EFFECT OF OXYTETRACYCLINE ON BIOGAS PRODUCTION AND MICROBIAL COMMUNITIES DURING ANAEROBIC DIGESTION OF COW MANURE BY FLUORESCENCE IN SITU HYBRIDIZATION AND REAL TIME POLYMERASE CHAIN REACTION

by

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ABSTRACT

Antibiotics used in veterinary practice end up in animal manure and present a threat to biogas production from substrates as such, for they are possibly detrimental to the fragile microflora of anaerobic digestion processes. In this study, inhibitory effects of a commonly used veterinary antibiotic, oxytetracycline on anaerobic digestion of cow manure is examined. Within the context, digester performances in terms of biogas and methane productions, and microbial dynamics were monitored. In this study, two batchwise experiment sets were conducted in serum bottles. In both sets OLR was maintained at 1.4 kgTVS/m^3d and HRTs were 60 and 30 days in Set 1 and Set 2 respectively. TS concentration was kept at 5%.

In both sets, total methane yields were between 87-90 L/kgTVS with a methane percentage of (58±5)%. In Set 1, OTC in concentrations of 50 mg/L, 100mg/L and 200 mg/L were added to serum bottles operating with non medicated manure. Biogas production continued untill 30 days in the control serum bottle up to a volume of 315 mL and inhibitions were 41%, 57% ve 61% in serum bottles containing 50, 100 and 200 mg/L OTC respectively. IC_{50} value of the drug was calculated as 68 mg/L. It was recorded that inhibitory effect of OTC regressed during the operation and serum bottle operated with 50 mg/L OTC could reach the values of control bottle at after 60 days. Thereby, in this serum bottle OTC toxicity fitted in with non-competitive inhibition model. In all of the bottles, the majority of the active *Archaea* was hydrogenotrophic and *Methanobacteriales* was the dominant order. Acetoclastic methanogens were represented by the members of *Methanosarcinaceae* family. It was encountered that acetoclastic methanogens decreased significantly by the increased concentrations of OTC, therefore inhibitions in biogas and methane productions were correlated with the decrease of this group. It was found out that changes in the total 16S rDNA copy numbers of bacteria, *Archaea* and methanogens were not effective in explaining the case of OTC toxicity.

In Set 2, a dairy cow was medicated with 20 mg/kg body weight OTC and manure samples collected on days 1, 2, 3, 5, 10, 15 and 20 after medication, were used as substrate in serum bottles. It was found out that, 10% of the OTC injected to the animal was

excreted in manure. Biogas production continued in the control serum bottle until day 30 of the operation to a final volume of 255 mL. The most severe inhibitions at this stage were monitored in serum bottles operated with manure collected on the 2nd and 3rd days after medication which were 60% and 57%. In this set majority of the active *Archaea* was also hydrogenotrophic and *Methanomicrobiales* was the abundant hydrogentrophic methanogen in the serum bottles. Acetoclastic methanogens were represented by the members of *Methanosarcinaceae* family. It could be observed that *Methanomicrobiales* was the most effected group from OTC in medicated manure in the early stages of the digestion, however as the operation progressed *Methanosarcinacea* was also effected. It was found that changes in the total 16S rDNA copy numbers of the domains *Archaea*, bacteria and methanogens were not significantly effected by the inhibitory effect of OTC in medicated manure.

ÖZET

Veteriner hekimliğinde kullanılan antibiyotikler, hayvan dışkısında sıklıkla tespit edilmişlerdir. Bu antibiyotikler havasız çürütme sistemlerindeki hassas mikroflaraya zararlı olduklarından, hayvan gübresinin biyogaz üretiminde sübstrat olarak kullanılması sırasında problem teşkil edebilirler. Bu çalışmada, sık kullanılan bir veteriner antibiyotiği olan oksitetrasiklinin (OTC), gübrenin havasız çürütülmesine olan etkisi araştırılmıştır. Bu kapsamda, biyogaz ve metan üretimleri ile mikrobiyel değişimler incelenmiştir. Çalışma doğrultusunda, iki deney seti kurulmuştur ve bu setler set 1 ve set 2 olarak adlandırılmışlardır. Set 1 de OTC içermeyen gübre substrat olarak kullanılmış ve OTC dışardan eklenmiştir. Set 2 de ise bünyesine OTC enjekte edilmiş ve enjeksiyondan sonra 1., 2., 3., 5., 10., 15. ve 20. günlerde toplanan inek gübresi substrat olarak kullanılmıştır. İki set için de organik yükleme hızları 1,4 kgTVS/m^3 gün olarak hesaplanmış ve hidrolik bekletme süreleleri 60 ve 30 gün olarak belirlenmiştir. Toplam katı oranı %5 de tutulmuştur.

İki sette de kontrol serum şişelerinde metan yüzdesi %(58±5) olmak üzere metan verimliliği 87-90 L/kgUKM ye ulaşmıştır. Set 1 de, kontrol serum şişesinde 30 gün işletme sonunda biyogaz üretimi sabitlenmiş ve 315 mL ye ulaşmıştır. Bu aşamada biyogaz üretiminde 50 mg/L, 100 mg/L ve 200 mg/L OTC içeren serum şişelerinde %41, %57 ve %61 inhibisyon görülmüştür. OTC nin IC_{50} konsantrasyonu 68 mg/L olrak hesaplanmıştır. Bu sette işletim süresi boyunca OTC inhibisyonunun azaldığı gözlemlenmiştir. Söz gelimi, 50 mg/L 60 gün işletmenin sonunda kontrol serum şişesindeki değerlere ulaşabilmiştir. Bu da 50 mg/L OTC içeren serum şişesinde yarışmasız inhibisyonu göstermektedir. Serum şişelerinde hidrogentrophik metanojenlerin çoğunlukta oldukları gözlemlenmektedir ve baskın grubun *Methanobacteriales* olduğu saptanmıştır. Asetoklastik metanojenler *Methanosarcinaceae* ailesi tarafından temsil edilmişlerdir. Bu sette *Methanosarcinaceae* ailesi OTC konsantrasyonuyla anlamlı şekilde azalmıştır ve biyogaz ve metan üretimlerinin bu grup ile korele olduğu saptanmıştır. Bakteriyel, Arkeal ve metanojenik16S rDNA gen kopya sayılarındaki değişimlerin OTC inhibisyonunu açıklayıcı olmadığı görülmüştür.

Set 2 de, ineğe enjekte edilen OTC nin %10 unun dışkıda atıldığı belirlenmiştir. Bu sette kontrol serum şişelerinde 30 gün işletme sonunda 255 mL biyogaz üretimi elde edilmiştir. Bu aşamada biyogaz üretimlerinde en çok inhibisyon 2. ve 3. günlerde toplanan gübreyle işletilen serum şişelerinde %60 ve %57 olarak görülmüştür. Bu sette aktif Arkea nın çoğunluğunun hidrogentrofik olduğu ve baskın grubun *Methanomicrobiales* olduğu görülmüştür. Dışkıdaki OTC nin en çok *Methanomicrobiales* grubuna etkili olduğu fakat işletmenin ilerleyen günlerinde *Methanosarcinaceae* nin de etkilendiği görülmüştür. Bu çalışmada, bakteriyel ve Arkeal 16S rDNA gen sayılarındaki değişimlerin OTC nin inhibitör etkisini açıklayıcı olmadığı tespit edilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units used
ATP	Adenosine Triphosphate	
CHP	Combined Heat and Power Station	
CSTR	Continious Stirred Tank Reactor	
CTC	Chlorotetracyline	
DNA	Deoxyribonucleic acid	
DGGE	Denaturating Gradient Gel Electrophoresis	
EDTA	Ethylenediaminetetraacetic Acid	
FISH	Fluorescent *in situ* Hybridization	
GHG	Green Hause Gasses	
HRT	Hydraulic Retention Time	
IC_{50}	Inhibitory Concentration	mgL^{-1}
OTC	Oxytetracycline	
PCR	Polymerase Chain Reaction	
PBS	Phosphate Buffer Saline	
PFA	Paraformaldehyde	
Q-PCR	Quantative Polymerase Chain Reaction	
TRFLP	Terminal Restriction Fragment Length Polymorphism	
RNA	Riboxynucleic Acid	
rRNA	Ribosomal RNA	
rDNA	rRNA coding DNA	

SSCP	Single Strand Conformation Polymorphism	
TS	Total Solids	mgL^{-1}
TC	Tetracycline	
TVS	Total Volatile Solids	mgL^{-1}
VA	Veterinary Antibiotic	
VFA	Volatile Fatty Acids	

1. INTRODUCTION

Nowadays, as non-renewable energy sources are depleting rapidly, anaerobic digestion process as a means of biogas production is becoming increasingly appealing as a topic, for it is considered to be a new and efficient way of producing renewable energy. Anaerobic digestion has been used for the stabilization of organic waste for a very long time (Tilche and Malaspina, 1998; Rajeshwari et al., 2000), and more recently, it's area of application has been extended to both treating organic waste and generating energy (Alvarez et al., 2000).

Currently, 88% of the world's energy demand is met by fossil fuels (IEA, 2006). However, over consumption in addition to the harmful effects of such sources have led authorities to search for alternative environmentally friendly energy sources. The production of biogas through anaerobic digestion offers significant advantages over other forms of bio energy production. It can reduce the green house gas (GHG) emissions to the atmosphere and the digestate can be reused as fertilizer for the crops. That's why, it is considered as one of the most energy-efficient and environmentally beneficial technology for bio energy production (Fehrenbach et al., 2008).

Many kinds of waste can be used as substrate for anaerobic digestion process. Sewage sludge, energy crops, solid waste and animal husbandry leftover are the most commonly used ones. The versatility of feedstocks, makes biogas production possible all around the world. Biogas production from agricultural waste is especially important because it is easily accesible, offers significant environmental benefits and it can contribute to the income of the farmers (Chynoweth, 2004). Appropriate agriculture origined substrates which can be used in anaerobic digesters are; energy crops, organic wastes, and animal manures. Sometimes, co digestion of these sources is also applied (Hashimoto, 1986; Rushbrook, 1990; Tafdrup, 1994; Callaghan et al., 2001). Within these feedstocks, manure can be considered one of the most important because although it has lower methane value compared to energy crops, it is easier to obtain and has lower production costs (Walla and Schneeberger, 2005). Especially in countries where livestock farming is extensively applied, like Turkey, biogas production from manure is likely to bring high

economic benefits. When manure is used in anaerobic digesters, methane yields as high as 166.3 Nl CH_4 kg/VS may be obtained (Amon et al., 2007). Unfortunately, despite all the benefits of agriculture originated wastes used for biogas production, some substances used in these kind of industries can be inhibitory to the anaerobic digestion process. Antibiotics are likely to be one of the most widely used of these substances. Antibiotics are defined as; naturally occuring organic products capable of killing microorganisms (Walsh, 2003). In livestock farming, antibiotics are used for veterinary practice, for the prevention or treatment of diseases and for growth promotion (Although the use of antibiotics as growth promoters has been banned since January 2006 in the European Union). When they are administrated, they are poorly metabolized and after excretion they are released to the environment. This kind of release may lead to their presence in sub therapeutic amounts in the environment, which is tought to trigger the proliferation of antibiotic resistance genes (Speer et al., 1992). In case of anaerobic digestion process for biogas production, the striking importance of antibiotics come from the fact that they are possible inhibitors to the fragile microorganisms carrying out the process.

Tetracyclines are one of the most commonly used of antibiotics, exhibiting activity against a wide range of gram negative and gram positive pathogens. The fact that they are relatively cheap and have little side effects resulted in their extensive use as a veterinary medicine. Also especially in United States they are added to animal feed to promote growth (Sarmah et al., 2006). Oxytetracycline is a good representative of this group, and like all the other tetracyclines, is favoured much. Oxytetracycline may enter anaerobic digesters through the use of antibiotic containing manure as substrate. Although it is a potential disruptor of the anaerobic digestion process, it's definite effects on biogas production and methane yield is still being investigated. The discoveries in this subject throughout years are highly contradictive. In some cases oxytetracycline is spiked into the system and in other cases, the drug is direcly given to the animal, from which the collected manure is utilized as substrate in biogas digesters. Biogas production seems unaffected when oxytetracycline is added to the reactors in some studies (Lallai et al., 2001). However there are also findings showing biogas is reduced when oxytetracycline is directly added to reactors (Alvarez et al., 2010). When medicated manure is used as substrate in biogas fermenters, inhibition of biogas and methane production is observed (Arikan et al., 2006).

These challenging results indicate that deeper investigations regarding the process and microbiology of biogas production systems are needed.

Biogas production from agricultural originated waste is a very fast growing market in Europe and in the world. The European energy production from biogas reached 6 million tons of oil equivalents in 2007, with nearly 20% increases per year. In Europe, each year 1,500 million tones of biomass is converted to biogas anaerobicly. Germany is the most biogas producing country in Europe, having 4000 agricultural biogas plants operated at the end of 2007 (Weiland, 2010). This kind of extent utilization of biogas may secure the supplement of future energy requirement and bring independence from fossil fuels. In order to benefit at the utmost level from this technology, insights of the process should be well understood. Although the general processes occurring in anaerobic biological digesters, such as hydrolysis, acidogenesis, acetogenesis, methanogenesis, are well known, the complex microbial ecology of the organic substrate, symbiotic relationships, the effect of microbial diversity on performance of anaerobic digestion systems are still needed to be enlightened. Understanding of the microbial ecology of the anaerobic digesters plays an important role in the controlling and operation of the biogas systems therefore increasing attention has been given into the study of microbiology in the past few years. Considerable effort has been made to understand the microbial community structure by using culture-dependent and culture-independent molecular approaches (Sekiguchi et al., 2001; Sekiguchi and Kamagata, 2004; Sekiguchi, 2006). Through these analyses, particularly those targeting the 16S rRNA gene, detailed pictures of the community compositions are being documented. Techniques like FISH and DGGE are efficient tools for determining the changing microbial populations during the anaerobic digestion process (Delbes et al., 2001; Collins et al., 2003 Gerardi, 2003). Despite all the improvement achieved on these subjects in the former years, there is need for extra information of the microbial ecology of anaerobic digesters.

Fate of antibiotics on anaerobic digestion processes have been studied many times, however the main focus of these studies have generally been physical and chemical aspects and microbiological data have unfortunately been over looked. Therefore, in this study, the inhibitory effect of oxytetracycline was evaluated in terms of their effects on methane production, and microbial population dynamics.

2. THEORETICAL BACKGROUND

2.1. Fundementals of Anaerobic Digestion

Anaerobic digestion is the biodegradation of organic matter to methane in the absence of oxygen, resulting with the formation of biogas (mixture of carbon dioxide and methane, a renewable energy source) and microbial biomass. It is a process naturally occurring in some kind of environments like rice paddies and swamp marshes, and has been integrated into treatment systems as a tool for handling organic waste. It has been widely used for the treatment of municipal sludge and is also applicable for the treatment of organic waste such as; fruit and vegetable processing wastes, agricultural wastes and packinghouse wastes (Parkin and Miller, 1983). Lately, focus of anaerobic digestion has shifted through bio energy production. The production of biogas through anaerobic digestion is considered one of the best ways of producing renewable energy, as it is environment friendly and economically beneficial. Despite all these benefits, poor operational stability prevents anaerobic digestion from being widely applied (Dupla et al., 2004).

2.1.1. Biochemistry of the Process

The anaerobic digestion process consists of four stages which are mediated by different groups of microorganisms. These microorganisms have symbiotic relationships, hence each group of them have their own unique requirement of nutrient and environmental conditions (Angelidaki et al., 1993). Three major groups of microorganisms have been identified with different functions in the whole degradation process are described in the following paragraphs. Hydrolysing and fermenting microorganisms initiate the attack on polymers and monomers found in the waste. Mainly, acetate and hydrogen is produced with varying amounts of volatile fatty acids (VFA) such as propionate and butyrates as well as some alcohols are produced at the end of this stage. The obligate hydrogen producing acetogenic bacteria convert propionate and butyrate into acetate and hydrogen and two groups of methanogenic Archaea produce methane from acetate or hydrogen (Ahring et al., 2003).

The carbon flow in an anaerobic reactor is mainly between the fermentative microorganisms and the methanogens. Only between 20% - 30% of the carbon is converted into intermediate products before being metabolized to methane and carbondioxide (Mackie and Bryant, 1981). The stages of the anaerobic digestion process are hydrolysis, acidogenesis, acetogenesis and methanogenesis. The last stage is considered the rate limiting stage of the process, in which the organic matter is reduced to methane. In the first two stages, organic polymers are hydrolyzed into their monomers and/or fermented into intermediate short chain fatty acids. In acetogenesis stage they are further converted into acetate and H_2/CO_2 and finally in the methanogenesis stage methane is produced from acetate or H_2/CO_2 (Liu et al., 2002). In the following sections, these stages which are hown on Figure 2.1., will be explained in detail.

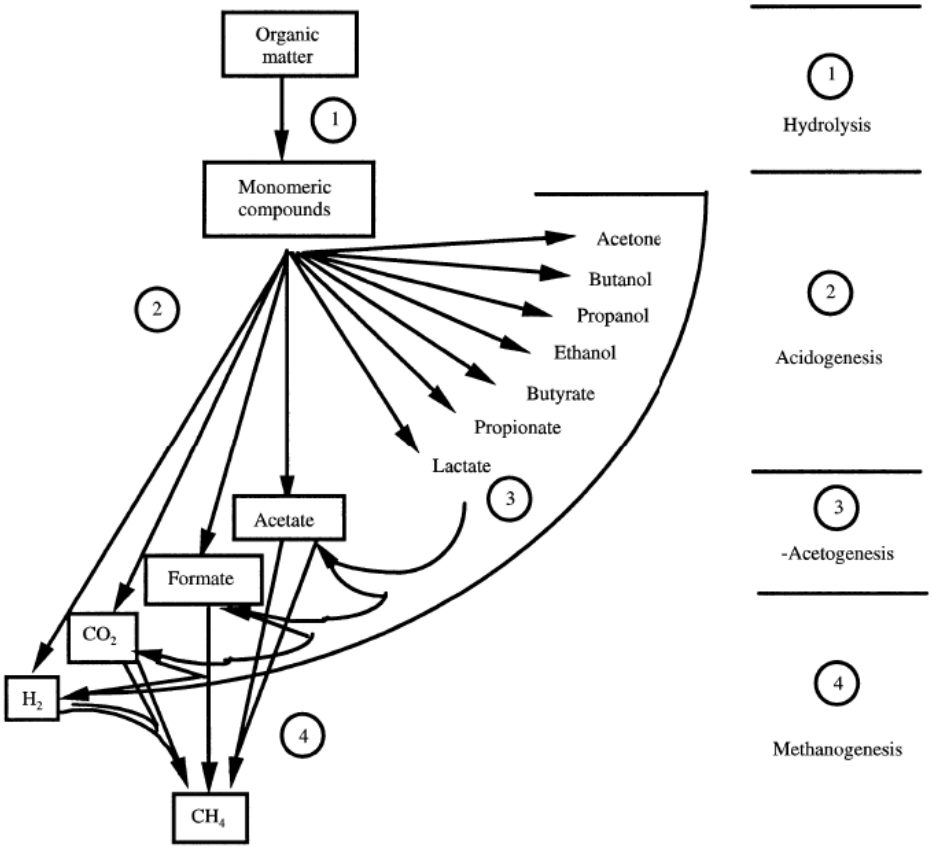

Figure 2.1. Anaerobic conversion of organic matter to methane (Garcia et al., 2000).

First step in anaerobic degredation of organic matter is hydrolysis. Complex wastes are required to be hydrolyzed into units as an initial step to be taken up by the microbial cells. The hydrolysis of macromolecules such as lipids, proteins and carbohydrates under anaerobic conditions is carried out by specific extracellular enzymes, the reaction rates of which are influenced by pH, cell residence time and the waste constituents in the digester produced by hydrolytic bacteria. The process is especially important in digesters operated with particulate matter like manure and may even be considered the rate limiting step (Pavlostathis and Giraldo Gomez, 1991). In the hydrolysis phase, undissolved compounds like cellulose, proteins and fats are cracked into monomers by extracellular enzymes of facultative and obligatory anaerobic bacteria, or through physicochemical reactions. The community that takes place in the hydrolysis step is quite heterogenic. For example: It was stated that *Clostridium* is responsible for degradation of compounds containing cellulose and starch while *Bacillus* play role in the degradation of proteins and fats (Noike et al., 1985; Lema et al., 1991). The types of hydrolytic microorganisms are reported namely as, the cellulytic (*Clostridium thermocellum*), proteoytic (*Clostridium bifermentas, Peptococcus sp.*), lipolytic (genera of clostridia and micrococci) and aminolytic (*Clostridium butyricum*, *Bacillus subtilis*) bacteria (Hungate, 1982; Payton and Haddock, 1986). The hydrolytic microorganisms are also capable of breaking down some intermediate products to simple volatile fatty acids (VFAs), carbon dioxide, hydrogen and ethanol (Eastman and Ferguson, 1981).

Following the hydrolysis stage, solubilized monomers are taken up by facultative and obligatory anaerobic bacteria and are converted to short chain organic acids, alcohols, hydrogen and carbon dioxide in acidogenesis. The concentration of the intermediately formed hydrogen ions determines the type of the end products of fermentation. If the partial pressure of the hydrogen is high, fewer reduced compounds like acetate is formed. Two groups of acid forming bacteria are known. The first group is acidogens or fermentative bacteria which are capable of metabolizing amino acids and sugars to the intermediary products, acetate and hydrogen. The catabolism of these organic compounds is carried out by a large number of both obligatory and facultatively anaerobic microorganisms and the process utilizes single amino acids, pairs of amino acids or a single amino acid with a non-nitrogenous compound. Single amino acids are converted by *Clostridia, Mycoplasmas* and *Streptococci* while butanol, butyric acid, acetone and iso-propanol are generally produced by the bacteria of the genera *Clostridum sp.* *Butyribacterium* produces butyrate, *Costridium acetobutylicum* mainly produces acetone and butanol and *Clostridium butylicum* produces butanol in addition

to hydrogen, carbondioxide and iso-propanol). The pathways of the degradation are as follows;

In the degradation of carbohydrates; propionic acid is formed by *Propionibacterium sp.* via succinate pathway and the acrylic pathway. Butyric acid is formed by *Clostridium sp.* Fatty acids are degraded by the beta oxidation reaction. Proteins are degraded by the Stickland reaction by *Clostridium botulinum* which takes two aminoacids at the same time (One for hydrogen donor and the other for hydrogen acceptor) and couples to acetate, ammonia and CO_2. Hydrogen sulfide can be formed if cysteine is degraged.

Acidogenesis state is followed by acetogenesis. Overall, two different types of acetogenic mechanisms can be pronounced. First of them is acetogenic hydrogenation which involve the production of acetate as an end product, either from the fermentation of hexoses or from CO_2 and H_2. Second of them is the acetogenic dehydrogenation which refers to the anaerobic oxidation of long and short chain volatile fatty acids. Obligate proton reducing or obligate hydrogen producing bacteria carry out the oxidation of fatty acids and are inhibited even by the minimal hydrogen partial pressure and can only survive with the presence of hydrogen consuming microorganisms such as the acetoclastic methanogens (Gavala et al., 2003).

Methanogenesis is the final and may be the most important step of the anaerobic digestion process which is mediated by a group of strictly anaerobic microorganism called *Archaea* (Woese et al., 1977). It is considered the rate-limiting step in the whole anaerobic digestion process due to the slow growth rate of the methanogens comparing to acidogens (Speece, 1983; Noike et al., 1985; Malina et al., 1992) and accordingly, the performance of anaerobic digesters and the quality of the digestate depend on the activity of methanogens.

Methanogenesis is an energy yielding metabolism for every methanogen, which is used for the synthesis of ATP, and occur via two conversion pathways. First one is the decarboxylation of acetic acid and the second one is the reduction of carbon dioxide in the absence of other electron acceptors such as oxygen, nitrate, and sulfate and only bicarbonate and protons act as terminal electron acceptors (Garcia et al., 2000; De Bok et

al., 2004; Stams et al., 2006). The substrates for methane fermentation can be divided into three groups (Deublein and Steinhauser, 2008);

- CO_2 type: CO_2, $HCOO^-$, CO
- Methyl type: CH_3OH, CH_3NH_3, $(CH_3)_2NH_2^+$, $(CH_3)_3NH^+$, CH_3SH, $(CH_3)_2S$
- Acetate type: CH_3COO^-

In mehanogenesis from CO_2 + H_2, electrons for the reduction of CO_2 to CH_4 comes from H_2. However, formate, carbon monoxide and some organic compounds like alcohols can give electrons for CO_2 reduction in some methanogens.

$$CO_2+4H_2\rightarrow CH_4+2H_2O \qquad \Delta G°=-131kJ \tag{2.1}$$

In methanogenesis from methyl compounds and acetate, methyl group substances which are listed above as the second class of methanogenic substrates are reduced to methane by two mechanisms. The formation of methane by reducing methyl group substances using an external electron donor such as H_2 is the first mechanism.

$$CH_3OH+H_2\rightarrow CH_4+H_2O \qquad \Delta G°= -113\ kJ \tag{2.2}$$

Also, the methyl group substances can be oxidized to CO_2 in order to generate the electrons needed to reduce other molecules of CH_3OH to CH_4 in the absence of H_2.

$$4CH_3OH\rightarrow 3CH_4+CO_2+2H_2O \qquad \Delta G°= -319\ kJ \tag{2.3}$$

Acetate is the final methanogenic substrate. The conversion mechanism of acetate to methane and carbondioxide called the acetotrophic reaction (Pavlostathis and Gomez, 1991).

$$CH3COO^-+H_2O\rightarrow CH_4+HCO_3^- \qquad \Delta G°= -31\ kJ \tag{2.4}$$

Each of the above reactions is exergonic and can be used to synthesize ATP. Concerning carbon for cellular biosynthesis, CO_2 is the precursor for all cellular components when growing on CO_2+H_2. If methanogenic substrates are acetate or

methylated compounds, these compounds are also used in the organic cell components with the fixation of some CO_2. Characteristics of methanogens take place in the process are expalined further down.

Methanogens are obligate anaerobes and they are very hard to culture. Most of them thrive under extreme coditions. All methanogens, belong to the Euarchaeota group of Archaea and they exist in diverse types of habitats and show different morphologies. Anoxic sediments, hydrotermal vents and the digestive tracks of animals are only some places they are present and these places are the main source of biogenic methane in nature. (Madigan, 2009). Also, methanogens are diverse in terms of cell wall chemistries. For example, Methanobacterium species and relatives have pseudomurein cell wall whereas *Methanosarcina* and relatives have cell walls that contain methanochondroitin. A summary of the characteristics of methanogens is shown on Table 2.1.

Woese et al. (1990) proposed a new classification for living organisms, dividing life on earth into three major domains: bacteria, *Archaea* and eukarya (Figure 2.2). The unique phylogenetic status and evolutinary divergence of *Archaea* suggest that they should exhibit wide physiological diversity. However, traditional culture-based studies have led to belief that opposite was the case. Two major lineages of *Archaea* are Crenarchaeota and Euryarchaeota (Figure 2.3). The first kingdom, Crenarchaeota derived from being phylogenetically close to ancestor or source of *Archaea* (Woese et al., 1990). It was believed to include only sulphur-dependent extreme thermophiles. Euryarchaeota is a heterogenous group comprimising a broad spectrum of organisms with varied patterns of metabolism from different habitats. It includes extreme halophiles, methanogens and some extreme thermophiles so far. Moreover, a third archaeal kingdom has been discovered which is reported isolation of several archaeal sequences evolutinary distant from all *Archaea* known to date by Barns et al. in 1994 and then in 1996. The new group was placed on phyologenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota.

Table 2.1. Characteristics of the methanogens (Madigan et al., 2002).

Order	Morphology	Substrate for methanogenesis
Methanobacteriales		
Methanobacterium	Long rods	H_2+CO_2, formate
Methanobrevibacter	Short rods	Methanol+CO_2, formate
Methanosphera	Cocci	Methanol+H_2
Methanothermus	Rods	H_2+CO_2, can also reduce S
Methanococcales		
Methanococus	Irregular cocci	H_2+CO_2,pyruvate +CO_2, formate
Methanomicrobiales		
Methanomicrobium	Short rods	H_2+CO_2, formate
Methanogenium	Irregular cocci	H_2+CO_2, formate
Methanospirillum	Spirilla	H_2+CO_2, formate
Methanoplasmus	Plate-shaped cells	H_2+CO_2, formate
Methanocorpusculum	Irregular cocci	H_2+CO_2, formate, alcohols
Methanoculeus		
Methanosarcinales		
Methanosarcina	Large irregular cocci in pacs	H_2+CO_2, methanol, methylamines, acetate
Methanolobus	Irregular coci in aggregates	
Methanohalobium	Irregular cocci	Methanol, methylamines; halophilic
Methanococcoides	Irregular cocci	Methanol, methylamines
Methanohalophilum	Irregular cocci	Methanol, methylamines, methyl sulfides; halophie
Methanosaeta	Long rods to filaments	Acetate
Methanopyrales		
Methanopyrus	Rods in chains	CO_2, hyperthermophile, growth at 110°C

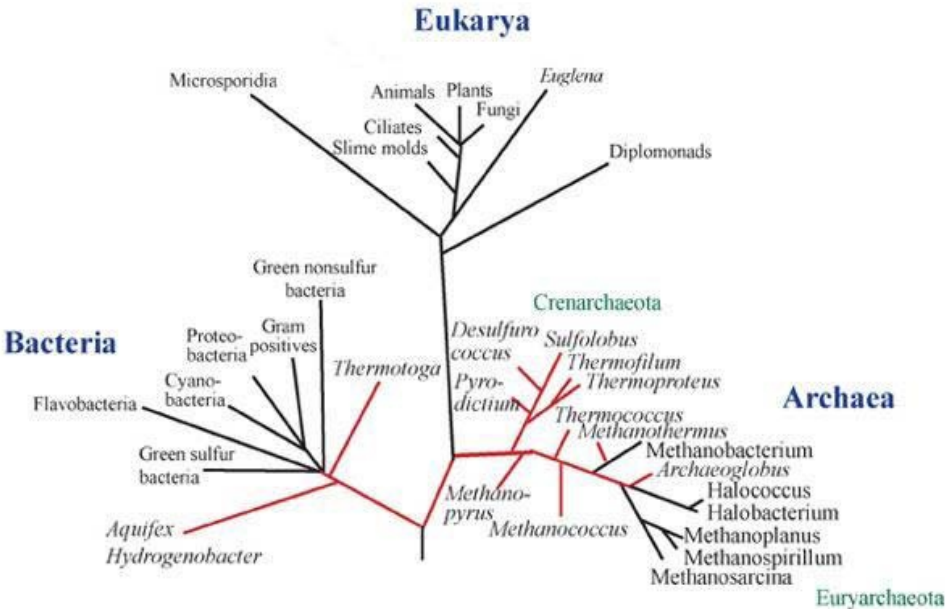

Figure 2.2. Universal phylogenetic tree (Woese et al., 1990).

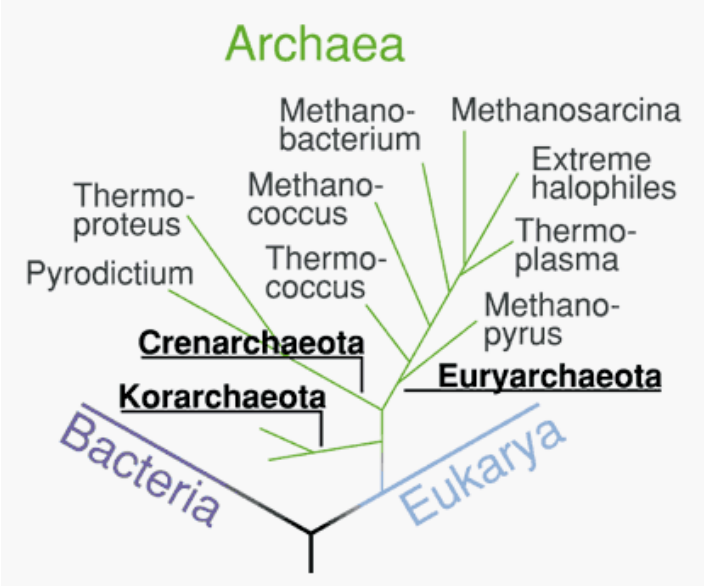

Figure 2.3. Major lineages of *Archaea*: Crenarchaeota, Euryarchaeota and Korarchaeota (Madigan et al., 2002).

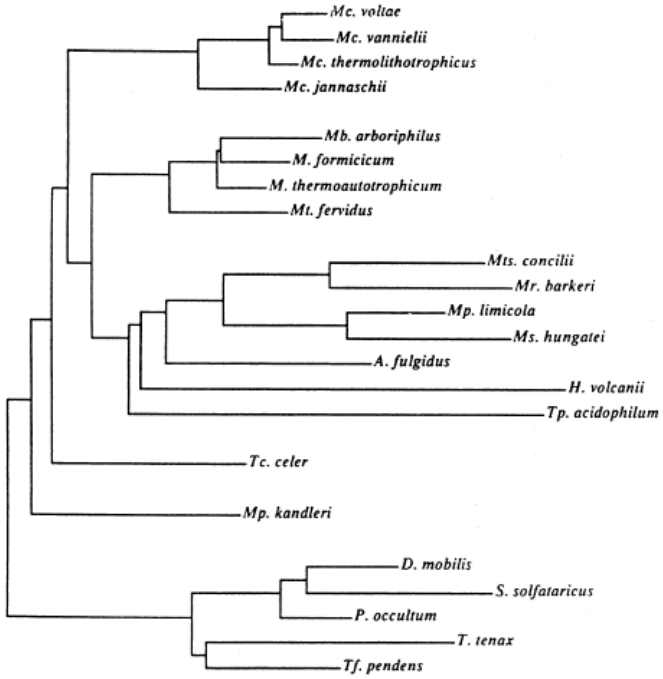

Figure 2.4. Representative phylogenetic tree for *Archaea*, based upon 16S rRNA sequences.

Phylogenetically, methanogens are Archaeabacteria, a group of microbes that are distinguished from true bacteria by a number of characteristiristics, including the possesion of membrane lipids composed of isoprenoids ether-linked to glycerol or other carbonhydrates (Langworthy, 1985), a lack of peptidoglycan containing muramic acid (Kandler et al., 1977), a distinctive ribosomal RNA sequences (Balch et al., 1979; Woese, 1987). This group also includes some extreme halophiles and some extremely thermophilic, sulfur-dependent microbes (Woese, 1987) and phylogenetically distinct from eukaryotes and true bacteria.

Methanogens are classified into five orders within the kingdom *Archaeobacteria*: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales,* and *Methanopyrales* (Figure 2.6). Organisms from different orders have less than 82% 16S rRNA sequence similarity. Methanogens belonging to different orders also possess different cell envelope structure, lipid composition, substrate range, and other biological properties.

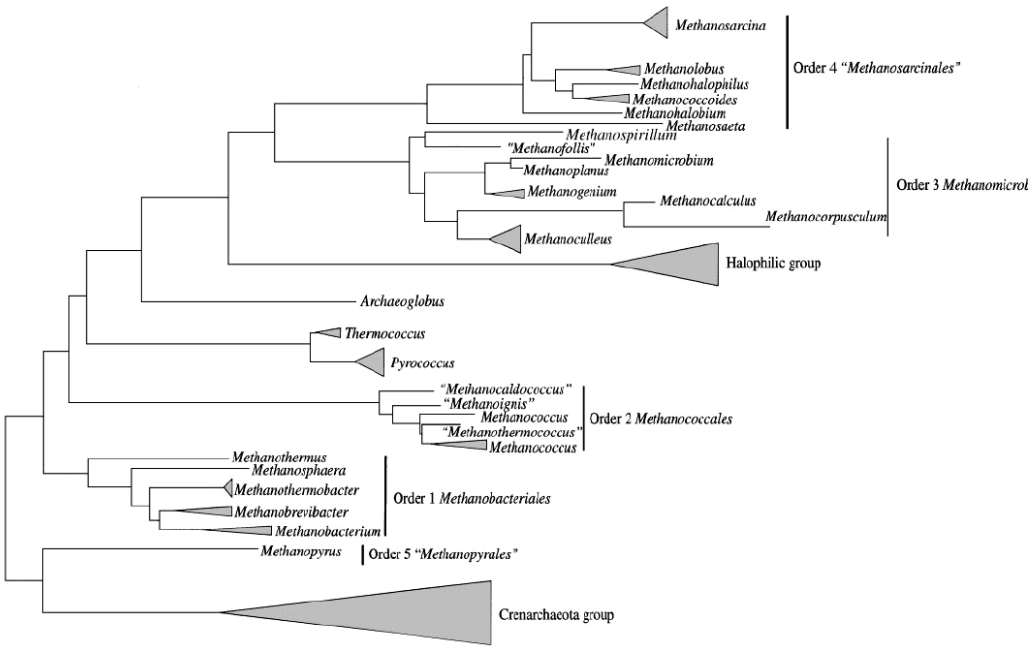

Figure 2.5. Updated phylogeny of methanogens, domain *Archaea* (Garcia et al., 2000).

Members of the order *Methanobacteriales* generally produce methane using CO_2 as elector acceptor and H_2 as the electron donor. Some species can also use formate, CO, or secondary alcohols as electron donors. The species *Methanosphaera* can only reduce methanol with H_2. In most genera, the cells are short to long rods with a length of 0.6–25 μm. They often form filaments up to 40 μm in length. They are widely distributed in anaerobic habitats, such as marine and freshwater sediments, soil, animal gastrointestinal tracts, anaerobic sewage digesters, and geothermal habitats (Liu, 2008). The order of *Methanobacteriale*s is divided into two families, *Methanobacteriaceae* and *Methanothermaceae*. The family *Methanobacteriaceae* contains three mesophilic genera, *Methanobacterium*, *Methanobrevibacter*, and *Methanosphaera*, and one extremely thermophilic species *Methanothermobacter*. The family *Methanothermaceae* is represented by one hyperthermophilic genus, *Methanothermus*, which has only been isolated from thermal springs.

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Methanococcales is an order of coccoid marine methanogens, which are slightly halophilic and mostly chemolithotrophic. They produce methane using CO_2 as the electron acceptor and H_2 or formate as the electron donor. The cells are irregular cocci with a diameter of 1–3μm. The order of *Methanococcales* has been divided into two families distinguished by their growth temperatures, *Methanocaldococcaceae* and *Methanococcaceae.*

Members of the order *Methanomicrobiales* are order of methanogens that use CO_2 as the electron acceptor and H_2 as electron donor. Most species can use formate, and many species also use secondary alcohols as alternative electron donors. Their morphology is diverse, including cocci, rods, and sheathed rods. They are widely distributed in anaerobic habitats, including marine and freshwater sediments, anaerobic sewage digestors, and animal gastrointestinal tracts. The order of *Methanomicrobiales* is divided into three families, *Methanomicrobiaceae, Methanospirillaceae* and *Methanocorpusculaceae*.

Methanosarcinales has the widest substrate range among methanogens. Most of them can produce methane by disproportionating the methylgroup containing compounds or by splitting acetate. Some species can reduce CO_2 with H_2, but formate is not used as an electron donor. Their cellular morphologies are diverse, including cocci, pseudosarcinae, and sheathed rods. All cells are nonmotile. They are widely distributed in marine and freshwater sediments, anaerobic sewage digestors, and animal gastrointestinal tracts. The order of *Methanosarcinales* is divided into two families, *Methanosarcinaceae* and *Methanosaetaceae.*

The order of *Methanopyrales* is represented by only one species, *Methanopyrus kandleri*. Cells reduce CO_2 with H_2 for methanogenesis. They are rod-shaped. *M. Kandleri* is hyperthermophilic with a growth temperature range of 84–110°C. It inhabits marine hydrothermal system.

Methanogens in anaerobic digesters can be seperated into two groups; acetate utilizers and hydrogen utilizers. The ratio of these two groups in anaerobic digesters may change according to operational parameters such as; temperature, pH, type of the substrate, alkalinity, HRT, SRT, reactor configuration and the influence of the seed sludge (Guyot et al., 1993). In some cases, community shifts or fluctuations may occur due to instabilities or differences in these conditions, even at species level (Demirel and Scherer, 2008).

Anaerobic digesters operated under thermophilic conditions exhibit less diversity compared to mesophilic ones (Karakashev et al., 2005). It was also reported that, as temperature in anaerobic digesters increase, population seems to favour to hydrogentrophic methanogens. In a reactor operated at 60°C, Methanobacterium was found to be the dominant specie and 50% of the clones belonged to the specie *Methanobacterium thermoautotrophicum* (Scherer at al., 2005).

Substrate also seems to have a fatal effect on the establishment of microbial community structure. According to Karakashev's study (2005), *Methanosaetaceae* was the dominant acetotrophic methanogen in digesters fed with sewage sludge however, in reactors fed with swine manure *Methanosarcinaceae* was dominant. This difference in community structure was reported to independent from the inoculum used, but could be

explained by the VFA and NH_3^+ concentrations. At high levels of NH_3 and VFA, like the case in manure digesters, the dominance of Methanosarcinaceae in was observed, while in sewage sludge digesters with low levels of NH_3 and VFA, Methanosaetaceae dominated. Acetate-utilizing methanogens having thin filaments with high surface seemed to be more sensitive to ammonia concentrations than hydrogenotrophic methanogens growing as rods or *Methanosarcinaceae* consisting of thick clumps. Therefore, *Methanosaeta* is not repoted to be dominating, particularly in swine manure biogas reactors (Schmidt et al., 2000; Mladenovska et al., 2003). Karakashev et al. (2006) also suggested that, in the absence of *Methanosaetaceae*, methane generation from H_2/CO_2 should be the dominant pathway. These results seem to be in correlation with the other studies (Angelidaki and Ahring 1993; Shigematsu et al., 2004). In addition, an instability caused by any of the factors mentioned above may less likely to affect *Methanosarcina* species due to its morphology. Also it was reported that number of methanogens belonging to group Methanobacteriales increased under unstable conditions (Padmasiri et al., 2007).

Another parameter which affect methanogenic structure in anaerobic digesters is reactor configuration. For example, UASB reactors are generally found rich in *Methanosaeta* species (Shin et al., 2001; Kolukırık, 2004). However, investigations on anaerobic digesters having different configurations show that effect of substrate is more defining than the effect of reactor configuration on methanogenic community structures (Schdmitt et al., 2000). HRT is also an important factor, being directly relevant with the growth rate of methanogens. Under short HRTs, hyrogen utilizing methanogens were found to dominate acetate utilizers by a factor of 10 to 10,000 (Scherer et al., 2000). Kinetic constants of some commonly detected methanogens are given in Table 2.2.

In anaerobic digesters, acetate concentration and hydrogen partial pressure have the most determining effects on acetotorphic methanogens. Under low acetate concentrations domination of *Methanosaeta sp.* occur, whereas under high acetate concentrations, *Methanosaeta sp*. are replaced by *Methanosarcina sp.* (Zheng and Raskin, 2000). Methanosarcina sp. Are mostly dominant in unstable reactors having high acetate concentrations (Stroot et al., 2000).

The change in hydrogen partial pressure has most direct effects on hydrogenotrophic methanogens as hydrogenotrophic methanogens are dependent hydrogen in anaerobic digesters. In the start up phase of anaerobic digesters, most of the methane production depends on hydrogen utilizing methanogens where hydrogen partial pressure is high (Montero, 2008).

Table 2.2. Kinetic constants of commonly detected methanogens in anaerobic digesters (Demirel and Scherer, 2008).

Bacteria	μmax(h^{-1})	K_s (mM)	Reference
M. concilii	0.032[a]	1.5	Schmidth and Ahring, 1999
M. mazei	0.06[a]	3.6	Schmidth and Ahring, 1999
Methanosarcina barkeri	0.019[b]	-	Yang and Okos, 1987;
	-	3	Schöneit et al., 1982;
	0.023	320 (as mgCOD/l)	Smith and Mah, 1978
Methanosarcina sp. MSTA-1	0.05-0.055	-	Clarens and Moletta 1990 Lundback et al., 1990
Methanobacterium formicium	0.053 0.082	-	Schauer and Ferry, 1980
Methannosarcina spp.	0.044-0.064	6.5-24.7	Mladenovska and Ahring, 2000.
Methanosarcina CALS-1	0.085	614 (as mgCOD/l)	Zinder and Koch, 1984
Methanosarcina thermophila	0.058	-	Zinder and Mah, 1979; Zinder et al., 1985
Methanosaeta soehngenii	0.08-0.29 (day^{-1})	0.4-0.7	Oude Elferink et al., 1994
Methanosaeta concilii	0.21-0.69 (day^{-1})	0.8-1.2	
Methanospirillum hungatei	0.053	-	Robinson and Tiedje, 1984
Methanobacterium bryantii M.o.H.	0.029	-	Dubach and Bachofen, 1985
Methanomicrobium paynteri	0.144	-	Dubach and Bachofen, 1985

[a] Growth on acetate [b] At an acetic acid concentration of 3.6 g/L

2.1.2. Operational Factors Affecting the Anaerobic Digestion Process

Temperature is one of the most important parameters which affect the anaerobic digestion process in many ways such as; ionization equilibrium, solubility of substrates, substrate removal rate and other constants such as specific growth rate, decay biomass yield, and half saturation constant. Although it is known that anaerobic digestion process can take place within a large temperature range, it is optimal at mesophilic (35ºC - 42 ºC) and thermophilic (45 ºC - 60 ºC) conditions. It is important to maintain a constant temperature during the anaerobic digestion process, for biogas production is negatively affceted by temperature fluctuations. In most cases thermophilic reactors exhibit less methanogenic diversity and therefore it can be said that thermophilic reactors are more vulnerable to temperature fluctuations (Karakashev et al., 2005; Leven et al., 2007). Mesophilic microflora are able to tolerate temperature fluctuations within ±3ºC without considerable reductions in methane production. Under thermophilic conditions, growth rate of anaerobic microorganisms are higher, therefore process is faster and more efficient. Under optimal operating conditions, a thermophilic reactor can be fed with higher organic loading rates at lower hydrolic retention times than mesophilic reactors, however higher temperatures makes the system imbalanced and susceptible to failure (Weiland, 2010).

The hydraulic retention time (HRT) which is a definig criterion on biogas production and waste stabilization, (Thakur, 2006) should be long enough to let microorganisms grow in the system. It is closely related with the growth rates of microorganisms in the system (Demirel and Scherer, 2008).

Without sludge withdrawal, SRT is equal to HRT which are the main parameters for designing an anerobic digester. Although with the new technology and improvements in heating and mixing equipments HRT of 15-20 days may be adequate in achieving an efficient operation, it is common practice to use 25-30 days as the design retention period. HRTs in systems operating with low temperatures can be kept longer (Medcalf and Eddy, 2003).

pH is another important factor that effect the performance of anaerobic digestion processes. Methanogenesis occur within a very limited pH range of 6.5- 8.0, being

optimum at pH 7.0-7.5. The process is disrupted severely if pH exceeds 8.5 or decreases below 6.0. The pH likely rises with ammonia accumulation and decreases with VFA accumulation which is produced by the acidogenic bacteria, however, the accumulation of VFA may not always result in a pH drop due to the buffering capacity of the substrate. Animal manure is tought to have an additional alkalinity which can neutrilaze acidification at VFA accumulation. Under optimal conditions, VFA acidity produced by the acidogenic bacteria is utilized by the bicarbonate produced by the methanogens nontheless, if VFA production is exceeded, buffering capacity can fail leadind to the demolishment of the whole system.

Mixing is a very important parameter in anaerobic digesters, especially operating with particulate substrate like manure. Thorough mixing of the substrate in the digester distributes organisms uniformly and also transfers heat, and thus is regarded as essential in high-rate anaerobic digesters (Sawyer and Grumbling, 1960; Meynell, 1976). Furthermore, agitation helps to reduce particle size as digestion progresses and to release biogas from the mixture. The importance of mixing in achieving efficient substrate conversion has been noted by many researchers (Casey, 1986; Smith et al., 1996), although the optimal mixing pattern is a subject of much debate. An intermediate degree of mixing appears to be optimal for substrate conversion (Smith et al., 1996).

Mixing can be accomplished by mechanical mixers, biogas recirculation, or by slurry recirculation. Mechanical mixers are reported to be most efficient in terms of power consumption (Brade and Noone, 1981). However, the internal fittings and equipment are not accessible for maintenance during digester operation, and long term reliability of operation is of paramount importance. In general, such reliability can be more readily attained with biogas or liquor recirculation systems, where there are no moving parts within the digester (Casey, 1986). Interestingly, in other literature sources it has been reported that biogas recirculation is the most efficient mode of mixing for anaerobic digesters (Morgan and Neuspiel, 1958; Kontandt and Roediger, 1977; Lee et al., 1995). Mixing of the anaerobic lab-scale digesters can vary between 20-100 rpm (Wu et al., 2010). In some studies, digesters were manually shaken once a day (El-Mashad and Zhang, 2010). Mixing in high rpms is difficult to be obtained in full scale digesters. Mixing in full scale digesters can also be performed by intermittent and minimal mixing which refer to

mixing for 10 minutes prior to feeding and withholding mixing for 2 h prior to feeding, respectively (Kaparaju et al., 2008). Schreding is also an important application in biogas digesters. Decreasing the particle size by implementing a macerating unit with knives was found to increase biogas production in digesters operating with manure (Hartman et al., 2000).

For the growth and survival of the existing groups of microorganisms in anaerobic digesters, certain macro and micro nutrients are essential. Macro nutrients are carbon, nitrogen and phosphorus whereas iron, nickel, cobalt, selenium, molybdenum, and tungsten can be pronounced among the micronutrients. Nickel is generally required for all methanogens and takes place in the synthesis of co factor F_{430}. For optimal biomass production, cobalt is required for the build up of cell component corrinoid factor III. The amount of micronutrients neccesary for the process is very low, changing between 0.05 and 0.06 mg/L. Iron may be an exception required in concentrations between 1 and 10 mg/L (Bichoff, 2009). Utilization of manure as a substrate decreases the need for micronutrients however, it has been shown that addition of micronutrients always enhance the performance of ana anerobic digester (Preißler et al., 2009).

The uniqueness and complexity of the anaerobic digestion process makes it vulnerable to system shut down caused by the inhibitors. A substance is called an inhibitor if it blocks the metabolism or biomass production. Inhibition of anaerobic digestion processes can easily be monitored by the methane content of the biogas or amount of volatile fatty acids accumulated in the system (Kroeker et al., 1979).

Ammonia is produced by the break down of nitrogenous compounds like urea or proteins (Kayhanian, 1999). Various mechanisms of ammonia inhibition has been suggested throught years including; a change in the intracellular pH, increase of maintenance energy requirement, and inhibition of a specific enzyme reaction (Whittmann et al., 1995). Inorganic nitrogen is found in the forms of ammonuim (NH_4^+) and free ammonia (NH_3^-), in anaerobic digesters. Methanogens are the least resistant group of microorganisms to ammonia inhibition (Kayhanian, 1994). However there are challenging information in literature on the susceptibility of aceticlastic and hydrogentrophic methanogens. In some cases it has been repoted that inhibitory effect was in general

stronger for the aceticlastic than for the hydrogenotrophic methanogens (Koster and Lettinga, 1984; Zeeman et al., 1985; Sprott and Patel, 1986; Bhattacharya and Saffermann, 1989; Robbins et al., 1989; Angelidaki et al., 1993), while others observed the higher tolerance of acetate consuming methanogens to high total ammonia nitrogen levels as compared to hydrogen consuming methanogens (Zeeman et al., 1985; Wiegant and Zeeman, 1986).

In anaerobic reactors, sulfate is reduced to sulfide by the sulfate reducing bacteria (SRB) (Koster et al., 1986; Hilton and Oleszkiewicz, 1988). According to Tursman and Cork, 1988, H_2S is the toxic form of sulfide because it can penetrate in to cells. Once inside the cytoplasm, H_2S may be inhibitory by denaturing native proteins through the formation of sulfide and disulfide cross-links between polypeptide chains (Conn et al., 1987), interfering with the various coenzyme sulfide linkages, and interfering with the assimilatory metabolism of sulfur (Vogels et al., 1988). This theory was supported by the studies of Speece (1983). By contrast, McCartney and Oleszkiewicz (1991) observed that sulfide toxicity increased with increasing pH. Other studies on sulfide inhibition indicated that more than one inhibition threshold might be present under different conditions.

In anaerobic reactors, complex organic material is first hydrolyzed fermented by acidogenic bacteria to volatile fatty acids (VFA) (Li et al., 1992). The VFAs are further oxidized to be converted into acetate, hydrogen and carbondioxide which are used by methanogens for the generation of methane (Öztürk et al., 1993). Much attention has been directed to the relationship between VFA concentration and the performance of an anaerobic fermenter. It has been shown that VFAs are important intermediary products in the metabolic pathway of methane production and cause microbial stress if exist in elevated levels, decrease pH, and lead to failure of the digester. Therefore, the concentration of VFAs is an important consideration for good performance of a digester (Wang et al., 1999).

The most common VFAs found in an anaerobic digester are; acetic acid, propionic acid, butyric acid and isovaleric acid. In most cases of digester failure acetic and propionic acid accumulate in the system and methane production is decreased. Among VFAs, acetate is directly degraded by aceticlastic methanogens. In addition, syntrophic association

between methanogens and proton-reducing bacteria converts VFAs to methane (Schnurer et al., 1999). The accumulation of VFAs might be caused by negative impact of bad environment conditions such as shock loading, nutrient depletion or infiltration of inhibitory substances. High concentrations of VFA (i.e.; butyric and propionic acid) in a system is making toxic impact on the microorganisms in the reactor. It is reported that inhibition of microbial growth was observed at 35 mg/l acetic acid and excess of 3000 mg/l propionic acid concentrations (Iannotti and Fischer, 1984). The same researchers indicated that butyrate has a toxic effect at 1000 mg/l concentrations minimum.

The most important light metal ions in anaerobic systems are; sodium, potassium, magnesium and calcium. They are required for microbial growth and affect specific growth rate like any other nutrient (Chen et al., 2007). Although moderate concentrations of these ions situmulate microbial growth in anaerobic systems, excessive amounts slow down the growth, and even higher concentrations can cause severe inhibition or toxicity (Soto et al., 1993).

Heavy metals such as; chromium, iron, cobalt, copper, zinc, cadmium, and nickel may cause toxic effect on anaerobic processes which are influenced by the oxidation – reduction potential, pH and ionic strength and the resultant speciation of the metals or metal complexes (Jin et al., 1998). Heavy metals are not biodegradable and can accumulate to potentially toxic concentrations (Sterritt and Lester, 1980).

Veterinary antibiotics are widely used in many countries worldwide to treat disease and protect the health of animals (Table 2.4). They are also incorporated into animal feed to improve growth rate and feed efficiency. As antibiotics are poorly adsorbed in the digestive system of the animals, the majority is excreted in faeces and urine. Given that anaerobic digestion of animal waste for biogas production is an increasing practice in many countries, there is a growing international concern about the potential impact of antibiotic residues on anaerobic digestion and biogas production. Frequent use of antibiotics has also raised concerns about increased spread of antibiotic resistant microorganisms. Among veterinary antibiotics, Tetracyclines are very much favoured in veterinary medicine. They are active against a range of organisms such as Myco-plasma and Chlamydia, as well as a number of gram-positive and gram-negative bacteria (Chopra

and Roberts, 2001). Tetracycline (TC), oxytetracycline (OTC) and chlortetracyclines (CTC) are widely used in animal feeds to maintain health and improve growth efficiency in many countries, because of the fact that they are cheap, easily administrated and has relatively few side effects (Moellering et al., 1990). These chemicals are characterized by a partially conjugated four-ring structure with a carboxyamide functional group (Mitscher, 1978) (Figure 2.8). The extreme use of tetracyclines have led to the contamination of tetracycline residues, which represent a serious problem to the environment.

Oxytetracycline was first isolated from different species of *Streptomyces rimosus* in the late 1940s. Tetracyclines are tought to kill microorganisms by entering the cell and binding to ribosomes, resulting with the termination of the protein synthesis (Goldman et al., 1983; Chopra et al., 1992; Schnappinger et al., 1996). They are very much favoured, and have been intensely used for years.

Oxytetracycline is commonly used in veterinary practice, especially in livestock farming, for therapeutic purposes and for growth promotion (Chopra et al., 1981). Although use of tetracyclines in order to promote growth was banned in Europe (EC directive 70/524; see http://europa.eu.int/ comm/ dg24/ health/ sc/ index_en.html), it is legal in most part of the world including USA (Institute of Medicine, Division of Health Promotion and Disease Prevention) and Australia. The intensive use of oxytetracycline in livestock farming is a problem when obtained manure is intended to be used as a substrate for biogas production. When oxytetracycline is administrated, it is poorly metabolized and excreted in manure. In the digestive system, it is degraded in to it's metabolites ; 4- epi-oxytetracycline (EOTC), a-apo-oxytetracycline (a-Apo-OTC) and b-apo-oxytetracycline (b-Apo-OTC). These compounds are possible inhibitors for the microbial communities in anaerobic digesters (Fedler and Day, 1985). Also, it has been shown that OTC could degrade into metabolites during the anaerobic digestion (Arıkan et al., 2006; Alvarez et al., 2010). However, the difference inhibitory effect of metabolites formed in the digestive track and during the process have not been studied before.

Table 2.3. Selected antibiotics approved for use in the US for use in livestock at therapeutic and at sub-therapeutic levels (NRC, 1999; Mellon et al., 2001).

Name of the Antibiotic	Disease prevention	Growth and feed Efficiency	Type of animal
Amoxicillina,b	Yes	No	Swine
Ampicillina,b	Yes	No	Swine
Apramycin	Yes	No	Swine
Arsenilic acid	Yes	Yes	Swine, chicken, turkeys
Bacitracin	Yes	Yes	Swine, beef cattle, quail, pheasant,chicken, turkeys
Bambermycins	No	Yes	Swine, turkeys
Chlortetracycline	Yes	Yes	Swine, beef cattle, chicken
Efrotomycin	No	Yes	Swine
Erythromycinc	Yes	Yes	Swine, beef cattle,poultry
Gentamycin	Yes	No	Swine
Lincomycin	Yes	No	Swine, poultry
Neomycin	Yes	No	Swine, beef cattle
Oleandomycin	No	Yes	Swine, chicken,
Oxytetracycline	Yes	Yes	Swine
Monensin	No	Yes	Beef cattle
Penicillin	No	Yes	Swine, chicken,
Spectinomycin	Yes	No	Swine
Streptomycin	Yes	No	Swine
Tetracycline	Yes	Yes	Swine
Tiamulin	Yes	Yes	Swine
Tylosin	Yes	Yes	Swine, beef cattle, chicken
Arsanilate sodium	No	Yes	Swine
Carbadox	Yes	Yes	Swine, beef cattle
Roxarsone	Yes	No	Swine, chicken, turkeys
Sulfamethoxypyridazined	Yes	No	Swine
Sulfachloropyidazined	Yes	No	Swine
Sulfamethazined	Yes	No	Swine
Sulfathiazoled	Yes	No	Swine

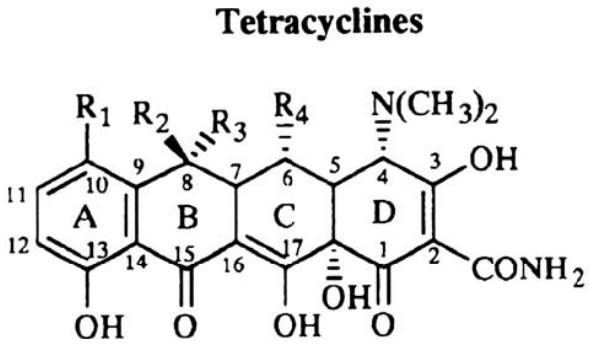

Figure 2.6. Molecular sturucture of Tetracyclines.

Table 2.4. Radical groups of tetracyclines.

	R_1	R_2	R_3	R_4
Tetracycline (TC)	H	CH_3	OH	H
Chlortetracycline (CTC)	Cl	CH_3	OH	H
Oxytetracyline (OTC)	H	CH_3	OH	OH

Fate and inhibitory effect of oxytetracycline in anaerobic digestion processes have been studied for quite sometime (Sanz, 1996; Lallai, 2001; Arıkan et al., 2006; Alvarez et al., 2010). Despite some contrary results, it can be said that oxytetracycline reduces biogas and methane yields in biogas digesters, in most cases without causing complete system failure. Some conclusions which can be drived from these works are; toxicity of OTC is increased by the presence of metabolites and that OTC alone is not as much competent to cause significant inhibition on the anaerobic digestion process. Combined effect of different tetracyclines are more severe effects than singular used ones and the inhibitory mechanism in biogas digesters is highly dependable on operation parameters, especially on inoculum characteristics.

Also, the studies in general, only target physical aspects of oxytetracycline inhibition and microbiological side of the phenomena is generally overlooked. Therefore, The key point to enlighten the unknown behind oxytetracycline inhibiton would be identifiying the microbial groups which are most immediately affected by oxytetracycline toxicity. In literature, it has been suggesed that although most antibiotics are effective only on bacterial populations, derivatives can exhibit activity also against methanogens. (Sanz et al., 1996). The most efficient and insightful way of investigating the microbial dynamics

in anaerobic reactors is by using molecular tools. Anaerobic digestion for biogas production has been investigated using molecular tools before (Karakashev et al., 2005; Blume et al., 2010) however there is lack of information reported regarding the process microbiology of more complex substrates like manure, especially under antibiotic inhibition. The microbial dimention of the oxytetracycline inhibition may be clarified by the usage of molecular tools, which are sensitive and accurate.

2.2. Biogas as a Renewable Energy Source

Global increasing energy demand has made energy generation from biogas a significantly important matter. Today, fossil fuels are on the edge of depletion as costs for energy consumption are multiplying day by day. In addition, environment is suffering from the adverse effects of fossil fuel consumption, especially from the release of green hause gasses (GHG). Utilization of biogas is not only cost effective, but environmnetally friendly and sustainable (Weiland, 2010). Biogas can be used in many ways such as; for the production of heat, steam, electricity, and hydrogen and for the utilization as a vehicle fuel. Many sources, such as crops, grasses, leaves, manure, fruit, and vegetable wastes or algae can be used, and the process can be applied in small and large scales, which allows the production of biogas at any place in the world.

To date, there are lots of biogas plants working in the world and in Europe, with Germany being the leader in the sector. All of these plants are co digesting other types of raw materials. During the last 10 years, large scale anaerobic digestion plants treating mixtures of waste hae spread throughout Europe and to the rest of the world. In late may of 2010, an energy company announced that world's largest biogas plant was going to be established in Germany, which will be producing 1 billion m^3 of raw biogas every year. More than 500 million cubic feet of biomethane will be processed at the biogas park and for this purpose, a little more than 120,000 metric tons of substrate will be fermented (http://www.thebioenergysite.com).

2.2.1. Feedstocks for Biogas Production

Any type of organic waste can be used as a substrate for the anaerobic digestion process as long as it contains carbohydrates, fats and lipids. The composition of the biogas and methane yield depends on the type of the feedstock, retention time and digestion system (Braun, 2007). The theoretical gas yield happens to change with the varying amount of carbohydrates, proteins, and fats. Only strong lignified organic substances, e.g., wood, are not suitable due to the slowly anaerobic decomposition. The real methane content in practice is generally higher than the theoretical values for a part of CO_2 is solubilized in the digestate (Weiland, 2003).

In history, anaerobic digestion has been mostly associated with animal manure and sewage sludge. Nowadays, most of the biogas plants use manure from pigs, cows, and chicken as substrate with the addition cosubstrates to increase the content of organic material for a higher biogas yield. With energy production as the main objective of anaerobic digestion, the type of feedstocks used for anaerobic digestion is highly relevant because the biogas yield obtained per cubic meter of reactor volume depends on the energy density and biologic degradability of the applied feedstocks. Biogas yields of different substrates are shown on Figures 2.9 - 2.11. The biogas yield from cow and pig manure is only between 25 and 36 m^3/t of fresh mass, because the organic dry matter (ODM) content is low (2–10%) and most of the energy-rich substances have already been digested by the animals. Therefore, the use of manure as the only substrate for biogas production is not considered economically feasible in most applications, which makes the digestion of cosubstrates necessary. More than 90% of the running biogas plants in Germany are operated with cosubstrates that mainly come from food and agricultural industries, markets, canteens, and the municipal sector. Co fermentation of energy crops was started in 1999 for the first time, and even today more than 50% of all biogas plants that were put into operation since 1999 use energy crops for codigestion. Only 7% of all biogas plants are operated with manure as the only substrate (Weiland,2003).The most common cosubstrates are harvest residues, e.g., top and leaves of sugar beets, organic wastes from agriculture-related industries, and food waste, collected from households and energy crops. Potential Biogas yields of common energy crops is shown on Figure 2.9. From all the organic compounds present in substrates, degredation of fats result with the highest biogas

yield, requiring the longest retention time due to its poor bioavailability. Carbohydrates and proteins are easier to degrate but result in lower biogas yields. Also, the origin and composition of the co substrates has to be well known in order to get the best efficiency from co digestion. To be sure that co substrate does not effect the process negatively, the existance of inhibitors and toxic compounds such as detergent residues, heavy metals etc. should be checked. Wrong addition of inhibiting substrate to the digester often leads to process collapsation, followed by an extended restart phase (Weiland, 2009). It has to be pointed out that co digestion requires extra equipment and investment costs. For a successful operation of a biogas plant with cosubstrates several important factors like; the composition of the co substrate, the energy potential of the co substrate, composition of the inhibitors and toxic compounds and legistative restrictions should be considered.

Table 2.5. Maximal Gas Yields and Theoretical Methane Contents of Different Substrates (Baserga, U., 1998).

Substrate	Biogas (Nm^3/t TS)	CH_4 (%)	CO_2 (%)
Carbohydrates	790–800	50	50
Raw Proteins	700	70-71	29-30
Raw Fats	1,200-1,250	67-68	32-33
Lignin	0	0	0

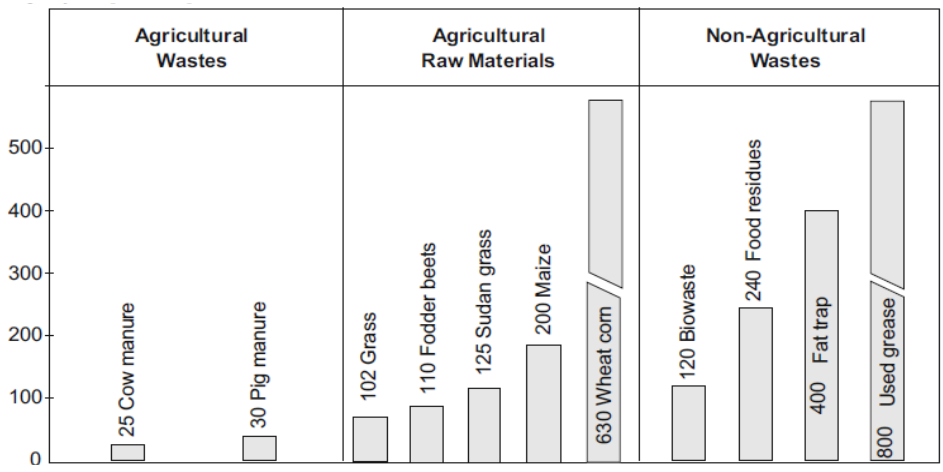

Figure 2.7. Biogas yields of different substrates (m^3/t Fresh Material) (Weiland, 2010).

Table 2.6. Mean biogas yields of some crops (Weiland, 2010).

Crop	Crop Yield (t FM/ha)	Biogas Yield (Nm^3/tVS)	Methane Content (%)
Sugar beet	40-70	730-770	53
Fodder beet	80-120	750-800	53
Maize	40-60	560-650	52
Corn crob mix	10-15	660-680	53
Wheat	30-50	650-700	54
Triticate	28-33	590-620	54
Sorghum	40-80	520-580	55
Grass	22-31	530-600	54
Red clover	17-25	530-620	56
Sunflower	31-42	420-540	55
Wheat grain	6-10	700-750	53
Rye grain	4-7	560-780	53

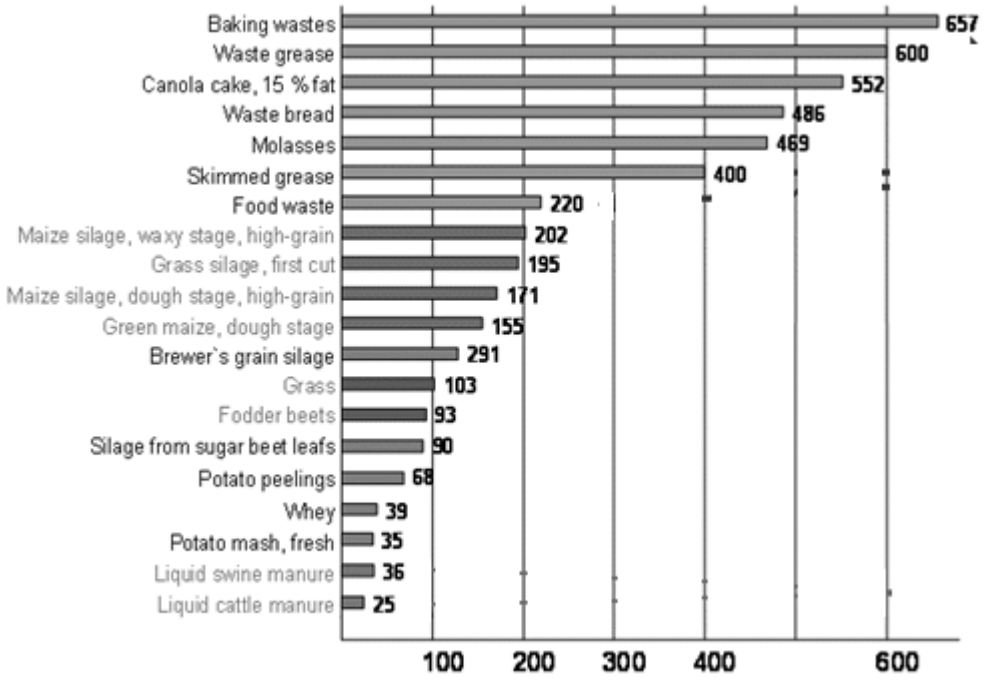

Figure 2.8. Potential Biogas yields of different substrates (m^3/t) (http://www.ormi.com).

2.2.2. Process Technology of Biogas Production

Different process types can be pronounced for biogas production mainly classified as dry and wet fermnetation pocesses. Wet digestion processes are operated with TS concentrtaion less than 10% which enables the application of completely stirred digesters. Dry digestion processes are operated with a TS concntration between 15% and 35%. Wet digestion processes are operated continuously while dry digestion systems are operated batchwise or continuously. According to Weiland, 2008, wet digestion processes dominate in the agricultural industry. Different configurations of agricultural biogas plants can be used (Schulz and Eder, 2001). The most preferred reactor type is the vertical continuously stirred tank fermenter which is used in nearly 90% of modern biogas plants in Germany (Gemmeke et al., 2009). Commonly, the fermenter's roof is covered with a membrane layer for the storage of the gas prior utilization. Stirring must be applied, using mechanical, hydraulic, or pneumatic mixing in order to provide the contact between microorganisms and the fedstock, to facilitate the upflow of gas bubbles, and to unify temperature conditions in the fermenter. Up to 90% of biogas plants use mechanical stirring equipment. Mechanical stirrers can be classified as slow and fast running mixers depending on their rotation speed. Fast running stirrers are generally run several times a day in series, whereas slow rotating paddles run continuously. Submerged motor propeller stirrers are often applied when adjusted to the height, tilt, and to the side (Gemmeke et al., 2009). Chainging with the type of the fermenter and the substrate, up to four stirrers may be used to prevent formation of the swimming layers on the top or sediments at the bottom. If the fermenter is operated at high TS, slow rotating paddle stirrers are preferred with a horizontal, vertical, or diagonal axis and large scale paddles. The motor is positioned outside the fermenter. Axial stirrers are mounted on shafts that are centrally put on the digester ceiling. They create a continuous stream in the digester that flows from the bottom up to the walls which provides homogenization of solid substrates. Pneumatic stirring uses the produced biogas for mixing, which is blown to the bottom of the digester. The system has the advantage that the necessary equipment is placed outside the fermenter, but it is not commonly used in agriculture, because the destruction of floating presents a problem (Gerardi, 2003). Hydraulic stirring by pumps are used only for some few reactor types. The typical size of completely mixed fermenter is in the range from 1,000 to 4,000 m^3 reactor volume. Horizontal digester types are plug flow systems that contain a low rotating

horizontal paddle mixer. They are mostly used for the first stage of two-stage reactor configurations because they can be operated at higher total solids concentrations of the input. The reactor volume is limited to a maximum of about 700 m^3 in for economic reasons.

Generally, wet fermenters are operated at mesophilic temperatures up to 42 Cº (Weiland, 2010). Although at higher temperatures, the degradation rate is faster and system can be operated at smaller HRTs with smaller reactor volumes required, increased selling of heat to local residencies have made thermophilic systems less efficient (Weiland,2010).

For dry fermentation batch processes are applied without mechanical mixing especially for the digestion of energy crops. The substrate is loaded in the reactor and is mixed with inoculum, preferrably from a previously operated digester, before the digestion process. The necessary share of solid inoculum has to be determined individually for each substrate (Weiland, 2006). For example, yard manure from cows requires relatively small ratios of solid inoculum however up to 70% of the input is necessary for energy crops (Kusch et al., 2005). The gas yiels are not much different from the wet fermenters (Heiermann et al., 2007). During the digestion process, process water is spread on the substrate to accelerate start up and inoculation as well as to control moisture content and temperature. After the digestion, the digestate is unloaded and a new batch is initiated. Applying a second stage methanogenic fermenter may be preferred, because the methane yields can be increased and the residual methane potential of the digestate can be lowered (Lethomäki, 2006).

For substrates containing more than 25% of TS, continuous dry fermentation may be applied (Weiland et al., 2009). For continuous dry fermentation, horizontal mechanically mixed fermenter or vertical plug flow fermenter may be used, which are known from anaerobic treatment of municipal organic solids (Schön 1994; De Baere and Mattheeuws, 2008). The vertical digester type requires no mixing inside the fermenter, and the substrate moves from the top to the bottom by gravitational forces. Before the substrate is fed to the fermenter top, it must be mixed with digestate coming from the bottom of the digester. The mixing of digestate with freshly fed feedstock prevents the accumulation of

VFA and enables a high organic loading rate of up to 10 kg ODM/ (m^3*d). The loading rate of wet fermentation processes is much lower being between 2 and 4 kg ODM/ (m^3*d).

In both dry and wet fermentation, due to the complexity of the parameters, process control is challenging. Methane production is generally used as an indicator of the process however alone it wont be enough to show process instability. Also hydrogen and redox potential are common control parameters but complicated dynamics and variability in reactors make the interpretation of the results difficult (Brauer and Weiland, 2009). Only VFA can be used as a precise indicator of the process stress. Weiland (2008) proposed a ratio of propionic acid: acetic acid >1 as an indicator for the upset of system, if the propionic acid concentration exceeds 1,000 mg/l. Ahring et al. (1995) suggested that the concentration of both butyrate and isobutyrate could be a trustable tool for indication of process failure, and Nielsen et al. (2007) recommended propionate as the key parameter for process control and optimization. VFA measurement has the disadvantage that manual sampling and analysis by gas chromatography or high pressure liquid chromatography is a relatively slow procedure. Instant determination of VFA by headspace chromatography is possible however difficult to practice (Boe et al., 2005). A fast control of the process stability is possible by determining the ratio of VFA to total inorganic carbonate by a simple titration test (Rieger and Weiland, 2006). If the ratio is <0.3, the process is stable, and an analysis for determining the individual VFAs is not necessary (Lossie and Pütz 2008).

The anaerobic digestion process can take place in a sinle or multi step process. In single phase digesting systems, the four steps of anaerobic degredation which were mentioned earlier, take place in a single reactor. However, in order to improve the stability and rate of degredation hydrolysis and acidogenesis steps are seperated from the others to be called; two phase digesting systems. In the first reactor hydrolysis and acidogenesis occur whereas in the second digester acetogenesis and methanogenesis take place. The methanogenic reactor is consequently fed with the outcome from the hydrolytic reactor. This way, hydrolysis and methanogenesis stages can be maximally optimised. Two-phase systems can especially be used for substrates that are non homogenous. Microorganisms are likely to hydrolyse the easily biodegradable compounds initially and the degredation of more resistant compounds may occur later, if ever. This allows the better acclimation of

biomass to the substrate. Accordingly, even with inhomogeneous substrates COD reductions up to %98 are achievable (Behmel, 1993). The quality of the biogas is also impoved in two phase systems. The gas from the hdrolysis digester contains 85–90% CO_2, 5–10% CH_4 whereas biogas from the methane digester is rich in CH_4 and little effort is necessary to remove trace gases, like H_2S.

2.2.3. Biogas Utilization

The conversion of biogas to heat and electricity is generally accomplished in Combined heat and power stations (CHPs) (Figure 2.12), using gas or dual fuel engines. In CHPs, electricity can be generated with an efficiency of 43%. Alternatives to the mostly used motor CHP are microgas turbines and fuel cells. With microgas turbines, low electric efficiency (25–31%) is achieved but they have a good part loading efficiency and long maintenance intervals (Schmid et al., 2005). Fuel cells result in a higher electric efficiency but need an efficient gas cleaning, because the catalyst for converting methane into hydrogen and the catalyst inside the fuel cell are very sensitive to impurities (Ahrens and Weiland, 2007). There are different fuel cell types operated at temperatures between 80 and 800 °C. The investment costs are much higher than for engine-driven CHP. Upgrading of biogas with injection into the grid or for the utilization as vehicle fuel has gained increasing importance because it enables more energy efficient usage. In many EU countries, the access to the grid is guaranteed by state ordinances. Countries like Germany, Sweden, and Switzerland have defined quality standards for biogas injection into the natural gas grid.

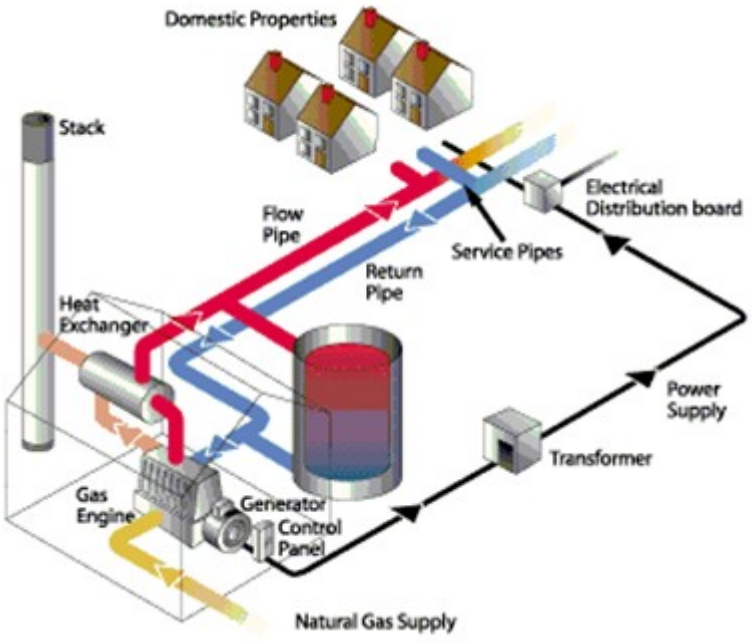

Figure 2.9. Scheme of a typical CHP system (http://www.ppsl-districtenergy.co.uk).

Prior to utilization, biogas which is a mixture of methane (CH4; 50%-85% by volume), carbondioxide (CO_2; 15%-50% by volume) and trace gases such as hydrogen sulfide (H_2S), carbonmonoxide (CO) or hydrogen (H_2), should be eliminated from all gas contaminants and the upgraded gas must have a methane content of more than 95% in order to fulfill the quality requirements of the different gas appliances. It should be ensured that bio methane does not contain bacteria and molds, for the sake of human health and welfare of the equipments (Wempe and Dumont, 2008). The content of biogas effects the selection of technology for cleaning and and utilization. Dewatering and removal of H_2S are the primary applications before the utilization of biogas. The type and composition of substrate are the major factors effecting the methane yield and methane production rates. Biogas produced by cofermentation of manure with energy crops or harvesting residues can contain levels of H_2S between 100 and 3,000 ppm., which are used for the utilization of biogas need mostly levels of H_2S below 250 ppm, in order to avoid excessive corrosion. Removal of H_2S is generally done by biological desulfurization (Schneider et al., 2002). The process is contingent on the oxidation of H_2S into elementary sulfur and sulfurous acid by sulfur oxidizing bacteria *Sulfobacter oxydans*. Desulfurization can also be achieved by adding a commercial ferrous solution into the digester. Ferrous compounds bind sulfur in

an insoluble compound in the liquid phase, preventing the production of gaseous hydrogen sulfide. However this method may not be preferred because it is not cost effective. Various technologies can be applied for increasing the methane content (Persson et al., 2006). Carbon dioxide is mostly removed from the biogas by water scrubbing or scrubbing with organic solvents like polyethylene glycol (Kapdi et al., 2005) as well as pressure swing adsorption using activated carbon or molecular sieves (Schulte-Schulze Berndt, 2005). Less common methods are; chemical washing by alkanol amines like monoethanolamine or dimethylethanolamine (Wünsche, 2008) or membrane technologies (Miltner et al., 2009) and cryogenic separation at low temperature (Petersson, 2008). When removing carbon dioxide from the gas stream, small amounts of methane may be lost also. These methane losses must kept at minimum for both environmental and economical reasons since methane is a greenhouse gas 23 times stronger than CO_2.

2.2.4. Utilization of the Digestate

The leftovers from the anaerobic digestion process, which are called the digestate, can be spread on farmlands as fertilizer (Wise, 1981). The digestate is rich in mineralized nitrogen and the C/N ratio is lowered which increases the short time N fertilization effect. When energy crops are used as substrate, N content is increased by three times (Gemmeke et al., 2009). The digestate penetrates into soil more easily therefore the loss of nitrogen to ammonia is lowered. Also the digestate is less odorous and purified from pathogens (Sahlström, 2003; Strauch and Philipp, 2000). These improved characteristics of the digestate make it highly suitable for utilization as fertilizer.

2.3. Molecular Methods Used In Anaerobic Digesters

Since the detection of first microorganism by Anton Von Leeuwenhoek and Robert Hook in the seventeenth century, scientists have been very curious in investigating the relations of the microbes within themselves and with their environment. These studies eventually lead to the proliferation of a new branch of science called “microbial ecology”. The science of microbial ecology explores how microbial communities interact with eachother and surrounding environment. Microbial activity and biodiversity are the two most important subjects in this valuable area of microbiology (Gray and Head, 2008).

During the studies in the area of microbial ecology, difficulties have been faced in identification, isolation and in *in situ* studies of the microorganisms. In the earlier days, microbial analysis were being carried out with culture dependent methods which were time consuming and lacked high sensitivity, however by the recognition of DNA and RNA based culture independent tools, microbiological studies gained speed and preciseness. More recently, molecular techniques have been practiced for the analysis of communities in anaerobic digesters (Godon et al., 1997; Sekiguchi et al., 1998). It has been shown by many researchers that microorganisms in anaerobic environments including bio reactors exhibit supreme diversity and their relationships and metabolic functions need to be clarified. By the use of molecular methods, the gap between microbiologists and engineers in the field of anaerobic digestion tends now to be bridged. Data obtained from molecular techniques may serve to model and optimize bioreactor systems. Quantitative data provided by molecular techniques such as FISH and Q-PCR are necessary to validate engineered models and to optimize biogas production. Ultimately, new knowledge obtained by molecular microbiology methods will be even more suitable than volatile suspended solids as the active biomass parameter (Talbot et al., 2000).

Culture independent methods can be divided into two categories; ones that include isolation and analysis of genetic material from environmental samples to detect which organisms are present and ones that include using nucleic acid based stains to microscopically visualize, numerate and identify microorganisms (Gray and Head, 2008). A scheme of culture indepentend approaches used in microbial ecology is shown on Figure 2.13. Most studies target conserved nucleic acids which are present in all organisms (e.g., small subunit rRNA) but a range of gene sequences associated with certain metabolic functions can also be investigated. This is useful when correlating the presence, diversity, and abundance of functional groups of organisms to environmental processes (Gray and Head, 2008).

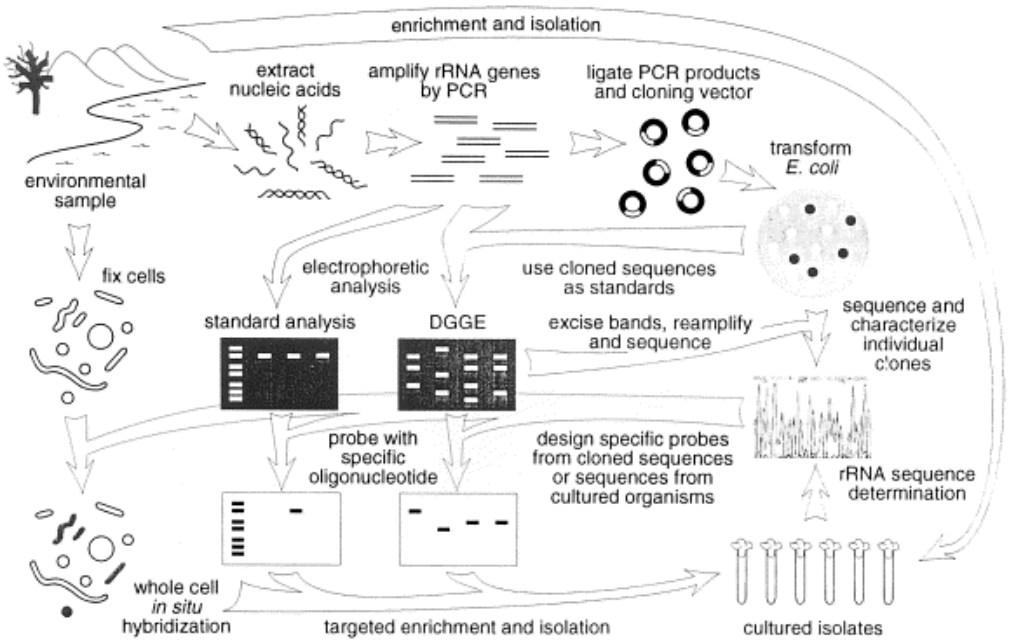

Figure 2.10. Summary of phylogenetic methodologies used in microbial ecology. (Scow et al., 2004).

Methods based on the extraction of nucleic acids are numerous and they depend on the enzymatic amplification of certain genes from complex genomic DNA of environmental samples. The comparative analysis of gene sequences provide information on the identity and abundance of the existing microorganisms. Fingerprinting tools like denaturing gradient gel electrophoresis, DGGE and terminal restriction fragment polymorphism (T-RFLP) analysis enable the observation of the general composition of microbial communities or may let us see the change in a microbial community in progressing time.

Quantification of different nucleic acid sequences is also possible by the real time quantitative polymerase chain reaction (Q-PCR) analysis method. This technique is based on analyzing the increase in concentration of specific nucleic acid sequences as they are enzymatically amplified *in vitro*. The initial number of the target gene is related to the time taken for the target gene to reach a set threshold concentration. Higher the initial concentration, the lower the time required to reach the threshold.

Counting and identification of single cells are achieved using a method called fluorescence *in situ* hybridization (FISH). In this method samples are immediately fixed after being taken from environment in order to preserve the cell morphology. They are then washed and hybridized with oligonucleotide probes spesific to certain gene sequences. The oligonucleotide probes are labeled with a fluorescent dye to enable visualization under a fluorescence microscope. In the following paragraphs, the mothods used in this thesis will be explained in detail.

Table.2.7. Summary of common molecular methods used in microbial ecology.

Approach	**Description**	**Remarks**
Cultivation	Study micro-organisms in defined circumstances.	Only a minor fraction of the micro-organisms can be cultivated.
PCR	Specific and sensitive amplification of genetic material (DNA/RNA).	Primers developed from known sequences and can cause bias.
Real-time PCR	Sensitive and sensitive quantitative amplification suitable for high-throughput over a wide dynamic range.	Sensitive and sensitive quantitative amplification suitable for high-throughput over a wide dynamic range.
Fingerprinting (DGGE/SSCP/TRFLP etc.)	Rapid overview of diversity. Ideal for comparisons of ecosystems in time or between different samples.	Bias in nucleic acids extraction and PCR. Only dominant populations can be visualised.
Sequencing	Gold standard for sequence retrieval.	Nucleic acids extraction, PCR and cloning can be biased.
FISH	Enumeration of micro-organisms *in situ*. Allows localization and quantification.	Laborious without automatisation and requires sequence information for probe development. Cell permeabilisation and fixation can cause bias.
In situ isotope tracking (e.g. SIP, MAR-FISH, isotope array)	Combination of cultivation and molecular techniques allowing the functional identification of active micro-organisms.	Not suitable for all environments and crossfeeding might prove difficult to interpretate.

2.3.1. Real Time Quantitative PCR (Q-PCR)

Real-time or quantitative PCR is based on the continuous monitoring of amplification in the PCR tube, with the help of fluorescence. In contrast to the conventional PCR, quantification occurs during the exponential phase of amplification (Malinen et al., 2003). Thus, the bias often observed in the PCR template-to-product ratios can be largely avoided (Suzuki and Giovannoni, 1996). This is most commonly achieved through the use of fluorescence-based technologies, including: (i) probe sequences that fluoresce upon hydrolysis (TaqMan; Applied Biosystems, Foster City, CA, USA) or hybridization (LightCycler; Roche, Indianapolis, IN, USA); (ii) fluorescent hairpins; or (iii) intercalating dyes (SYBR Green). 1) Q-PCR Analysis can be done to observe absolute levels (i.e., numbers of copies of a specific RNA per sample) or relative levels (i.e., sample 1 has twice as much mRNA of a specific gene as sample. 2) For absolute quantification, a RNA standard curve of the gene of interest is prepared in order to calculate the number of copies. In this case, a serial dilution of a known amount (number of copies) of pure RNA is made and subjected to amplification. The unknown signal is compared with the standard curves so as to calculate the starting concentration. Alternatively, a computation method for absolute quantitation has been proposed that does not use standard curves (Jia, 2009).

2.3.2. Fluorescence in situ hybridization (FISH)

FISH is based on the microscopic analysis of already defined (at least its SSU rRNA gene sequence) groups of bacteria by a fluorogenic oligonucleotide (or probe) targeting SSU rRNA molecules inside cells (Giovannoni et al., 1988; Amann et al., 1990). First, microbial cells are first fixed with appropriate chemical fixatives and then hybridised under optimal conditions on a glass slide or in solution with oligonucleotide probes. These probes are generally 15–25 nucleotides in length and are labelled covalently at the 5'end with a fluorescent dye. After washing steps, specifically stained cells are detected by epifluorescence microscopy or flowcytometry. The determination of composition and number of bacteria can be achieved by rRNA-targeted oligonucleotide probes without cultivation, directly in their natural environment. rRNA gene fragments were used as phylogenetic stains firstly in 1989 (De Long et al., 1989). Since the pioneering study of De Long, fluorescence in situ hybridization technique has become a common tool for

identification of microorganisms in environmental samples (Amann et al., 2001). Several hundred rRNA-targeted oligonucleotide probes suitable for FISH have been described, together with a large online database providing an encompassing overview of over 700 published probes and their characteristics (Loy et al., 2003). Such probes can be readily developed and tested to detect lineages of uncultured microbes in environmental samples (Pernthaler et al., 1997; Ravenschlag et al., 2001). The signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. This allows a quantification of rRNA concentrations both in single cells and in the environment (Poulsen et al., 1993). Raskin et al. (1994a) evaluated the methanogenic group composition in anaerobic digesters by oligonucleotide probe hybridization. Several studies (Merkel et al., 1999; Imachi et al., 2000; Tagawa et al., 2000; Upton et al., 2000; Wu et al., 2001) include FISH results using these same oligonucleotides but experimental conditions are variable. These probes are still reasonably accurate to target most of the defined phylogenetic groups of methanogenic *Archaea.*

The main advantage of FISH is that it does not require any DNA or RNA amplification and allows microscopic inspection of intact cells in the samples. The other important advantages of FISH technique are; it is an easy and fast technique and it allows direct visualization of organisms without cultivation. Tt is generally quantative and it allows possible to detect active microorganisms in the sample (Sanz and Kohling, 2006).

Despite the advantages above, FISH technique has its own limitations. The most significant one is that; not all bacterial and archaeal cells can be permeabilisied by oligonucleotide probes using standart fixation protocols (Amann et al., 1995).

The other disadvantages of FISH are; priori knowledge of the studied ecosystem and the microorganisms to be detected is necessary, meaning combining with other techniques is obligate. Also, in case a particular microorganism is to be detected and quantified, the rRNA sequence of the microorganism must be known (in case corresponding probe has not yet been published). In addition, it is not always possible to design a specific probe for a certain group of microorganism, especially if metabolic criteria are applied. Finally, the design and assessing optimum conditions for hybridization

for a new probe is a difficult dedication and quantification of microorganisms can be tedious and subjective (manual counting) or complex (image analysis).

2.3.3 Pattern Analysis and Denaturing Gradient Gel Electrophoresis (DGGE)

Pattern analysis or fingerprinting is often carried out by evaluating banding patterns of PCR products on gels (Dahllöf, 2002). Several fingerprinting techniques, such as DGGE, TGGE, restriction fragment length polymorphism (RFLP), and single strand conformation polymorphism (SSCP), have been developed to screen clone libraries, to estimate the level of diversity in environmental samples, to follow changes in community structure, to compare diversity and community characteristics in various samples and simply to identify differences between communities (Dahllöf, 2002; Hofman-Bang et al., 2003).

DGGE is a gel electrophoresis method that separates genes/ DNA fragments of the same size (obtained after PCR of DNA extracted from an environmental sample) that differ in base sequence, at least by one nucleotide into distinct bands on a chemical denaturing gradient polyacrylamide gel. DGGE is nowadays a routine technique that is used to assess the diversity of microbial communities, to monitor their dynamics (Muyzer and Smalla, 1998; Muyzer, 1999) and to screen clone libraries. This method can be used to obtain qualitative and semi-quantitative estimations of biodiversity. Also, the DGGE pattern obtained provides a rapid identification of the predominant species in the microbial community.

2.4. Aim of the study

Utilization of biogas as a renewable energy source is an emerging application around the world. Accordingly, studies investigating the insights of the phenomena has been gaining undeniable importance, including the ones focusing on the effects of inhibitory compounds in the process, such as oxytetracycline which is commonly used as a veterinary antibiotic. Although it is clear that microbiolgy of biogas production process is primarily related with the quality and quantity of the produced biogas, there is lack of information on this side of the matter. This study aims to determine the inhibitory effect of a commonly used veterinary compound, oxytetracycline, on the anaerobic digestion of cow manure with the evaluation of digester performance and microbial population by molecular tools.

3. MATERIALS AND METHODS

In this study, two batchwise experiment sets were conducted in 120 mL serum bottles, which are going to be named as Set 1 and Set 2. In Set 1, non medicated manure was used as substrate and OTC was added externally in differing concentrations. The experiment set was operated for 60 days. In Set 2, a dairy cow was medicated with OTC. It's manure was collected for 20 days and after OTC measurement, used as substrate. The experiment set was operated for 30 days. In both sets, seed sludge obtained from an already operating lab scale manure digester was used as inoculum. The details of the experiments are explained further down.

3.1. Sampling and Characteristics of Manure, Manure Slurry and Seed Sludge

Manure samples were obtained from the barn of Veterinary Faculty of Istanbul University, Istanbul, Turkey. For Set 1, manure of a 3.5 year old, 450 kg Holstein dairy cow, which lacked antibiotics, was used as substrate and OTC was added externally. In Set 2, same cow was medicated with 20 mg/kg oxytetracycline injectable solution which was injected equally into the right and left sides, between *musculus semitendinosus* and m*usculus semimembranosus* muscles. Following medication, its manure was collected from the rectum for the following 20 days. Prior to conducting of the serum bottles, OTC concentration in medicated manure was determined. Seed sludge was obtained from an already operating lab scale anaerobic manure digester and had TS and TVS concentrations of 24.2 g/L and 17.7 g/L respectively. Characteristics of manure and manure slurries are given in Table 3.1.

Table 3.1. Characteristics of manure and manure slurry samples used in this study

Sampling Day	TS, %	TVS, %	TKN, mg/kg	Alkalinity, $mgCaCO_3/L$	TotalC/N***
0-control*	18	15	12000	1400	4.16
0-control**	20	16	11500	1500	4.67
1	14	12	10500	1500	4.20
2	15	13	14000	1600	4.62
3	14	12	12500	1500	4.18
5	12	10	13000	1200	3.70
10	14	12	14000	1600	3.92
15	15	12	11000	1200	3.54
20	15	12	11000	1200	4.22

*Set 1 **Set 2 *** 95% of total carbon was organic C and 90% of total nitrogen was organic N.

3.2. Serum Bottle Tests

In both sets, prior to contucting the serum bottles, manure samples were diluted with tap water and mixed with seed sludge for inoculation (1:4), and the final TS concentration was set to 5%. The experiments were arranged in 120 mL serum bottles (Figure 4.1). OTC was added at concentrations of 50, 100, 200 mg/L in Set 1. In Set 2, manure collected on 1^{st}, 2^{nd}, 3^{th}, 5^{th}, 10^{th},15^{th} and 20^{th} days after medication were used in serum bottles. pH of the manure slurry-sludge mixture were set to 6.8 by using HCl and KOH. Anaerobic conditions were provided by flushing the bottles with nitrogen gas for 2 minutes. The headspaces were sealed with rubber septums and a screw cap, and covered with parafilm to prevent oxygen intake and escape of gasses. All serum bottles were placed on a mechanical shaker which operated at 120 rpm. The temperature was maintained at 37±1C˚, in a temperature controlled room. The serum bottle tests were performed in triplicate and carried out for 60 days. Samples were taken from the serum bottles for chemical and molecular analysis.

Figure 3.1. Serum bottle.

3.3. Analytical Measurements

On days 5, 10, 20, 30, 45 serum bottles were sacrificed and samples were taken for analytical measurements and molecular analyses. Total Solids, Alkalinity and COD tests were done according to Standard Methods (APHA, 1997). Total Kjeldahl Nitrogen (TKN) was determined colorimetrically, using Nessler Method. At the end of every five days gas pressure of serum bottles were measured with a 7000 mbar manometer. Gas compositions were measured using HP Agilent 6850 gas chromatograph (GC) with a thermal conductivity detector (HP Plot Q column 30 m x 530 μm) at days 10, 20, 30, 45, 60. Methane production values were obtained by multiplying methane percentages of biogas with with gas pressures of the serum bottles. Methane and biogas productions were calculated as volume in ambient conditions. Total volatile fatty acids (VFA) were measured with GC (Perkin Elmer Clarus 600) equipped with a FID detector was used. The column used was Elite FFAP (30 m, 0.32mm ID).

3.3.1. Oxytetracycline Measurement

Acetic acid glacial (BDH-GPR), Oxalic acid dihydrate (Merck), Methanol and acetonitril (LiChrosolv) were commercially supplied. Oxytetracycline was purchased from Agros Chemicals. Methanol and Acetonitril were HPLC grade. The other chemicals were of analytical grade. Double distilled water was used throughout the analysis.

HPLC instrument was a Shimadzu, (Schimadzu LC-10 AD) HPLC equipped with an UV detector; (UV VIS Detector, SPD 10-A) operating at 357 nm. The analytical column used in this study was Inertsil ODS-3 HPLC column, 25 cm x 4.6 mm ID, 5 μM. An autosampler, SIL-10 AD was used for injection. The injection volume was 20 μl. Degassing of the solvents was done by sonication, in a Transonic ultrasonic bath, ELMA D-78224 Singen/Htw prior to use. All of the results were analysed by the system software; Class VP (Schimadzu Scientific Instruments Inc.)

The Inertsil ODS-3 analytical HPLC column was used at ambient temperature. The mobile phase consisted of 75% 0.1M oxalic acid buffer and %25 Methanol: Acetonitril (1:1.5) solution which was delivered isocratically at a flow rate of 1 ml/min. The mobile

phase was degassed prior to use. The total run time was 30 min. Wavelenght for the detection of oxytetracycline was 357 nm.

Before every analysis, analytical column was conditioned with the mobile phase, until a clean baseline was observed. After an acceptable baseline was achieved, standards and then the samples were analyzed.

Stock standart solution of OTC was prepeared by dissolving 100 mg of OTC in HPLC grade methanol and stored at -20 Cº. A total of five working standart solutions were prepeared in methanol at concentrations 1 mg/L, 10 mg/L, 20 mg/L, 100 mg/L and 200 mg/L. All solutions were protected from direct sun and artificial light in order to prevent photodegredation of OTC. In order to plot a calibration curve, serial dilutions of OTC standart solution prepeared in 100% methanol, were analysed by HPLC.

Prior to the extraction of samples, extraction efficiency was determined. 5 g of non medicated wet manure was spiked with OTC solution in methanol, incubated for 4 hours in dark and extracted as mentioned earlier. Spiking levels were; 1g/kg, 200 mg/kg, 20 mg/kg and 5 mg/kg. Recovery rate was calculated by equation 3.1.

$$\text{Recovery Rate} = \frac{\text{Amount of OTC detected by HPLC}}{\text{Amount of OTC added to manure}} \times 100 \qquad (3.1)$$

Extraction was done according to a method modified from Yuan et al. (2010). 5 g wet manure was put into 50 mL polycarbonate centrifuge tubes with 0.5 g Oxalic acid ($C_2O_4H_2 \cdot 2H_2O$), 4 mL acetic acid and 7.5 mL of 90% methanol and shaked at 100 rpms for 30 minutes. The tubes were further centrifuged at 11000 rpm for 10 minutes. This procedure was repeated for 3 times and the supernatants were collected in 50 mL volumetric flasks. Flasks were diluted to 50 mL with double distilled water and centrifuged again at 14000 rpm for 3 minutes and filtrated through 0.2 µm Millipore filters. The extracts were kept in 2 mL amber vials at -20 ºC until the day of HPLC analysis.

3.4. Molecular Techniques

3.4.1. Fluorescence In Situ Hybridization

After the gas composition and gas pressure values were obtained, 5 mL of the samples were transferred to Falcon tubes and diluted 1:1 with absolute ethanol and stored at -20 ^{0}C and fixed with Paraformaldehyte (PFA) within 3 days.

For the standard PFA fixation, 1 mL ethanol-sample mixture was transferred to 1.5 mL Eppendorf tubes and washed with 0.5 mL 3X Phosphate Buffer Saline (PBS) for two times and resuspended in 0.25 mL 3x PBS and 0.75 mL freshly prepared 4% PFA and incubated for 3 hours at +4 ^{0}C. After incubation, cells were washed once with 3x PBS and resuspended in 1 mL 1:1 ethanol:1x PBS mixture and stored at -20 ^{0}C until hybridization.

For the hybridization, oligonucleotid probes targeting 16S ribosomal RNAs (rRNAs) listed in Table 3.2 were used. The methanogen targeted probe sequences and classification of the methanogens are given in Figure 3.2 (Raskin et al., 1994). Their optimum hybridization conditions are given in Table 3.3.

Table 3.2. 16S rRNA-targeted oligonucleotide probes used in this study.

Probe	**Target Group**	**Probe Sequence (5'-3')**	**Labelling (5')**	**Reference**
UNIV1393	Virtually all known organisms	ACGGGCGGTGTGTAC	CY3	Raskin et al., 1994
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	CY3	Stahl et al., 1988
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	CY3	Amman et al., 1990a
MB310	*Methanobacteriales*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin et al., 1994
MG1200	*Methanomicrobiales* relatives	CGGATAATTCGGGGCATGCTG	CY3	Raskin et al., 1994
MS1414	*Methanosarcina* + relatives	CTCACCCATACCTCACTCGGG	CY3	Raskin et al., 1994
MSMX	*Complete acetoclastic methanogens*	GGC TCG CTT CAC GGC TTC CCT	CY3	Raskin et al., 1994
NON338	Non sense probe	ACTCCTACGGCAGGCAGC	CY3	Raskin et al., 1994

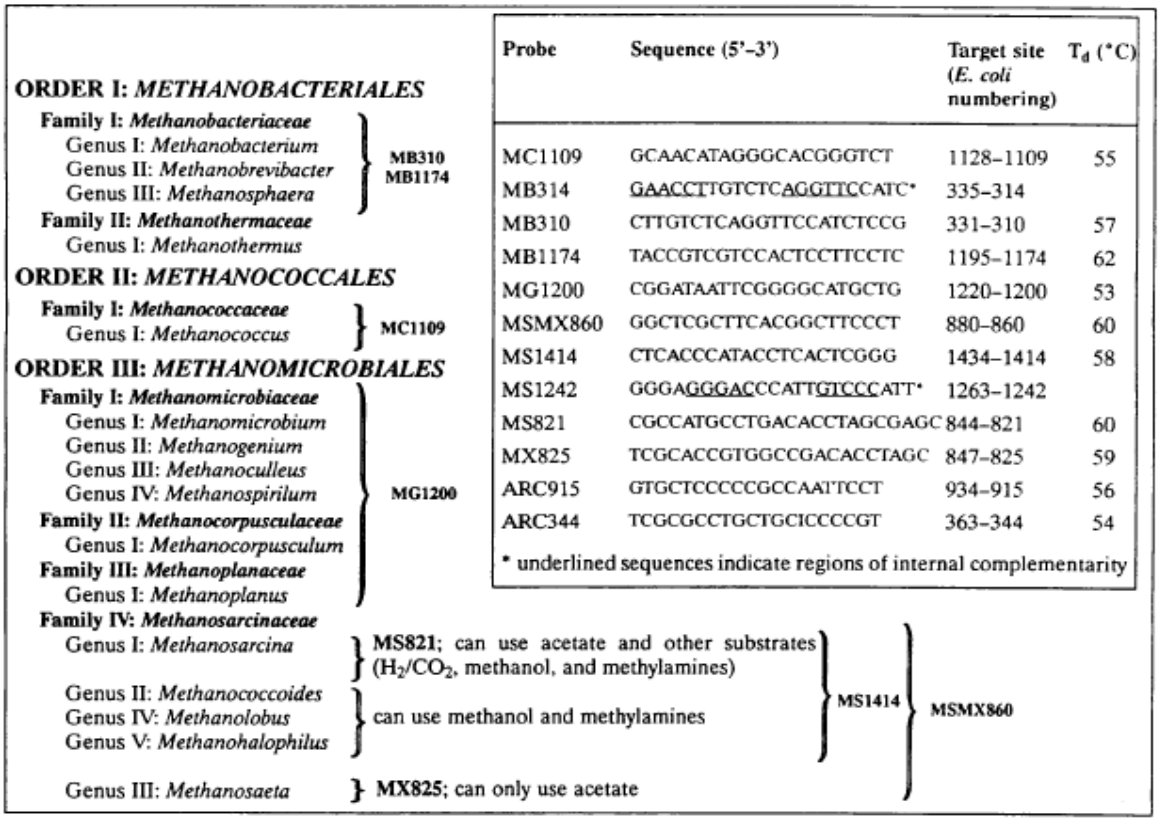

Figure 3.2. Classification of methanogens in relationship to the oligonucleotide probes characterized.

Table 3.3. Optimum hybridization conditions for oligonucleotide probes (Kolukırık, 2004).

Probe	Formamide concentration	Hybridization temperature (^{0}C)	Washing temperature (^{0}C)	NaCl concentration
UNIV1393	10%	37	37	450 Mm
ARC915	35%	46	48	84 Mm
EUB338	10%	46	46	450 mM
MB310	20%	46	48	225 Mm
MG1200	30%	46	48	112 mM
MS1414	35%	46	48	84 mM
MSMX	35%	46	48	84mM

For each hybridization, two negative controls were prepared; one for assessing non-specific bindings (with Non338 probe), and the other (lacking a probe) monitoring autofluorescence. In addition to negative controls, one positive control was prepared to

assess success of cell permeabilization and rRNA content of the cells (with universal probe UNIV1392).

20-25 µl of the fixed samples were transferred to new microfuge tubes. The amount was determined by the microorganism density in the sample. The samples were than washed 2 times with 3X PBS and once with ddH_2O. After washing, the pellet was resuspended in 0.5 mL ddH_2O. The slides were dehydrated through ethanol series (50%, 80%, 96%) for 3 minutes. 17 µl hybridization buffer (2 mg/ml Ficoll, 2 mg/ml Bovine serum albumen, 2 mg/ml polyvinyl pyrolidone, 5 mM EDTA,Tris HCl, pH 7.2, 25 mM NaH_2PO_4, NaCl, pH 7.0, 0.1% SDS) and 3µl targeted probes were added and incubated at the optimal hybridization temperature for the given probe for 4 hours. Following hybridization, the cells were washed twice in a wash buffer containing 20 mM Tris-HCl (pH 7.2), 0.01% SDS, 4.5 M NaCl before a final wash in MilliQ water. The cells were resuspended in 200 µl of MilliQ water, and then dried. 10 µl of DABCO (1,4-diazabicyclo[2.2.2]octane) [Sigma D-2522]: 0.233g DABCO 800 µl ddH_2O 200 µl TRIS-HCl (pH=7.2) was added to the cells, and a coverslip was applied and sealed with nail polish before epifluorescence microscopy**.**

In DAPI staining, the total cells present in the samples were previously determined by counting 4,6-diamine phenylindol (DAPI) stained cells. Hybridization procedure of a regular sample was followed except the hybridization time in incubator (Schönholzer et al., 2002; İnce et al., 2007). Hybridization time needed for DAPI is 15-20 minutes at 46°C. Slides were examined under Olympus BX 50 epifluorescence microscope equipped with a 100 W high-pressure mercury lamp, U-MWIB and U-MWG filter cubes. Images were captured using a Spot RT charged coupled device (CCD) camera having special software supplied by the camera manufacturer (Diagnostic Instruments Ltd., UK) The dilution percent needed is determined by counting DAPI added cells. For all times, counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated. Average of the counts gave the representative number of total microorganisms in each sample. Images were processed and analyzed using Image-Pro Plus version 6.3 image analysis software (Media Cybernetics, USA).

Different fluorochromes are excited and emitted at different wavelengths. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used in this study are given in Table 3.4.

Table 3.4. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used.

Fluorochrome	Color of Fluorescence	Maximum Excitation Wavelength (nm)	Maximum Emission Wavelength (nm)	Filter Cube Used
CY3	Red	552	565	U-MWG
DAPI	Blue	365	397	U-MWU

3.4.2. DNA Extraction

Approximately 200 µL sample taken from the serum bottle was added up to lysing matrix tubes provided by the kit. The tube contains mixture of ceramic and silica particles to lyse all microorganisms in sample. Then lysing matrix tubes were spinned in Ribolyser (Fast Prep TM FP120 Bio 101 Thermo Electron Corporation) for 30 seconds at speed of 5.5 m/s. The tubes were then centrifuged at 14000xg for 30 seconds. After centrifugation supernatants were transferred to clean 1.5 ml appendorf tubes and added 250 µl PPS reagent. To mix the composition tubes were shaked by hands for 30 seconds. After mixing the tubes centrifuged again at 14000xg for 5 minutes to pellet the precipitate. Supernatants were transferred to 2 mL eppendorf tubes and 1 mL of binding matrix suspension was added to supernatant. Tubes were inverted by hand for 2 minutes to allow binding of DNA to matrix. To settle the silica matrix tubes were incubated 3 minutes at room temperature. 500 µl of supernatant was removed carefully without disturbing settled silica matrix. Then the binding matrix was resuspended in the remaining supernatant. All mixture was filtered and filter was placed to a new tube. Filter was washed by 500 µl SEWS-M wash solution. After washing, filter was dried by centrifugation at 14000xg for 2 minutes. Filter was removed to a new tube and 50 µl DES (DNase/Pyrogen free water) was added. The filter with DES was vortexed and then centrifuged at 14000xg for 1 minute. Application-ready DNA was obtained in the tube. 1/100 diluted genomic DNA was run on the 1% (w/v) agarose gel, prestained with ethidium bromide (EtBr) in 1x Tris-acetate-EDTA (TAE)

buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8). Gel was visualized by using a gel documentation system, Mitsubishi 91.

3.4.3. Real Time PCR (Q-PCR)

Roche LightCycler DNA Master SYBR Green I kit and Roche Light Cycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) were used for the Q-PCR assays. The primers used in this study are in Table 3.5.

Table 3.5. Information regarding the 16S rDNA spesific primers used in this study.

Primer	**Target Gene**	**Target Organism**	**Annealing Temperature**	**Standard Gene Sequence**	**Reference**
Bac519f Bac907r	16S rDNA	Bacteria	53C°	16S rDNA of *Rhodopseudomonas palustris* CGA009 (NC_005296)	Lane,1991
Arc349f Arc806r	16S rDNA	*Archaea*	55C°	16S rDNA of *Methanobrevibacter smithii* ATCC 35061 *(*NC_009515)	Takai and Horikoshi, 2000
Met348f Met786r	16S rDNA	Methanogens	55C°		Sawayama et al., 2006

To observe the results of the reaction, Light Cycler Software 4.05 program provided by Roche was used. The program consisted of 4 sections; denaturation (95 °C), amplification (95 °C, 56 °C, 72 °C), melting (95 °C, 53 °C, 95 °C) and cooling (40 °C).

3.5. Statistical Analyses

Statistical analyses were made using PASW Statistics 18 (Polar Engineering and Consulting, USA) and Canonical Components Analysis (Canoco 4.5, Biometris, The Netherlands).

4. RESULTS AND DISCUSSION

4.1. Results of Set 1: Effect of Externally Added OTC

In Set 1, serum bottles were operated with non medicated cow manure as substrate and OTC was externally added in concentrations; 50, 100 and 200 mg/L. A serum bottle was prepeared as control which lacked OTC. The OLR was maintained at 1.4 kg TVS/m^3d and reactor operation was monitored for 60 days. Gas Pressure was measured every 5 days and gas composition was measured every 10 days. Samples for molecular analysis were taken on days 10, 20, 30 and 45.

4.1.1. Biogas and Methane Production

In the control serum bottle, cumulative biogas production reached a steady-state on day 30 (Figure 4.1) which was 315±5.2 mL. Biogas production showed reducing trend against the increasing concentration of OTC and in serum bottles containing 50 mg/L, 100 mg/L and 200 mg/L OTC, 41%, 57% and 61% inhibition was observed. Likewise, methane production in control serum bottle reached a steady-state on day 20 of digestion. At this stage, inhibitions were 49%, 55% and 65% in 50 mg/L, 100 mg/L and 200 mg/L OTC containing serum bottles (Figure 4.2). It could be seen that inhibition in biogas and methane productions regressed in the further operation days. In 50 mg/L OTC containing serum bottle, cumulative biogas and methane productions could reach that of the control serum bottle when the operation was prolonged to 60 days. This pattern suggests that OTC toxicity in this serum bottle applied for the non-competitive inhibition model where substrate utilization slows down in the operation by the increasing concentration of the inhibitor. In serum bottles with 100 mg/L and 200 mg/L OTC, inhibition in biogas production was recorded as 30% and 32% by the end of 60 days. In all of the serum bottles no significant change in methane percentages could be observed which was (58±5)% in all of the serum bottles.

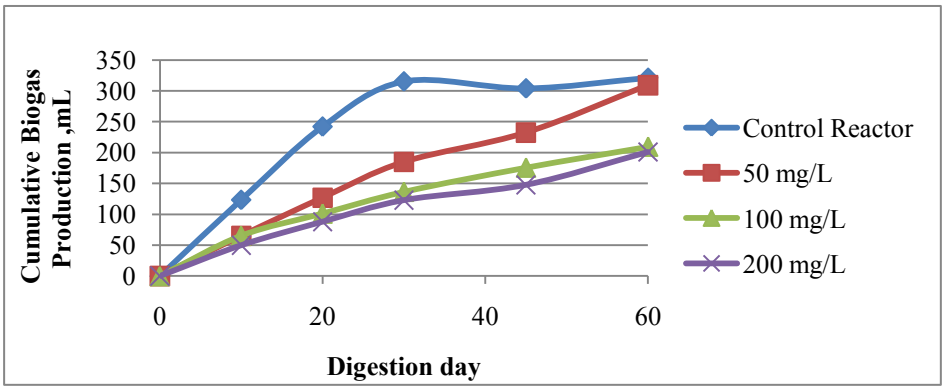

Figure 4.1. Mean cumulative biogas production in serum bottles.

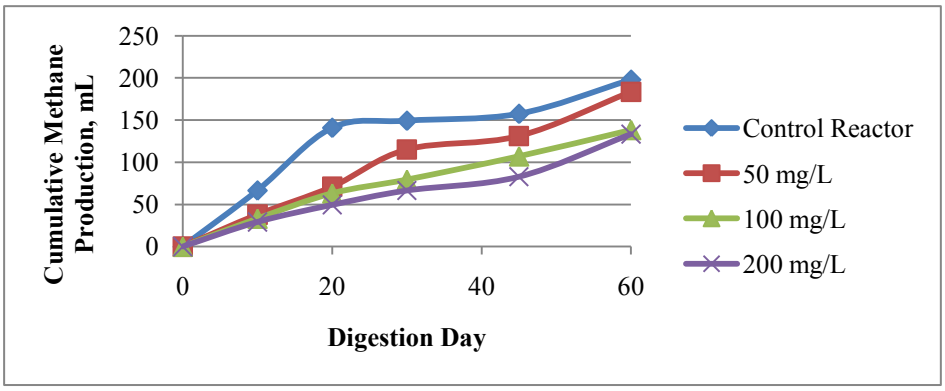

Figure 4.2. Mean cumulative methane production in serum bottles.

Biogas and methane yields are given in Table 4.1. Under the operation conditions of 1.4 kgTVS/m^3d OLR and HRT of 30 days, a total biogas yield of 190.11±3.13 L/kg TVS could be obtained in the control serum bottle and no significant increase could be pronounced when the HRT was extended to 60 days. In manure digesters usually OLR is kept higher (Comino et al., 2010) and HRTs are between 20-45 days (Weiland, 2010). In this study, biogas and methane production progressed untill day 30 of the operation in the control serum bottle, however in order to monitor further changes in OTC containing serum bottles operation was prolonged.

Results regarding biogas production, methane production and biogas and methane yields shows that, addition of OTC in concentrations 50 mg/L, 100 mg/L and 200 mg/L caused process disturbance without leading to complete collapse of the system. The IC_{50}

value of OTC was calculated as 68 mg/L in this assay. A comparative summary of OTC inhibition in biogas production repoted in literature can be seen in Table 4.2.

Table 4.1. Biogas and methane yields in serum bottles (L/kg TVS).

OTC Concentration (mg/L)	**Digestion Day 20**		**Digestion Day 30**		**Digestion Day 60**	
	Biogas Yield	**Methane Yield**	**Biogas Yield**	**Methane Yield**	**Biogas Yield**	**Methane Yield**
Control	**146.02±3.04**	**85.05±2.44**	**190.11±3.13**	**90.10±1.55**	**193.63±5.00**	**119.44±1.98**
50	**76.57±6.36**	**44.04±2.23**	**111.49±5.11**	**63.59±5.19**	**186.51±2.28**	**111.16±1.27**
100	61.07±3.27	38.14±1.80	82.30±2.20	47.95±1.08	126.38±0.72	83.51±0.34
200	53.04±1.72	29.83±3.55	74.12±2.97	40.11±2.40	121.18±1.41	80.50±1.14

Table 4.2. Comparison of similar studies on biogas yields via anaerobic manure digestion.

Manure Type	**Temperature, °C**	**HRT, days**	**Digester Type**	**Biogas Yield, L/kgTVS**	**Reference**
Llama	25	50	Semi-continious	90	Alvarez and Lidden, 2009
Llama:sheep (1:1)	25	50	Semi-continious	140	Alvarez and Lidden, 2009
Llama:cow:sheep (1:1:1)	25	50	Semi-continious	120	Alvarez and Lidden, 2009
Cow	25	50	Semi-continious	100	Alvarez and Lidden, 2009
Sheep	25	50	Semi-continious	120	Alvarez and Lidden, 2009
Cow:sheep (1:1)	25	50	Semi-continious	100	Alvarez and Lidden, 2009
Llama:cow (1:1)	25	50	Semi-continious	90	Alvarez and Lidden, 2009
Llama:cow:sheep (4:1:1)	25	50	Semi-continious	90	Alvarez and Lidden, 2009
Llama:cow:sheep (1:1:4)	25	50	Semi-continious	120	Alvarez and Lidden, 2009
Llama:cow:sheep (1:4:1)	25	50	Semi-continious	70	Alvarez and Lidden, 2009
Cattle	38	60	Batch	208-268	Amon et al., 2007
Calves	35	64	Batch	257*	Arıkan et al., 2006
Cow	37	30	Batch	190	This study
Cow	37	60	Batch	194	This study

*Methane productivity, 60% of total biogas.

Results reported in literature, on the effect of externally OTC in manure digestion is generally in agreement with the results of this study. Gamel-El-Dim et al. (1986) reported 32%, 40% and 49% reduction in biogas production for the batch anaerobic digestion of cow manure containing OTC when externally added in concentration 12.5 mg/L, 37.5 mg/L and 75 mg/L, which is comparable with our results. Sanz et al. (1996) reported that

reduction of methane production was increased to 80% from 20% when CTC concentration was raised to 150 mg/L from 5 mg/L. Alvarez et al. (2010) studied the effect of OTC and CTC at concentrations of 10, 50 and 100 mg/L, added to 500 mL batch reactors digesting swine manure. Reduction in methane production was 45.2%, 56.5% and 64.1% when 10 mg/L, 50 mg/L and 100 mg/ L of both antibiotics were added. Despite these studies, Lallai et al. (2002) did not observe any reduction in methane production in batch reactors containing 125 and 250 mg/L OTC. The reason for this inconsistincies may be due to the slight differences in operation parameters such as; the source of inoculum or source of substrate, inoculum/ manure ratio, reactor size and type of the operation. Further comparison with the literature is given in Table 4.3.

Table 4.3. Summary of OTC inhibition in literature in anaerobic digesters.

Manure Type	Antibiotic	Medication	Concentration, mg/L	Inhibition %	Reference
Pig	OTCand metabolites	Oral	N.D.*	25.0	Masse et al., 2000
Pig	OTC+CTC	External	10	45.2	Alvarez et al., 2010
Pig	OTC+CTC	External	50	56.5	Alvarez et al., 2010
Pig	OTC+CTC	External	100	64.1	Alvarez et al., 2010
Calves	OTC and metabolites	Oral	3.1	27	Arıkan et al., 2006
Cow	OTC	External	50	41	This study
Cow	OTC	External	100	57	This study
Cow	OTC	External	200	61	This study

*N.D :Not defined

4.1.2. Volatile Fatty Acid Production

On days 20, 30, 45 and 60 of the digestion, VFAs were measured. As can be seen from Table 4.4, acetic acid was the only VFA in the control bottle and was also present in OTC containing serum bottles. Other VFAs detected in serum bottles were propionic, isobutyric and isovaleric acids which were in minor concentrations. In the OTC containing serum bottles, there was an increase in the concentration of propionic acid which was detected up to approximately 500 mg/L in 100 and 200 mg/L containing serum bottles. In OTC containing serum bottles, VFAs reached their maximum concentration on days 20, 30 and 45 of the operation and it can be seen that on day 60 they were totally consumed.

Propionic, isobutyric and isovaleric acids are common VFAs detected in anerobic digesters operating with manure (Roy et al., 2000). Their accumulation have been considered as an indicator of the instability of the reactors (Lyberatos and Skyatas, 1999), and they have been detected up to 6000 mg/L in digesters operated with solid waste and/or manure without causing any process failure (Masse et al., 2000; Cuetos et al., 2008).

Table 4.4. Distribution of VFAs detected in the serum bottles (mg/L).

OTC Concentration, mg/L	Digestion Time, day	Acetic Acid	Propionic Acid	Isobutyric Acid	Isovaleric Acid
Control	20	20	-	-	-
Control	30	20	-	-	-
Control	45	26	-	-	-
Control	60	23	-	-	-
50	20	24	509	57	58
50	30	37	424	11	41
50	45	32	494	-	14
50	60	29	-	-	-
100	20	26	488	38	63
100	30	28	402	46	55
100	45	49	292	56	32
100	60	42	-	-	-
200	20	31	493	38	38
200	30	47	503	36	51
200	45	34	516	62	65
200	60	36	12	64	34

According to these results, although we may say that consumption of VFAs was less rapid with the increasing concentration of OTC, which may indicate a possible disturbance of the process, an inhibition caused by volatile fatty acid accumulation can not be pronounced due to the continuation of methanogenesis and stability of the methane percentages in biogas. Also, concentrations of the VFAs detected were below their reported toxic concentations (Angelidaki et al., 1995) which probably means that the hydrogen pressure in the system was low enough to prevent VFA accumulation due to the consumption by hydrogenotrophic methanogens (Cuetos et al., 2008). The low VFA concentation is also an indicator that the system can tolerate higher organic loading rates (Ahring, 2003). However in this study, it was aimed to show the effect of OTC on digester performance thereby, operation with higher OLRs were not preferred in this part of the study.

4.1.3. FISH and Q-PCR Results

Changes in microbial population dynamics were determined using Fluorescence *in situ* hybridization (FISH) and quantitative real time pcr (Q-PCR). FISH was used to determine the number of active cells of bacteria, *Archaea* and different phylogenetic groups of methanogens in control and OTC containing serum bottles. Q-PCR was used to determine the total 16S rDNA copy numbers of bacteria, *Archaea* and methanogens.

Samples were taken from serum bottles for *FISH* analysis on digestion days 10, 20 and 30 in order to monitor the total number of active cells of different groups of microorganisms during the operation. 16S rRNA specific probes targeting Bacteria (EUB338), *Archaea* (ARC915) and phylogeneticly different methanogenic groups, were used for hybridization (*Metahanobacteriales*; MB310; *Methanomicrobiales*; MG1200, *Methanosarcinaceae*; MSMS1414 and *Methanosarcinales*; MSMSX). Samples were initially stained by DAPI before hybridization to observe intact cell concentration. For each sample hybridization, two negative controls were used; one of these controls was used to assess nonspecific binding (with Non338 probe), and the other (lacking a probe) was used to monitor autofluorescence. Also one positive control was used with UNIV 1392 probe. Whole microbial community in the samples was also stained using DAPI stain to visualize intact cells in the samples. 10 random fields of views were used for each quantification study. The results are expressed as total numbers in mL.

Changes in numbers of bacterial and *Archaeal* cells during the operation are given in Table 4.5 and can also be seen from Figures 4.3-4.4. Percentage of methanogens in active *Archaea* was determined by dividing the total active cell number of the group in question to the total cell number of *Archaea*.

It can be said that number of *Archaea* and bacteria was negatively effected by the increased concentration of OTC during the operation. In both of the groups, activity increased on day 20 of the operation. It is stated in literature that effect of OTC on bacteria is biostatic and selective which means that it stops protein and nucleic acid synthesis in order to terminate cell fusion and is more effective on certain groups of bacteria (Schadewinkel-Scherkl and Scherkl, 1995). In this study, total number of bacteria is

determined and changes in the sub groups are unfortunately unknown, which were likely in relation with the changes in *Archaeal* subgroups.

Table 4.5. Number of active cells/mL in serum bottles.

	Digestion Day 10		Digestion Day 20		Digestion Day 30	
OTC Concentration (mg/L)	Bacteria	*Archaea*	Bacteria	*Archaea*	Bacteria	*Archaea*
Control	3.36E+07	1.54E+08	3.77E+07	6.42E+07	3.48E+07	2.49E+07
50	3.35E+07	1.53E+08	3.48E+07	1.03E+08	1.50E+07	2.46E+07
100	2.29E+07	1.44E+08	3.38E+07	6.31E+07	1.29E+07	1.95E+07
200	2.26E+07	1.09E+08	3.00E+07	3.25E+07	1.04E+07	1.94E+07

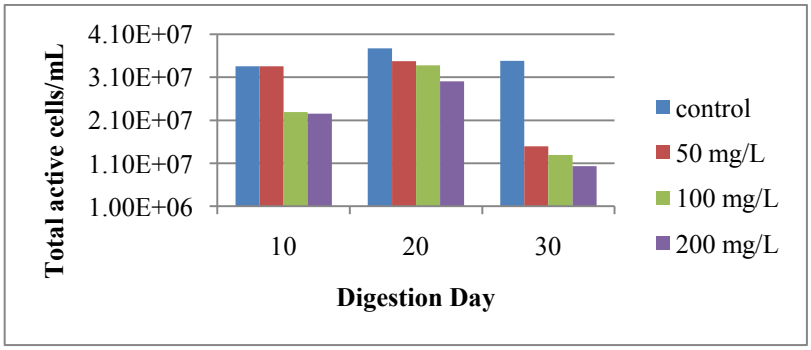

Figure 4.3. Changes in the total cell number of bacteria.

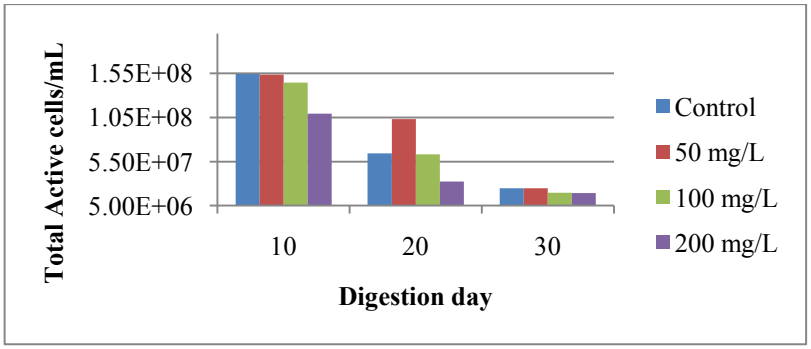

Figure 4.4. Changes in the total active cell number of *Archaea.*

Table 4.6. Percentage of methanogens in *Archaeal* population (Digestion day 10).

	OTC concentration (mg/L)			
	Control	50	100	200
Methanobacteriales	53.9	62.3	63.2	55.1
Methanomicrobiales	22.8	13.9	13.9	24.4
Methanosarcinaceae	23.0	23.6	22.7	20.1
Methanosarcinales	23.3	23.8	23.0	20.5

Table 4.7. Percentage of methanogens in *Archaeal* population (Digestion day 20).

	OTC concentration (mg/L)			
	Control	50	100	200
Methanobacteriales	43.5	84.2	55.0	51.9
Methanomicrobiales	34.3	9.7	40.5	40.0
Methanosarcinaceae	18.5	6.1	4.5	7.3
Methanosarcinales	22.2	6.4	4.8	8.2

Table 4.8. Percentage of methanogens in *Archaeal* population (Digestion day 30).

	OTC concentration (mg/L)			
	Control	50	100	200
Methanobacteriales	29.8	80.6	54.9	52.7
Methanomicrobiales	58.6	11.8	38.3	43.8
Methanosarcinaceae	11.6	7.5	6.8	3.4
Methanosarcinales	12.2	9.7	8.2	4.8

According to FISH results, most of the *Archaea* consisted of methanogens which are the common case in anaerobic digesters. Percentages of methanogens in *Archaea* are presented in Tables 4.6-4.8. In control serum bottle, 76.7% of the *Archaea* was represented by hydrogenotrophic methanogens on the day 10 of the operation. In hydrogenotrophic methanogens, 53.9% belonged to the order *Methanobacteriales* and 22.8% belonged to order *Methanomicrobiales*. Acetoclastic methanogens were 23.3% of the active *Archaea* and belonged to the order *Methanosarcinales* which was nearly completely consisted of the family *Methanosarcinaceae*. By the end of 30 days, percentage of hydrogenotrophs were 88.4%, where 29.8% belonged to the order *Methanobacteriales* whereas 58.6% was *Methanomicrobiales* in control serum bottle.

In OTC containing serum bottles, percentage of hydrogenotrophic methanogens in *Archaea* were 76.2%, 77.1% and 79.5% in serum bottles containing 50, 100 and 200 mg/L OTC on day 10 of the digestion. In these serum bottles *Methanobacteriales* was dominant over *Methanomcirobiales*. At this stage, *Methanosarcinales* constitued 23.6%, 22.7% and 20.1% percent of the active *Archaea* in serum bottles containing 50, 100 and 200 mg/L OTC. During the operation, there was a decrease in acetoclastic methanogens and hydrogenotrophic methanogens were increased up to 92%, 93% and 95% on day 30 of the operation as concentration increased to 200 mg/L from 50 mg/L. At this stage, *Methanobacteriales* consisted 80.6%, 54.9% and 52.7% whereas *Methanomicrobiales* consisted 11.8%, 38.3% and 43.8% in serum bottles containing 50 mg/L, 100 mg/L and 200 mg/L OTC respectively. *Methanobacteriales* was most abundant in serum bottle containing 50 mg/L OTC where it was detected up to 84.2% and 80.6% on days 20 and 30 and its percentage was lower in higher OTC containing serum bottles. By the end of day 30, percentage of *Methanosarcinales* was 9.7%, 8.2% and 4.8% in serum bottles with 50 mg/L, 100 mg/L and 200 mg/L of OTC respectively.

FISH results were also interpreted with canonical correspondance analysis and results can be seen from Figure 4.5. From the results of cannonical correspondance analysis, it can be said that all of the microbial groups in the serum bottles were negatively affected by OTC toxicity. Decrease in methane and biogas productions were found out to be closely related with the dissappeareance of the acetoclastic methanogens represented by the *Methanosarcinales* order in serum bottles. There, it can be said that *Methanosarcinales* order was the most affected group from OTC toxicity during the operation. The case is also reported in other studies earlier regarding tettracycline antibiotics (Stone, 2009). According to canonical correspondance analysis, the number of total active bacteria seemed not to effect biogas and methane production directly, however the case is likely does not apply for the subgroups due to their syntrophic relations with the methanogens. The situation was also similar for the total number of *Archaea*, meaning that changes in subgroups were more effective on explaining OTC inhibition. Additionally, *Methanomicrobiales* in this set was positively correlated with the digestion day which means that its abundance significantly increased through the operation.

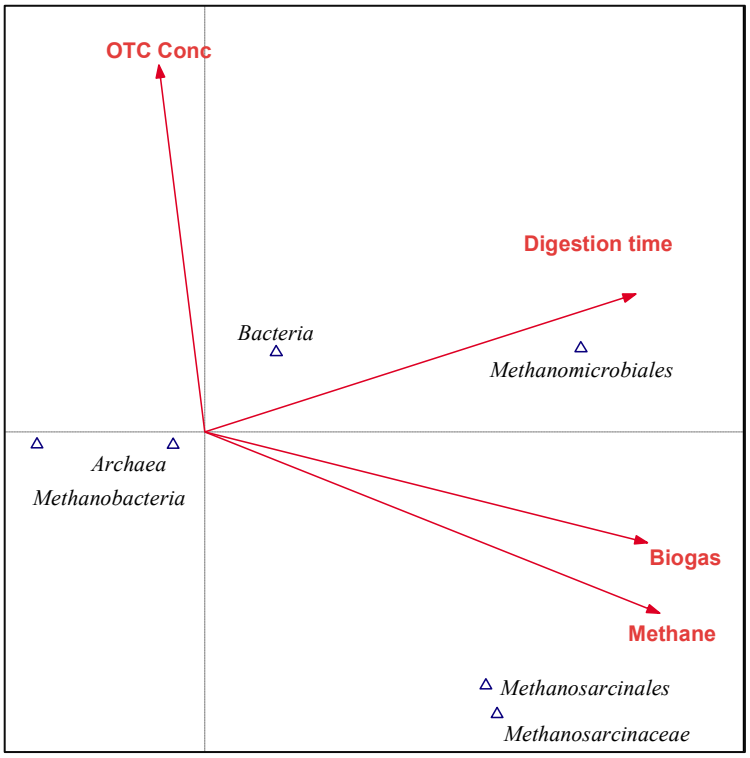

Figure 4.5. Canonical correspondence analyses of FISH results.

The microbiology of manure digesters have been investigated with molecular tools before, and the most commonly represented methanogens in lab scale and full scale manure digesters belong to the orders *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales* (Karakashev, 2005; Karakashev, 2006). Acetate utilizers are almost completely represented by the members of *Methanosarcinaceae* and sole acetate utilizer *Methanosaetaceae* is generally absent in manure digesters (Schmidt et al., 2000; Mladenovska et al., 2003). The case also applied for the serum bottles used in this assay, which is explained by some authors with the disturbance caused on *Methanosaetaceae* by ammonia nitrogen likely to be present in high concentrations in manure. (Karakashev et al., 2005; Schmidt et al., 2000; Mladenovska et al., 2003). The morphology of *Methanosarcina* enables this methanogen to resist ammonia toxicity caused by the degradation of proteins in manure (Demirel and Scherer, 2009) According to most authors, methanogenesis is generally accomplished through hydrogenotrophic pathway in manure digesters (Angelidaki and Ahring, 1993; Shigematsu et al., 2004; Karakashev et al., 2006) which was also true in this study.

According to FISH results, members of *Methanobacteriales* was found to be dominant in the serum bottles, which utilize H_2 and CO_2 and can also use formate (Bonin and Boone, 2004). In the control serum bottle, replacement of *Methanobacteriales* with *Methanomicrobiales* in the later stages of the operation is hard to explain because ecological significance of different hydrogen and formate utilizing methanogens and the competition for common substrates among these populations have been studied less extensively than the competition for acetate in acetoclastic methanogens (Raskin et al., 1996).

To date, there is limited amount of information in literature on the effect of OTC on active methanogenic population in anaerobic digestion of manure. According to FISH results, dominance of *Methanobacteriales* could be pronounced in OTC containing serum bottles. Tolerance of *Methanobacteriales* to toxic substances has been mentioned in the literature before (Gözdereliler, 2008; Köksel, 2010). However, although in some studies the resistance of *Methanobacterium* species to certain antibiotics such as vancomycin and penicillin-G were mentioned (Hammes et al., 1979) information regarding tetracyclines are unfortunately rare. In this study, biogas and methane production was associated with *Methanosarcinales* order, which was represented by the *Methanosarcinaceae* family. This finding is also supported by the work of Sanz et al. (1996), in which it was found out that different species of tetracycline group of antibiotics effected acetoclastic methanogens. Additionally, Stone et al. (2009) studied the effect of a type of tetracycline antibiotic, on different groups of methanogens and stated that it exhibited inhibiting activity against acetoclastic methanogens. However methodology of all these works were different from the course of this study and a comparison can not be made in all terms. The interpretations of changes in methanogens are also likely to closely related with the fate of OTC in anaerobic digestion and for better estimations, concentration of OTC during the process should be determined.

For the Q-PCR assays, 16S rDNA sequence specific primers were used to quantify total copy numbers of bacterial, *Archaeal* and methanogenic 16S rDNA gene. On Table 4.9., reaction parameters of the standard curves used in this assay is presented. Results can be seen from Figures 4.6-4.8.

Table 4.9. Q-PCR reaction parameters of the standard curves used for the quantification of bacteria, *Archaea* and methanogens.

Q PCR Assay	Error	Slope	Y Intetrcept	Efficiency
Bacteria	0.159	-3.502	41.68	1.930 (96.5%)
Archaea	0.143	-3.461	34.8	1.945 (97.2%)
Methanogens	0.00115	-3.838	32.78	1.822 (91%)

All of the reaction parameters of Q-PCR standard curves are in the ranges that assumed to produce reliable results (Zhang and Fang, 2006).

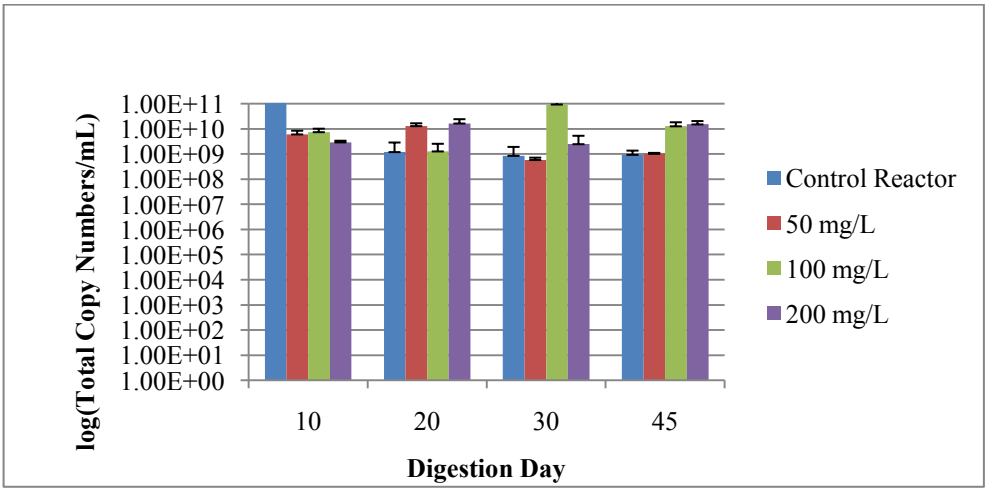

Figure 4.6. Total copy number of the 16S rDNA gene of bacteria.

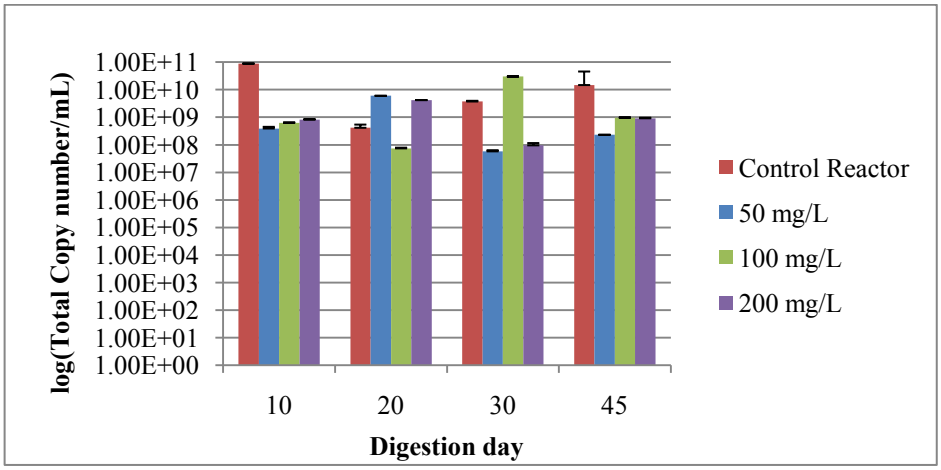

Figure 4.7. Total copy number of the 16S rRNA gene of *Archaea.*

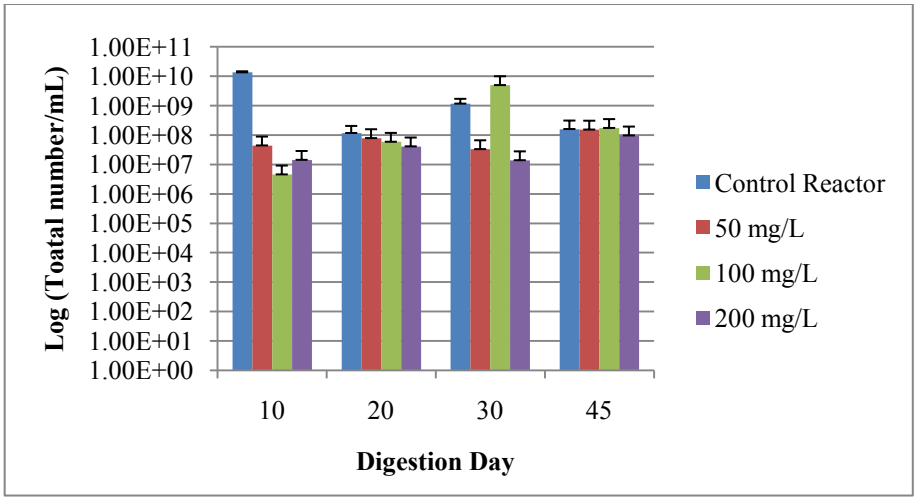

Figure 4.8. Total copy number of 16S rDNA gene of methanogens.

According to Q-PCR results, it could be said that OTC did not have a significant effect on the total bacterial, archaeal and methanogenic 16S rDNA copy numbers throughout the operation. The total 16S rDNA gene copy numbers of domains bacteria and *Archaea* has also been quantified in order to monitor the changes in anaerobic digesters previously. It has been shown that changes in total numbers were not as significant as the changes in subgroups (Blume et al., 2010). Also, RNA based quantification may also yield better results.

4.2. Results of Set 2: Effect of OTC in Medicated Manure

In Set 2, serum bottles were prepared with medicated cow manure following the HPLC analysis for OTC measurement. A serum bottle prepared with non medicated manure was used as control assay. OLR of this set was maintained at 1.4 kgTVS/m^3 d. Operation was monitored for 30 days. Gas Pressure was measured every 5 days and gas composition was measured every 10 days. Samples for molecular analysis were taken on days 10, 20 and 30.

4.2.1. Results of OTC Measurement

The analytical conditions maintained were mentioned earlier. Retention time of OTC was found to be 7.3±0.1 min. In order to confirm the correctness of the method, duplicate analysis of five working standard solutions covering the range from 1 to 100 mg/L were made. 20 µL of these standards were injected into the HPLC system and its concentrations were calculated by the software. A calibration curve was plotted with concentration against area. Results of the assay are presented in Figure 4.9.

The accuracy of extraction was verified by extracting a known amount of OTC spiked into non medicated manure, and analyzing with HPLC. The spiking concentrations were 5, 20, 200 and 1000 mg/g manure. After each extraction OTC was collected in 50 mL of extract. The extract was injected into HPLC. All of the analyses were conducted triplicate. The extraction efficiencies are given in Table 4.10.

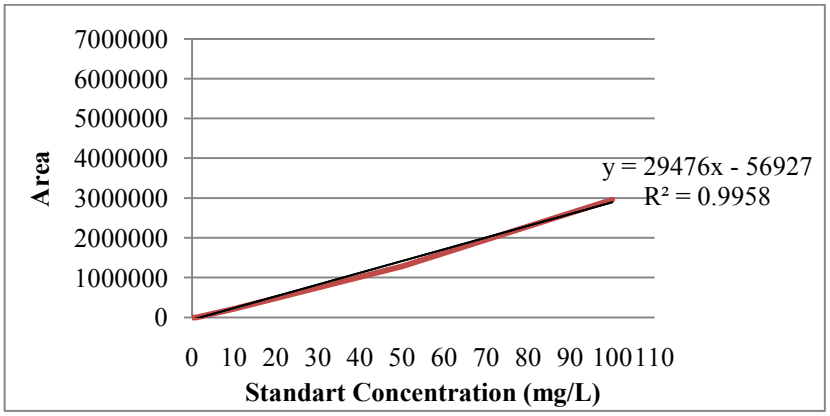

Figure 4.9. Calibration curve for standard solutions.

Table 4.10. Extraction efficiencies.

Amount Collected in 50 mL extract (mg/L)	Concentration in Manure (mg/kg)	Recovery Rate (%)
100	1000	99±0.02
20	200	92±0.1
2	20	85±0.1
0.5	5	77.6±0.04

After plotting the calibration curve and calculating the extraction efficiency. samples were extracted and analyzed. Following extraction, samples were stored at -20ºC until the day of HPLC analysis.

The results of HPLC analysis are on Figure 4.10. The highest amount of OTC in manure was detected after the first day of collection which was 10.38 mg/kg. On day 2, concentration of OTC in manure was decreased nearly %50. On day 4, there was a slight increase in the OTC concentration. After the 13th day, OTC could no more be detected. OTC concentrations in manure samples used in this study are on Table 4.11.

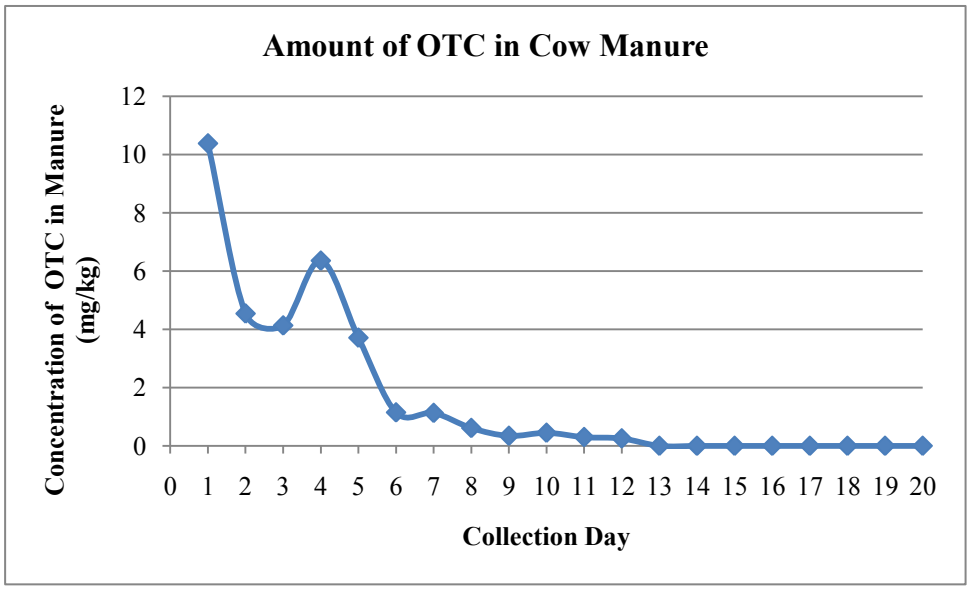

Figure 4.10. Amount of OTC excreted in manure.

According to these results, it can be seen that about 10% of OTC administrated was excreted in manure. The other percentage is either metabolized in the body or present as metabolites. In literature, it is said that 10-90% of TC, OTC and CTC can be absorbed after administration and up to 50% percent can be excreted in feces (Agwuh and Gowan, 2006). It is also remarked that the diet has determining effect in the absorbtion of these drugs and that protein and fat diets may reduce absorbtion up to 50% (Agwuh and Gowan, 2006). However, information in literature on faecal excretion in manure generally stands for oral administration. In this study however, relatively low percentage is found to be excreted in manure after intramuscular administration.

OTC from manure samples was detected in differing amounts in literature. For example De Liguero et al. (2003) reported 871 mg/kg OTC found in swine manure. in which the the drug was mixed with the feed, which is nearly 80 times higher than the amount detected in this study. Tylova et al. (2009) detected CTC in concentration 5.88 mg/kg in manure samples collected from different farms. In previous studies, tetracyclines were reported to be present at a wide range of concentration in manure; changing from 0.1 to 173 mg/kg (Hamsher et al., 2002; Jakobsen and Halling Sorensen, 2006). The differences are likely due to administration of the drug, sampling and storage conditions, the diet, general health and the type of the animal.

4.2.2. Biogas and Methane Production

OTC concentrations in manure samples and serum bottles used in this assay are given in Table 4.11. In control serum bottle, biogas production reached a maximum of 254.9 ±13 mL on day 30 (Figure 4.11). Methane percentages of the biogas was more or less stable and were (58±5)% in all of the bottles. Highest inhibition in biogas prduction at this stage was observed in serum bottles operated with manure collected after the 2nd and 3rd day of medication which were around 60%. In these serum bottles, OTC concentration corresponded to 1.40 and 1.39 mg/L. The lowest inhibition was observed in reactors operated with manure collected on the 15th and 20th days after medication. Methane production values showed a similar pattern with biogas production values (Figure 4.12). Control serum bottle reached a maximum on day 20 of the operation. Highest inhibitions at this stage were detected in serum bottles operated with manure collected after the 2nd and 3rd days of medication, which were 57% and 62%. As can be seen from Table 4.12, a maximum of 155.22±7.91 L/kg TVS biogas yield and 86.96±6.41 L/kg TVS methane yield could be obtained in the control serum bottle after 30 days of operation. Most severe inhibition in biogas and methane yields could be observed in serum bottles operated with manures collected on day 2 and day 3 after the medication of the cow. The inhibition decreased in serum bottles operated with manures collected after the day 5.

Table 4.11. Concentration of OTC in manure samples and serum bottles used in this study.

Manure Collection Day	OTC Concentration (mg/kg)	OTC concentration in serum bottles (mg/L)
1	10.40	3.34
2	4.50	1.40
3	4.10	1.39
5	6.30	1.01
10	0.45	0.15
15	N.D.*	N.D.*
20	N.D.*	N.D.*

*N.D.: Not Detected

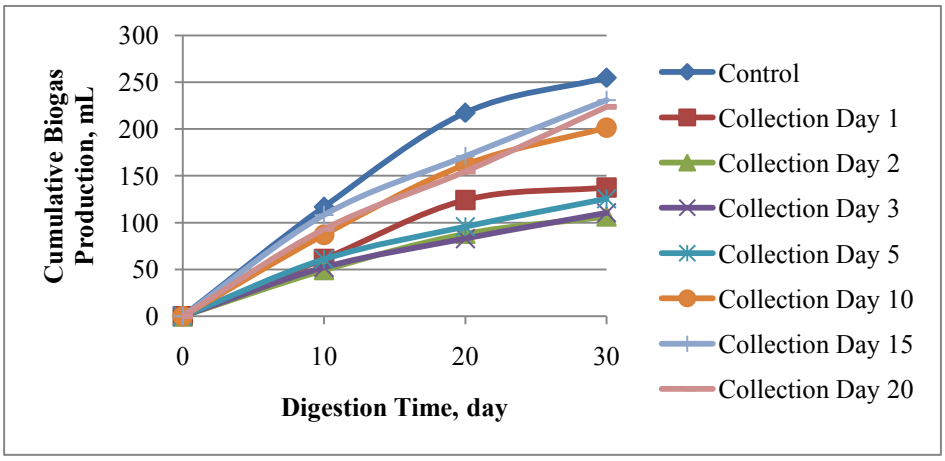

Figure 4.11. Cumulative biogas production.

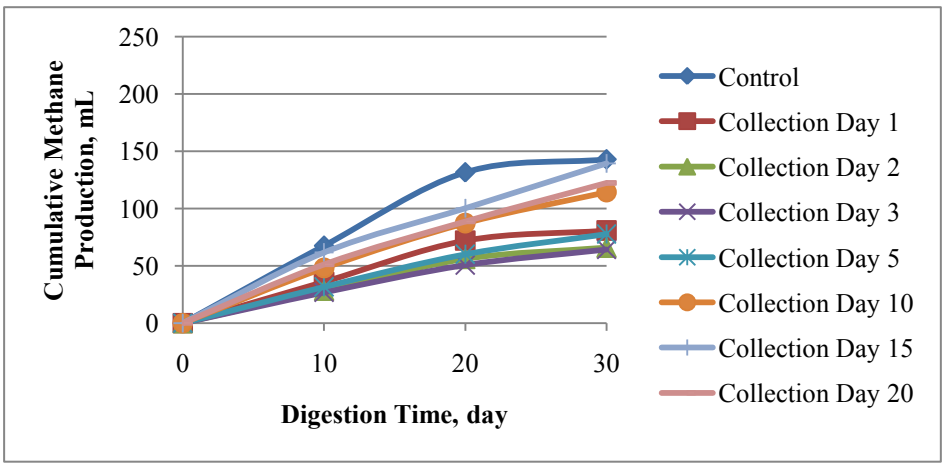

Figure 4.12. Cumulative methane production.

Table 4.12. Total methane and biogas yields in serum bottles.

Collection Day	Digestion Day 20		Digestion Day 30	
	Biogas Yield. L/kgTVS	Methane Yield. L/kgTVS	Biogas Yield. L/kgTVS	Methane Yield. L/kgTVS
Control	**132.43±5.72**	**80.07±0.92**	**155.22±7.91**	**86.96±6.41**
1	70.46±6.46	40.83±0.56	78.04±6.29	45.82±2.13
2	51.73±0.68	32.85±1.26	62.57±4.75	38.62±3.24
3	48.32±1.54	29.45±1.12	64.30±3.88	37.35±2.16
5	54.10±2.17	33.96±2.16	71.00±4.09	43.93±3.53
10	95.67±12.12	51.52±17.68	119.08±11.97	67.53±10.02
15	103.08±20.61	60.38±15.27	139.17±23.01	83.93±15.67
20	94.66±13.65	54.19±8.58	136.85±18.56	74.77±1.23

In Set 2, OTC concentration causing the maximum inhibition in serum boottles was 1.4 mg/L whereas in Set 1 same amount of inhibition was caused by 100 mg/L OTC. Therefore, it can be drawn that externally added OTC in serum bottles is less capable of disturbing the system than OTC already present in manure due to medication of the animal.

The inhibitory effect of tetracyclines in manure, on the anaerobic digestion process has been studies earlier. In most of these works, the drug was administrated orally by the animal. Arıkan et al. (2006) repoted 27% reduction in cumulative biogas production during the anaerobic digestion of cow manure, in which the OTC concentration was 3.1 mg/L in the slurry. Likewise, in the work of Stone et al. (2009), it was found out that 28 mg/L CTC in manure slurry of a swine inhibited methane production 28.4%.

4.2.3. Volatile Fatty Acid Production

On days 20 and 30 of the digestion concentration of VFAs were measured. Results are presented in Table 4.13.

Table 4.13. Distribution of VFAs in serum bottles containing medicated manure (mg/L).

Sampling Day	Digestion Time	Acetic Acid	Propionic Acid	Isobutyric Acid	Isovaleric Acid	Isocaproic Acid
0	20	38	0	0	0	0
1	20	39	80	0	0	5
2	20	30	95	0	0	0
3	20	33	0	0	0	0
5	20	35	0	0	0	0
10	20	24	17	0	0	0
15	20	32	0	14	30	5
20	20	28	0	0	6	0
0	30	31	0	0	0	0
1	30	32	0	0	0	0
2	30	33	0	0	0	0
3	30	31	138	16	11	0
5	30	33	0	0	0	0
10	30	28	0	0	0	0
15	30	36	0	0	13	0
20	30	55	0	0	0	0

Acetic acid was dominant in all of the serum bottles. Compared to other anaerobic digesters operated with manure, concentration of VFAs can be considered low and VFA accumulation can not be pronounced. Therefore, it could be said that VFAs were consumed throughout the operation.

4.2.4. FISH and Q-PCR Results

Microbial community dynamics of the serum bottles were studied by FISH and Q-PCR. FISH was used for monitoring the active cells belonging to bacteria, *Archaea* and different phylogenetic groups of methanogens. Q-PCR method was used for quantification of bacterial, *Archaeal* and methanogenic 16S rDNA copy number.

FISH analyses were done according to the reactor performance results. Samples were taken from the control serum bottle and serum bottles operated with 2^{nd} day manure where highest inhibition in methane and biogas production was observed, 10^{th} day manure where moderate inhibition was observed and 15^{th} day manure which showed the least inhibition, on operation days 10, 20 and 30. The results are expressed as cell number/mL.

The numbers of bacterial and *Archaeal* cells are given in Figures 4.13 and 4.14. Number of bacterial and *Archaeal* cells were lowest in serum bottle with the highest inhibition in biogas and methane productions and showed a similar pattern with that of the reactor performance results. When serum bottles showing the highest inhibition in Set 1 and Set 2 are compared, it is seen that number of bacterial cells were higher in set 2 on operation days 10 and 20. *Archaeal* cells were lower on day 10 and nearly equal on day 20. On day 30 number of both bacterial and Archaeal cells were lower in Set 2 when compared with Set 1.

Table 4.14. Number of active bacterial and *Archaeal* cells in serum bottles (cells /mL).

Manure Sampling Day	Digestion Day	Bacteria	*Archaea*
Control	10	6.83E+07	8.82E+07
2nd day	10	3.37E+07	4.90E+07
10th day	10	3.83E+07	7.34E+07
15th day	10	5.97E+07	7.61E+07
Control	20	9.22E+07	1.90E+08
2nd day	20	4.00E+07	6.36E+07
10th day	20	6.24E+07	9.10E+07
15th day	20	8.64E+07	1.92E+08
Control	30	6.73E+06	1.72E+07
2nd day	30	2.45E+06	1.38E+07
10th day	30	6.22E+06	2.24E+07
15th day	30	8.90E+06	1.07E+07

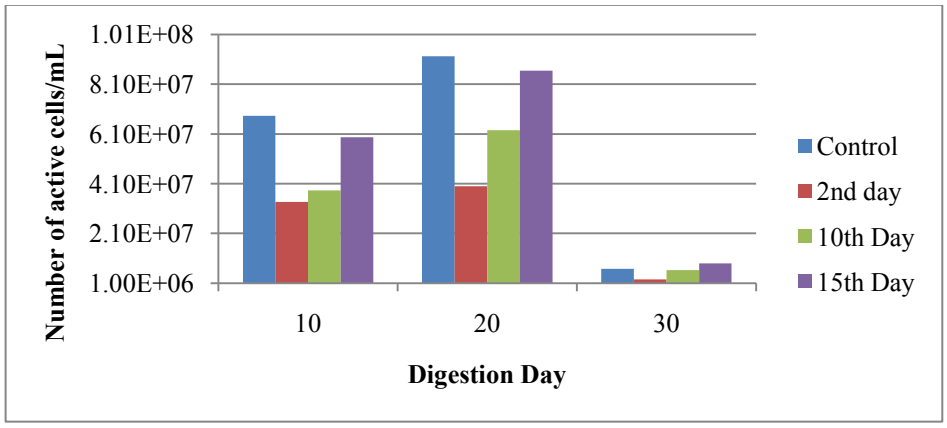

Figure 4.13. Changes in total number of active bacteria.

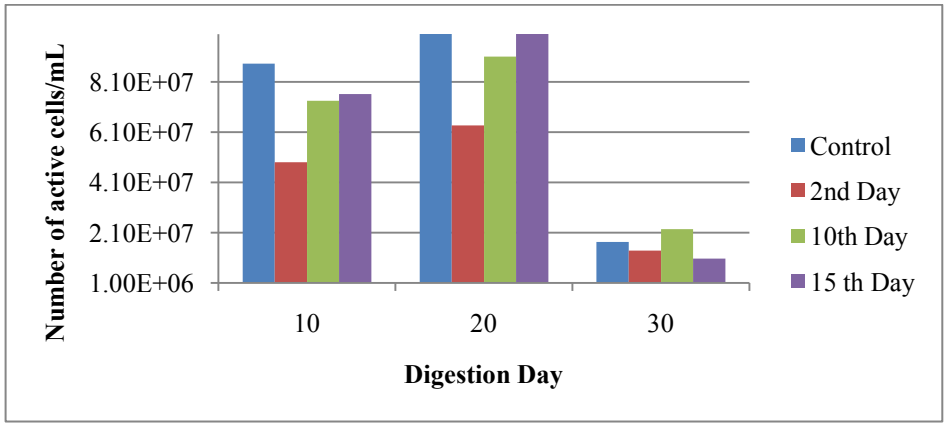

Figure 4.14. Changes in total number of active *Archaea.*

The percentages of methanogens in *Archaea* are given in Tables 4.16-4.18. In the control serum bottle of this set, percentage of hydrogentrophic methanogens were 97.4% on day 10 of the operation. During the operation this percentage decreased and there was an increase in the percenage of acetoclastic methanogens. The dominant methanogenic group was *Methanomicrobiales* in the control bottle. On the day 10 day of the operation, *Methanomicrobiales* constitued 92.5% and 5% was represented by the order *Methanobacteriales*. Acetoclastic methanogens were merely 3%. In control serum bottle percentage of *Methanomicrobiales* decreased during the operation whereas percentage of Methanobacteriales increased. Acetoclastic methanogens were represented by *Methanosarcinaceae* family like Set 1, which increased to 21% from 3% during the operation in the control serum bottle.

In serum bottle operated with manure collected on 2nd day after medication, which showed highest inhibition values in biogas and methane production, 55% of *Archaea* was hydrogenotrophic whereas 45% was acetoclastic on day 10 of the digestion. Members of the order *Methanobacteriales* constitued 19.2% whereas members of *Methanomicrobiales* constitued 36% of the *Archaeal* cells. Percentage of the *Methanosarcinaceae* was 37.2% in this stage of the operation. In this serum bottle, during the operation *Methanomicrobiales* increased to 60.8% while *Methanobacteriales* constitued approximately 20% in the first 20 days however decreased to 10.4% in the end of the operation. Acetoclastic methanogens decreased to 28% from 45% .

In serum bottle operated with manure collected on the 10th day after medication, which showed moderate inhibition in biogas and methane production, *Methanomicrobiales* constitued 59.3%, *Methanobacteriales* 21.0% and *Methanosarcinales* 19.2% of the *Archaeal* cells on day 10 of the operation. During the operation, percentage of *Methanobacteriales* and *Methanomicrobiales* dropped whereas *Methanosarcinales* increased. In serum bottle operated with manure collected after the 15th day of medication, which showed the least inhibition, *Methanomicrobiales* was 67.6% *Methanobacteriales* was 15.4%. *Methanosarcinales* was 17.0% of the active *Archaeal* population. In this serum bottle like the case in control serum bottle and serum bottle operated with manure collected on the 10th after medication, *Methanomicrobiales* decreased. There was approximately 10% increase in *Methanobacteriales* and 10% decrease in *Methanosarcinales.*

Table 4.15. Percentage of methanogens in *Archaea* on day 10.

	Manure Sampling Day			
	Control	2	10	15
Methanobacteriales	5.3	19.2	21.0	15.4
Methanomicrobiales	92.1	36.0	59.3	67.6
Methanosarcinaceae	2.8	37.2	15.2	15.7
Methanosarcinales	2.9	45.0	19.2	17.0

Table 4.16. Percentage of methanogens in *Archaea* on day 20.

	Manure Sampling Day			
	Control	2	10	15
Methnanobacteriales	29.0	20.3	21.9	11.0
Methanomicrobiales	50.1	37.7	45.9	62.1
Methanosarcinaceae	20.2	27.9	31.1	23.0
Methanosarcinales	20.8	42.0	32.2	27.0

Table 4.17. Percentage of methanogens in *Archaea* on day 30.

	Manure Sampling Day			
	Control	2	10	15
Methanobacteriales	28.7	10.4	16.7	26.0
Metanomicrobiales	34.8	60.8	50.0	46.5
*Methanosarcina*ceae	17.3	28.4	31.1	21.5
Methanosarcinales	21.2	28.8	33.3	27.4

When FISH results of Set 2 are summarized, it could be said number of bacteria and *Archaea* showed reducing trend against the increasing degree of inhibition and the order *Methanomicrobiales* was abundant in all off the serum bottles. When looked at the percentages of methanogens, it could be seen that on day 10, the effect of OTC in medicated manure was most on the order *Methanomicrobiales*. Decrease of *Methanosarcinacea* and increase of *Methanomicrobiales* was encountered in serum bottle showing the highest inhibition in methane and biogas productions. Also, *Methanobacteriales* decreased in serum bottles showing high and moderate inhibitions. The common incident in the control bottle serum bottle and the serum bottle with the least inhibition was the increase in acetoclastic methanogens.

FISH method has been applied to manure digesters previously in literature (Karakashev et al., 2005; Karakashev et al., 2006). However, these studies does not focus on determining the inhibitory effect of toxic compounds like oxytetracycline. In this assay, *Methanomicrobiales* was the dominant hydrogenotrophic methanogen in all of the serum bottles, which is considered the second most abundant hydrogenotrophic methanogen in manure digesters and in the rumen after the order *Methanobacteriales* (Sharp et al., 1998; Dworkin, 2006). The dominance of one group of methanogen over another is likely to be linked with the changes in earlier stages. Therefore determination of the pattern of bacterial population is of paramount importance. However, focus of this study was mainly on the changes in methanogens under OTC toxicity. In order to be able to interpret the certain effect of excreted OTC on microbial population in manure digestion, these results should be enriched with the measurements of the metabolites.

For the real time pcr assays, 16S rDNA sequence specific primers were used to quantify total bacteria, total *Archaea* and total methanogens present in the serum bottles. Reaction parameters of the standart curves are given in Table 4.18. Results of the Q-PCR assay can be seen from Figures 4.15-4.17.

Table 4.18. Quantitative real-time PCR (Q-PCR) reaction parameters of the standard curves used for the quantification of bacteria, *Archaea* and methanogens

Q PCR Assay	Error	Slope	Y Intetrcept	Efficiency
Bacteria	0.0179	-3.502	41.68	1.959 (98%)
Archaea	0.143	-3.458	42.42	1.946 (97.3%)
Methanogens	0.000358	-3.834	39.65	1.823 (91%)

Total numbers of the 16S rDNA can be seen from Figures 4.15-4.17.

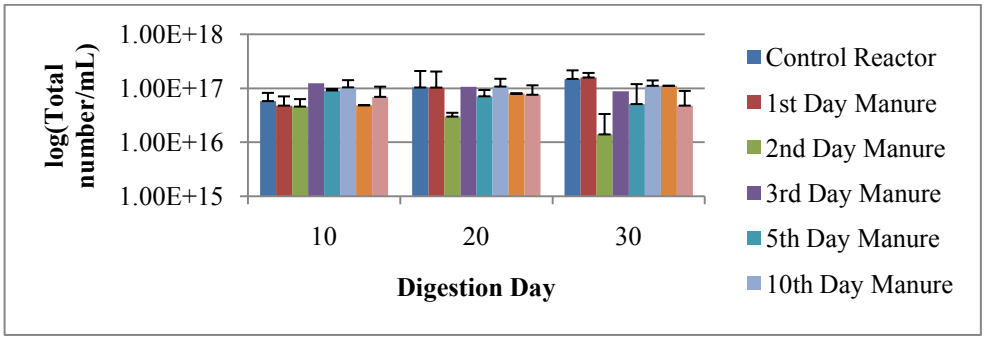

Figure 4.15. Total number of bacterial 16S rDNA gene copy number

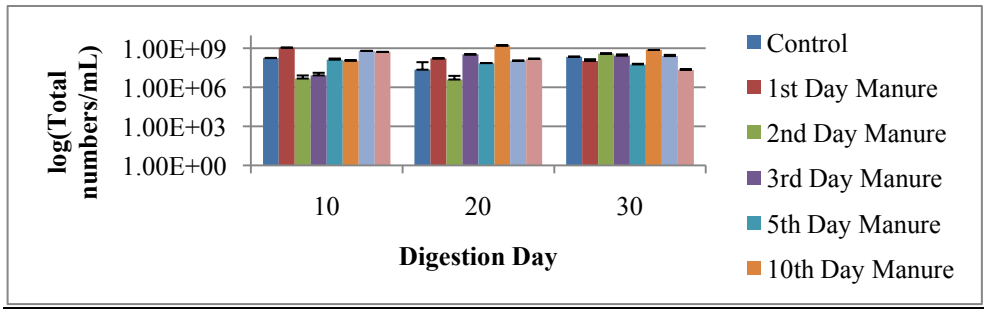

Figure 4.16. Total number of *Archaeal* 16S rDNA gene copy number

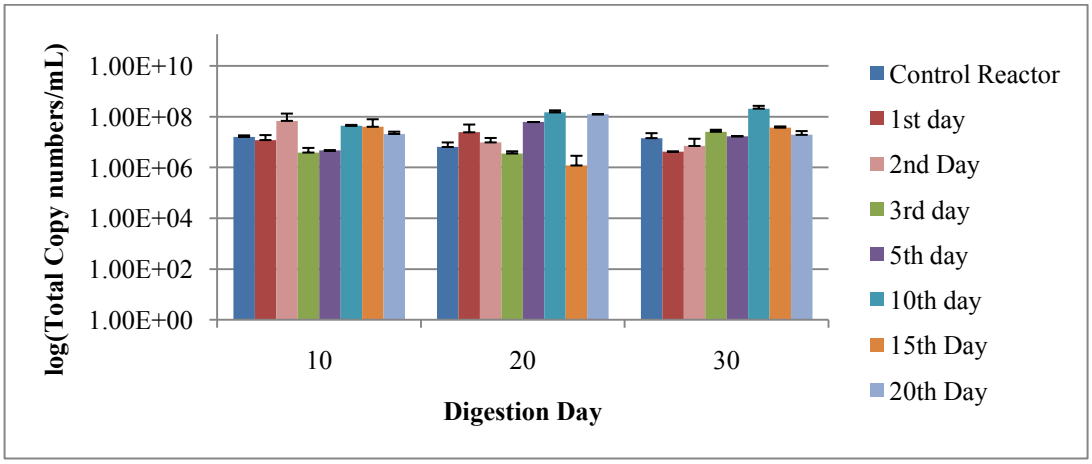

Figure 4.17. Total number of methanogenic 16S rDNA gene copy number.

Total 16S rDNA copy numbers of bacteria, *Archaea* and methanogens were not effected significantly by OTC inhibition in medicated manure. This may show that changes in subgroups might be more effective in explaining the case of OTC toxicity (Klocke et al., 2008; Blume et al., 2010). Also, rRNA based quantification would yield better results related with OTC inhibition.

5. CONCLUSIONS

In this study, effect of oxytetracycline on biogas production and microbial dynamics in batchwise operated serum bottles with cow manure was investigated. Two experiment sets were conducted, namely Set 1 and Set 2, and operated at an OLR of 1.4 kgTVS/m^3d and HRT of 60 and 30 days respectively. TS concentration was kept at 5%. In Set 1, OTC in concentrations of 50 mg/L, 100 mg/L and 200 mg/L were added externally to serum bottles containing non medicated cow manure. In Set 2, a dairy cow was medicated intramuscularly with 20 mg/kg bodyweight OTC and its manure collected on days 1, 2, 3, 5, 10, 15, 20 after the medication, were used as substrate in serum bottles.

According to the findings of this study, biogas production continued untill day 30 in the control serum bottles. In both set the maximum methane yields were between 87-90 l/kgTVS with (58±5)% of biogas being methane. In Set 1, IC_{50} concentration was evaulated as 68 mg/L where 50 mg/L, 100 mg/L and 200 mg/L reduced biogas production 41%, 57% and 61% respectively. Toxicity of OTC seemed to regress during the operation where the serum bottle containing 50 mg/L OTC could almost reach the biogas and methane production values of the control serum bottle. Therefore, it could be said that OTC toxicity in the serum bottle containing 50 mg/L OTC fitted in with non-competitive inhibition model. In Set 2, it was obtained that 10% of the administrated OTC was excreted in manure. The most severe inhibitions in biogas production were monitored in serum bottles containing manure collected on days 2 and 3 after medication which were 60% and 57%. The concentration of OTC in serum bottles with the highest inhibitions were approximately 1.4 mg/L, which meant that OTC in medicated manure was more than 100 times effective in causing inhibition compared to externally added OTC. Also, it was found out that inhibitory effect of OTC decreased in manure samples collected after the day 5 to 20 following medication and in real cases, manure samples should not be used for biogas production in the first 5 days after intramuscular medication.

In this study, it was found out that majority of the active *Archaea* in the serum bottles were represented by hydrogenotrophic methanogens and acetoclastic methanogens were represented by the members of *Methanosarcinaceae* family. The effect of externally

added OTC was mostly on acetoclastic methanogens. The effect of excreted OTC in manure effected *Methanomicrobiales* the most on day 10 of the operation whereas it was encountered that during the operation acetoclastic methaogens decreased in the serum bottles with highest inhibition and increased in the control serum bottle and serum bottles with less inhibition.

It was found out that total 16S rDNA gene copy numbers of bacteria, *Archaea* and methanogens were not signifantly effected by OTC, either externally added or present in manure.

6. FURTHER RECOMMENDATIONS

This study is a part of an ongoing Tubitak Project (109Y275). In this study, effects of OTC on the anaerobic digestion of cow manure has been studied. Two seperate experiment sets were conducted in order to monitor the different effects of externally added OTC and OTC in medicated manure. The focus has been oriented to biogas and methane productions, changes in active methanogenic populations and quantification of the bacterial, *Archaeal* and methanogenic 16S rDNA copy numbers. For further investigtations, changes in active bacterial populations should also be determined. Also, presence of tetracycline resistance genes should be investigated.

In this study, 16S rDNA gene copy number of bacteria, *Archaea* and methanogens were quantified. RNA based quantification of these domains with subgroups should also be done.

For further analyses, digester volumes used in this study can be up scaled for better representation of the real case of OTC inhibition. Effect of OTC during the anaerobic digestion of manure and cosubstates should also be determined. Additionally, measurement of OTC and its metabolites through the operation should be made.

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APPENDIX A: EPIFLUORESCENCE MICROGRAPHS OF SET 1

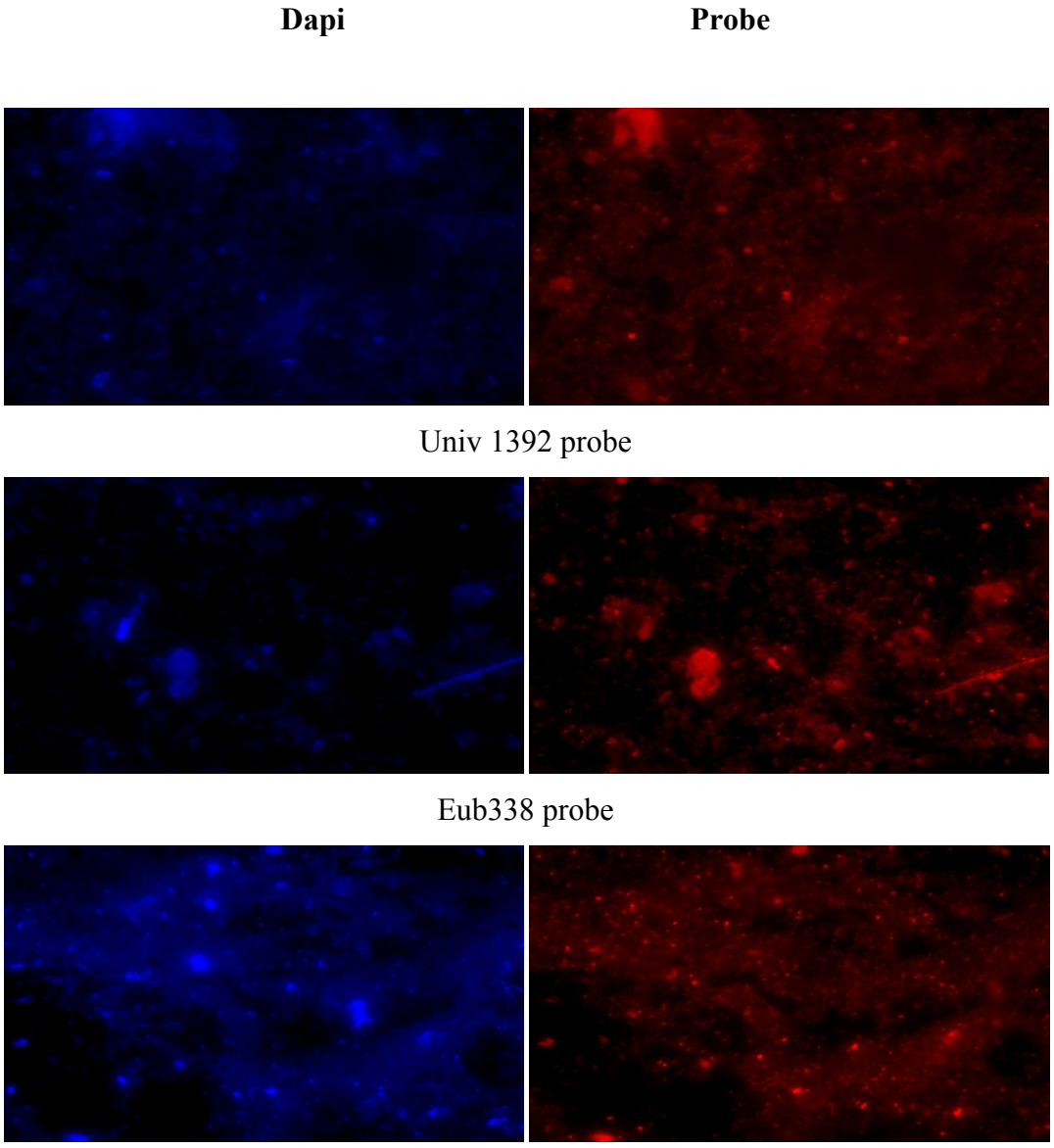


Figure A.1. Epifluorescence micrographs taken from Set 1.

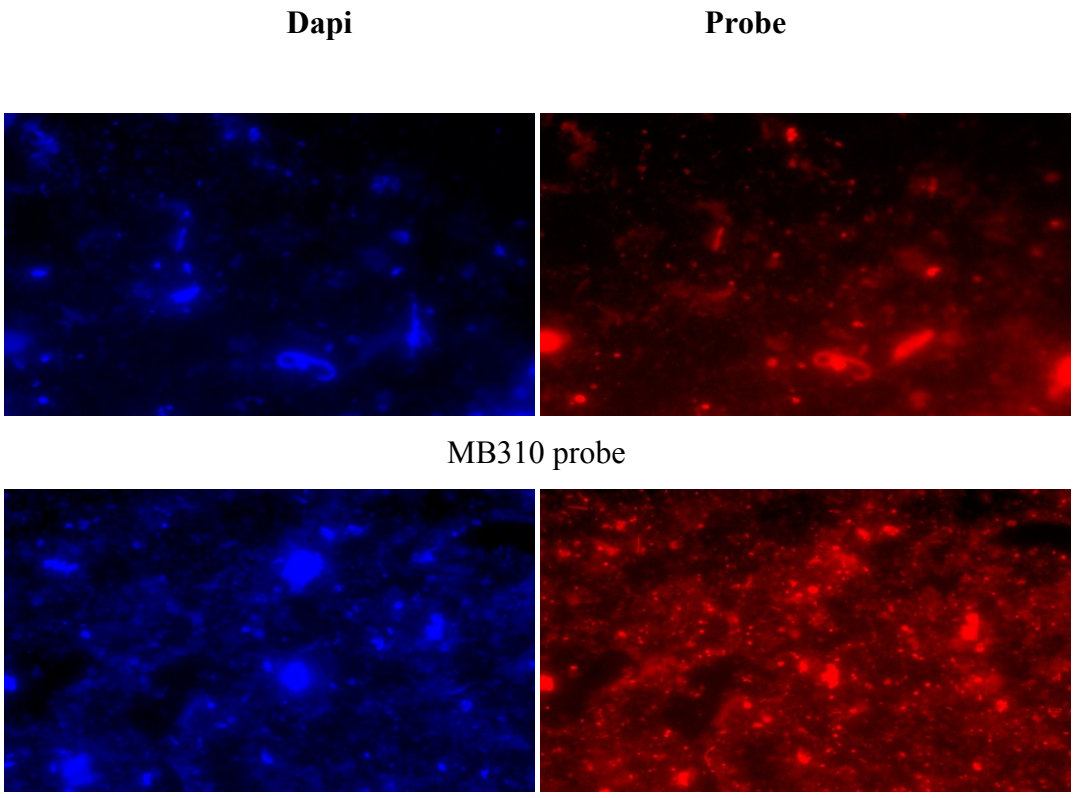


Figure A.1. (continued) Epifluorescence micrographs taken from the control serum bottle.

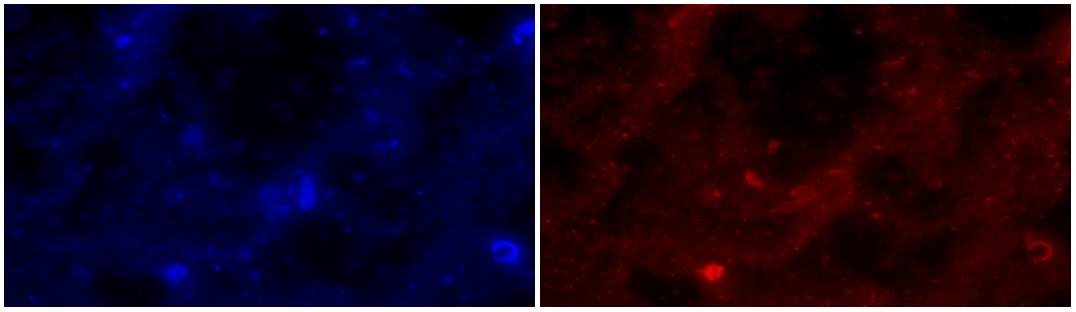


Figure A.2. Epifluorescence micrographs taken from serum bottle with 200 mg/L OTC.

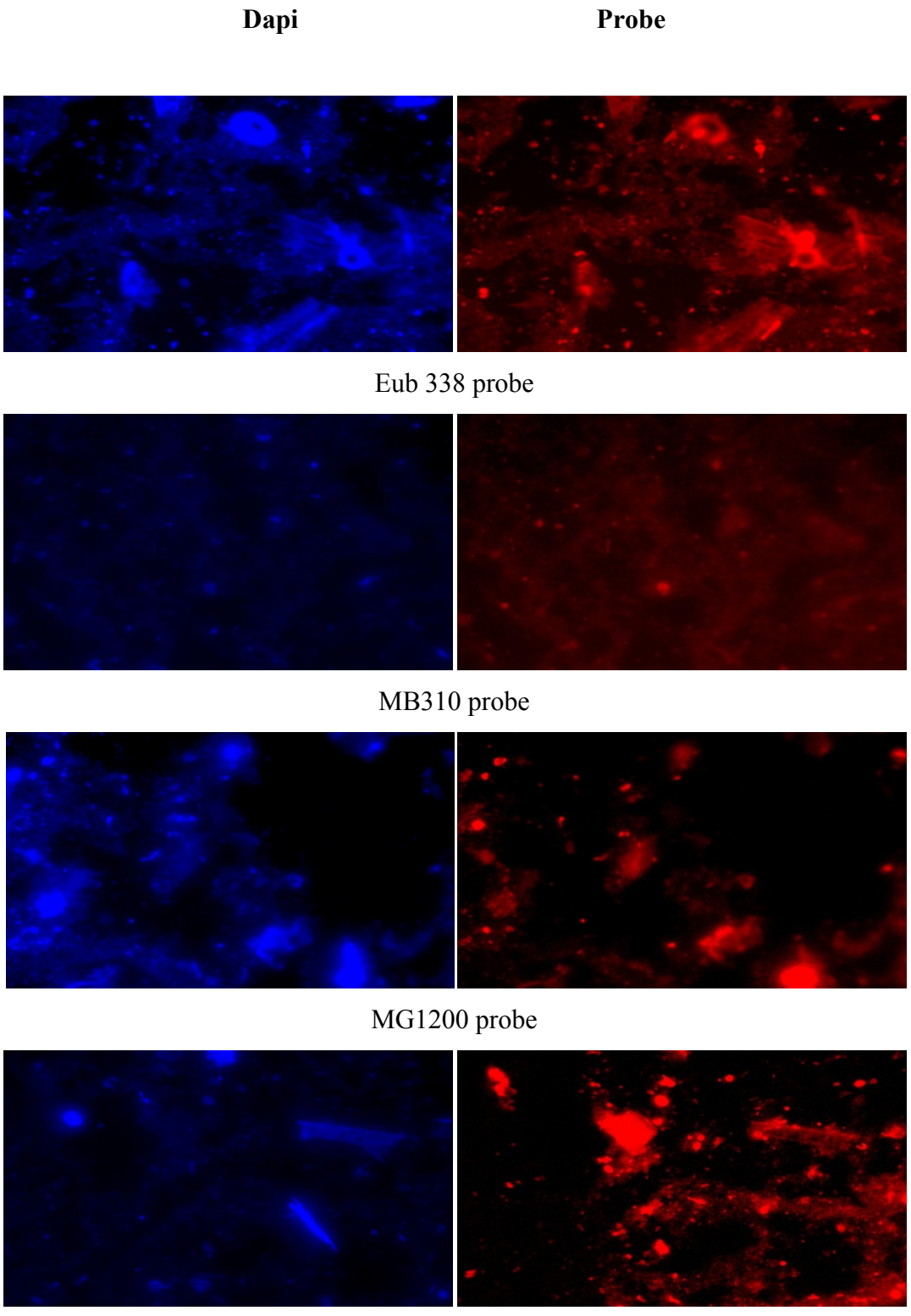


Figure A.2. (continued) Epifluorescence micrographs taken from serum bottle with 200 mg/L OTC.

APPENDIX B: EPIFLUORESCENCE MICROGRAPHS OF SET 2

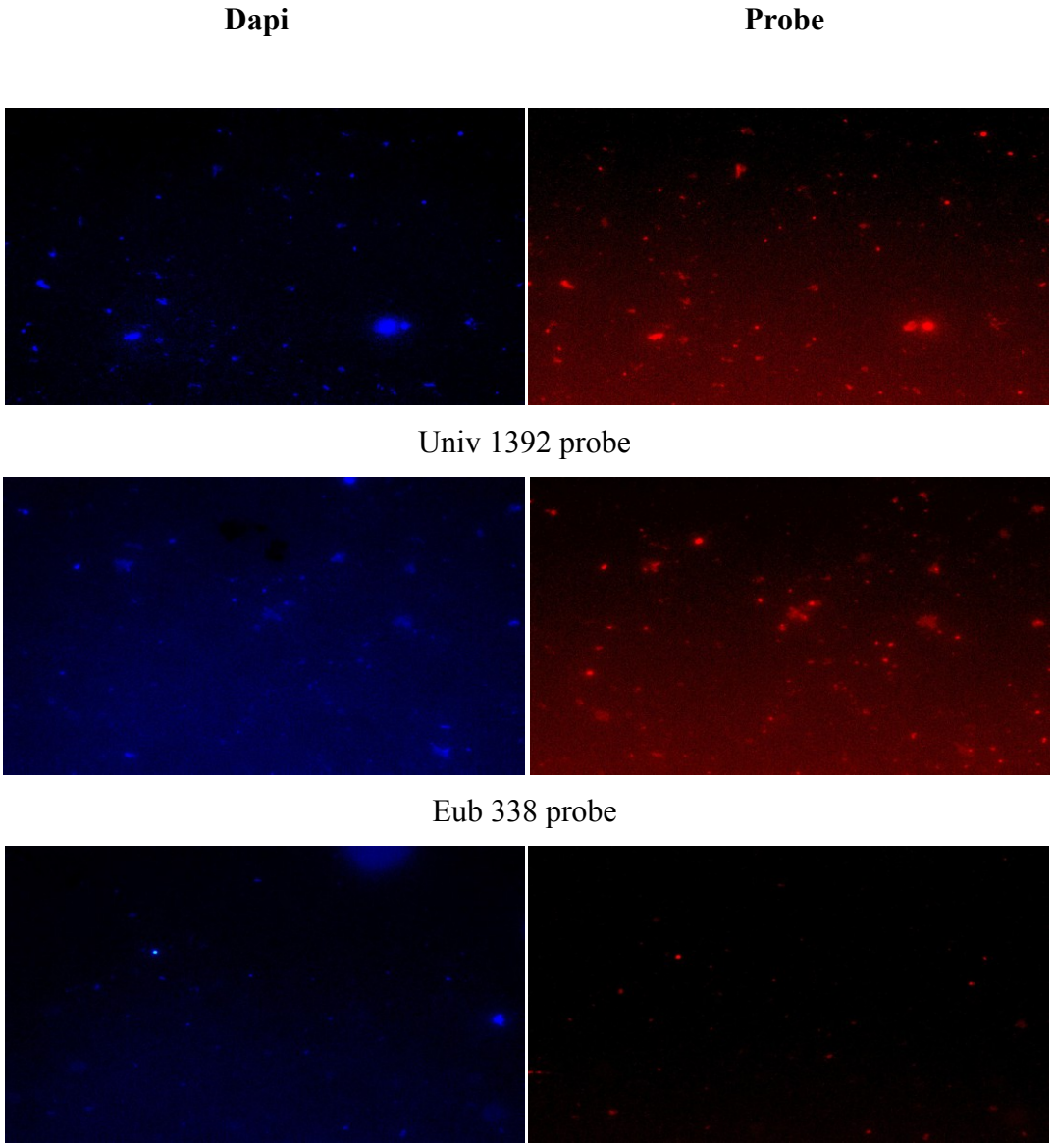


Figure B.1. Epifluorescence micrographs taken from the control serum bottle.

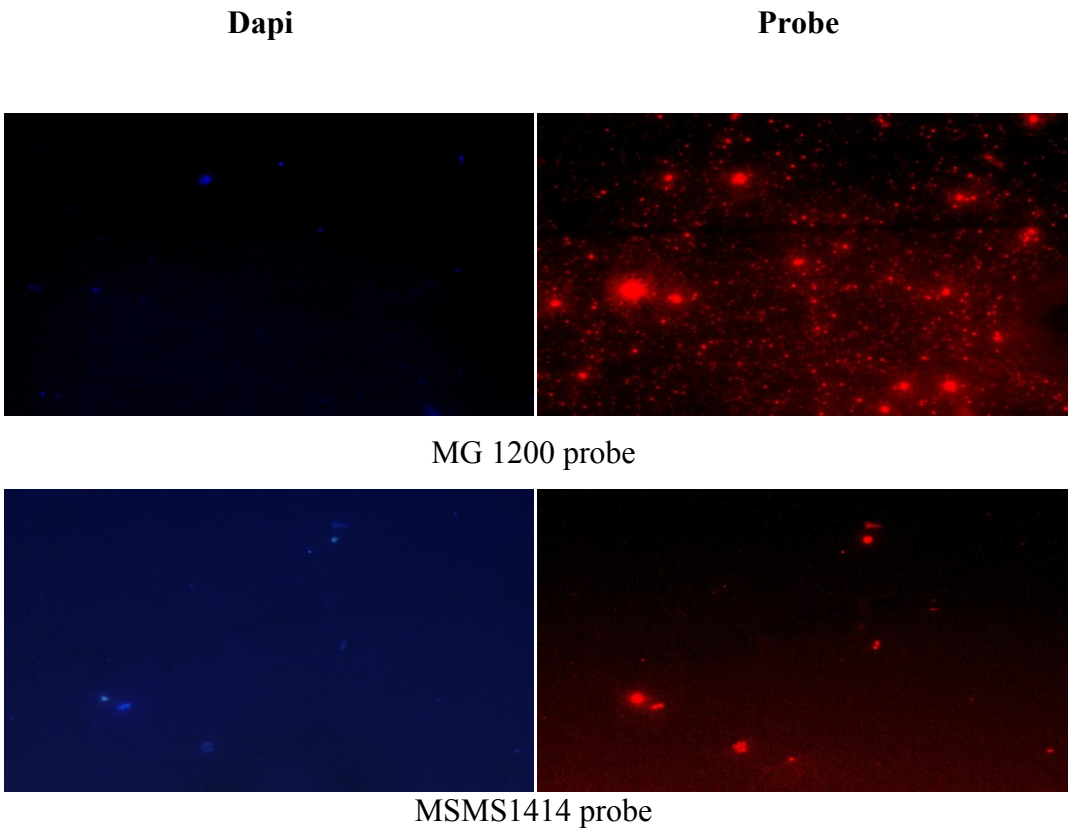


Figure B.1. (continued) Epifluorescence micrographs taken from the control serum bottle.

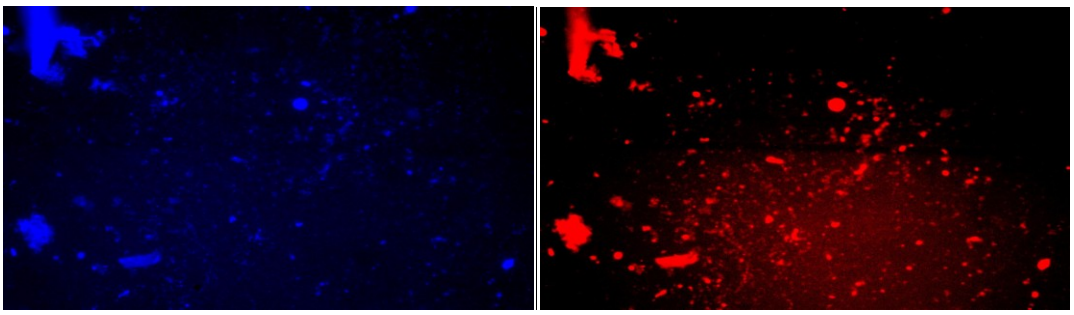


Figure B.2. Epifluorescence micrographs taken from serum bottles operated with 2nd day manure.

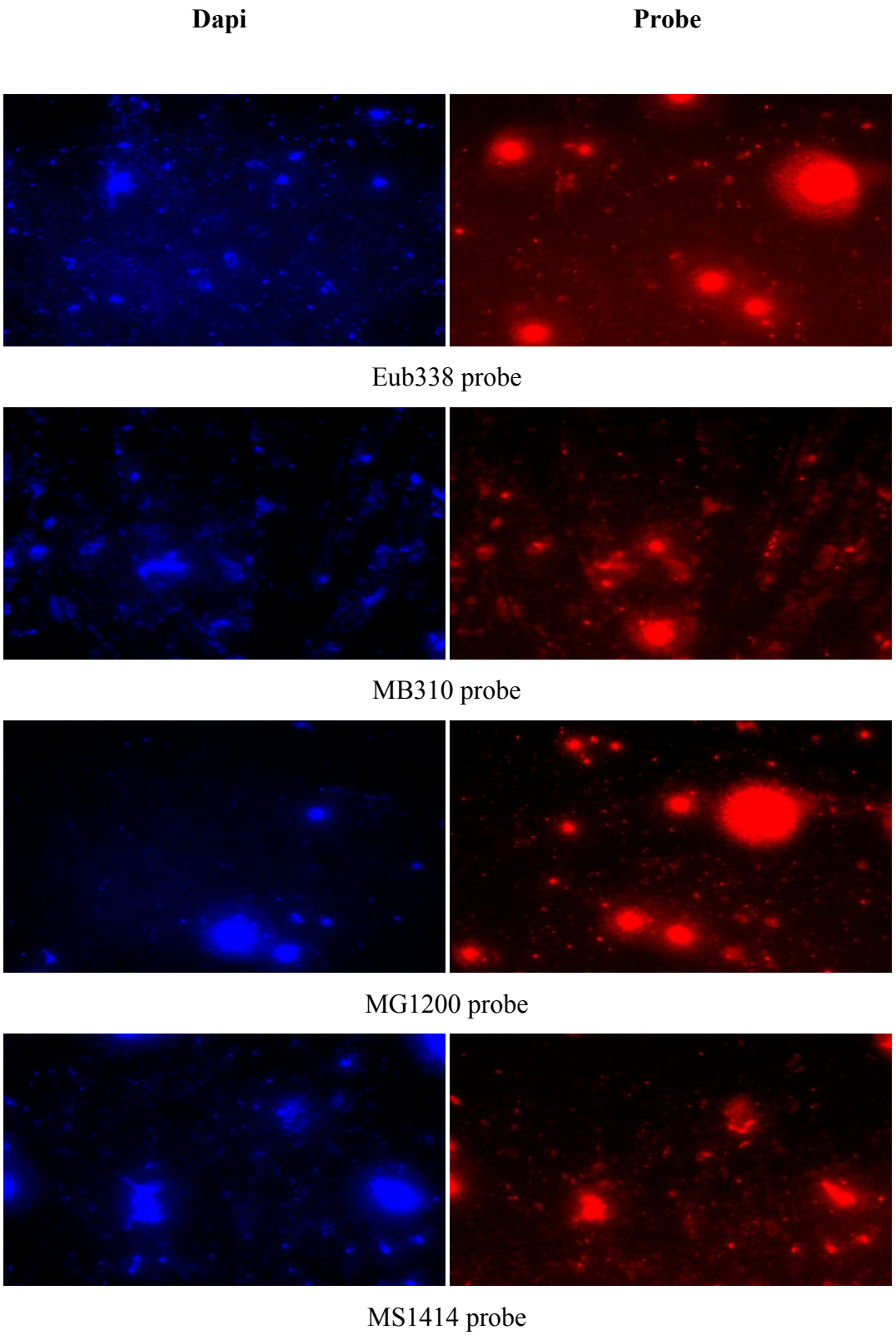


Figure B.2. (continued) Epifluorescence micrographs taken from serum bottles operated with 2nd day manure.

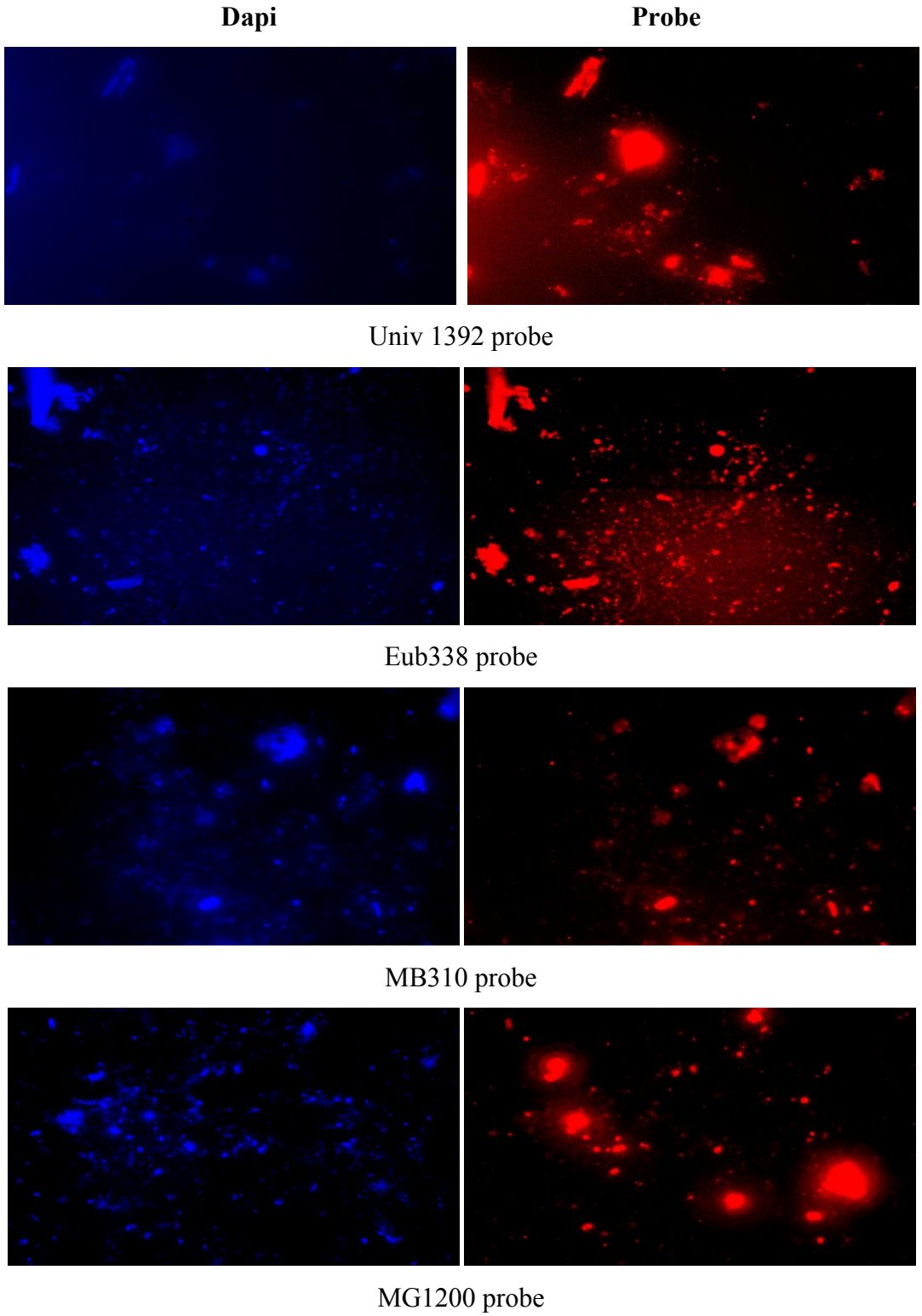
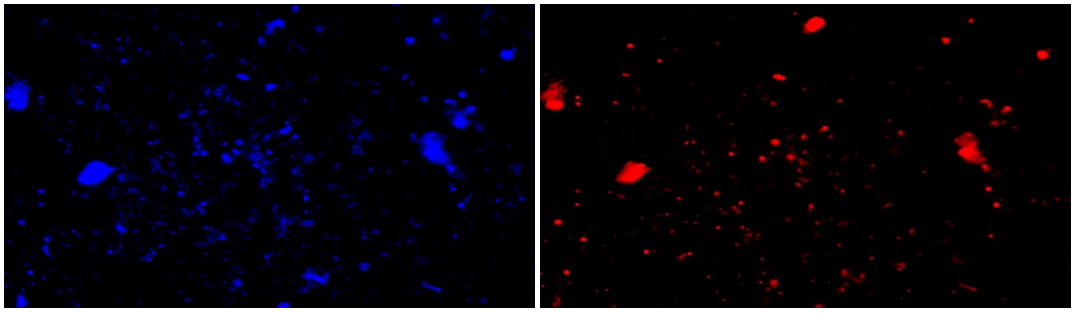


Figure B.3. Epifluorescence micrographs taken from serum bottles operated with 15th day manure.



MS1414 probe

Figure B.3. (continued) Epifluorescence micrographs taken from serum bottles operated with 15th day manure.