day and reached to 685 mg/L at the last day of this phase. During this period, addition of propionic acid, only butyric and valeric acid were observed lower than 10 mg/L. In

Phase e, where the ERY addition was stopped, surprisingly acetic acid accumulation was started and increased from 56 mg/L to 570 mg/L day to day until reactor operation was terminated. The propionic acid concentration was decreased at the beginning of phase a however increased again and reached to 1185 mg/L at the end of the operation. Butyric acid and valeric acid concentrations in Phase e were similar to Phase d, lower than 20 mg/L.

During Phase d and e, no VFA was detected in the effluent of the control reactor as mentioned in the previous section.

Differently from this study, Amin *et al.* (2006) found that acetic, butyric and iso-butyric acids were accumulated when ERY concentration was increased from 1 mg/L to 200 mg/L. However they mentioned that while butyric acid utilization was mostly affected in the long-term operation, propionic acid degradation was inhibited in SMA tests.

Also the results obtained from this study support previous works which inhibition of acetate, butyrate and propionate degradation was observed in the presence of macrolide antibiotic at pH 7-8.5 (Sanz *et al.*, 1996; Shimada *et al.*, 2008).

The results obtained from this study showed that, when ERY concentration was increased to 3 mg/L in long-term operation, propionic acid degradation was firstly affected. Despite the general opinion about ERY, this compound is more effective on Gram-positive bacteria (Oleinick, 1975), propionic acids are mostly utilized by Gram-negative bacteria such as Syntrophobacter species (Stams and Dong, 1995). So, i the presence of ERY-sensitive propionate oxidizers, homoacetogenic bacteria and acetoclastic methanogens can be caused this inhibiton.

4.4.2.3 Antibiotic measurement

Antibiotic measurement was carried out for wastewater and sludge samples taken from different phases of ERY reactor. The results of the measurement is given in Figure 4.45.

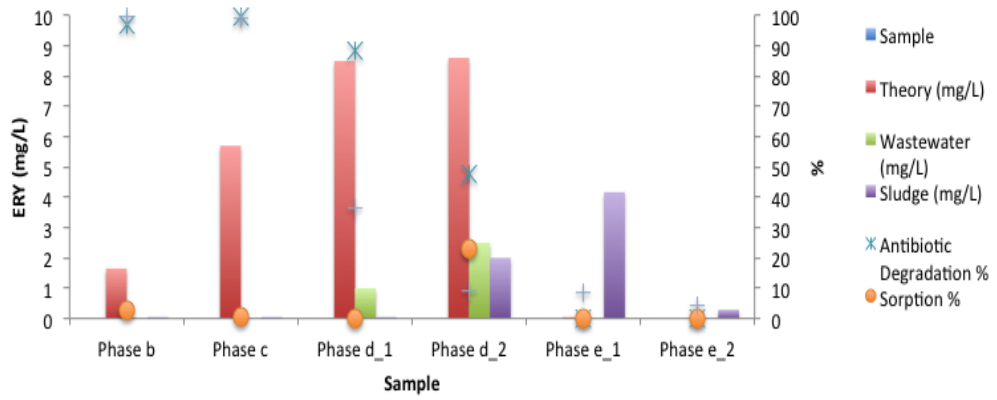

Figure 4.45: Antibiotic measurement results of ERY reactor.

As seen in Figure 4.45, in Phase d_2 (at the end of the antibiotic feeding period), ERY sorption in the sludge increased. In the same time, removal of antibiotic impaired gradually with COD removal efficiency. Instead of feeding of ERY was stopped, the reactor performance was not recovered, however the eRY concentration within sludge decreased to 0 mg/L suddenly.

4.4.2.4 Specific methanogenic activity tests

Long-term effects of ERY on the methanogens were examined by specific methanogenic activity (SMA) test. For this purpose, the inhibitory effect of this compound on different trophic groups was tested. Also SMA test provide the information on the potential loading capacity of the reactors treating similar wastewaters.

Utilization of Acetate

SMA test was performed to determine the acetate utilization capacity of the anaerobic sludge taken from different operation phase of the ERY reactor. Biogas production in the test tubes was monitored during 6 days. The biogas and methane production profiles of the SMA tests are given in Figure 4.46. The inhibitory influence of the ERY on acetoclastic methanogens was clear. The methane production capacity decreased obviously at Phase d in which 3 mg/L of ERY was added to reactor. In the Phase e, where antibiotic addition was terminated, the sludge capacity was going to worse instead of getting better.

The acetoclastic activity of the seed sludge of chronic tests was found 77 mL of methane as given in section 4.2. After ERY addition started, the activity decreased

60 mL at Phase b and c. A dramatical decrease was observed at Phase d, the acetoclastic methanogenic activity was 40 mL methane. Also this decrease continued after addition of ERY was stopped, and the activity was determined as 30 mL methane at Phase e. Contrary of SMX influence, ERY inhibited anaerobic systems even in the low concentrations, this influence cannot be recovered. The methane percentage was the same in all test bottles as 70%.

In this study, at the end of the operation, acetoclastic methanogenic capacity decreases as approximately 40% according to seed sludge. However, Amin *et al.* (2006) mentioned that the removal of acetic acid in the SMA test was not effected in even in the presence of high ERY concentration such as 200-500 mg/L.

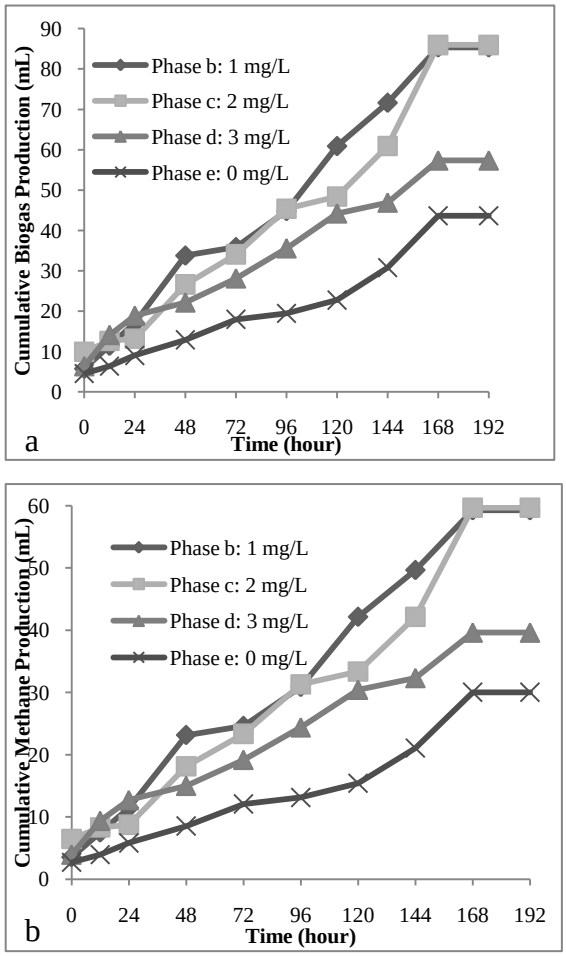

Figure 4.46: Specific methanogenic activity (SMA) test using 4000 mg/L of acetate (a) cumulative biogas production profiles (b) cumulative methane production.

Utilization of VFA Mixture

Influence of ERY on all methanogenic activity was examined via VFA mixture utilization. The profiles of the cumulative biogas and methane production were similar to acetate fed set as seen in Figure 4.47. However, an important decrease between phase b and c was observed in this set. Theoretical methane production from VFA mixtures fed to the system is 315 mL. Even in the low concentration of ERY had a clear influence on methane production. While CMP value was 89 mL at Phase b, it gradually decreased and reached to 19 mL at Phase e. Also the highest CMP value was also 19% of this theoretical value as seen in Figure 4.47b. Also methane content of the biogas was also decreased stepwise manner. Whereas, 56% methane was measured at Phase b, this value declined to 50%, 49% and 48% at Phase c, d and e, respectively.

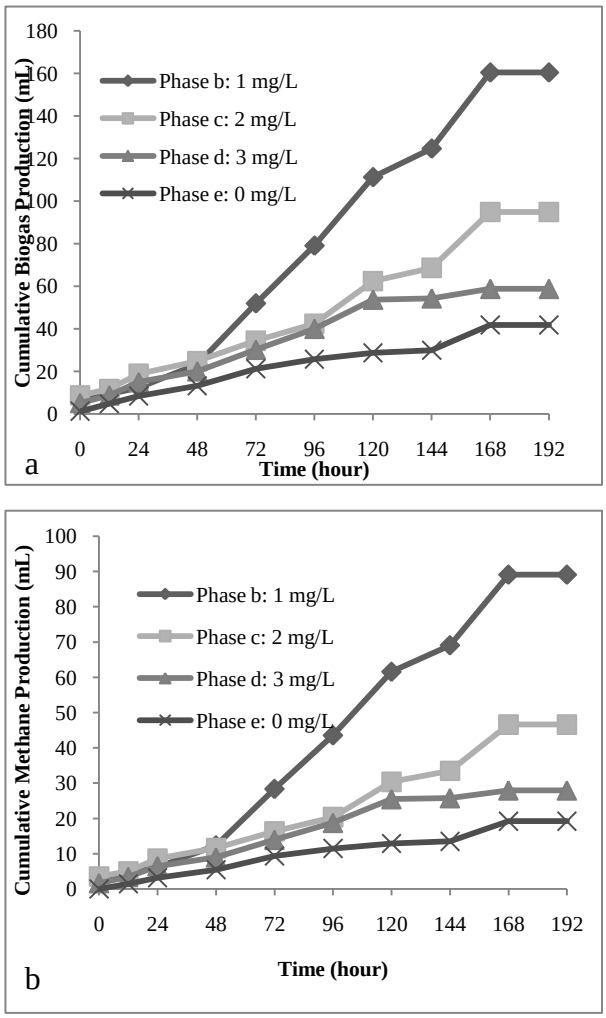

Figure 4.47: Specific methanogenic activity (SMA) test using 3000 mg/L of VFAs (a) cumulative biogas production profiles (b) cumulative methane production.

The SMA results indicated that all methanogens were affected by ERY. However when compared to acetate utilization set, two possible explanations are:

i) ERY has more influence on hydrogenotrophic methanogens
ii) Propionate and acetate oxidizers could be inhibited.

However, limited information is available on the importance of this pathway in the anaerobic degradation. Previous studies have shown that the indirect production of methane from acetate via homoacetogenic bacteria and hydrogenotrophic methanogens is significant for streaa conditions such as thermophilic conditions or in systems with high ammonia levels (Griffin *et al.*, 1998; Angenent *et al.*, 2002). Furthermore, the decreased utilization of other VFAs in the presence of erythromycin (propionic and butyric acid) is consistent with the fact that these substrates are used by bacteria, and furthermore, agrees with the general observation that erythromycin is more effective against Gram-positive bacteria (Oleinick, 1975). VFAs are not directly used by methanogens, but thet-y are a direct relation between proton reducing syntrophic bacteria. Different groups of syntrophic bacteria utilize specific VFAs, hence accumulation of particular VFAs is a proof for the erythromycin sensitivity of relevant syntrophs.

The reactor operation data also supported the SMA results, because in the Phase d, propionate accumulation was observed.

4.4.2.5 Microbiological Approach

16S rDNA and rRNA microbiological analysis were done to determine the effects of ERY on existing and active microorganisms, relatively. In this scope, genomic DNAs and total RNAs were extracted from the samples collected form different phases of the ERY reactor and RNAs were converted to cDNAs by reverse transcriptase PCR (RT-PCR). Amplicons were obtained using 16S rRNA gene spesific primers for further analysis on DGGE. Furthermore, GDNAs and cDNA were used to quantify total bacteria, archaea and methanoges by Q-PCR.

Predominant Bands in the DGGE Patterns

To evaluate the species having role on the ERY fed system, predominant bacterial and archaeal band patterns obtained from different Phases of the ERY reactor were

compared to 16S rDNA clone libraries of the seed sludge. While16S rDNA band patterns reflected the existing species; 16rRNA samples indicated the active ones. Also their abundance was evaluated according to intensity of each band on DGGE gel because the intensity of the bands might correspond at least semiquantitatively with the abundance of the corresponding species (Heuer *et al.*, 1997). The comparison results of bacterial and archaeal 16S rDNA DGGE band patterns were given in Table 4.11 and 4.12, respectively.

62 different bacterial OTUs were detected in the 16S rDNA DGGE profiles. According to comparison results to 16S rDNA clone library, 11 of bacterial OTUs from bacterial DGGE profile of the RRY reactor matched to the library OTUs. Clostridum species (FR836437, FR83648 and FR836443) existed in the system as expected because the system was fed with a synthetic wastewater including starch and glucose. Clostiridium species have role on hydrolysis steps for polysaccharides and also β-oxidation process (Hungate, 1982; Payton and Haddock, 1986). Most of them existed in the every Phase with same abundance, however only at Phase c and d_2, two of them were not detected. Also, Paludibacter propionicigenes (AB078842), which is responsible to produce propionate, was detected most of the steps. So, the results are not surprise for this system fed by a synthetic wastewater including starch and glucose. Other OTUs detected in the samples almost belong to the uncultured clones or unclassfied bacterial cultured species.

Similar to SMX reactor results, the intensities of matched archaeal DGGE predominant bands were higher than 16S rDNA bacterial DGGE bands. The comparison results of the archaeal 16S rDNA DGGE band patterns and the clone library is given in Table 4.12, 16 of the 47 archaeal OTUs in the DGGE profile were matched to 16S rDNA archaeal clone library. According to results, acetoclastic methanogen species (FR836463, FR836464, FR836468, U89773) existed in each phase. Also hydrogenotrophic species were detected in different phases. However their abundance in each phase was different and there was no correlation between the operation data. Uncultured methanogenic euryarchaeote (FR836462), a hydrogenotrophic species Methanobacterium beijingense strain 8-2 (AY350742) and an archeon clone (AJ745135) were detected in every step. So, ERY could be unpredictable inhibition effect on the archaela species. Also SMA test results supported these results. Methanogenic activity in terms of acetoclastic and

hyrrogenotrophic decreased with respect to ERY concentrations and could not be recovered when ERY addition was stopped.

Table 4.11: Affiliation of bacterial clones and their abundance at the different phases of the ERY reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
B1	2.80	FR836435	*Clostridium sp. BG-C66* (FJ384378)	96						
B2	0.93	FR836436	*Clostridium sp. Kas301-1* (AB114242)	96						
B3	1.87	FR836437	*Clostridium intestinale* (AM158323)	95	•		•		•	
B4	4.67	FR836438	Uncultured Clostridium sp. clone AUCLO214 (AY648564)	96	••					•
B5	1.87	FR836439	Uncultured Firmicutes bacterium clone QEDS2BE07 (CU921543)	93						•
B6	2.80	FR836440	Uncultured Firmicutes bacterium clone QEDN7DC08 (CU926749)	97	•	•		•	•	•
B7	14.95	FR836441	Uncultured bacterium clone BS7_65 (GQ458219)	98						
B8	3.74	FR836442	*Syntrophomonas zehnderi strain OL-4* (DQ898277)	89						
B9	33.64	FR836443	*Clostridium sp. Kas107-1* (AB114241)	96	•	•	•	•		•
B10	5.61	FR836444	Uncultured bacterium clone C3-25 (AM181973)	91	•					•
B11	4.67	FR836445	*Bacterium CBIC19I3* (HM240274)	95						

Table 4.11 (continued): Affiliation of bacterial clones and their abundance at the different phases of the ERY reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
B12	3.74	FR836446	Uncultured bacterium UASB_TL84 (AF254390)	94	•	•				
B13	0.93	FR836447	Uncultured Candidatus Cloacamonas sp. clone 8SN (EU887773)	92						
B14	5.61	FR836448	Uncultured bacterium clone BS1_25R (GQ458208)	97		•			•	
B15	2.8	FR836449	Uncultured bacterium clone aaa97f10 (DQ816701)	96						
B16	0.93	FR836450	*Acinetobacter sp. 1B3* (EU337120)	96						
B17	2.8	FR836451	Uncultured Sarcina sp. clone JPL-2_O10 (FJ957591)	89	•					
B18	0.93	FR836452	Uncultured bacterium clone 30c07 (EF515566)	87						
B19	0.93	FR836453	*Paludibacter propionicigenes* (AB078842)	92	•	•		•	•	
B20	0.93	FR836454	*Bacterium 061128-OL-KR37-AA 3-0 10000x -1A* (FJ037613)	92		•		•	•	•
B21	0.93	-	*Propionibacteriaceae bacterium FH044* (AB298766)	99						
B22	1.87	FR836458	Uncultured bacterium clone D14R15C96 (FM956760)	98						

Table 4.12: Affiliation of archaeal clones and their abundance at the different phases of the ERY reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
A1	1.45	FR836460	Uncultured archaeon clone OK3 (GQ406364)	94						
A2	2.9	FR836461	Uncultured archaeon clone I17_Rya (HM639831)	98			••	••		
A3	9.42	FR836462	*Methanogenic euryarchaeote Annu4* (FJ896263)	95	••••	•••••	••••	••••	•••••	••
A4	2.9		*Methanobacterium formicicum strain S1 (*DQ649309)	99						
A5	2.9	FR836464	Uncultured Methanosarcinales archaeon clone:KuA4 (AB077214)	98						••
A6	4.35	FR836470	Uncultured euryarchaeote clone MVP-8A-21 (DQ676281)	98						••
A7	12.32	FR836463	*Methanothrix soehngenii* (X51423)	97			••	••		
A8	0.72		Uncultured archaeon clone A6T20L208 (DQ262482)	99				••		
A9	7.25		*Methanospirillum hungatei* (M60880)	99						
A10	0.72	FR836465	*Methanobacterium beijingense strain M4* (EU544027)	97		••••				
A11	6.52	FR836467	*Methanosphaerula palustris strain E1-9c* (EU156000)	95		••	••	•••		•••
A12	0.72		*Methanobacterium sp. OM15* (AJ550160)	99						
A13	2.17		*Methanobacterium beijingense strain 8-2* (AY350742)	100	•••	••	••	••	••	••
A14	3.62	FR836468	Uncultured Methanosarcinales archaeon clone S4 (GU475184)	95		••	•••	•••		••

Table 4.12 (continued): Affiliation of archaeal clones and their abundance at the different phases of the ERY reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
A15	2.9	FR836469	Uncultured archaeon clone Sed-ARC-34 (EU255763)	98						
A16	3.62	FR836471	Uncultured euryarchaeote clone MVP-8A-26 (DQ676246)	96		••	•••	••	••••	••
A17	9.42	FR836472	*Methanogenic archaeon Prasan1* (HM630570)	97			••	•••		•••
A18	7.25	FR836473	*Methanobacterium beijingense strain 4-1* (AY552778)	96		••	••	••	••	••
A19	2.17		*Methanosarcina siciliae* (U89773)	99		••	••	••	•••	
A20	4.35		Uncultured archaeon clone EOTU21 (FJ896263)	99						
A21	1.45	FR836474	Uncultured archaeon clone OKA6 (GQ465437)	97					••	
A22	1.45		Uncultured archaeon clone ATB-KM-2942-A18 (EF680353)	99						
A23	6.52		*Archaeon LL25A3* (AJ745135)	99	••	•••	•••	•••	•••	•••
A24	2.17		Uncultured archaeon clone WA10 (AB494239)	99						
A25	0.72	FR836475	*Methanogenic euryarchaeote Annu1* (HM630571)	95						

Also to determine the active species and their shift and/or changes in the ERY reactor with respect to the antibiotic concentration and operation time, the amplicons obtained from 16S rRNAs were compared with the bacterial and archaeal clone libraries. The comparison results were given in Table 4.13 and 4.14, respectively. 13 of the bacterial OTUs were matched to the 16S rRNA DGGE profiles. However, 3 of these OTUs were not the same with 16S rDNA DGGE comparison which is given in Table 4.11.

According to 16S rRNA DGGE results, the uncultured Clostridum species, which were detected in 16S rDNA results were detected in the first phases however all of them excluding uncultured Sarcina clone (FR836451) disappeared at the end of the operation. Despite some of the Clostridium species were detected as DNA level, these species were directly inhibited by ERY. Clostidium species have an important role on the fermentation of carbonhydrates and VFA production (Hungate, 1982; Payton and Haddock, 1986). These finding supported the reactor results which the COD removal efficiency decreased with respect to the antibiotic concentration and the reactor performance could not be recovered even ERY addition was stopped. Uncultured Paludibacter clone (FR836453) which is responsible to propionate production disappeared at Phase e. Other predominant bacterial clones were mostly uncultured species. These uncultured bacterial clones may probably be species responsible to fermentation and homoacetogenesis, especieally VFA-oxidizing syntrophs (Speece, 1996, Madigan *et al.*, 2009). In the archaeal 16S rRNA samples, mostly uncultured species was detected as seen in Table 4.14. Detected acetoclastic methanogens appeared in the beginning or the middle of the operation. No active acetoclastic species appeared at the end of the ERY reactor operation. Also hydrogenotrophic species didn't seem active existing of Methanobacterium beijingense strain 8-2 (AY350742) which was detected in all phases and their abundances were high. Other detected active species in the last phases were uncultured species.

As mentioned before, degradation of organic compounds to CO_2 and CH_4 relies greatly on methanogenic archaea for ultimate stage (Madigan *et al.*, 2009). So, the results was supported SMA test and VFA data. Additionally, VFA concentration in the effluent indicated that acetate and propionate accumulation in the reactor started in Phase e and SMA test results also indicated that methanogenic activity decreased gradually even antibiotic addition to reactor stopped.

Table 4.13: Affiliation of bacterial clones and their abundance within the active community at the different phases of the ERY reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
B1	2.80	FR836435	*Clostridium sp. BG-C66* (FJ384378)	96						
B2	0.93	FR836436	*Clostridium sp. Kas301-1* (AB114242)	96						
B3	1.87	FR836437	*Clostridium intestinale* (AM158323)	95	•••••					
B4	4.67	FR836438	Uncultured Clostridium sp. clone AUCLO214 (AY648564)	96	•••	••		••	•	
B5	1.87	FR836439	Uncultured Firmicutes bacterium clone QEDS2BE07 (CU921543)	93		•••				
B6	2.80	FR836440	Uncultured Firmicutes bacterium clone QEDN7DC08 (CU926749)	97	•••••	••		•	•	
B7	14.95	FR836441	Uncultured bacterium clone BS7_65 (GQ458219)	98	••••••	•••••••	•••••••		••••	••
B8	3.74	FR836442	*Syntrophomonas zehnderi strain OL-4* (DQ898277)	89	•					
B9	33.64	FR836443	*Clostridium sp. Kas107-1* (AB114241)	96		••				
B10	5.61	FR836444	Uncultured bacterium clone C3-25 (AM181973)	91						
B11	4.67	FR836445	*Bacterium CBIC19I3* (HM240274)	95						

Table 4.13 (continued): Affiliation of bacterial clones and their abundance within the active community at the different phases of the ERY reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
B12	3.74	FR836446	Uncultured bacterium UASB_TL84 (AF254390)	94	••					
B13	0.93	FR836447	Uncultured Candidatus Cloacamonas sp. clone 8SN (EU887773)	92						
B14	5.61	FR836448	Uncultured bacterium clone BS1_25R (GQ458208)	97	•••	•••				•
B15	2.8	FR836449	Uncultured bacterium clone aaa97f10 (DQ816701)	96						
B16	0.93	FR836450	*Acinetobacter sp. 1B3* (EU337120)	96						
B17	2.8	FR836451	Uncultured Sarcina sp. clone JPL-2_O10 (FJ957591)	89		•••	•••	••		•••
B18	0.93	FR836452	Uncultured bacterium clone 30c07 (EF515566)	87						
B19	0.93	FR836453	*Paludibacter propionicigenes* (AB078842)	92	•••	•••	••	••		
B20	0.93	FR836454	*Bacterium 061128-OL-KR37-AA 3-0 10000x -1A* (FJ037613)	92		••	••	••	••	•
B21	0.93	-	*Propionibacteriaceae bacterium FH044* (AB298766)	99						
B22	1.87	FR836458	Uncultured bacterium clone D14R15C96 (FM956760)	98		••				

Table 4.14: Affiliation of archaeal clones and their abundance within the active community at the different phases of the ERY reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
A1	1.45	FR836460	Uncultured archaeon clone OK3 (GQ406364)	94				•••		
A2	2.90	FR836461	Uncultured archaeon clone I17_Rya (HM639831)	98				•••		
A3	9.42	FR836462	*Methanogenic euryarchaeote Annu4* (FJ896263)	95				••••••••	••••	•••
A4	2.90		*Methanobacterium formicicum strain S1* (DQ649309)	99						
A5	2.90	FR836464	Uncultured Methanosarcinales archaeon clone:KuA4 (AB077214)	98				••		
A6	4.35	FR836470	Uncultured euryarchaeote clone MVP-8A-21 (DQ676281)	98						
A7	12.32	FR836463	*Methanothrix soehngenii* (X51423)	97			•••			
A8	0.72		Uncultured archaeon clone A6T20L208 (DQ262482)	99						
A9	7.25		*Methanospirillum hungatei* (M60880)	99						
A10	0.72	FR836465	*Methanobacterium beijingense strain M4* (EU544027)	97	••••	••••	•••••			
A11	6.52	FR836467	*Methanosphaerula palustris strain E1-9c* (EU156000)	95	••••••	•••••	•••••			
A12	0.72		*Methanobacterium sp. OM15* (AJ550160)	99						
A13	2.17		*Methanobacterium beijingense strain 8-2* (AY350742)	100	•••••	••••	•••••	•••••	••••••••	••••••
A14	3.62	FR836468	Uncultured Methanosarcinales archaeon clone S4 (GU475184)	95						

Table 4.14 (continued): Affiliation of archaeal clones and their abundance within the active community at the different phases of the ERY reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
A15	2.9	FR836469	Uncultured archaeon clone Sed-ARC-34 (EU255763)	98	•••		•••		•••	
A16	3.62	FR836471	Uncultured euryarchaeote clone MVP-8A-26 (DQ676246)	96				••		
A17	9.42	FR836472	*Methanogenic archaeon Prasan1* (HM630570)	97	••••	•••••	•••••	•••	••••••	••••
A18	7.25	FR836473	*Methanobacterium beijingense strain 4-1* (AY552778)	96	••••	••••	••••	•••		
A19	2.17		*Methanosarcina siciliae* (U89773)	99				•••		
A20	4.35		Uncultured archaeon clone EOTU21 (FJ896263)	99						
A21	1.45	FR836474	Uncultured archaeon clone OKA6 (GQ465437)	97						
A22	1.45		Uncultured archaeon clone ATB-KM-2942-A18 (EF680353)	99						
A23	6.52		*Archaeon LL25A3* (AJ745135)	99	•••••	••••	•••••	•••	••••••	•••••
A24	2.17		Uncultured archaeon clone WA10 (AB494239)	99						
A25	0.72	FR836475	*Methanogenic euryarchaeote Annu1* (HM630571)	95						

Cluster analysis of DGGE banding pattern

DGGE technique was used in this study to assess the stability and/or change in microbial community and active species at different phases of the ERY reactor. A total of seven sludge samples collected from different phases of the reactor were compared in terms of bacterial and archaeal communities: The seed sludge of the chronic reactors and the sludges collected from different phases of the ERY reactor. Result of DGGE analysis suggested that bacterial community was respectively, more diverse than archaeal diversity in the reactor; 62 bacterial and 47 archaeal bands were remarked in the ERY reactor samples.

Bacterial and archaeal diversities and species richness in seed sludge and sludges obtained from ERY reactor were measured by the Shannon-Weaver diversity index and Margalef's species richness, respectively. The values were given in Table 4.15 and 4.16.

In seed sludge, while 33 of different bacterial species were detected, this number decreased to 23 at the end of operation as seen in Table 4.15. Bacterial species number dramatically decreased to 7 at the first part of Phase d, in which ERY concentration was firstly increased to 3 mg/L, however an increase was detected during Phase d. Also archaeal species number changed during the operation time; at the Phase b, in which 1 mg/L of ERY was firstly added, the archaeal species number dramatically decreased to 9. However it increased gradually to 18 at the end pf Phase d. Also the archaeal spesices number slightly decreased to 16 at the end of the operation. However the change in both bacterial and archaeal OTUs was not steady. After the addition of SMX to the reactor, bacterial and archaeal species were generally stable until the 45 mg/L of SMX concentration (phase h). In the last phase (phase h), both of them firstly increased and then dramatically decreased.

Eichner *et al.* (1999) mentioned that microbial diversity in the biological systems decreased after the shock loadings. The results indicated that ERY addition even in low concentrations affected to archaeal species than bacterial ones. However bacterial and archaeal community were recovered during the operation, however this situation could not reflect the operation of the reactor.

Table 4.15: Shannon-Weaver diversity indices, Margalef's species richness measure, and eveness measure estimates for bacterial DGGE profiles of sludges taken from different phases of the ERY reactor.

	Seed_Chronic Reactors	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
Taxa	33	29	32	7	19	23	23
Shannon-Weaver diversity	3.449	3.254	3.365	1.925	2.913	2.975	3.1
Evenness	0.9537	0.8933	0.904	0.9797	0.9693	0.8514	0.9656
Margalef's richness	4.954	4.364	4.847	1.385	3.205	3.547	3.772

Table 4.16: Shannon-Weaver diversity indices, Margalef's species richness measure, and eveness measure estimates for archaeal DGGE profiles of sludges taken from different phases of the ERY reactor.

	Seed_Chronic Reactors	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
Taxa	13	9	14	18	18	17	16
Shannon-Weaver diversity	2.526	2.088	2.559	2.845	2.854	2.744	2.749
Evenness	0.962	0.8966	0.9233	0.956	0.9639	0.915	0.9765
Margalef's richness	1.901	1.265	1.984	2.512	2.518	2.332	2.3

Pearson product-moment correlation coefficients with UPGMA clustering were used to quantify the similarity of the community fingerprints between each sample. The bacterial and archaeal 16S rDNA phylogenetic trees obtained from ERY reactor with seed sludge sample were given in Figure 4.48 and 4.49, respectively.

The analysis of DGGE band patterns from the ERY reactor sludge samples revealed that the samples differentiated with ERY addition.

The changes in bacterial community during the operation reflect the clusters obtained from DGGE band patterns, which were divided into two clusters. The clusters were defined by less than 70% pattern similarity. One of them included seed sludge Phase b, c and e_2. This cluster also divided sub-clusters. Seed sludge belonged to one sub-cluster as solo and it was similar to Phase b, c and e_2 samples as 79.1%, 79.8%, and 70.6%, respectively.

Phase d_1, d_2 and e_1 also belonged to the second cluster. This cluster also divided into two sub-clusters based on their population origin, which were defined by a similarity pattern varied between 93.3% and 96.4%. As seen in dendogram (Figure 4.48), the pattern similarity of the samples did not change very distinctly in the clusters. However there was a community change during the operation. After the reactor operated with 3 mg/L of ERY, the bacterial community was shifted. Also in the beginning of Phase e, in which ERY addition was terminated, the bacterial community was similar to Phased. However at the end of the operation (Phase e_2), the bacterial community was similar to Phase c as 91.2% as seen in the dendogram (Figure 4.48).

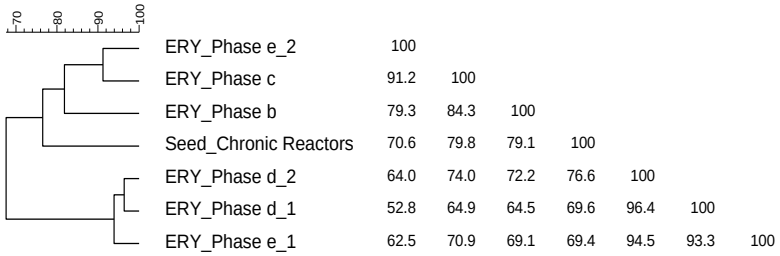

Figure 4.48: Cluster analysis and similarity matrix of bacterial 16S rDNA gene DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

Archaeal cluster analysis obtained from DGGE banding pattern was different form bacterial cluster analysis of the ERY reactor. The archaeal banding patters revealed two main clusters defined by less than 60% similarity (Figure 4.49). In one of these clusters, only Phase b sample was defined. It was most similar to Phase c sample as 65.7%. The second cluster also divided into two sub-clusters. One of them included seed sludge and Phase e_2 and the samples defined by the similarity as 81.2%. The second sub-cluster divided more sub-clusters. Phase c, d_1, d_2 and e_1 samples belonged to this cluster and their similarity varied between 72.6% and 93.1%. Differently from the bacterial dendogram, the archaeal pattern similarity of the samples changed more distinctly.

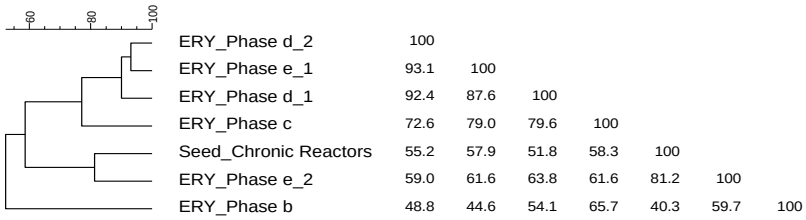

Figure 4.49: Cluster analysis and similarity matrix of archaeal 16S rDNA gene DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

DGGE analysis was also performed to find out the active community changes during the operation of the ERY reactor. The bacterial and archaeal phylogenetic dendograms obtained from DGGE band patterns were represented in Figure 4.50 and 4.51, respectively.

The bacterial band patterns obtained from 16S rRNA gave the different result from 16S rDNA band patterns. Phase e_2 differentiated from other samples obviously. As seen in Figure 4.50, Phase e_2 samples was defined by a similarity as 85.6% to the closest sample, Phase e_1. Also the similarity in the dendogram represented active bacterial community varied between 72.3% and 97.9%. The most similar samples based on bacterial 16S rRNA were Phase c and d_1.

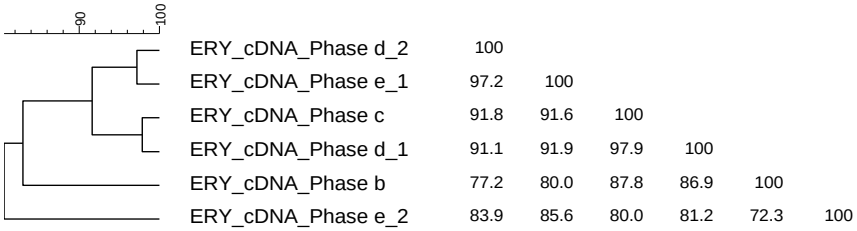

Figure 4.50: Cluster analysis and similarity matrix of bacterial 16S rRNA DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

In the archaeal phylogenetic tree of 16S rRNA, the dendogram structures and similarities were different from the dendogram of archaeal 16S rDNA. As seen in Figure 4.49, the distribution of the active archaeal community reflected the clusters, which were divided into two like dendogram of 16S rDNA. One of this cluster included only Phase d_2 sample, which was defined by a similarity as 79.8% to the closest sample, Phase d_1. In the contrary of this result, Phase b sample differentiated from other samples and represented a cluster lonely. The second

cluster also divided into two sub-clusters. While Phase e_1, and e_2 belonged to one of this sub-cluster with a similarity defined as 96.2%, Phase b, c and d_1 samples represented the second one with a similarity changed from 95.8% to 98.4%

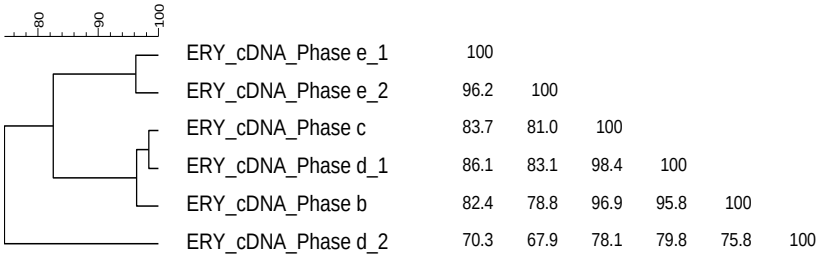

Figure 4.51: Cluster analysis and similarity matrix of archaeal 16S rRNA DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

As seen in the results obtained from cluster analysis;

1- Similarities in the 16S rDNA dendograms were more distinct from 16S rRNA results.
2- The cluster analysis reflected to ERY concentration within the system and operation time.

A change in the microbial community can be explained by environmental conditions (LaPara *et al.,* 2001). Also it was found that the flexibility of microbial community leads to more stable reactor operation (Fernandez *et al.*, 2006). However compared the microbiological data with reactor efficiency, the change in the microbial community was not enough to continue reactor stability in this study.

Quantification by Q-PCR

Quantitative changes of 16S rDNA and 16S rRNA concentrations in terms of bacteria, archaea and methanogens were also determined by Q-PCR during operation time. The concentration profiles showed the temporal variations in the microbial community and the active population at different phases of the ERY reactor as given in Figure 4.52-4.57.

While bacterial 16S rDNA concentration increased at Phase b and c compared to seed sludge and reached to 3.53 X 10^{10} copies/mL, the bacterial amount decreased at Phase d, in which ERY concentration was 3 mg/L. The concentration of bacteria at Phase e, in which the ERY addition was terminated, increased again 1.97 X 10^{10} copies/mL. Bacterial change during the operation of the ERY reactor was not

remarkable. However archaeal and methanogenic community changes were related to reactor performance and SMA results. There was a slight increase in Phase b and c. While archaeal community was detected as 1.52 X 10^8 copies/mL and 2.38 X 10^9 copies/mL at Phase b and c, respectively, methanogenic community was quantified as 6.52 X 10^7 copies/mL and 1.22 X10^9 copies/mL at Phase b and c, respectively. As seen in Figure 4.53 and 4.54, archaeal and methanogenic community dramatically decreased approximately 90% at the beginning of Phase d.

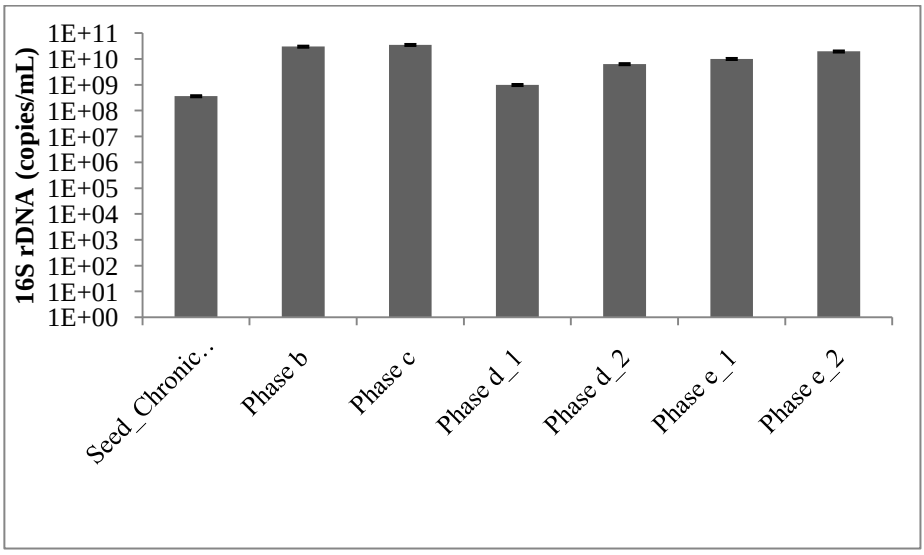

Figure 4.52: Quantitative changes in bacterial 16S rDNA concentrations at different phases of the ERY reactor.

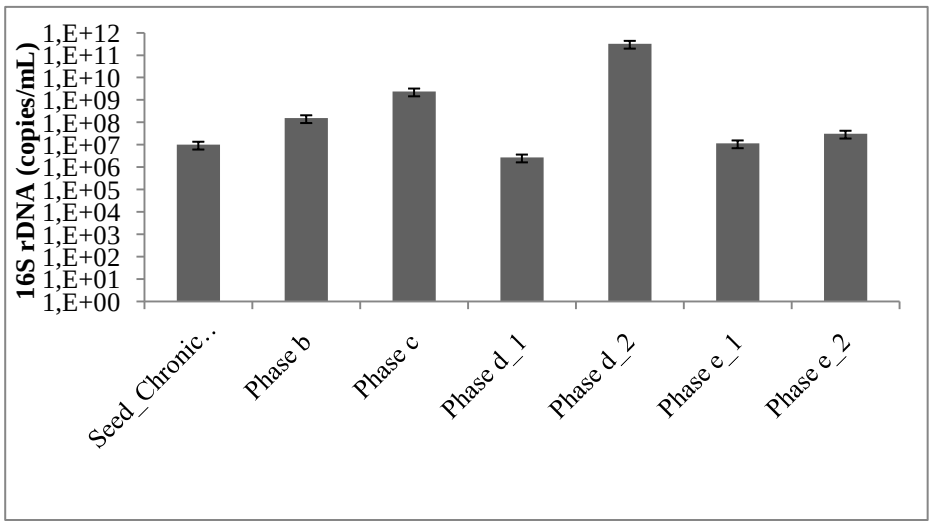

Figure 4.53: Quantitative changes in archaeal 16S rDNA concentrations at different phases of the ERY reactor.

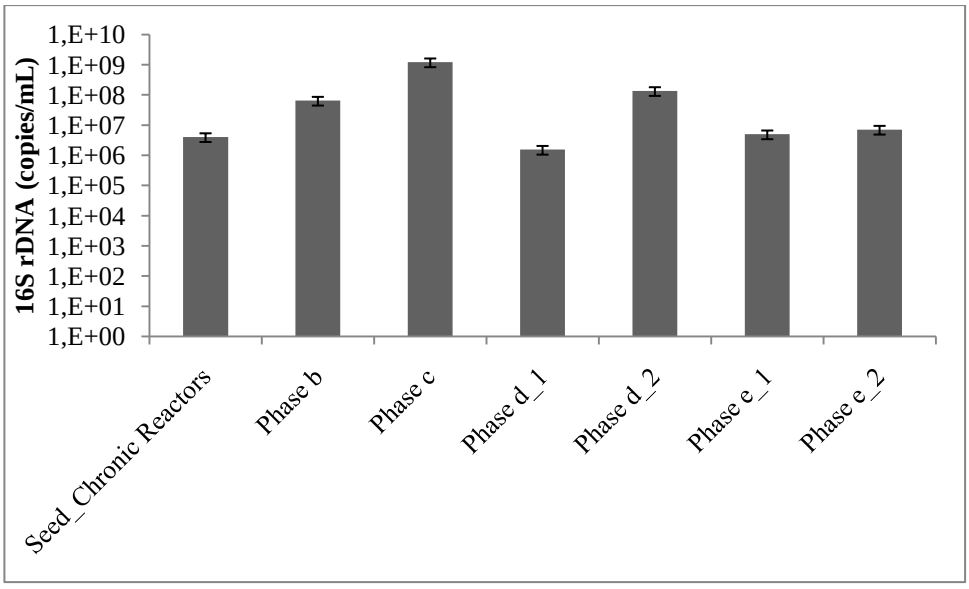

Figure 4.54: Quantitative changes in methanogenic 16S rDNA concentrations at different phases of the ERY reactor.

Besides quantification of 16S rDNA concentration, 16S rRNAs were quantified to determine the effects of ERY on the active microbial groups. Quantification results of bacteria, archaea and methanogens were given in Figure 4.55-4.57, respectively.

Bacterial 16S rRNA expression level showed that the activity of bacterial community was affected at the Phase c and d_1 of the operation, while ERY concentration was added as 2 and 3 mg/L.

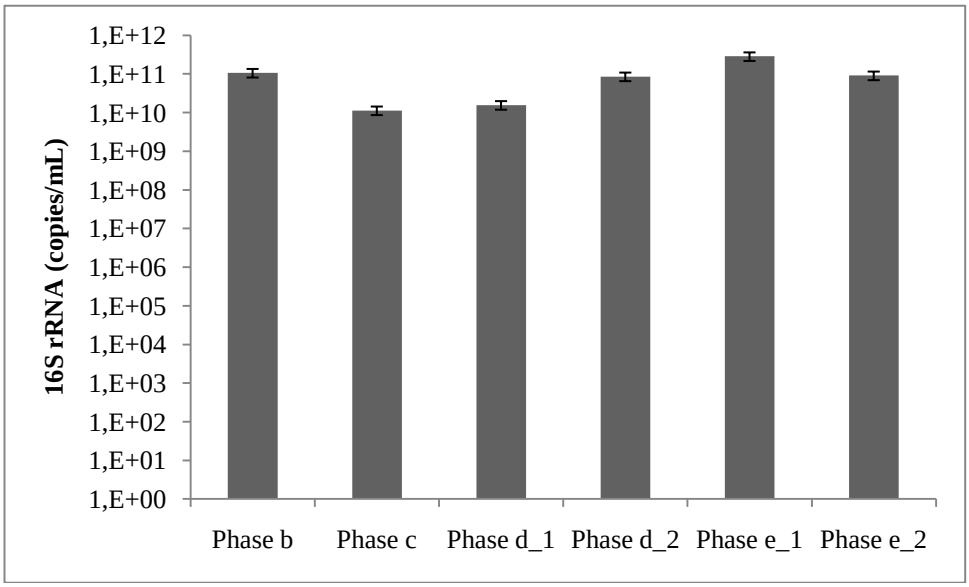

Figure 4.55: Quantitative changes in bacterial 16S rRNA concentrations at different phases of the ERY reactor.

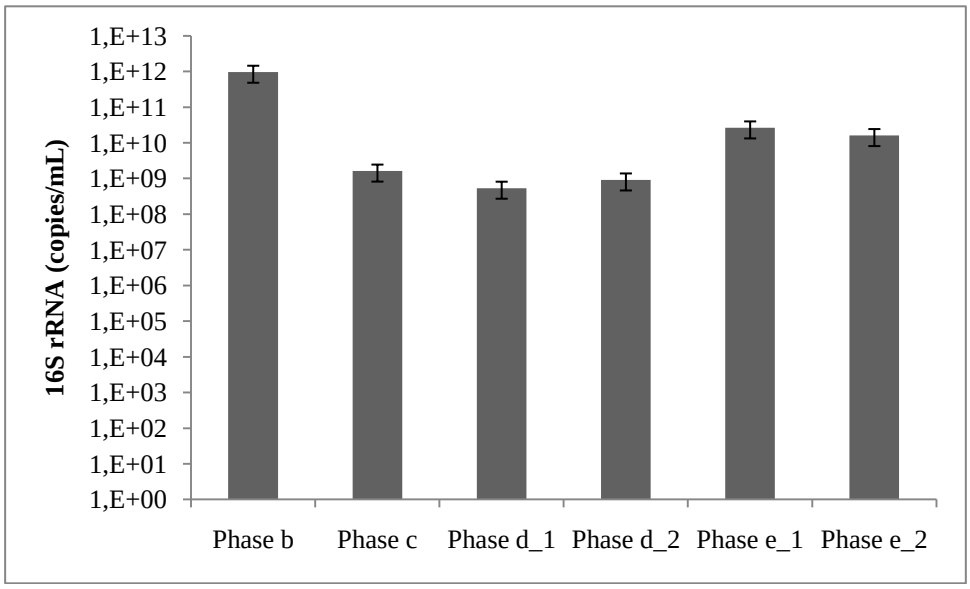

Figure 4.56: Quantitative changes in archaeal 16S rRNA concentrations at different phases of the ERY reactor.

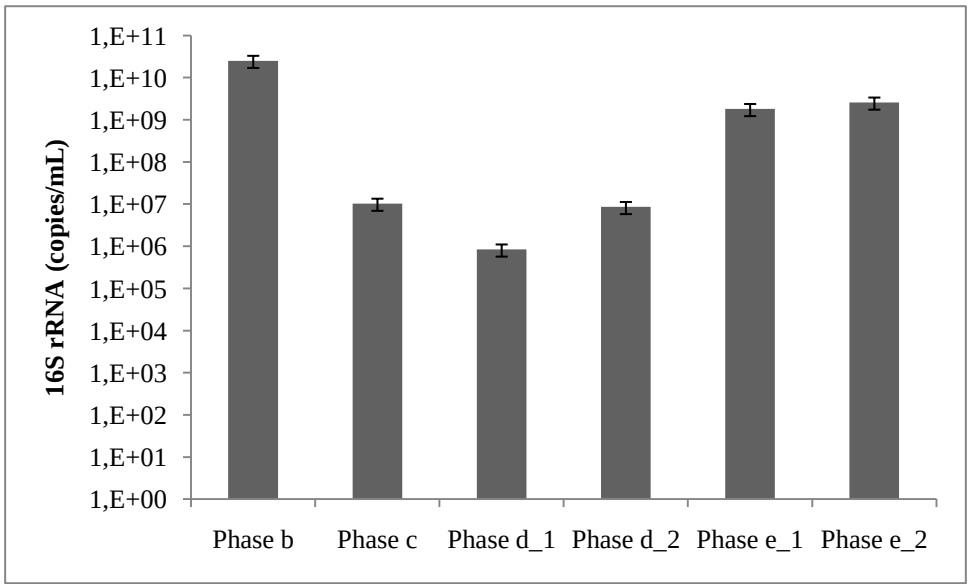

Figure 4.57: Quantitative changes in methanogenic 16S rRNA concentrations at different phases of the ERY reactor.

However, during Phase d, the expression level of the bacterial community was increased and then bacterial 16S rRNA expression was conserved after ERY addition stopped. The reactor operation data and SMA test results changed during the operation time reflected 16S rRNA expression level of archaea and methanogens in the system noticeably. The remarkable decrease in the expression of archaeal and methanogenic 16S rRNA was observed at Phase d. Also CMP values of the SMA tests dramatically decreased during 3 mg/L of ERY addition to the system. In Phase

e, expression level of archaeal and methanogenic 16S rRNA increased as CMP value of Phase e in the SMA test.

Enzyme expression

To detect the inhibition effect of ERY on the anaerobic pathways three enzymes were selected based on mRNA level from the different metabolic pathways of the anaerobic degradation. Formylterahydrofolate synthetase (FTHFS), methyl-coenzyme M reductase (mcrA) and acetyl-coA synthetase (ACAS) were selected to determine the inhibition on homoacetogenesis, methanogenesis and specifically acetoclastic methanogenesis, respectively.

Despite acetate and other VFAs were not observed in effluent and active methanogenic species were detected in the system at the Phase b and c, FTHFS expression was surprisingly not observed in the mRNA level during all operation time.

Expression of mcrA gene was detected in every phase of the ERY reactor except of Phase d_2 as seen in Figure 4.58. The expression level of mcrA gene in the ERY reactor was similar during Phas b and c as 7 X 10^3 and 8 X 10^3 copies/mL. While a slight decrease was observed as 37.5% in the beginning of the Phase d (Phase d_1 sample), no mRNA encoded by mcrA gene was detected at the end of Phase d. However the expression level again increased surprisingly and reached to 6.47 X 10^4 copies/mL at the end of the Phase e while the reactor could not produce biogas. Also SMA test result obtained from both sole acetate fed and VFA mixture fed test bottles showed that the methanogenic activity was increased at Phase e.

ACAS gene expression results are given in Figure 4.59. The expression profile of ACS gene was similar to expression of mcrA and ACAS was detetcted at Phase d_2 additively. Surprisingly, the expression level of ACAS was higher than mcrA during all operation. However, in DGGE analysis of archaeal 16S rRNA, Methanosaeta species could not detected in every Phase.

The main reason of this discrepancy between DGGE analysis and Q-PCR results could be the potential bias of the molecular tools with differences in DNA extraction efficiency, gene copy number or PCR amplification and primer biases, must be considered. The biases were discussed in detail (Reysenbach *et al.*, 1992; Farrelly *et al.*, 1995; Suzuki and Giovannoni, 1996).

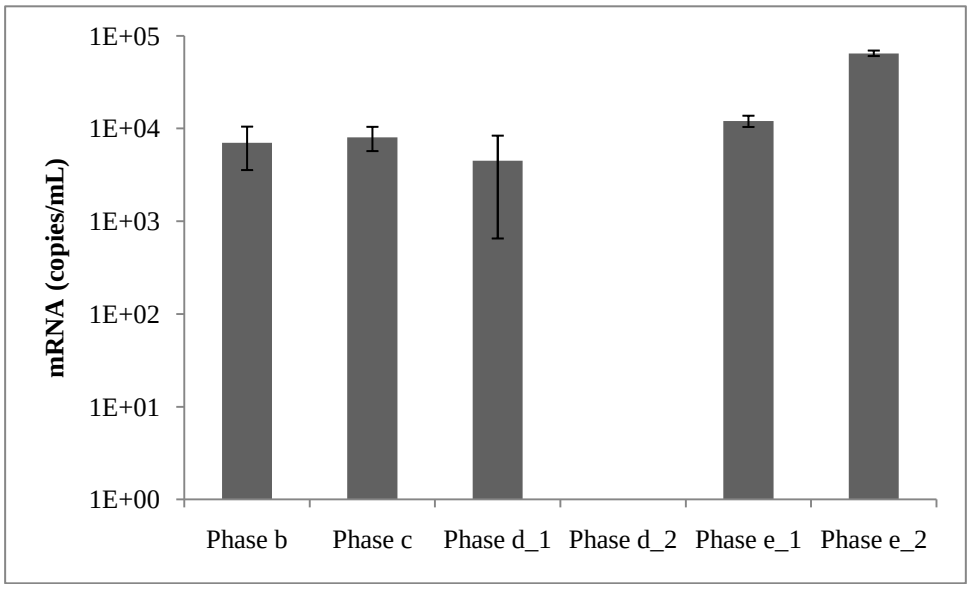

Figure 4.58: Quantitative changes in methyl-coenzyme M reductase expression level at different phases of the ERY reactor.

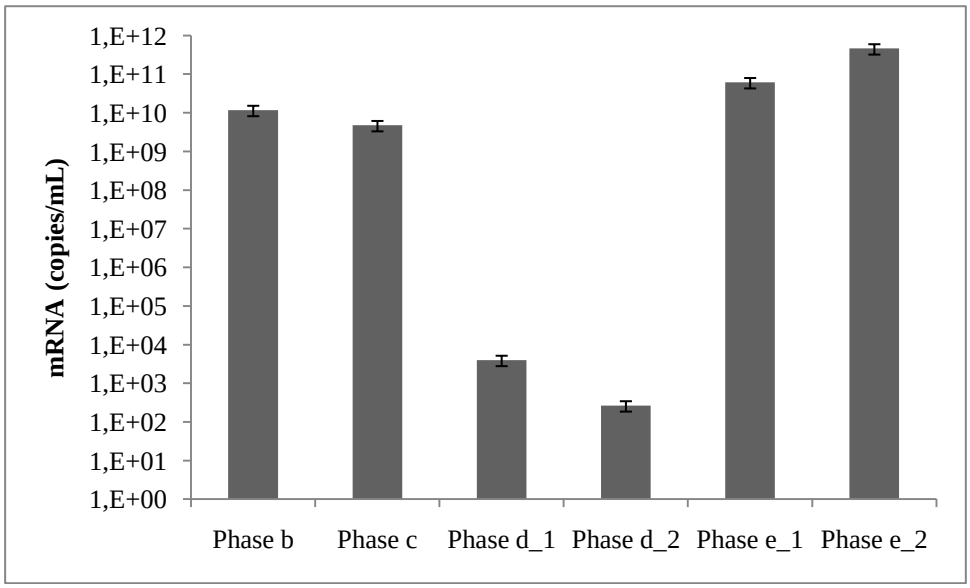

Figure 4.59: Quantitative changes in acetyl-coA synthetase expression level at different phases of the ERY reactor.

4.4.3 Tetracycline

4.4.3.1 Performance of the Tetracycline (TET) reactor

To examine the chronic effect of TET on anaerobic systems, an ASBR fed by synthetic wastewater and TET was operated with a control reactor, which was fed by only synthetic wastewater. The performance of control and TET reactor are given in Figure 4.60 and 4.61. After the start-up, the OLR was increased gradually from 1.4 g COD/L*d. OLR was increased to 2.2 g COD/L*d in first 22 days as explained in the

section 6.4.2. Then ERY reactor was operated with OLR of 2.2 g COD/L*d ay steady-state until 78th day. Also control reactor as mentioned previous sections was operated. Average effluent soluble COD, COD removal efficiency, and biogas production of ERY reactor between 22nd and 78th were observed as 74.9±19, 96.5%, and 1046±10 mL. The data obtained from control reactor was given in Section 4.4.1 and 4.4.2. Besides, the methane percentage of TET reactor was 66.2%±3.2. This corresponds to an average specific methane production of 0.32 L CH_4/g COD_removed at TET reactor before the feeding of antibiotic to the system. This value is confirming to reported in the literature (Tchobanoglous *et al.*, 2003). VSS concentration and pH were stable during all operation.

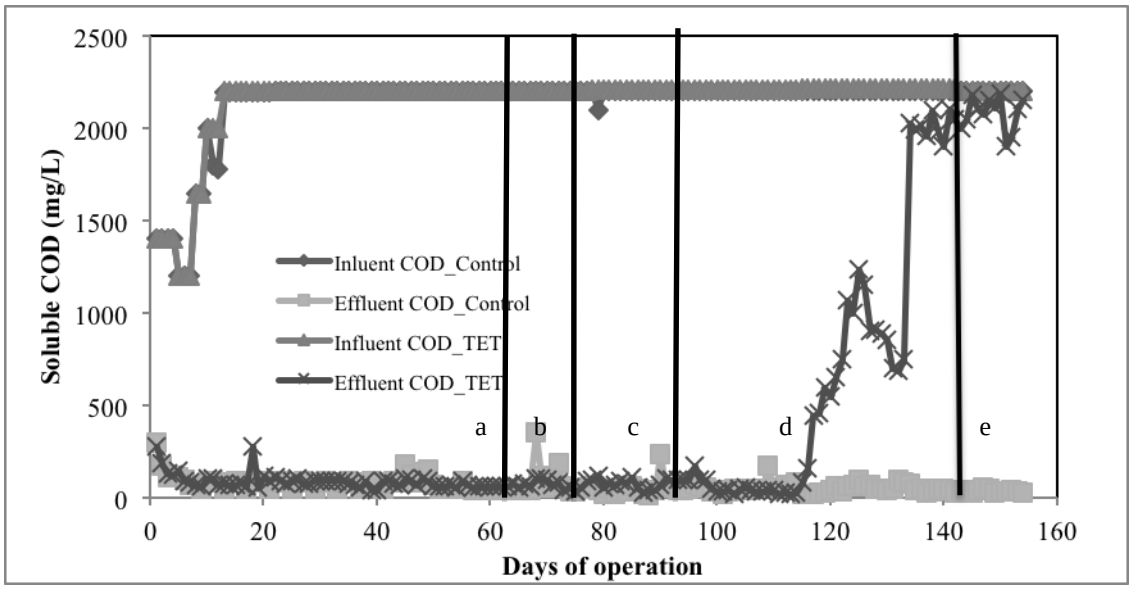

Figure 4.60: Performance of Control and TET reactors in terms of influent and effluent soluble COD concentration (Phase a: 0 mg/L TET, Phase b: 1 mg/L TET, Phase c: 2 mg/L TET, Phase d: 3 mg/L TET, Phase e: 0 mg/L TET).

1 mg/L of TET addition to the reactor was started on 78th day continued until 91st day. During this period (Phase b), no significant changed in COD removal efficiency and biogas production was observed compared to control reactor ($P>0.05$). In Phase b, while biogas production was 951±12 mL/d, average effluent soluble COD and COD removal efficiency were detected as 71.2±28.0 mg/L and 96.8%. However, a slight decrease in the methane content of biogas was determined. The methane content of biogas in TET reactor was 59.8% during Phase b.

On the 91st day, TET concentration in the reactor was increased to 2 mg/L an operated the reactor with this concentration until 115th day (Phase c). Also noticeable

difference between control and TET reactors in terms of COD removal efficiency and biogas production was not detected (P>0.05). Effluent soluble COD, COD removal efficiency and biogas production was 57.4±36.8 mg/L, 97.4% and 864±21 mL/d, respectively. The average biogas production decreased as 6% as seen in Figure 4.61. The methane content of biogas during Phase c was similar to Phase b as 58.5%.

The reactor was operated with 3 mg/L o TET from 115th day to 143rd day and this concentration was significantly affected to reactor performance (P<0.001). The COD removal efficiency and daily produced biogas decreased dramatically in a stepwise manner from 96.4% and 853 mL to 9.1% and 71 mL, respectively. The average specific methane production decreased also 0.23 L CH_4/g COD_removed in TET reactor.

At the end of Phase d, TET addition was stopped to the reactor to recover the performance again, but the COD removal efficiency and biogas production couldn't be increased again and the reactor operation was ended on day 154 (Phase e).

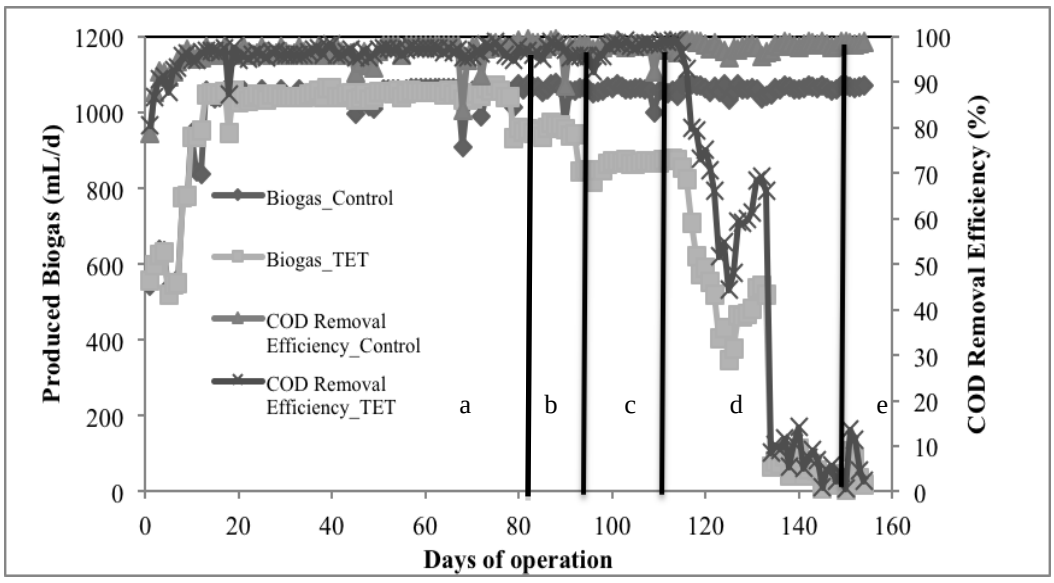

Figure 4.61: Performance of Control and TET reactors in terms of biogas production and COD removal efficiency (Phase a: 0 mg/L TET, Phase b: 1 mg/L TET, Phase c: 2 mg/L TET.

A limited study about effect of TET on anaerobic wastewater treatment systems in the literature was found. The compound and its derivates are commonly used as promotor in animal growth, so most of the studies have focused on the anaerobic digestion of manure, which contains this compound (Masse *et al.*, 2000; Arikan et al, 2006; Arikan *et al.*, 2007; Arikan 2008; Stone *et al.*, 2009; Wu *et al.*, 2011; Shi *et*

al., 2011; Hu *et al.*, 2011). However the result of those studies are different from the results obtained from this study. Masse *et al.* (2000) operated psychrophilic ASBR treating swine manure and they mentioned that 25% removal efficiency in TET was achieved and any significant inhibition was not detected in the system as well on 21 g/L of TET. In another study, the fate and effect of oxytetracycline, which is an antibiotic from tetracycline group, on anaerobic digestion of manure was focused (Arikan *et al.*, 2006), this compound was degraded biologically under mesophilic conditions and converted to transformation products. Also the concentration of those transformation products was decreased by the time. The author indicated that 27% biogas production was decreased with 22 mg/kg/day. Stone *et al.* (2009) mentioned that 28 mg/L of chlortetracycline inhibited the methane production as approx 28% in an anaerobic reactor digesting manure. 70% of tetracycline was degraded by biologically in a pilot scale anaerobic digestor composting swine manure (Wu *et al.*, 2011). The author explained that TET was also converted to transformation products and also they were degraded in the reactor as mentioned before by Arikan *et al.* (2006). Wu and his colleagues did nit mention any inhibition in the system. In another study, no influence of TET on composting was detected while the compound was biologically degraded more than 93% (Hu *et al.*, 2011).

Contrary of the knowledge obtained from literature, TET inhibited the anaerobic reactor treating wastewater even lower concentration as 3 mg/L in this study. This compound also considered being non-biodegradable under anaerobic conditions in wastewater treatment systems (Gartiser *et al.*, 2007). However, it should be taken consider that TET has an unstable character because of its unique chemical structure and may withstand abiotic degradation with different environmental conditions such as pH, temperature or chemical oxidation and photolytic reactions and then it is converted to transformation products (Kuhne *et al.*, 2001; Halling-Sorenson *et al.*, 2003).

Additionally, a few studies have been focused on the effect of TET on activated sludge systems. They indicated that TET is non-biodegradable and non-toxic under aerobic conditions up to 40 mg/L TOC, it inclines to sorption into sludge (Prado *et al.*, 2009a and b). This discrepancy between literature and this study can be explained by specific experimental conditions such as SRT, temperature variations,

ions and salinity, the different waste typed should be taken into consideration (Cirja *et al.*, 2008).

4.4.3.2 Volatile fatty acids

No VFA in the effluent was detected until Phase d like the ERY reactor explained in Section 6.4.2. Differently from SMX and ERY reactors, not only propionic acid but also acetic acid utilization pathway was inhibited by TET. At the first day (on day 116) after TET concentration was increased to 3 mg/L, acetic acid and propionic acid accumulation started as 27 and 28 mg/L, respectively. Acetic acid concentration in the effluent increased to 110 mg/L at the end of Phase d and reached to 457 mg/L at the end of the operation, Phase e.

Propionic acid accumulation has also similar trend however, the concentration was higher than acetic acid. Its concentration was measured as 750 mg/L at the end of Phase d. However propionic acid concentration decreased in the Phase d and it was detected as 385 mg/L at the end of operation.

Also butyric and valeric acids were observed in the effluent of the TET reactor at Phase d and e. While butyric acid concentration varied between 4 and 20 mg/L, valeric acid concentration increased from 14 mg/L to 70 mg/L slowly until the end of operation.

A clear effect of TET on acetoclastic methanogens, which utilize acetate to produce methane, was observed in the VFA results. Specifically TET ceases aminoacyl-tRNA interaction with acceptor site on the mRNA-ribosome complex to inhibit the protein synthesis via binding ribosome of bacteria (Chopra and Robertes, 2001; Loftin *et al.*, 2005). So the direct effect of TET would not expected. However, Sanz *et al.* (1996) indicated that chlortetracycline, which is an antibiotic from the same group of TET, inhibited acetoclastic methanogens and was considered a potential inhibitor of archaea. Furthermore, Stone *et al.* (2009) speculated that chlortetracycline might have contributed to inhibition of acetoclastic methanogens. In the same study, the VFA concentration during the operation of anaerobic digestor increased.

4.4.3.3 Antibiotic measurement

As seen in Figure 4.62, sorption of TET increased with operation time and TET concnetration in the influent. Also COD removal efficiency of the reactor decreased with respect to time and TET concentration. However TET was removed in the system in early phases, even though it mentioned that TET is not biodegradable under anaerobic conditions (Gartiser et al., 2007).

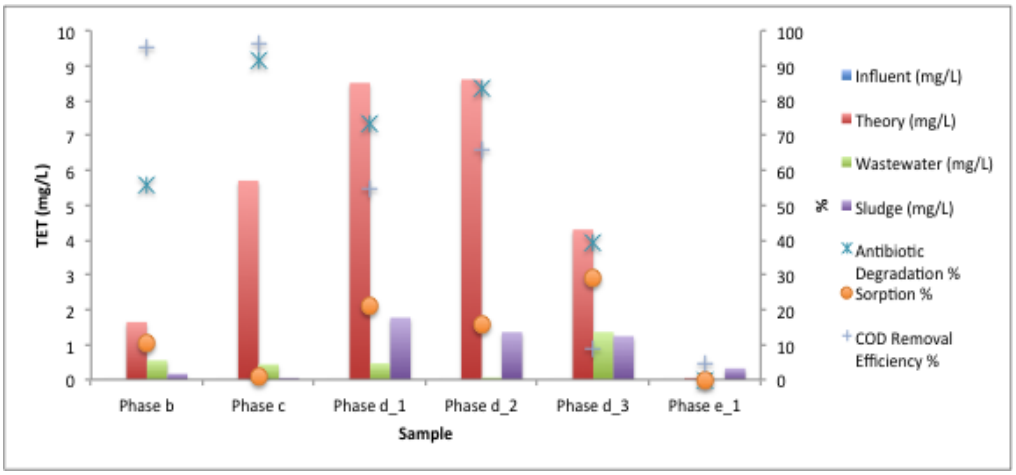

Figure 4.62: Antibiotic measurement results of TET reactor

4.4.3.4 Specific methanogenic activity tests

To evaluate long-term effect of TET on different trophic group, specific methanogenic activity (SMA) test was performed with only acetate and acetate-butyrate-propionate mixture.

Utilization of acetate

To determine TET effect on acetoclastic metahogenic activity, SMA test was performed with sludges taken from different operation phases of the TET reactor. Acetate was used as sole carbon sources. CBP and CMP profiles obtained from the SMA test are given in Figure 4.63. CMP value of the seed sludge was determined as 77 mL. As seen Figure 6b, there was a decrease in CMP values even in 1 mg/L TET fed sludge and detected as 55 mL. Approximately 30% reduction was occurred after TET addition (Phase b). While a gradually reduction on CBP and CMP values was occurred during operation with respect to TET concentration, they increase after TET addition was stopped at Phase e. CBP values were determined as 86 and 80 mg/L at

Phase b and c, respectively. A dramatic decrease was observed in CBP, thereby CMP; at Phase d. CBP was reduced more than 50% and detected as 41 mL at Phase d. However, after TET addition in the reactor was stooped, the activity of the sludge increased as 69 mL of CBP (Phase e).

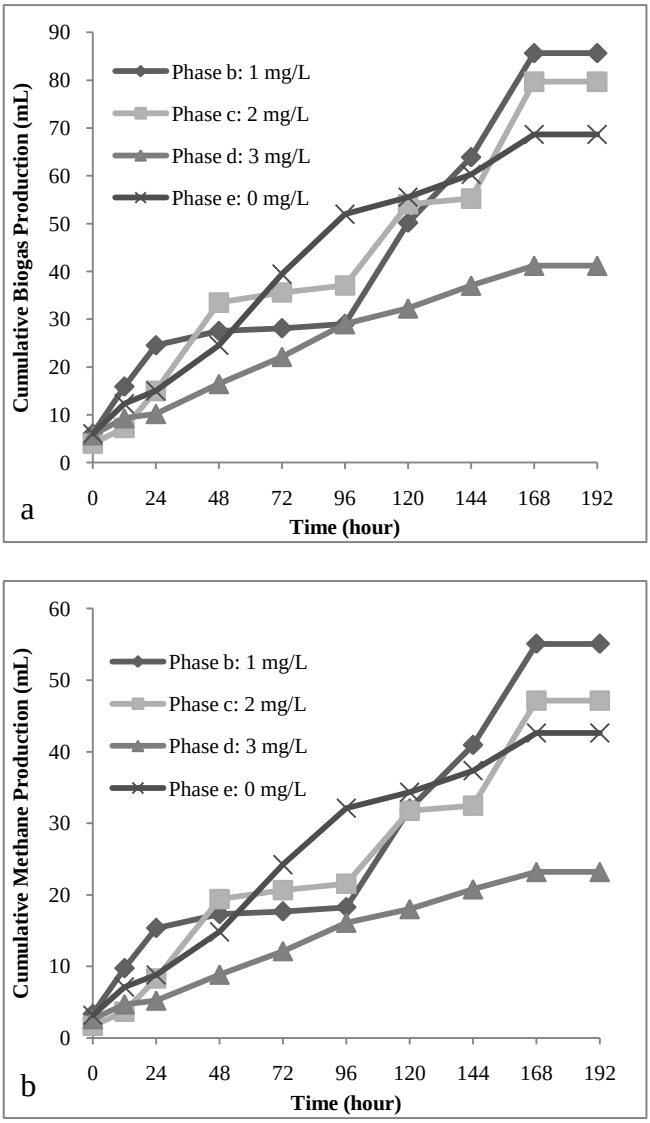

Figure 4.63: Specific methanogenic activity (SMA) test using 4000 mg/L of acetate (a) cumulative biogas production profiles (b) cumulative methane production.

The methane content of the biogas was also decreased gradually depending on TET concentration. While the methane percent was 65%, 60% and 58% at Phase b, c and d, respectively, the value increased to 63% again at Phase e, in which TET addition was terminated.

The SMA results, except for Phase e, supported the reactor performance and VFA data obtained from TET added ASBR. Also, the reactor efficiency decreased

gradually with TET concentration and 3 mg/L of TET addition was almost terminated the COD removal. VFA analysis reflected that fatty acid accumulation, especially acetate, was started in the beginning of Phase d. Also anaerobic digestor studies in the literature mention that tetracycline group antibiotics inhibit acetoclastic methanogens (Sanz *et al.*, 1996; Stone *et al.*, 2009).

The results obtained from this study also showed inhibitory affect of TET on acetoclastic methanogenic activity. Even the increase in CBP and CMP values of sludge from Phase e is a proof to reflect that while TET addition is stopped, acetoclastic methanogens can recover themselves. The differences between the reactor operation and SMA test in Phase e may be explained that SMA test focused only acetoclastic activity. However, all syntrophic groups in terms of bacterial and archaeal species were evaluated together.

Utilization of VFA Mixture

To evaluate the TET effect on homoacetogens and all methanogens, SMA test was performed with VFA mixture. The CBP and CMP profiles of SMA test are given in Figure 4.64. Even 1 mg/L of TET addition to the ASBR was affected utilization capacity of VFA. While CMP value of the seed sludge for utilization of VFA was 315 mL, the values dramatically decreased to 93 mL (70% reduction). The decrease continued until the Phase e and CBP and CMP values of the sludge taken from Phase d were 29 mL and 14 mL, respectively. The results were supported to the TET reactor data, which almost ceased at the end of Phase d. Contrary of the ERY reactor, the VFA utilization activity increased to 105 mL of CBP after TET addition was terminated at Phase e. The results were indicated that TET also inhibited the bacterial group as expected. In all SMA test tubes, methane content of biogas was same as 50%.

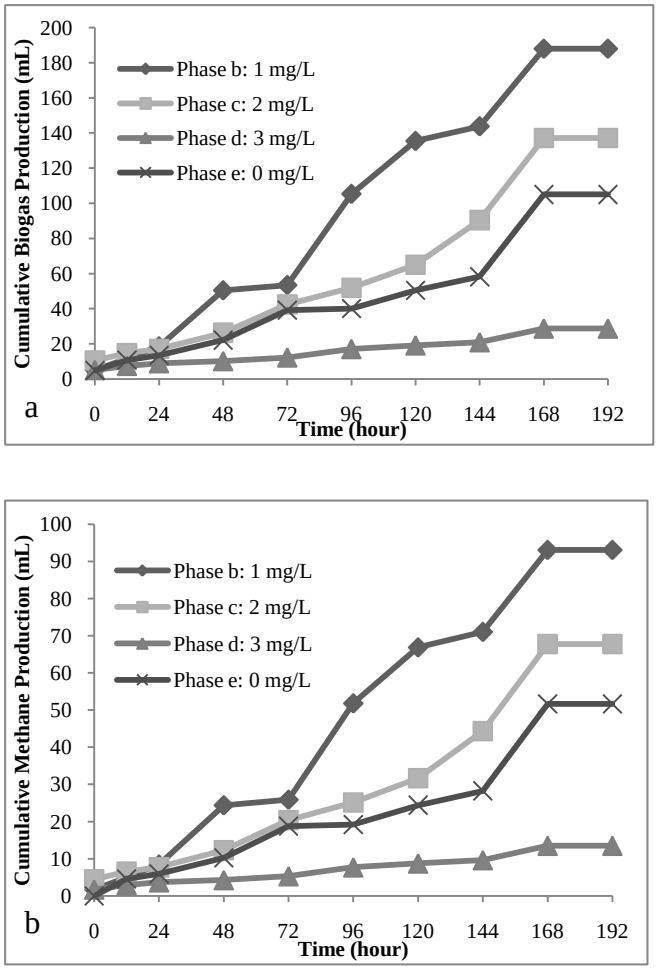

Figure 4.64: Specific methanogenic activity (SMA) test using 3000 mg/L of VFAs (a) cumulative biogas production profiles (b) cumulative methane production.

4.4.3.5 Microbiological approach

Molecular tools based on 16S rDNA and 16S rRNA were used to find out the effects of TET on existing and active microorganisms, relatively. In this scope, genomic DNAs and total RNAs were extracted from the samples collected form different phases of the TET reactor and RNAs were converted to cDNAs by reverse transcriptase PCR (RT-PCR). Amplicons were obtained using 16S rRNA gene spesific primers for further analysis on DGGE. Furthermore, GDNAs and cDNA were used to quantify total bacteria, archaea and methanoges by Q-PCR.

Predominant bands in the DGGE patterns

To find out the microorganisms having role on the TET reactor, predominant bacterial and archaeal 16S rDNA/rRNA band patterns of the different phases of TET reactor were compared to 16S rDNA clone libraries of the seed sludge. The reason to use both 16S rDNA and 16S rRNA is evaluation of the existing and active species in

the system, respectively. Also their abundance was evaluated according to intensity of each band on DGGE gel because the intensity of the bands might correspond at least semiquantitatively with the abundance of the corresponding species (Heuer *et al.*, 1997). The comparison results of bacterial and archaeal 16S rDNA DGGE band patterns were given in Table 4.17 and 6.18, respectively.

According to comparison results to 16S rDNA clone library, 12 of bacterial OTUs from bacterial DGGE profile of the TET reactor matched to the library OTUs. 3 uncultured Clostridium clones (FR836437, FR836438 and FR836443) were detected in the TET reactor at different phases. While all of them existed at Phase b and c, the clone with accession number as FR836443 was detected at the last Phase e_2. Clostridium spp. have the important role both the fermentation of starch and also production of volatile fatty acids by β-oxidation pathway (Hungate, 1982; Payton and Haddock, 1986). Also, Paludibacter propionicigenes (AB078842), which is responsible to produce propionate, was detected at Phase b, c and e. So, the results are not surprise for this system fed by a synthetic wastewater including starch and glucose. At last three phases, Uncultured Syntrophomonas clone, which is probably responsible to degrade the long-chain fatty acids, were made out (Sousa *et al.*, 2007). Other OTUs detected in the samples almost belong to the uncultured clones or unclassfied bacterial cultured species.

The comparison results of the archaeal 16S rDNA DGGE band patterns and the clone library is given in Table 4.18. 13 of archaeal OTUs in the DGGE profiles of TET reactor were matched to 16S rDNA archaeal clone library. According to results, acetoclastic methanogen species (FR836463, FR836468, U89773) existed in the system. Hydrogenotrophic methanogens were observed in different phases, however their abundance was less existing of Methanobacterium beijingense strain 8-2 (AY350742). This hydrogenotrophic specie was detected with uncultured Methanosphaerula clone (FR836467).

Table 4.17: Affiliation of bacterial clones and their abundance at the different phases of the TET reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
B1	2.80	FR836435	*Clostridium sp. BG-C66* (FJ384378)	96						
B2	0.93	FR836436	*Clostridium sp. Kas301-1* (AB114242)	96						
B3	1.87	FR836437	*Clostridium intestinale* (AM158323)	95	•	•				
B4	4.67	FR836438	Uncultured Clostridium sp. clone AUCLO214 (AY648564)	96	••					
B5	1.87	FR836439	Uncultured Firmicutes bacterium clone QEDS2BE07 (CU921543)	93						••
B6	2.80	FR836440	Uncultured Firmicutes bacterium clone QEDN7DC08 (CU926749)	97	••	•		•	•	•
B7	14.95	FR836441	Uncultured bacterium clone BS7_65 (GQ458219)	98		••				
B8	3.74	FR836442	*Syntrophomonas zehnderi strain OL-4* (DQ898277)	89				•	•	•
B9	33.64	FR836443	*Clostridium sp. Kas107-1* (AB114241)	96	•	•				•
B10	5.61	FR836444	Uncultured bacterium clone C3-25 (AM181973)	91						
B11	4.67	FR836445	*Bacterium CBIC19I3* (HM240274)	95						

Table 4.17 (continued): Affiliation of bacterial clones and their abundance at the different phases of the TET reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
B12	3.74	FR836446	Uncultured bacterium UASB_TL84 (AF254390)	94	•	•			•	
B13	0.93	FR836447	Uncultured Candidatus Cloacamonas sp. clone 8SN (EU887773)	92						
B14	5.61	FR836448	Uncultured bacterium clone BS1_25R (GQ458208)	97						
B15	2.8	FR836449	Uncultured bacterium clone aaa97f10 (DQ816701)	96						
B16	0.93	FR836450	*Acinetobacter sp. 1B3* (EU337120)	96						
B17	2.8	FR836451	Uncultured Sarcina sp. clone JPL-2_O10 (FJ957591)	89		••				
B18	0.93	FR836452	Uncultured bacterium clone 30c07 (EF515566)	87						
B19	0.93	FR836453	*Paludibacter propionicigenes* (AB078842)	92	•	•			•	•
B20	0.93	FR836454	*Bacterium 061128-OL-KR37-AA 3-0 10000x -1A* (FJ037613)	92		•				
B21	0.93	-	*Propionibacteriaceae bacterium FH044* (AB298766)	99						
B22	1.87	FR836458	Uncultured bacterium clone D14R15C96 (FM956760)	98		•				•

Table 4.18: Affiliation of archaeal clones and their abundance at the different phases of the TET reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
A1	1.45	FR836460	Uncultured archaeon clone OK3 (GQ406364)	94						
A2	2.90	FR836461	Uncultured archaeon clone I17_Rya (HM639831)	98			••	••		
A3	9.42	FR836462	*Methanogenic euryarchaeote Annu4* (FJ896263)	95	••••	••••	••••	•••	••••	••
A4	2.90		*Methanobacterium formicicum strain S1* (DQ649309)	99						
A5	2.90	FR836464	Uncultured Methanosarcinales archaeon clone:KuA4 (AB077214)	98						
A6	4.35	FR836470	Uncultured euryarchaeote clone MVP-8A-21 (DQ676281)	98		••			••	•
A7	12.32	FR836463	*Methanothrix soehngenii* (X51423)	97				••		
A8	0.72		Uncultured archaeon clone A6T20L208 (DQ262482)	99						
A9	7.25		*Methanospirillum hungatei* (M60880)	99						
A10	0.72	FR836465	*Methanobacterium beijingense strain M4* (EU544027)	97						
A11	6.52	FR836467	*Methanosphaerula palustris strain E1-9c* (EU156000)	95		••	•••	•••		
A12	0.72		*Methanobacterium sp. OM15* (AJ550160)	99						
A13	2.17		*Methanobacterium beijingense strain 8-2* (AY350742)	100	••		•••	•••	••	••
A14	3.62	FR836468	Uncultured Methanosarcinales archaeon clone S4 (GU475184)	95	••	••	•••	•••		••

Table 4.18 (continued): Affiliation of archaeal clones and their abundance at the different phases of the TET reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
A15	2.9	FR836469	Uncultured archaeon clone Sed-ARC-34 (EU255763)	98					•••	
A16	3.62	FR836471	Uncultured euryarchaeote clone MVP-8A-26 (DQ676246)	96		••		•••	••••	••
A17	9.42	FR836472	*Methanogenic archaeon Prasan1* (HM630570)	97			••	•••		••
A18	7.25	FR836473	*Methanobacterium beijingense strain 4-1* (AY552778)	96			••			••
A19	2.17		*Methanosarcina siciliae* (U89773)	99	••		••	••	•••	••
A20	4.35		Uncultured archaeon clone EOTU21 (FJ896263)	99						
A21	1.45	FR836474	Uncultured archaeon clone OKA6 (GQ465437)	97						
A22	1.45		Uncultured archaeon clone ATB-KM-2942-A18 (EF680353)	99						
A23	6.52		*Archaeon LL25A3* (AJ745135)	99	••	•••	•••	•••	••	••
A24	2.17		Uncultured archaeon clone WA10 (AB494239)	99						
A25	0.72	FR836475	*Methanogenic euryarchaeote Annu1* (HM630571)	95						

To find out the active OTUs and their changes in the TET reactor with respect to antibiotic concentration and operation time, the PCR amplicons obtained from cDNAs of 16S rRNA were compared to bacterial and archaeal 16S rDNA clone libraries constructed from the seed sludge. The comparison results are represented in Table 4.19 and 6.20, respectively. 13 of the bacterial OTUs were detected in the 16S rRNA DGGE profiles. However, 2 of these OTUs (FR836444 and FR836448) were not the same with 16S rDNA DGGE comparison which is given in Table 4.17. According to 16S rRNA DGGE results, 3 of the uncultured Clostridum species (FR836437, FR836438 and FR836443) were detetcted in the TET fed system. However only FR836443 was detetcted at the last phase. The other two uncultured Clostridium clones were only observed in the Phase b, c and d_1 and also their abundance was getting lower. Clostidium species converte carbonhydrates to VFAs by fermentation and also β-oxidation process (Hungate, 1982; Payton and Haddock, 1986). These finding supported the reactor results which the COD removal efficiency decreased with respect to the antibiotic concentration and the reactor performance could not be recovered even TET addition was stopped. Addition of Clostridum spcies, Uncultured Syntrophomonas clone (FR836442) was only onserve at Phase d_2, while it was detected at Phase d_2, e_1 and e_2 by 16S rDNA level. Uncultured Paludibacter clone (FR836453), which is responsible to propionate production, was detected only at Phase e_1. Other detected bacterial clones were mostly uncultured species. These uncultured bacterial clones may probably be species responsible to fermentation and homoacetogenesis, especieally VFA-oxidizing syntrophs (Speece, 1996; Madigan *et al.*, 2009). In the archaeal 16S rRNA DGGE profiles, hydrogenotrophic OTUs (FR836465, FR836467, FR836473, AJ550160, AY350742) were dominant mostly each step, acetoclastic methanogenic OTUs (FR836468 and U89773) were observed only at Phase e. While acetoclastic methanogens are capable of growth under low acetate concentration bu have a high affinity for acetate (Jetten *et al.*, 1992), their abundance and especialy decrease with respect to time and TET concentration. Stone *et al.* (2009) showed that acetoclastic methanogens were affected by chlortetracycline during anaerobic swine manure digestion. The authors also mentioned that activity and abundance of hydrogenotrophic species increased. The results indicated that hydrogenotrophic methanogens were not inhibited by TET even in the last phase. However VFA and SMA test results reflected there was a general inhibition in the system without recovering system perfomance even if antibiotic addition was stopped.

Table 4.19: Affiliation of bacterial clones and their abundance within the active community at the different phases of the TET reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
B1	2.80	FR836435	*Clostridium sp. BG-C66* (FJ384378)	96						
B2	0.93	FR836436	*Clostridium sp. Kas301-1* (AB114242)	96						
B3	1.87	FR836437	*Clostridium intestinale* (AM158323)	95	••••					
B4	4.67	FR836438	Uncultured Clostridium sp. clone AUCLO214 (AY648564)	96	••	••••	••			
B5	1.87	FR836439	Uncultured Firmicutes bacterium clone QEDS2BE07 (CU921543)	93						
B6	2.80	FR836440	Uncultured Firmicutes bacterium clone QEDN7DC08 (CU926749)	97	••	••	••	••	•	•
B7	14.95	FR836441	Uncultured bacterium clone BS7_65 (GQ458219)	98	•••••••					•
B8	3.74	FR836442	*Syntrophomonas zehnderi strain OL-4* (DQ898277)	89				•		
B9	33.64	FR836443	*Clostridium sp. Kas107-1* (AB114241)	96						•
B10	5.61	FR836444	Uncultured bacterium clone C3-25 (AM181973)	91			••	•		•
B11	4.67	FR836445	*Bacterium CBIC19I3* (HM240274)	95						

Table 4.19 (continued): Affiliation of bacterial clones and their abundance within the active community at the different phases of the TET reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
B12	3.74	FR836446	Uncultured bacterium UASB_TL84 (AF254390)	94				••	•	•
B13	0.93	FR836447	Uncultured Candidatus Cloacamonas sp. clone 8SN (EU887773)	92						
B14	5.61	FR836448	Uncultured bacterium clone BS1_25R (GQ458208)	97		••••	•••	••	•	•
B15	2.8	FR836449	Uncultured bacterium clone aaa97f10 (DQ816701)	96						
B16	0.93	FR836450	*Acinetobacter sp. 1B3* (EU337120)	96						
B17	2.8	FR836451	Uncultured Sarcina sp. clone JPL-2_O10 (FJ957591)	89				••		
B18	0.93	FR836452	Uncultured bacterium clone 30c07 (EF515566)	87						
B19	0.93	FR836453	*Paludibacter propionicigenes* (AB078842)	92					•	
B20	0.93	FR836454	*Bacterium 061128-OL-KR37-AA 3-0 10000x -1A* (FJ037613)	92				•		
B21	0.93	-	*Propionibacteriaceae bacterium FH044* (AB298766)	99						
B22	1.87	FR836458	Uncultured bacterium clone D14R15C96 (FM956760)	98			•••			

Table 4.20: Affiliation of archaeal clones and their abundance within the active community at the different phases of the TET reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
A1	1.45	FR836460	Uncultured archaeon clone OK3 (GQ406364)	94	••••			••		
A2	2.90	FR836461	Uncultured archaeon clone I17_Rya (HM639831)	98				••		
A3	9.42	FR836462	*Methanogenic euryarchaeote Annu4* (FJ896263)	95					•••	••••
A4	2.90		*Methanobacterium formicicum strain S1* (DQ649309)	99						
A5	2.90	FR836464	Uncultured Methanosarcinales archaeon clone:KuA4 (AB077214)	98						
A6	4.35	FR836470	Uncultured euryarchaeote clone MVP-8A-21 (DQ676281)	98						
A7	12.32	FR836463	*Methanothrix soehngenii* (X51423)	97						
A8	0.72		Uncultured archaeon clone A6T20L208 (DQ262482)	99						
A9	7.25		*Methanospirillum hungatei* (M60880)	99						
A10	0.72	FR836465	*Methanobacterium beijingense strain M4* (EU544027)	97	•••••••	••••••••	••••	••••••		
A11	6.52	FR836467	*Methanosphaerula palustris strain E1-9c* (EU156000)	95		•••••	•••••			
A12	0.72		*Methanobacterium sp. OM15* (AJ550160)	99		•••		••		
A13	2.17		*Methanobacterium beijingense strain 8-2* (AY350742)	100	•••••	•••••	••••	•••••	•••••••••	••••••••
A14	3.62	FR836468	Uncultured Methanosarcinales archaeon clone S4 (GU475184)	95					•••••	

Table 4.20 (continued): Affiliation of archaeal clones and their abundance within the active community at the different phases of the TET reactor.

Clone	Clone Frequency (%)	Accession No (This study)	Closest Relative	Similarity (%)	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
A15	2.9	FR836469	Uncultured archaeon clone Sed-ARC-34 (EU255763)	98	•••				•••	•••
A16	3.62	FR836471	Uncultured euryarchaeote clone MVP-8A-26 (DQ676246)	96		•••	•••	••		
A17	9.42	FR836472	*Methanogenic archaeon Prasan1* (HM630570)	97	••••	••••	•••	•••	•••••••	•••••
A18	7.25	FR836473	*Methanobacterium beijingense strain 4-1* (AY552778)	96	••••	•••••	•••	••••	••••••	•••••••
A19	2.17		*Methanosarcina siciliae* (U89773)	99						•••••
A20	4.35		Uncultured archaeon clone EOTU21 (FJ896263)	99						
A21	1.45	FR836474	Uncultured archaeon clone OKA6 (GQ465437)	97				••		•••
A22	1.45		Uncultured archaeon clone ATB-KM-2942-A18 (EF680353)	99						
A23	6.52		*Archaeon LL25A3* (AJ745135)	99	•••••	•••••	••••	•••	•••••	••••••
A24	2.17		Uncultured archaeon clone WA10 (AB494239)	99						
A25	0.72	FR836475	*Methanogenic euryarchaeote Annu1* (HM630571)	95						

Cluster analysis of DGGE banding pattern

DGGE technique was used in this study to assess the stability and/or change in microbial community and active species at different phases of the TET reactor. A total of seven sludge samples collected from different phases of the reactor were compared in terms of bacterial and archaeal communities: The seed sludge of the chronic reactors and the sludges collected from different phases of the TET reactor. Result of DGGE analysis suggested that bacterial community was respectively, more diverse than archaeal diversity in the reactor as found in sludge of SMX and ERY reactors; 62 bacterial and 46 archaeal bands were remarked in the TET reactor samples.

Bacterial and archaeal diversities and species richness in seed sludge and sludges obtained from the TET reactor were measured by the Shannon-Weaver diversity index and Margalef's species richness, respectively. The values were given in Table 4.21 and 6.22.

In seed sludge, while 33 of different bacterial species were detected, this number decreased to 19 at Phase b, in which 1 mg/L of TET was fed to system, as seen in Table 4.21. While, at Phase c, the number increased to 38 because of presumptive acclimation, it decreased to 12 dramatically when TET concentration was increased to 3 mg/L. As discussed in the reactor operation section, 4.4.3.1, the reactor performance also decreased in this period, Phase d. At the end of the operation, the bacterial OTUs reached to 23 despite the reactor efficiency was near to zero. Also situation of the archaeal OTUS was similar to bacterial profile. In the first TET addition period, the number decreased from 13 to 7. After a probable acclimation period, the archaeal OTU number increased to 16. At the end of the operation, a slight change was observed in the archaeal species number as 13.

Table 4.21: Shannon-Weaver diversity indices, Margalef's species richness measure, and eveness measure estimates for bacterial DGGE profiles of sludges taken from different phases of the TET reactor.

	Seed_Chronic Reactors	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
Taxa	33	19	38	12	15	20	23
Shannon-Weaver diversity	3.449	2.802	3.504	2.449	2.65	2.736	3.055
Evenness	0.9537	0.8671	0.8747	0.9645	0.9433	0.7711	0.9222
Margalef's richness	4.954	3.03	5.535	2.17	2.574	2.996	3.686

Table 4.22: Shannon-Weaver diversity indices, Margalef's species richness measure, and eveness measure estimates for archaeal DGGE profiles of sludges taken from different phases of the TET reactor.

	Seed_Chronic Reactors	Phase b	Phase c	Phase d_1	Phase d_2	Phase e_1	Phase e_2
Taxa	13	7	11	15	16	13	13
Shannon-Weaver diversity	2.526	1.895	2.359	2.67	2.748	2.474	2.539
Evenness	0.962	0.9499	0.9619	0.9628	0.9757	0.9132	0.974
Margalef's richness	1.901	1.038	1.589	2.12	2.211	1.776	1.953

Pearson product-moment correlation coefficients with UPGMA clustering were used to quantify the similarity of the community fingerprints between each sample. The bacterial and archaeal 16S rDNA phylogenetic trees obtained from TET reactor with seed sludge sample were given in Figure 4.65 and 4.66, respectively.

DGGE analysis found out that bacterial and archaeal differentiation in TET samples occurred according to TET concentration in the reactor and the operation time.

The changes in bacterial community during the operation reflect the clusters obtained from DGGE band patterns, which were divided into two clusters. The clusters were defined by less than 60% pattern similarity. One of them included seed sludge, Phase b, c and e_2. This cluster also divided sub-clusters. Seed sludge belonged to one sub-cluster as solo and it was similar to Phase b, c and e_2 samples as 68.3%, 78.4%, and 62.2%, respectively.

Phase d_1, d_2 and e_1 also belonged to the second cluster. This cluster also divided into two sub-clusters based on their population origin, which were defined by a similarity pattern varied between 88.9% and 97.6%. As seen in Figure 4.65, while the pattern similarity of the samples belonging to second cluster did not change very distinctly, the distance between the first cluster samples were higher. It was clear that a shift in the bacterial community profile occurred during the operation time. After the reactor operated with 3 mg/L of TET, the bacterial community was changed. Also in the beginning of Phase e, in which TET addition was terminated, the bacterial community was similar to Phase d. However at the end of the operation (Phase e_2), the bacterial community was similar to Phase c as 84.4% as seen in the Figure 4.62. The bacterial community profile of TET samples was similar to ERY bacterial DGGE profile.

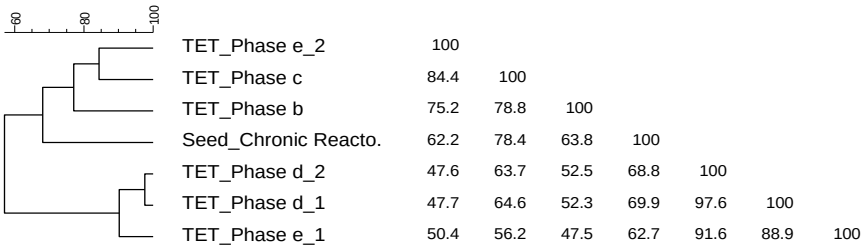

Figure 4.65: Cluster analysis and similarity matrix of bacterial 16S rDNA gene DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

Archaeal cluster analysis obtained from DGGE banding pattern was different form bacterial cluster analysis of the TET reactor. The archaeal banding patters revealed two main clusters defined by less than 55% similarity (Figure 4.66). In one of these clusters, samples of the seed sludge and Phase b were defined. They were the most similar to Phase e_2 sample as 49.6% and 67.1%, respectively. The second cluster also divided into two sub-clusters. One of them included only Phase e_2 and the this sample was the most closest to Phase c by the similarity as 67.8%. The second sub-cluster divided more sub-clusters. Phase c, d_1, d_2 and e_1 samples belonged to this cluster and their similarity varied between 76.8% and 93.8%. Differently from the bacterial dendogram, the archaeal pattern similarity of the samples changed more distinctly.

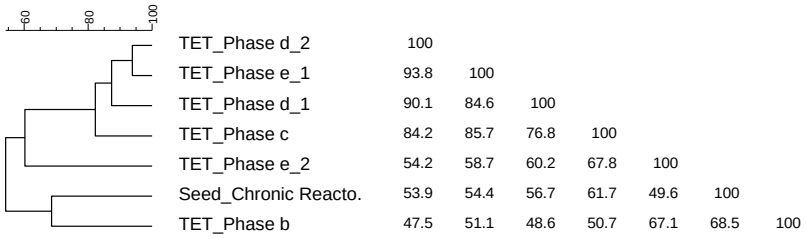

Figure 4.66: Cluster analysis and similarity matrix of archaeal 16S rDNA gene DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

DGGE analysis was performed with 16S rRNA amplicon obtained from cDNAs of the sludge samples to reveal the active community changes in the TET reactor during the operation. The bacterial and archaeal dendograms reflecting the active community change are given in Figure 4.67 and 4.68, respectively.

The bacterial band patterns obtained from 16S rRNA gave slightly different result from 16S rDNA band patterns. As seen in the bacterial 16S rDNA dendogram, two main clusters defined by less than 80% similarity. One of these clusters included only Phase b sample, which differentiated from other samples obviously. The closest samples to Phase b was surprisingly Phase d_2 sample by a similarity as 74.3%. The second cluster also divided into two sub-clusters. One of the sub-clusters included Phase c and d_1 samples, which were define a similarity 94.0%. The second sub-cluster also divided into two more clusters. While Phase d_2 sample was represented in one of these sub-clusters, Phase e_1 and e_2 samples were placed in the second one and defined a similarity each other as 93%.

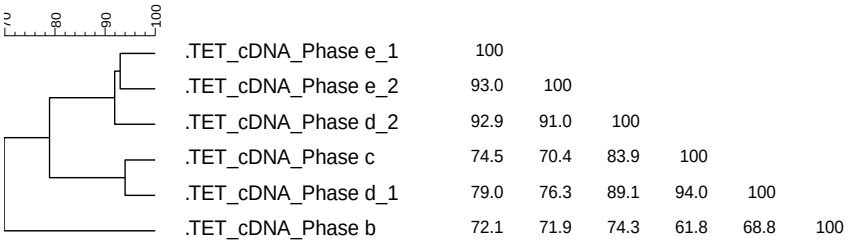

Figure 4.67: Cluster analysis and similarity matrix of bacterial 16S rRNA DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

In the archaeal phylogenetic tree of 16S rRNA, the dendogram structures and similarities were different from the dendogram of archaeal 16S rDNA. As seen in Figure 4.65, the distribution of the active archaeal community reflected the clusters, which were divided into two sub-clusters like dendogram of 16S rDNA. The

similarity of the clusters defined lees tan 80%. One of these clusters included only Phase e samples, e_1 and e_2, which were defined by a similarity as 91.9%. The second sub-cluster also divided into clusters in which b, c, d_1 and d_2 samples were defined with a similarity varied from 84.7% to 97.3%.

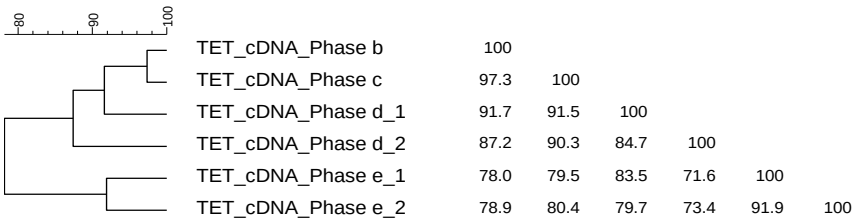

Figure 4.68: Cluster analysis and similarity matrix of archaeal 16S rRNA DGGE banding patterns based on Pearson product-moment correlation coefficients and UPGMA.

As seen in the results obtained from cluster analysis;

1- Similarities in the 16S rDNA dendograms were more distinct from 16S rRNA results.
2- The cluster analysis reflected to effect of TET concentration within the system and operation time.
3- 16S RNA DGGE dendogram showed that TET effect on the archaeal community at the end of the operation.

Environmental conditions and toxic compound loadings to ecosystems are the important reason of shift in the microbial community (Eichner *et al.*, 1999; LaPara *et al.*, 2001). Also it was found that the flexibility of microbial community leads to more stable reactor operation (Fernandez *et al.*, 2006). However compared the microbiological data with reactor efficiency, the change in the microbial community was not enough to continue reactor stability in this study.

Quantification by Q-PCR

Quantitative changes of 16S rDNA and 16S rRNA concentrations in terms of bacteria, archaea and methanogens were also determined by Q-PCR during operation time of the TET reactor. The concentration profiles showed the temporal variations in the microbial community and the active population at different phases of the TET reactor as given in Figure 4.69-4.74.

While bacterial 16S rDNA concentration decreased at Phase b compared to seed sludge and reached to 1.3 X 10^8 copies/mL, the bacterial amount increased to 3.4 X

10^{10} copies/mL (approx. 260 fold) at Phase c, in which TET concentration was 2 mg/L in the system. The concentration of bacteria at Phase e_2, in which the TET addition was terminated, was found as 1.5 X 10^{10} copies/mL. Bacterial change during the operation of the TET reactor was not remarkable. Also similar trend was observed in archaeal and methanogenic community changes. There was an increase in Phase c. While archaeal community was detected as 4.0 X 10^{6} copies/mL, 9.0 X 10^{6} copies/mL and 8.1 X 10^{8} copies/mL at the seed sludge, Phase b and c, respectively, methanogenic community was quantified as 1.0 X 10^{7} copies/mL, 1.2 X 10^{6} copies/mL and 5.6 X 10^{9} copies/mL at the seed sludge, Phase b and c, respectively. As seen in Figure 4.70 and 4.71, archaeal and methanogenic communities were accounted as 1.5 X 10^{9} copies/mL and 1.1 X 10^{8} copies/mL, respectively, at the end of the operation.

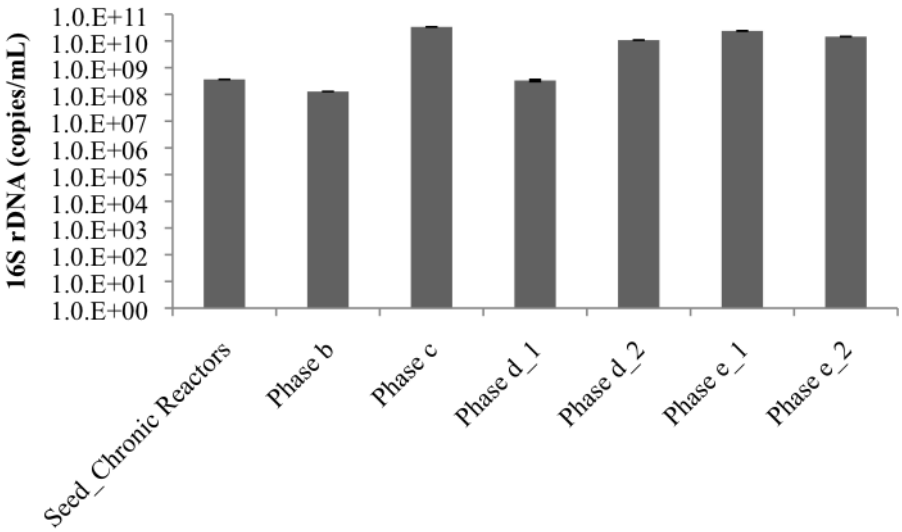

Figure 4.69: Quantitative changes in bacterial 16S rDNA concentrations at different phases of the TET reactor.

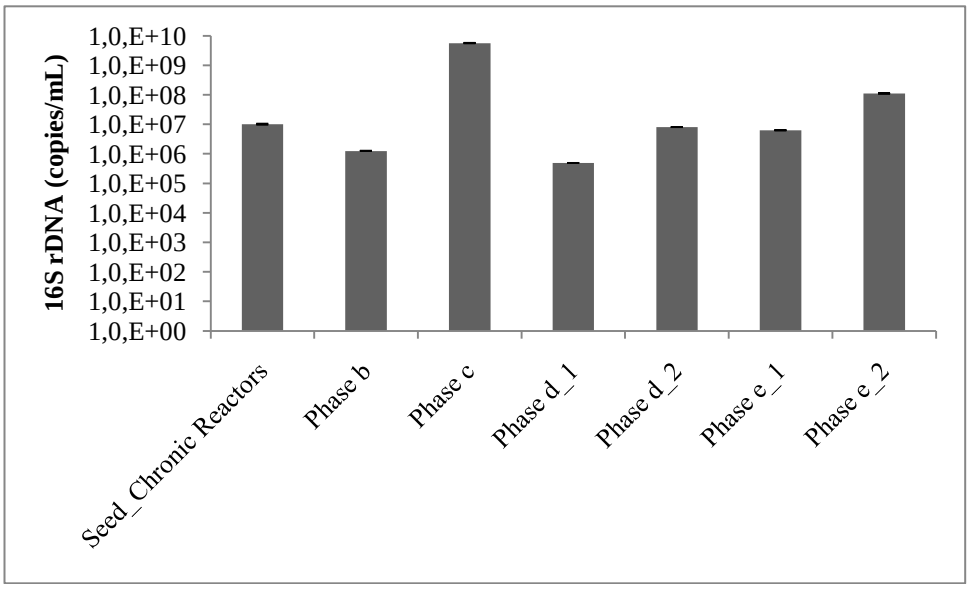

Figure 4.70: Quantitative changes in archaeal 16S rDNA concentrations at different phases of the TET reactor.

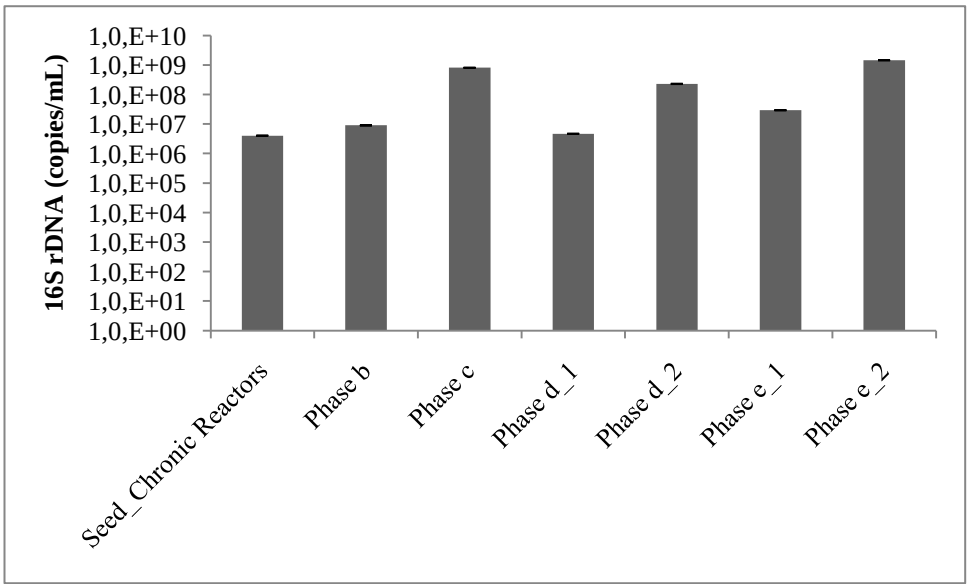

Figure 4.71: Quantitative changes in methanogenic 16S rDNA concentrations at different phases of the TET reactor.

Besides quantification of 16S rDNA concentration, 16S rRNAs were quantified to determine the effects of TET on the active microbial groups. 16S rRNA quantification results of bacteria, archaea and methanogens were given in Figure 4.69-6.71, respectively. Bacterial 16S rRNA expression level increased from 1.4 X 10^{11} copies/mL (Phase b) to 4.0 X 10^{11} copies/mL (Phase d_2). A slight decrease at Phase c was detected as 4.2 X 10^{10} copies/mL. Bacterial 16S rRNA quantification results could not reflect the reactor performance. While 16S rRNA expression level of archaea had a similar trend to bacterial one, effect of TET on active methanogenic

community was more noticeable when compared to change between Phase b and e_2 (35% reduction). However none of these results could not shoew the rwactor performance and SMA results.

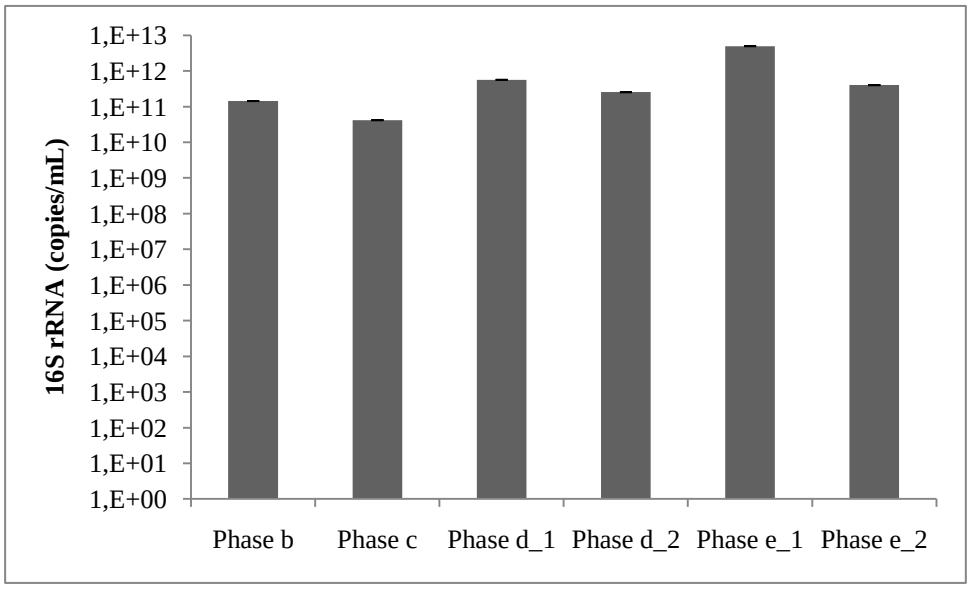

Figure 4.72: Quantitative changes in bacterial 16S rRNA concentrations at different phases of the TET reactor.

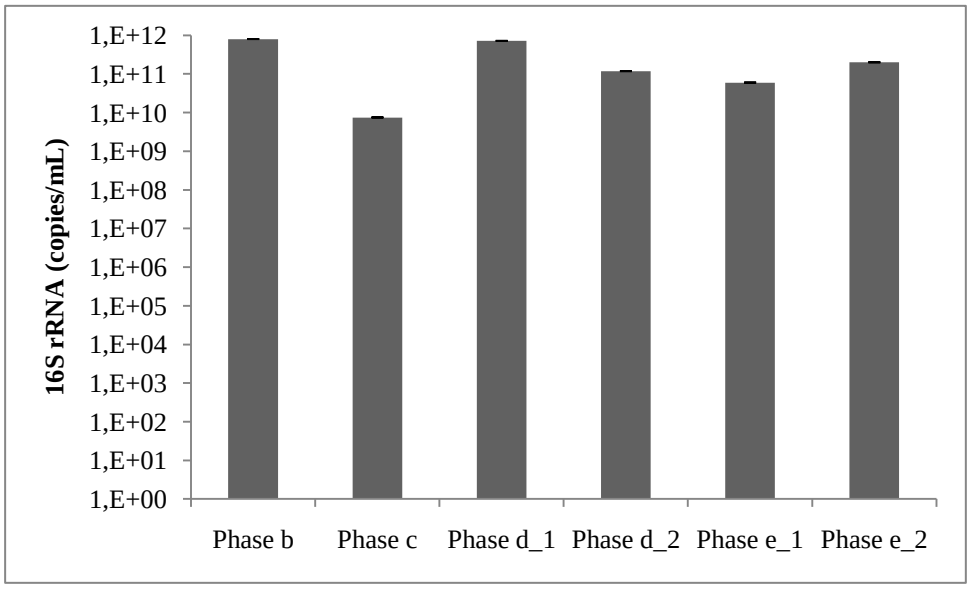

Figure 4.73: Quantitative changes in archaeal 16S rRNA concentrations at different phases of the TET reactor.

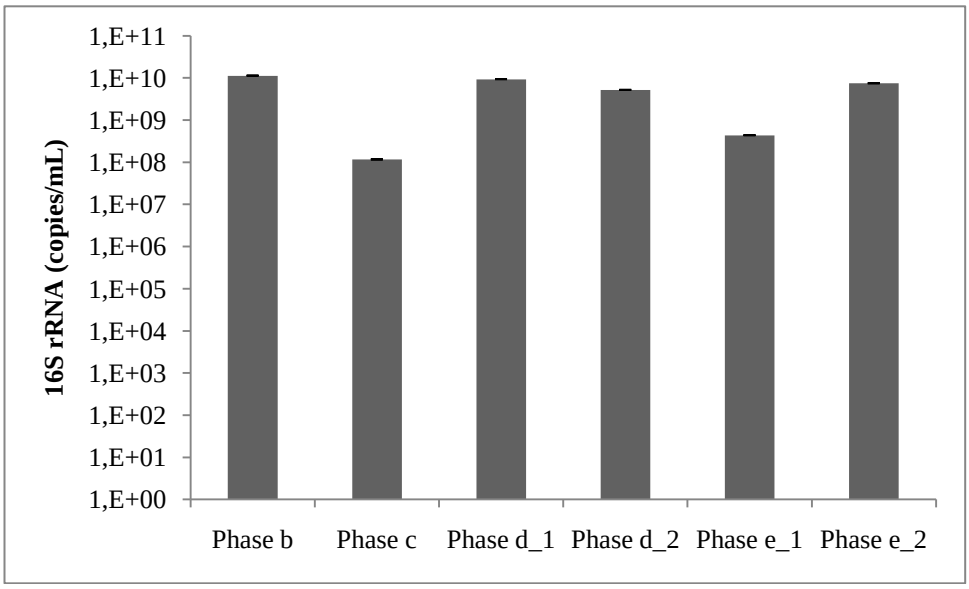

Figure 4.74: Quantitative changes in methanogenic 16S rRNA concentrations at different phases of the TET reactor.

Enzyme Expression

To detect the inhibition effect of TET on the anaerobic pathways, three enzymes were selected from the different metabolic pathways of the anaerobic degradation and quantified their expression level based on mRNA method. Formylterahydrofolate synthetase (FTHFS), methyl-coenzyme M reductase (mcrA) and acetyl-coA synthetase (ACAS) were selected to determine the inhibition on homoacetogenesis, methanogenesis and specifically acetoclastic methanogenesis, respectively.

Despite acetate and other VFAs were not observed in effluent and active methanogenic species were detected in the system at the Phase b and c, FTHFS expression was surprisingly not observed in the mRNA level during all operation time.

Expression of mcrA gene was not detected at Phase e of the TET as seen in Figure 4.75. The expression level of mcrA gene in the TET reactor decreased from 3 X 10^5 and 5 X 10^4 copies/mL while TET concentration was increased from 1 mg/L to 3 mg/L. After TET addition was stopped to the reactor, no mRNA encoded by mcrA gene was detected at Phase e. Also reactor performance data and SMA test results supported this situation.

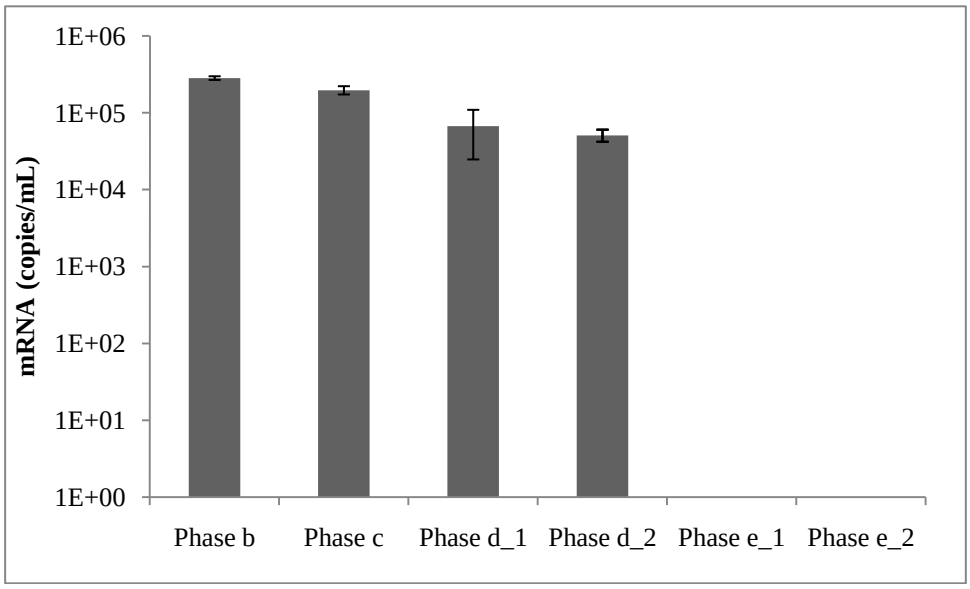

Figure 4.75: Quantitative changes in methyl-coenzyme M reductase expression level at different phases of the TET reactor.

ACAS gene expression results are given in Figure 4.76. ACAS was detected only at Phase b, c, and d_1 and also the expression level decreased gradually whereas TET concentration was increased from 1 mg/L to 3 mg/L. Similar to SMX and ERY results, the expression level of ACAS was higher than mcrA during all operation of the TET reactor. Also in the SMA test results showed that acetoclastic activity decreased during the operation time.

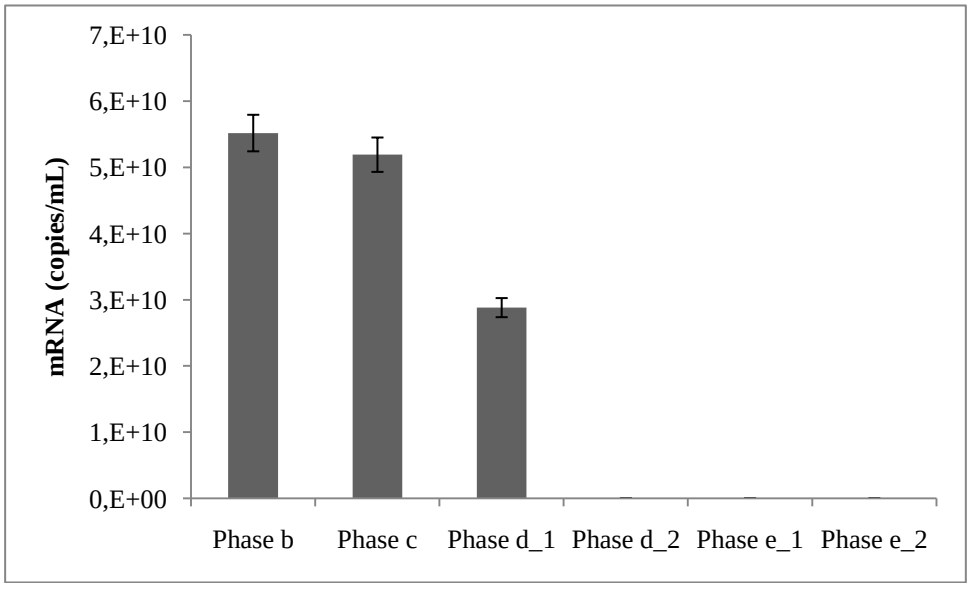

Figure 4.76: Quantitative changes in acetyl-coA synthetase expression level at different phases of the TET reactor.

5. CONCLUSION AND RECOMMENDATIONS

In the scope of this dissertation, acute and chronic effects of three selected antibiotics on anaerobic treatment in terms of conventional parameters and microbial community were studied. As a results of this study, the following highligted items were obtained:

- This study reflected that the sludge tolarated higher antibiotic concentration in the short-terms studies. The significant short term inhibitory effect was started after 50 mg/L and the metabolic activity completely stopped after 500 mg/L of the antibiotic addition.
- Short-term inhibitory effect of TET was more different than SMX and ERY. While VFA accumulation in the SMX and ERY bottles started after 250 mg/L of the antibiotic, it observed in TET bottles after 25 mg/L.
- In the acetate fed acute test bottles, while no aceteta was detected in any effluent, the methane production decreased with respect to the antibiotic concentration. It was speculated that antibiotics could bind the acetate and this complex was sorped to the sludge.

Also in the chronic test, the results obtained from the ASBRs which were fed by different antibotic compounds reflects that the inhibitory effects of these compound were conflicting in the long term operation:

- SMX was fed up to 45 mg/L with synthetic wastewater and then the reactor collapsed. VFA accumulation in the SMX reactor started after 35 mg/L of the compound addition in terms of propionic acid and acetic acid. Contrary of the reactor operation data, specific methanogenic activity increased with respect to the SMX concentration and it reached to the maximum value at the end of the operation.
- ERY concentration was increased to 3 mg/L in the ASBR and then the reactor performance desreased dramatically. Even ERY addition was stopped,

the reactor performance couldn't recover again. While propionic acid accumulation started after 3 mg/L of ERY, acetic acid was detected in the system after ERY addition was terminated. SMA test results showed that the activity of the sludge decreased with respect to the ERY concentration, however the potential methanogenic activity increased again after ERY addition was stopped.

- Also TET was fed to the ASBR up to 3 mg/L and like ERY, performance of the reactor in terms of COD removal efficiency and biogas production was suddenly decreased after this concentration. After that TET addition was stopped however the reactor performance couldn't improve. Also propionic acid and acetic acid accumulation started in the system at the same time. Even TET addition was stopped, the accumulation continued. Methanogenic activity tests reflected that the potential acitivity of the sludge also decreased in the parallel with performance of the reactor.
- Microbiological analysis reflected that the dominanat bacterial species were Clostridum in the all three reactor. While hydrogenotrophic methanogens were commonly detected in the SMX and TET reactors, acetoclastic methanogens with hydrogenotrophic species were dominant in the ERY reactor.
- No FTHFS gene expression on mRNA level was detetcted in all there systems. Expression levels of mcrA and ACAS decreased with respect to antibiotic concnetration in the three reactors. While this expression level increased in the ERY reactor after antibiotic addion was stopped, it continued to decrease in TET reactor even at no TET addition phase.

In the view of the findings of this study, the following recommendations are made for future studies:

- Two phase anaerobic reactor may reflect the inhibition effect on the system more detailed in terms of microbial structure.
- Synergistics effects also should be studies in the future to mimic real systems.
- Antibiotic resistance gene abundance can be determined to reveal relation between antibiotic concentration and resistance genes.

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