MICROBIAL COMMUNITY DYNAMICS DURING ANAEROBIC DIGESTION OF OTC MEDICATED COW MANURE USING DGGE AND CLONE LIBRARIES

by

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ABSTRACT

Veterinary antibiotics used in cattle medication may have inhibitory effects on microorganisms in anaerobic digestion of manure for biogas production as a renewable energy. Since microbial community plays an important role in digestion process, defining effects of antibiotics on microbial diversity can lead to improvements in understanding of interactions and processes, thereby obtaining better performance in terms of higher digestion capacity and biogas production. In this study, effect of OTC as a common veterinary antibiotic on biogas production and microbial communities was investigated with two microcosm tests: in the first part, OTC was externally added to manure slurries and in the second part, as in practical applications, a cow was medicated with OTC and manure samples of medicated cow was used as substrate in the microcosms.

In the first part, 90±2 L/kgTVS methane yield was observed after 30 days in control microcosms. Inhibitions in biogas productions were 41%, 57% and 61% in the 50 mg/L, 100 mg/L and 200 mg/L OTC added digesters, respectively. For 50 mg/L OTC added microcosms, retardation in the biogas production was observed and almost the same amount of biogas with control was produced after 60 days. Bacterial community similarities of OTC added microcosms to control were lower (64-92%) than archaeal community similarities (94-99%).

In the second part, methane yield of the control microcosms were 87±6 L/kgTVS. In medicated manure collected on day 1, 10 mg/kg OTC was measured, and the concentrations decreased to undetectable limit after 12 days. Biogas inhibitions in the manures collected on the first 5 days were between 50-60% whereas that of was 23% for day 10. Samples collected on day 15 and 20 produced similar amount of biogas with the control. Archaeal community similarities of manures collected on first 10 days to control were lower (73-93%) than bacterial community similarities (93-98%).

Mostly related species with biogas production was found as *Methanobacteriales spp.* in a syntrophic relation with *Clostridium spp.* Almost all bacterial and archaeal species were negatively affected by OTC.

ÖZET

Sığır tedavilerinde kullanılar antibiyotikler, dışkının biyogaz amaçlı anaerobik yıkımında rol alan mikroorganizmalar üzerine inhibör etkide bulunabilirler. Yıkım sürecinde mikrobiyal komünitenin önemli bir etkisi olduğundan, antibiyotiklerin mikrobiyal çeşitliliğe etkilerinin araştırılması, proses ve etkileşimlerin anlaşılmasında ilerlemelere yön verebilir. Bu çalışmada yaygın bir antibiyotik olan OTC'nin biyogaz üretimine ve mikrobiyal komüniteye etkileri iki mikrokozmos seti ile araştırılmıştır. Birinci sette OTC, dışkı sulularına dışarıdan eklenmişken ikinci kısımda, uygulamaları yansıtması açısından, bir ineğe OTC enjekte edilmiş ve OTC'li dışkılar substrat olarak kullanılmıştır.

İlk kısımda kontrol mikrokozmoslarında 30 gün işletme sonunda 90±2 L/kgUKM metan verimi gözlemlenmiştir. Biyogaz üretimindeki inhibisyonlar 50, 100 ve 200 mg/L OTC eklenmiş mikrokozmoslarda sırası ile %41, %57 ve %61 olarak bulunmuştur. 50 mg/L OTC, biyogaz üretiminde gecikmeyle sebep olmuş ve 60 gün işletme sonunda kontroldeki değere ulaşmıştır. OTC eklenmiş mikrokozmoslardaki bakteriyel komünitenin kontrole benzerliği (%64-92), arkeal komüniteye gore (%94-99) daha düşüktür.

İkinci kısımda, kontrol mikrokozmosları 87±6 L/kgUKM metan verimliliğine ulaşmışlardır. İlk gün toplanan gübrede 10 mg/kg OTC ölçülmüş ve konsantrasyonlar 12 gün boyunca azalarak devam etmiştir. İlk 5 gün toplanan dışkıların biyogaz üretimine %50-60 inhibisyonu gözlemlenmiş ve bu oran 10. gün toplanmış dışkıda %23 olarak bulunmuştur. 15. ve 20. günlerde toplanmış dışkılar, kontrol ile aynı miktarda biyogaz üretmişlerdir. İlk 10 gün toplanan dışkıların yıkıldığı mikrokozmoslarda arkeal komünitenin kontrole benzerliği (%73-93), bakteriyal komüniteye (%93-98) göre daha düşüktür.

Biyogaz üretimi ile ilişkili türler *Clostridum spp.* ile sintrofik bir ilişki içerisinde olan *Methanobacterium spp.* olarak bulunmuştur. Arkeal ve bakteriyel türlerin tamamına yakını OTC'den olumsuz olarak etkilenmiştir.

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LIST OF SYMBOLS/ABBREVATIONS

Symbol	Explanation
APS	Ammonium Persulfate
DGGE	Denaturing Gradient Gel Electrophoresis
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
EOTC	4-epi-Oxytetracycline
FISH	Fluorescent *in situ* Hybridization
gDNA	Genomic DNA
HPLC	High Pressure Liquid Chromatography
HRT	Hydraulic Retention Time
HS-HTP	N-7-(mercapN-7-(mercaptoheptanoyl)-L-threonine-03-phosphate
IC_{50}	The Half Maximal Inhibitory Concentration
NRB	Nitrate Reducing Bacteria
OHPA	Obligate Hydrogen Producing Bacteria
OLR	Organic Loading Rate
OTC	Oxytetracycline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcriptase PCR
SRB	Sulfate Reducing Bacteria
SRT	Solids Retention Time
TAE	Tris-Acetic Acid-EDTA
TEMED	Tetra Methyl Ethylene Diamine
TGGE	Thermal Gradient Gel Electrophoresis
TS	Total Solids
TVS	Total Volatile Solids
UV	Ultraviolet
VFA	Volatile Fatty Acids

1. INTRODUCTION

Recently, because of population increase and industrial developments, cheap and green energy production has become priority of all nations. Almost 90% of global energy consumption comes from fossil fuels. Depletion of natural energy sources and effect of these sources on climate change, forced all nations including Turkey to use of renewable energy sources (GRID-Arendal, 2001). Energy production from renewable energy sources except hydraulic energy made up 4% of world energy production as the same value reaches only to 1% in Turkey (Öztürk, 2005). Green energy production from renewable sources suggested to be a priority in Turkey, in the frame of European Union Integration Program. One of the renewable energy sources is biomass. Energy production from biomass is an ideal energy source under the defined criteria of the law, use of renewable energy sources for electricity production, in Turkey where agriculture and animal husbandry performed extensively (Karaosmanoğlu, 2004). Main component of biomass is manure. Anaerobic biodegradation of manure produces energy as biogas. Turkey's energy production potential from manure may provide 25% of annual energy demand which is 190 TWh in 2007.

In 27 countries of European Union (EU-27), more than 1500 million tons of manure is produced every year (Holm-Nielsen et al., 2009). 65% of this livestock manure is used in agriculture in Europe as a fertilizer. In use of manure as fertilizer, pre-treatment with anaerobic digestion is suggested (Chau, 1998). Secondly, anaerobic digestion of manure in biogas plants has gained popularity in Europe. Factors affecting the performance of biogas plants using manure as substrate are still being studied. The factors that affect the biogas production and anaerobic fermentation is claimed as natural conditions, transition to summer or winter feeding, composition of feed, feed quality and quality of bedding (Sarapatka, 1994). Additionally, veterinary antibiotics used in animal husbandry became a problem in digester systems. It is estimated that in 2001, total antimicrobial use in animals was more than 35 million pounds (app.16 kilotons) (Mellon et al., 2001). The problem is their low metabolisation. (Löscher et al., 2002). Type and concentration of antibiotics given to animals are thought to have an effect on the animal manure. The fraction of antibiotics in the manure may produce a toxic substance and inhibit the digestion step (Massé et al., 2000).

Tetracyclines are claimed to be the most widely used veterinary antibiotics in the world. They exhibit broad-spectrum antimicrobial activity against a variety of pathogen bacteria and used in human therapy and livestock industry (Thrile-Bruhn, 2003). Metabolisation and absorption of tetracyclines by the organism is known to be in very small portions and most of the tetracyclines can be found in the excreta without any change (Sarmah et al., 2006). As a member of tetracyclines family, oxytetracycline is a common antibiotic used in livestock animals due to the broad spectrum of activity and low cost.

Most of the studies of oxytetracycline effect on the anaerobic digestion of manure were based on the gas outputs. However, determination and identification of the microbial community and effects of antibiotics on this should be studied. The main problem in identification of community was unavailability of the microorganisms to be cultured from the gastrointestinal track or feces with classical *in vitro* methods since the growth conditions are not defined. However, recent molecular methods, such as polymerase chain reaction based amplification of 16S rDNA have dramatically expanded the availability of identification from the fecal materials. Experiments were successful in the identification of microbial species in human feces (Matsuki et al., 1999). These methods should be applied in the determination and identification of the microbial community in the manure.

In conclusion, microbial characterization of the manure in changing oxytetracycline environments is crucial. Since the petroleum based energy sources are running out, finding the mechanisms in biogas production and increasing its efficiency is very necessary. This study will provide a help to researchers of anaerobic digestion of manure in determining microorganisms survived and taking a role in the digestion and explaining the steps which oxytetracycline would be an inhibitor.

2. THEORETICAL BACKGROUND

2.1. Fundamentals of Anaerobic Digestion

Anaerobic digestion is a new technology to transform biomass into energy (Deublein and Steinhauser, 2008) and has gained interest due to increasing energy prices (Lettinga, 1995). In the anaerobic digestion, generally all types of biomass can be used as substrate as long as they contain carbohydrates, proteins, fats, cellulose and hemicelluloses as main components, such as liquid manure, sewage sludge, industrial waste water, waste grease and fat, plankton, sediments and also wood (Deublein and Steinhauser, 2008). All of these have led the anaerobic processes to be known for the unique ability to convert highly objectionable wastes into useful products (McCarty, 2001).

The process of anaerobic digestion results in lower energy release compared to other terminal electron accepting processes and therefore lower sludge yields. Lower sludge release means that most of the energy in the original substrates is stored in the biological fuel, energy rich biogas. These features make anaerobic digestion a net energy producer (Lettinga, 1995).

2.1.1. Biochemistry of Anaerobic Digestion

The microbiology and biochemistry of anaerobic digestion is a complex process which includes a number of microbial populations, linked by their individual substrate and product specificities (Hutnan et al., 1999). Organic pollutants are hydrolyzed and/or fermented into intermediate short-chain fatty acids in the first two phases of anaerobic digestion, then, they are degraded to acetate and H_2/CO_2. Acetate and H_2/CO_2 are converted into CH_3 in the last phase (Liu et al., 2002).

There are several models defining the biochemical steps in anaerobic digestion such as three-stage-model (Gerardi, 2003), six stage model (Lester et al., 1986) and nine-stage model (Harper and Pohland, 1986). The nine-step model is described below.

1. Hydrolysis of organic polymers to intermediate organic monomers
2. Fermentation of organic monomers
3. Oxidation of propionic and butyric acids and alcohols by obligate H_2 producing acetogens
4. Acetogenic respiration of bicarbonate by homoacetogens
5. Oxidation of propionic and butyric acids and alcohols by sulphate reducing bacteria (SRB) and nitrate reducing bacteria (NRB)
6. Oxidation of acetic acid by SRB and NRB
7. Oxidation of hydrogen by SRB and NRB
8. Acetoclastic methane formation
9. Methanogenic respiration of bicarbonate

In anaerobic digestion process there are numerous interactions between four major metabolic groups that are generally accepted as present in anaerobic digesters; hydrolytic-fermentative bacteria, proton-reducing acetogenic bacteria, hydrogenotophic methanogens, and acetolastic methanogens (Zinder et al., 1984). These microorganisms have spesific biochemistry to gain metabolic energy from the methanogenic pathway. (Whitman et al., 1982). Most of the methanogens have different metabolisms than each other. *Methanobrevibacter spp.* is only able to use H_2 + CO_2 for growth, whereas *Methanosaeta spp.* only uses acetate as their energy substrate. *Methanosarcina spp.* are more versatile that they use H_2+CO_2, methanol, methylated amines, acetate and pyruvate for growth and biogas production (Whitman et al., 1982; Jetten et al., 1992). Only specific substrates are utilised by methanogens so the anaerobic breakdown of organic matter is carried out by communities of different physiological types of anaerobic bacteria (Stams, 1994; Schink, 1997). Figure 2.1. shows substrate conversion patterns associated with anaerobic digestion.

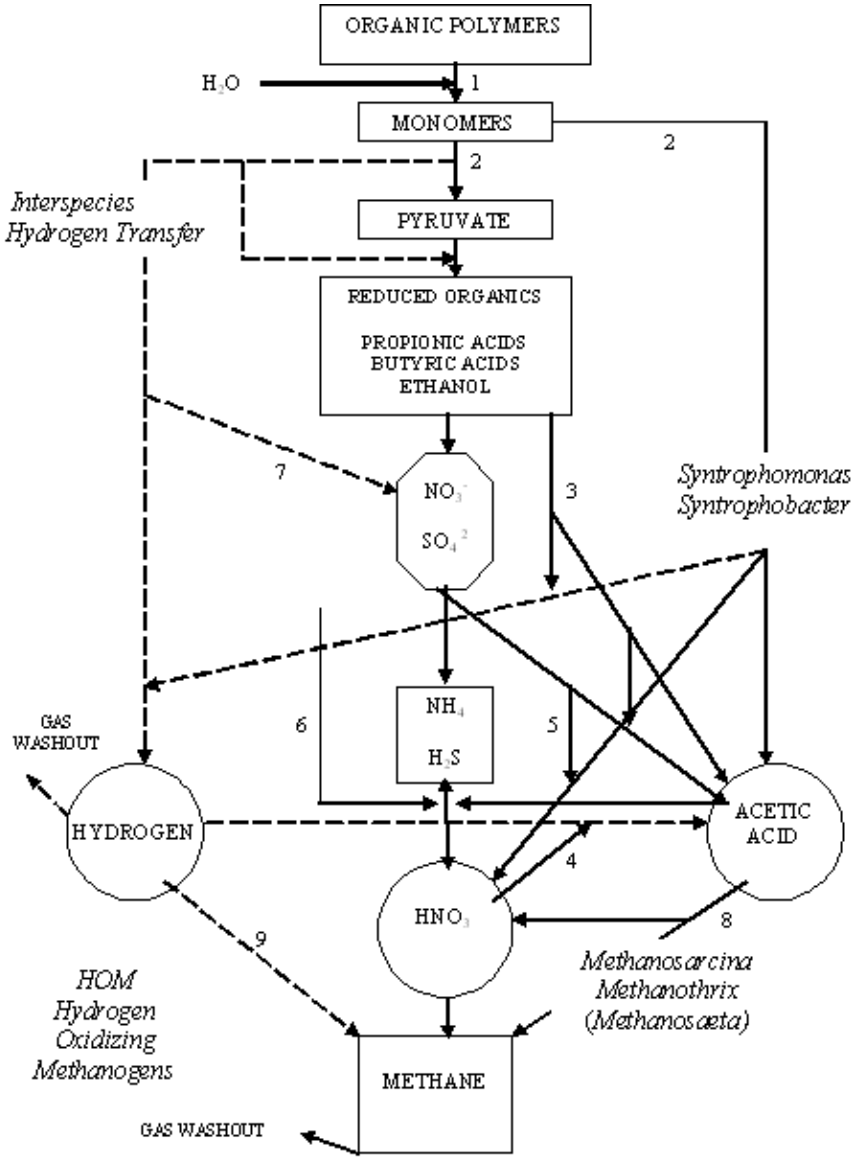


Figure 2.1. Substrate conversion patterns associated with the anaerobic digestion (Harper and Pohland, 1986).

2.1.2. Process Microbiology

There are several groups taking part in the anaerobic degradation of organic matter. These microorganisms are as follows:

1. Hyrolytic fermentative bacteria
2. Acidogenic (acid forming) bacteria
3. Hydrogen-producing acetogenic bacteria

4. Hydrogen-utilizing acetogenic bacteria
5. Carbon dioxide-reducing methanogens
6. Acetoclastic methanogens

Firstly, complex wastes are required to be degraded or hydrolyzed into units by microbial cells. Under anaerobic conditions, the hydrolysis of macromolecules is carried out by extracellular enzymes. The reaction rates of these enzymes are affected by the pH, cell residence time and the by-products produced by the hydrolytic bacteria.

In the anaerobic digestion process, hydrolytic bacteria are important for producing the simpler substrates which will be used in the later steps (Stronach et al., 1986). The degradation of compounds containing cellulose was stated to be done by *Clostridium spp.*, however, fats and proteins are more likely to be degraded by *Bacillus spp.* (Lema et al., 1991; Noike et al., 1985). The most abundant hydrolytic microorganisms are grouped as cellulytic (*Clostridium thermocellum*), proteoytic (*Clostridium bifermentas, Peptococcus spp.*), lipolytic (genera of *Clostridia* and *Micrococci*) and aminolytic (*Clostridium butyricum, Bacillus subtilis*) bacteria (Hungate, 1969; Payton and Haddock, 1986). In hydrolysis, pH and cell residence time are the notable factors.

After hydrolysis, monomers are converted by facultative and obligatory anaerobic bacteria to short chain organic acids, alcohols, hydrogen and carbon dioxide. The type of end products depends on the intermediately formed hydrogen ions. Propionic acid is formed by *Propionibacterium sp.* via succinate and acrylic pathway for carbohydrates. Butyric acid is formed by *Clostridium sp.* For fatty acids, beta oxidation occurs. For proteins, Stickland reaction occurs by *Clostridium botulinum*. Obligate hydrogen producing bacteria (OHPA) is responsible for the conversion of the short chain fatty acids other than butyric, propionic and other higher fatty acids to acetic acid, carbon dioxide and hydrogen. This process is called as β-oxidation in which in every reaction a molecule is removed from fatty acids with more than two carbons until all fatty acids are converted to acetate molecules. Propiponic and butyric acid conversions need energy input and inhibited at high hydrogen partial pressure, the step is slow and can be rate limiting step for the soluble part of anaerobic digestion. Acetic acid is produced from propionic acid by *Methanobacterium bryantii, Desulfibrio,* and *Synthrophobacter wolinii*. The common

organisms convert butyric, caproic and valeric acids to acetic acid are *Synthrophomonas wolfei* and *Syntrophus buswellii* (Malina and Pohland, 1992; Gujer and Zehnder, 1983).

Methanogenesis is the last step of anaerobic digestion where intermediates are converted to methane by *Archaea.* Growth rate of methanogenic archaea is slower than the acidogens, thus, methanogenesis is the rate limiting step in the whole process (Speece and Parkin, 1983; Noike et al., 1985; Malina and Pohland, 1992). Therefore, activity of methanogens is vital for maintaining efficient anaerobic digestion and preventing the accumulation of hydrogen and short chain fatty acids. Phylogenetically, methanogens belong to domain archaea. Archaea is consisted of microorganisms different from Bacteria (Eubacteria) and Eukarya. Archaeal cells possess of membrane lipids composed of isoprenoids ether-linked to glycerol or other carbohydrates, lack of peptidoglycan, and a distinctive RNA sequence (Woose, 1987). rDNA based 3 domain system is given in Figure 2.2.

Archaea includes also some extreme halophiles and extreme thermophilic, sulfur-dependent microbes (Woose, 1987). Methanogens in the archaea are given in Figure 2.3. The methanogenic archaea are strictly anaerobes and gain energy by using hydrogen, carbonmonoxide, formate and a few alcohols and producing CH_4 and CO_2. Alternatively, methanogens reduce methyl groups to methane. Some methanogens are able to use hydrogen as a substrate to reduce the methyl. The methanogenic classification belonging to substrate type is given in Table 2.1.

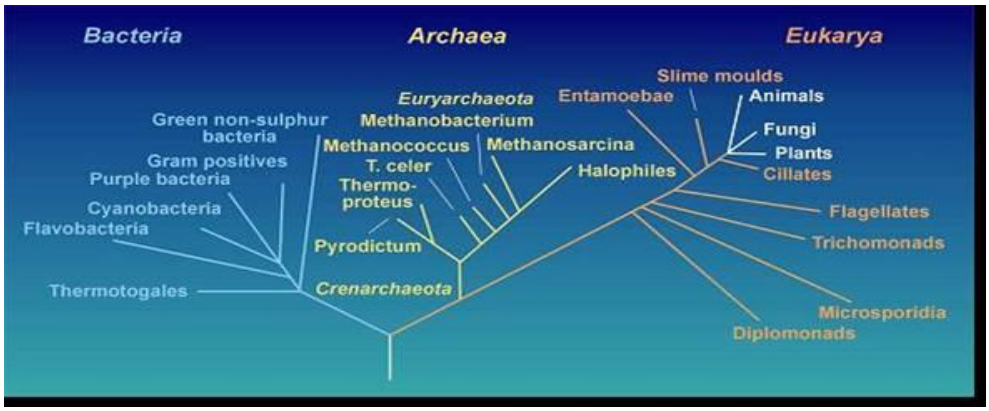


Figure 2.2. 3-Domain system (Madigan et al., 2002).

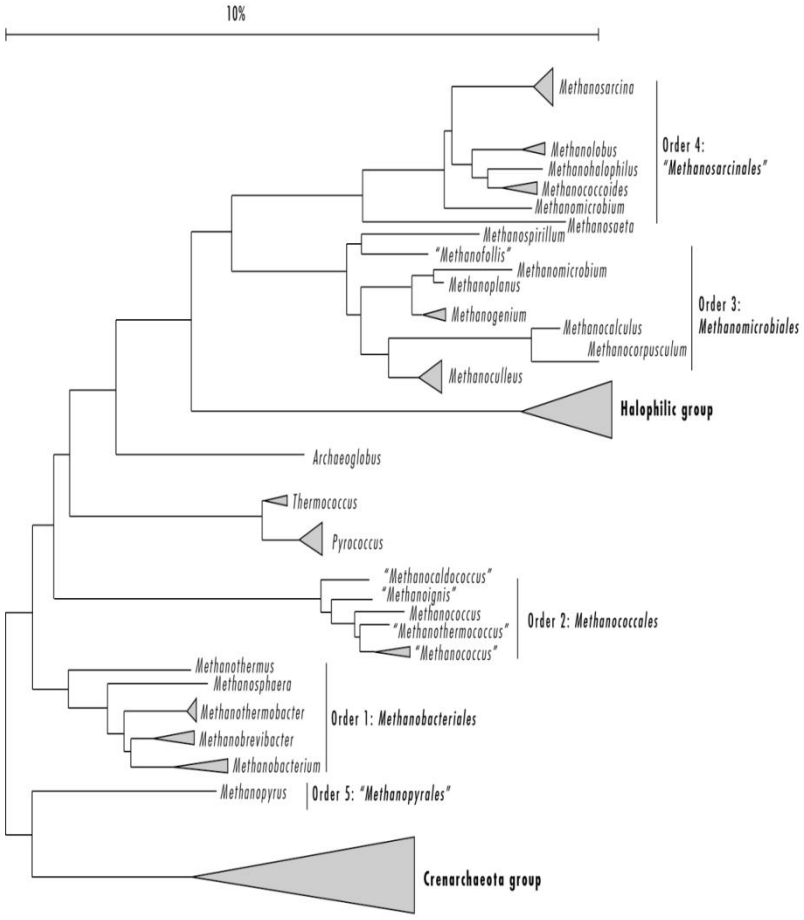


Figure 2.3. Phylogeny of methanogens, domain *Archaea*. (Non-methanogens are indicated by their group names, large triangles) (Garcia et al., 2000).

Table 2.1. Substrates converted to methane by various methanogenic archaea (Madigan et al., 2002).

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

In the first substrate type, carbon dioxide, formate and carbon monoxide are reduced to methane. The general equation for this conversion was given in Eq 2.1. This conversion is mostly hydrogen dependent, however, other substrates in this class can

supply the electrons for CO_2 reduction. This class is also known as hydrogenotrophic methanogens. These microorganisms include *Methanobacteriales, Methanomicrobiales, Methanococcales* and *Methanosarcinaceae.*

$$CO_2+4H_2\rightarrow CH_4+2H_2O \qquad \Delta G°=-131kJ/mol \qquad (2.1)$$

The second substrate group is methyl substances. The organisms responsible for methane formation using methyl substances are called ormethylotrophic methanogens and are limited to *Methanosarcinales*, except for *Methanosphaera spp*., which belong to the order *Methanobacteriales*. CH_4 production from methyl compounds can occur via 2 pathways. First mechanism is the formation of methane by reducing methyl group substances with an external electron donor such as H_2. Conversion of methanol in this way is given in Equation 2.2 Secondly, the methyl group substances can also be converted to methane without H_2. Equation 2.3 shows the conversion of methanol to methane in the absence of H_2.

$$CH_3OH+H_2\rightarrow CH_4+H_2O \qquad \Delta G°=-113kJ/mol \qquad (2.2)$$

$$4CH_3OH\rightarrow 3CH_4+CO_2+2H_2O \qquad \Delta G°=-319kJ/mol \qquad (2.3)$$

Acetate is the last type of substrate used by methanogens. Since acetate is the major product of fermentation, it is found in anaerobic digesters commonly, and represent a large portion of methane production. Acetate is catabolized by cleavage, with the carboxyl group being oxidized to CO_2 and the methyl group being reduced to CH_4. Methane producing reaction is catalyzed by the enzyme methyl-CoM reductase which converts methyl-CoM and HS-HTP to methane and a heterosulfide consisting of HS-HTP and CoM-SH. This reaction is usual for all methanogens, not depending on the first substrate type. The subsequent reduction of the heterodisulfide to CoM-SH and HS-HTP is coupled to the generation of a proton motive force. This is the most important step for energy conservation. In the first step, acetate is converted to acetylcoenzyme A (acetyl-CoA), which the expenditure of energy is required in the process. This conversion can occur by two different reactions. Conversion of acetate to acetyl-CoA requires one energy-rich

phosphate bond of ATP in *Methanosarcina spp*. However, in *Methanosaeta spp.* acetate is activated using an acetyl-CoA synthetase. This reaction requires two energy-rich phosphate bonds of ATP. This means that *Methanosaeta spp.* use more energy for acetate activation than *Methanosarcina spp*. Only two genera of methanogens can catabolize acetate, which are *Methanosarcina* and *Methanosaeta*.

2.2. Environmental and Operational Factors Affecting Anaerobic Digestion

One of the factors affecting anaerobic processes is the temperature. System is affected by the temperature in several ways including ionization equilibrium, solubility of substrates, substrate removal rate and other constants such as specific growth rate, decay biomass yield, and half saturation constant. Temperature is a very important parameter in the metanogenesis step of the anaerobic digestion (Strover et al., 1994). Methanogenesis can be carried out both in the mesophilic and the thermophilic temperature environment. In municipal wastewater treatment plants, anaerobic digestion is carried out in the mesophilic range at temperatures from 25°C up to 40°C with the optimum at approximately 35°C. Thermophilic digestion operates at temperature ranges of 50–65°C. Thermophilic digestion can be preferred if pathogen removal is necessary. On the other hand, in the thermophilic operation, toxicants are much likely to affect the system. Mostly bacteria is not sensitive to temperature until the metanogenesis step, however, methanogenic A*rchaea* is highly sensitive to even small temperature changes (Noike et al., 1985; Speece, 1983).

The hydraulic retention time (HRT) depends on substrate characteristics and environmental conditions. It must be long enough to permit sustaining anaerobic bacteria in digesters. The retention times of mesophilic and thermophilic digesters range between 25 - 35 days.

Solids retention time in anaerobic digesters is the same with hydraulic retention time if recycling or supernatant withdrawal is not applied. SRT can be also the basis for the reactor volume. The digestion process is a function of time required by microorganisms to digest the organic material, so SRT and volume of the digesters should be chosen correctly. The shortest SRT in anaerobic digesters is 10 days at 35°C. Shorter SRTs can result in washout of microorganisms. For digesters with solids retention time values longer than 10

days at the same temperature, volatile solids destruction changes are relatively small. Usually SRT in digesters is about 30 days for mesophilic digestion and longer for low-temperature digestion (Medcalf and Eddy, 2003).

The contact between the reactor contents and the biomass is supplied by mixing. Also, mixing reduces the negative effects of VFAs and other digestion products. Another advantage of mixing is that it avoids settling which could lead to reduction of substrate and microorganism contact. It is an energy consuming process but is applied to many treatment systems.

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).

Another important parameter affecting digestion is the pH. It affects the solubility of substances and the reaction behavior of microorganisms. As a result of this, digestion performance is directly in a relation with the pH. In the one-phase reactors, methanogenic pH requirements are taken into account. Most methanogens function in a pH between 6.5 and 7.5. *Methanomicrobium* can grow in pH 6.1. Deviations from optimum range may result in excess production and accumulation of acidic or basic conversion products such as organic fatty acids or ammonia, respectively. It has been shown that pH below 6.0 are inhibitory to methanogenic bacteria while acid forming bacteria can live at this pH and keep producing volatile fatty acids despite low pH, therefore making the environmental conditions worse (Pohlan and Suidan, 1987). Acidogenic microorganisms produce organic acids which can lead a pH decrease if alkalinity is not high enough. This pH reduction by the acidogenic microorganisms is buffered by the bicarbonate produced by methanogens under normal conditions. On the other hand, under adverse environmental conditions, the buffering capacity of the system can be upset, eventually stopping the production of methane. Acidity is inhibitory to methanogens than of acidogenic bacteria. An increase in

volatile acid level thus serves as an early indicator of system failure (Malina and Pohland, 1992).

Not only nitrogen and phosphorus, but also trace elements called micronutrients are required for methanogen's metabolisms (Speece and Parkin, 1983). Iron, nickel, magnesium, calcium, sodium, barium, tungstate, molybdate, selenium and cobalt are considered as necessary for various conditions of active methanogenesis (Henze and Harremoes, 1983). Selenium, tungsten and nickel are significant in the enzyme systems of acetogenic and methanogenic bacteria (Stronach et al., 1986).

A number of inhibitors are responsible for the failure of anaerobic digesters. Inhibition in the metanogenesis step generally results in a decrease in methane production and an increase in VFA concentrations. Metanogenesis is much sensitive to environmental conditions than other steps. Bacteria are affected permanently while Archaea can be acclimated to these compounds (Speece and Parkin, 1983).

The biological production in the anaerobic digestion may cause sulfides via sulfur containing inorganic compounds. Sulfate can inhibit metanogenesis due to both the competition for acetate and hydrogen by SRBs and the production of sulfide from the sulfate reduction by SRBs. Soluble sulfide concentrations less than 100 mg/L can be tolerated with a slight or no acclimation. Soluble sulfide concentrations between 100 and 200 mg/L do not show inhibitory effect after an acclimation period. Sulfate concentrations higher than 200 mg/L had a direct inhibitory effect on anaerobic systems (Stronach et al., 1986).

Although it is an important buffer, high concentrations of ammonia can cause failure in digestion systems. Ammonia can be found as ammonium ion (NH_4^+) or dissolved ammonium gas (NH_3). These compounds are in equilibrium with each other at neutral pH. However, higher pH causes to shift the equilibrium to ammonia gas. Ammonia concentrations less than 1000 mg/L reported to have no adverse effect on methanogens, whereas up to 3000 mg/L ammonia may have inhibitory effects at higher pHs.

Two important fermentation types occur complementary on each other: butyric and propionic acid fermentations. In the burtyric acid fermentation, buryrate, acetate, hydrogen and cabondioxide are produced. In propionic acid fermentation, propionate, acetate and some valerate are produced. Propionic acid fermentation does not produce a significant gas production (Dinopolou et al., 1988). Accumulation of VFA produced by acidogenic bacteria is the most common inhibition. High VFA accumulation indicates to metanogenic failure in the system. High concentrations of VFA (butyric and propionic acid) in a system is toxic for microorganisms. Microbial growth inhibitions were reported in 35 mg/L acetic acid and higher than 3000 mg/L propionic acid concentrations. In the same study, butyrate was found as toxic at 1000 mg/L concentration (Ianotti and Fischer, 1983). Unionized VFA accumulation usually occurs at lower pH when the equilibrium goes to the left.

Heavy metal 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. Heavy metal ions inhibit metabolisms of microorganisms and inactivate their certain enzymes, however, trace amounts of heavy metals are essential for the microorganism activity.

Organic chemicals can be inhibitory to anaerobic microorganisms at high concentrations even they are energy source at low concentrations. These organic chemicals are poorly soluble in water and may accumulate in the digesters. There are several parameters affect organic chemical inhibition such as toxicant concentration, biomass concentrations, toxicant exposure time, sludge age, feeding, acclimation and also temperature (Yang and Speece, 1986). The inhibition concentration ranges are widely depending on the organic chemical type.

A common stress in the anaerobic degradation of the manure is veterinary antibiotics used in animal husbandry. These antibiotics are very important in veterinary medicine to prevent common diseases or curing them (Arıkan, 2008; Thiele-Bruhn, 2003; Thile-Bruhn and Beck, 2005). Most common antibiotic groups in the veterinary medicine are tetracyclines, sulfonamides, beta lactams, aminoglicozydes and macrolyds (Kumar et al., 2004).

Veterinary antibiotics have a low metabolisation in the animal depending on antibiotic type. About 23% of oxytetracycline was excreted in the manure without any change in a study (Arıkan et al., 2006). In a study, antibiotics were obtained to be as high as 216 mg/L (Kumar et al., 2005). In another study, tetracycline, sulfonamide, and fluorokinolone were detected as 46 mg/kg, 91 mg/kg and 8.3 mg/kg, respectively (Martinez-Carballo et al., 2007). Treatment of antibiotic containing manure includes several processes such as ozonation (Ötker and Balcıoğlu, 2008).

Oxytetracycline (OTC) is a widely used antibiotic of tetracyclines group. OTC was first isolated from *Streptomyces rimosus* in 1940s. Tetracyclines have a broad spectrum of activity against bacteria (Giguere et al., 2007). Tetracyclines are protein synthesis inhibitors that binding to ribosome results in the inhibition of protein synthesis. Information on OTC is given in Table 2.2. Figure 2.4 illustrates the structure of OTC.

Table 2.2. Information on OTC.

Systematic name	(4S,4aR,5S,5aR,6S,12aS) -4-(dimethylamino)-3,5,6,10,11,12a-hexahydroxy -6-methyl-1,12-dioxo-1,4,4a,5,5a,6,12,12a-octahydrotetracene -2-carboxamide
Formula	$C_{22}H_{24}N_2O_9$
Molecular mass	460.434 g/mol
Half-life	6-8 hours
Excretion	Renal

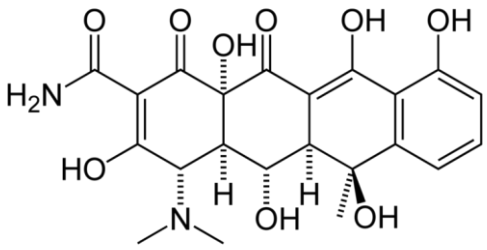


Figure 2.4. Structure of OTC.

OTC applications in veterinary medicine are common, especially in livestock farming for therapeutic purposes and growth promotion. In Europe, use of tetracyclines as

growth promoters was banned in 1970s (EC directive 73/264) (Castanon, 2007). However, the use as a promoter is still legal in some countries including U.S.A. and Australia. Widely use of OTC results in the accumulation in manure, and when the manure is used as a substrate in biogas production, can be a problem. Oxytetracycline is poorly metabolized and excreted in manure. In the digestive system of animals, OTC is degraded into 4-epi-Oxytetracycline (EOTC), a-apo-oxytetracycline (a-Apo-OTC), and b-apo-oxytetracycline (b-Apo-OTC). These metabolites can also be inhibitors for the microbial communities playing role in the anaerobic digestion.

Antibiotics in the animal manure are inhibitors of the biogas production in the anaerobic digesters. It is found that, 10 mg/L oxytetracycline containing animal manure produces 27% less biogas (Arıkan et al., 2006). In another study, 32% , 40% and 49% inhibition in biogas were observed in the manures containing 12.5 mg/L, 37.5 mg/L and 75 mg/L OTC, respectively (Gamel-El-Din, 1986).

The problem with the antibiotics is their inhibitory effect on the microbial community taking role in the process. Also, the antibiotics can affect the substrate degradation and lead the volatile fatty acids to accumulate, thus, system balance can be lost (Amin et al., 2006). There are several studies also with the granular sludge. In these studies, different inhibitions were observed with the existence of different antibiotics. In one of them, chlorotetracycline had a higher inhibition on the use of acetate and butyrate than propionate. IC_{50} value of chlorotetracycline was found as 40 mg/L. These inhibition concentration values are various by means of antibiotics. IC_{50} values of the chloramphenicol, higromycin B, Rifamphycin and neomycin were found as 26 mg/L, 210 mg/L, 250 mg/L and 500 mg/L, respectively (Sanz et al., 1996). In the studies with the granular sludge, since methanogens are usually deep in the granules, may have survived and not suffered the antibiotics. The species or genus affected by the antibiotics in the process is unknown. There are a number of studies with the engineering parameters, especially with the biogas production, however the microbial effects were not studied (Massé et al., 2000; Arıkan et al., 2006; Arıkan, 2008; Lallai et al., 2002).

2.3. Biogas from Anaerobic Digestion Systems

2.3.1. A New Energy Source: Biogas

Until recently, fossil fuels have been used as the main energy source. As a result of this, greenhouse gases accumulated in the atmosphere have caused a change in the climate. Carbon dioxide in the atmosphere increased to 375 ppm in the last 50 years from 313 ppm. The increase in carbon emission can be seen in Figure 2.5 (Rohde, 2007). Not only in carbon dioxide, but also methane in the atmosphere has increased in the last 50 years. From 1200 ppb to 1600 ppb methane in the atmosphere has dangerously increased (The Scientific Basis, 2003).

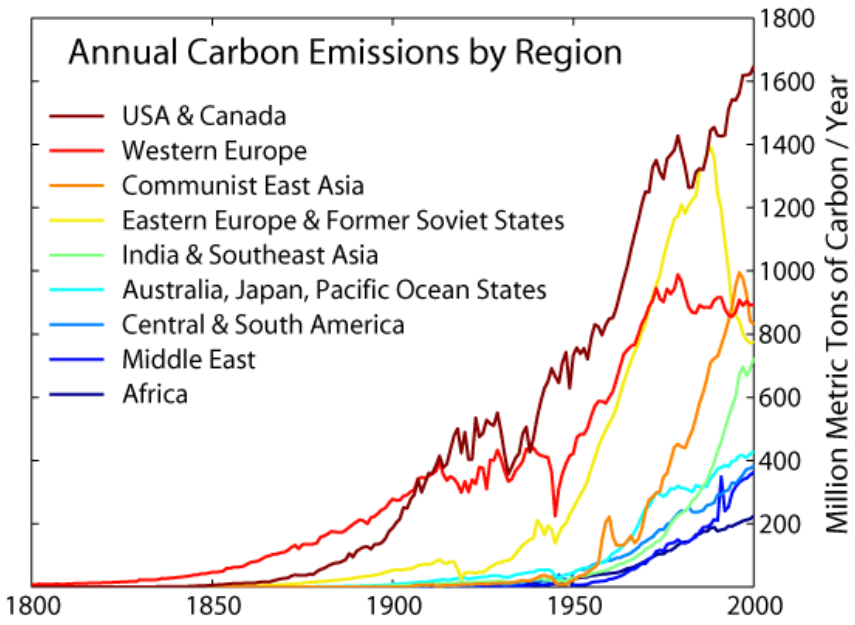


Figure 2.5. Annual carbon emissions by region (1800-2000) (Rohde, 2007).

Recently, the governments have been heading towards alternative energy sources which have less carbon emission, to decrease the greenhouse gases in the atmosphere and prevent the climate change. In the Kyoto protocol, research on renewable energy sources was suggested (United Nations, 1998).

Biomass energy is much useful than other renewable energy sources. Biomass can be found almost everywhere and can be stored. Because of this, biomass is a non-stop energy source comparing to other sources such as wind energy. Another advantage of biomass is its availability for both electricity and heat (Karaosmanoğlu, 2004). Biogas systems can be used as heating source by small services such as farms. In larger services, a biogas facility can be used as electricity generator. Thus, biogas can be used as household, commercial and also industrial applications.

One of the sources of biomass to be used for biogas production is the animal manure. Table 2.3 shows numbers of biogas plants and yearly energy productions in European Union countries. Animal manure is a very rich source by means of organic matter. Chemical Oxygen Demand can be higher than 45000 mg O_2/L. The biomass of manure, a very rich source, fades in the environment away when it is not managed with the anaerobic digestion. Anaerobic digestion of the manure converts organic matter to biogas, a usable energy.

Table 2.3. Numbers of biogas plants and yearly energy productions in EU countries (Öztürk, 2005).

Country	**Biogas Plants**	**Biogas Production**	
		TWh/yıl	**PJ***
Austria	>90	1410	32900
Denmark	39	45150	1.052.090
Germany	2200	-	-
Greece	2	0.001	33
The Netherlands	3	1.960	45.630
Italy	70	6.880	160.250
Norway	4	0.620	14.450
Portugal	16	1.520	35.300
Spain	6	0.026	625
Sweden	12	19.430	452.700
Switzerland	59	1.790	41.700
England	31	-	-

*:1052090 J =1.05 PJ,

Averagely 15% of global energy use is covered by biomass. This percentage can be higher in developing countries up to 38%. In Turkey, energy is generated only from hydraulic and thermal reactors and alternative energy production is almost zero. About 12

million cattle and 30-40 million sheep is present in Turkey and digestion of manures defecated by these animals can generate 25% of annual energy production. Biomass energy is expected to increase the energy use per capita in Turkey which is very low comparing to European and OECD countries (Öztürk, 2005).

2.3.2. Feedstocks for Biogas Production

For biogas production, any kind of biomass which contains carbohydrates, fats, proteins, cellulose and hemicelluloses as main component can be used as a substrate in biogas production via anaerobic digestion. The composition of biogas and methane yield is related with substrate, digestion system and retention time (Braun, 2007). The theoretical gas yield depends on the carbohydrate, protein and fat content of the substrate. In Table 2.4 maximal gas yields and theoretical methane contents are given. The real methane content in practice is usually higher than the values given in the table, because a part of carbon dioxide is solubilized in the digestate.

Table 2.4. Maximal gas yields and theoretical methane contents (Baserga, 1998).

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

[a] Only polymers from hexoses, not inulins and single hexoses

Recently, most of the agricultural plants digest manure from pigs, cows and chicken with the addition co-substrates to increase the methane yield. These co-substrates are harvest residues and food waste from households and energy crops (Weiland, 2010). Table 2.5 shows biogas yields of several substrates.

Table 2.5. Biogas yield values of several substrates (Kuhn, 1995).

Substrate	DM[a] (%)	ODM[b] (% DM)	C/N	Biogas yield (m^3CH_4/kg ODM)
Chicken manure	15	77	7	0.2–0.4
Pig manure	5–7	77–85	5–10	0.2–0.3
Raw glycerol (biodiesel)	> 98	90–93	–	0.69–0.72
Rotten potatoes	25	79	25	0.5–0.6
Clover	20	80	12	0.4–0.5
Apple slops	25	86	30	0.3–0.4
Spent grain	20–22	87–90	10	0.6–0.7
Bread (waste)	90	96–98	42	0.7–0.75
Cacao peels	95	91	2.8	20–22
Molasses	80	95	14–27	0.3
Whey	95	–	27	0.5–0.6
Rape seed slops	92	97	9–12	0.58–0.62
Green waste	60–75	30–70	40–80	0.2–0.6
Flotating sludges (fat)	5–24	83–98	–	0.6–0.8
Feces (intestinal)	12–15	80–84	17–21	0.2–0.3
Rumen (pressed)	20–45	90	11–20	0.6–0.7
Animal meal	8–25	90	–	0.5–0.8
Fat (separators)	35–70	96	–	0.7 (1.0)
Grass	21–23	76–80	22–24	0.45–0.5

[a] dry matter
[b] organic dry matter

2.3.3. Process Technology of Biogas Plants

Various process types are applied for biogas production which can be classified as wet and dry fermentation.

In wet fermentation, digesters are operated below 10% TS that allows the application of completely stirred tank digesters. The digested material can be pumped and spread on fields for fertilization. Solid substances such as energy crops are mixed with liquid manure in order to achieve suitable TS concentration. Dry digestion processes are operated with TS concentrations between 15% and 35%. Dry digestion processes are operated both continuously and batch while the wet digestion processes are operated only continuously. Today, wet digestion processes are much used than dry digestion processes in the agricultural sector (Weiland, 2008).

Many types of biogas plants are applied (Schulz and Eder, 2001). The most common wet fermentation reactor is the vertical continuously stirred tank fermenter. It is applied nearly 90% of modern biogas plants in Germany (Gemmeke et al., 2009). Often, the fermenter is covered with a gas tight single or double membrane roof to store the gas in the fermenter top before utilization. Stirring must be applied using mechanical, hydraulic or pneumatic mixing in order to contact microorganisms with the substrate, to facilitate the up-flow of gas bubbles and to achieve constant temperature in the whole digester. Almost 90% of biogas plants use mechanical stirring equipment (Gemmeke et al., 2009).

Horizontal digesters are also applied in anaerobic digestion for biogas. These are plug-flow systems equipped with a low rotating horizontal paddle mixer. Since higher TS content can be operated, paddle mixers are used in the first stage of a 2-stage reactor. Because of technical and economical aspects, reactor volume is limited to 700 m^3 (Weiland, 2010).

2-stage digesters are also available for biogas production (Figure 2.6). Advantage of these digesters is the separation of hydrolysis and methanogenesis stages. Ideal pH value for hydrolysis is 5.5-6.5 and for methanogenesis 6.8-7.2 (Vieitez and Gosh, 1999). A disadvantage of 2-stage is that the control of operation and process parameters are difficult. Also, in an improper hydrolysis stage, methane and hydrogen can be formed in extent amounts which cause energy losses and when the gas from hydrolysis is emitted to the atmosphere, it can negatively affect the climate (Oechsner and Lemmer, 2009).

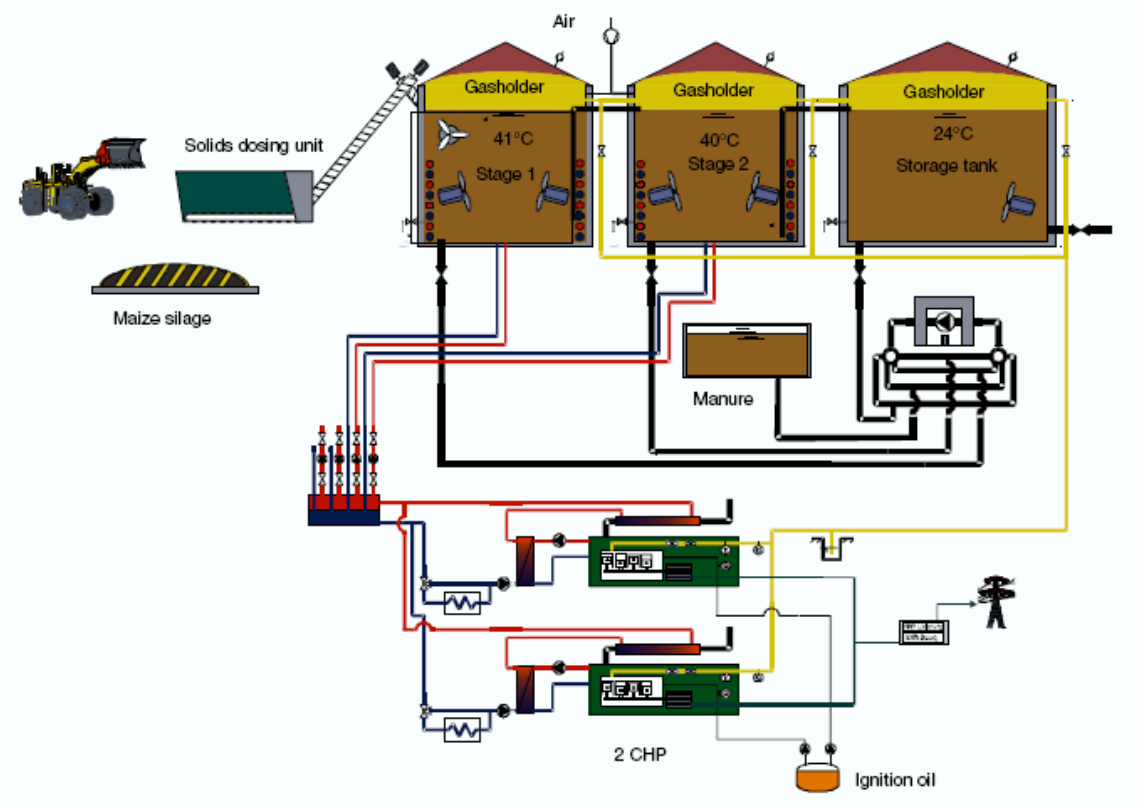


Figure 2.6. A 2-stage agricultural biogas digester (Weiland, 2010).

The main components of biogas are methane and carbon dioxide with smaller amounts of hydrogen sulfide and ammonia. Biogas is saturated with water vapor. Biogas must be desulfurized and dried before utilization to prevent the damage on biogas utilization units. Biogas produced by the co-fermentation of manure can contain H_2S between 100 and 3,000 ppm. H_2S concentration should be decreased to at least 250 ppm by biological desulfurization (Schneider et al., 2002). In this process, small amount of air is injected to the raw biogas to oxidize H_2S. *Sulfobacter oxydans* bacteria must be present to convert H_2S into elementary sulfur and sulfurous acid. Since *S. oxydans* is found in the digester, it does not have to be added. (Schneider et al., 2002).

2.4. Molecular Methods Used in Microbial Ecology of Anaerobic Digesters

2.4.1. Characterization of Microbial Communities using Molecular Tools

Classical microbiology techniques used in identification of environmental microorganisms are mostly based on cultivation dependent methods on selective growth media. These methods have certain limits which prevent an efficient identification of the community. Since there are many groups of microorganism difficult to grow, this technique is not able to address whole microorganisms.

In early years of modern microbiology, the most common method for identification of microorganisms was cultivation dependent method. The main limitation of this method was cultivability of a small fraction of all microorganisms. Microorganisms living in anaerobic environment are hard to grow because of low growth rates, syntrophic interactions and unknown growth requirements. Also cultivation dependent methods cause cultivation shift by favoring a normally not favorable microorganisms by changing competitions. Therefore a microbial community cannot be cultured as whole and cultured microorganisms do not reflect microbial community. The cultivable microorganisms make up 0.1%-10% of all microorganisms on earth (Amann et al., 1995; Hugenholtz et al., 1998; Muyzer et al., 1993; Muyzer, 1999; Lim et al., 1999; Gouillou et al., 1999).

Despite the developments in the microscopy, direct microscopic analyses have many limitations in identifying microorganisms. The small size of prokaryotic organisms, the absence of distinguishing phenotypic characters, and the fact that most of these organisms cannot be cultured are the most important factors that limit the evaluation of the biodiversity (Pace, 1997). In last 20 years, a significant number of studies dealing with microbial biodiversity involve the use of molecular tools and have often focused on investigating the dynamics of the composition and structure of microbial populations and communities in defined environments, and the impact of specific factors, such as pollution by xenobiotics on microbial diversity (Morris et al., 2002; Ranjard et al., 2000).

Molecular biology tools increased understanding of composition, dynamics and interactions within microbial ecosystems. Molecular phylogeny has provided a new basis

for the direct identification and quantification of microorganisms (Olsen and Woese, 1993). Nucleic acids are biomarkers and hereditary molecules most probably because of their important role in protein synthesis, making them one of the earliest evolutionary functions in all cellular life-forms. (Woose, 1987).

Particularly, 16s rRNA, and its encoding genes are ideal biomarkers. 16S rRNA is found in all prokaryotes and has conserved and variable sequence regions. (Woose, 1987). It is possible to design general and specific primers and probes for the study of evolution to species level (Amann et al., 1995). The rRNA is highly conserved in nucleotide sequence as well as in secondary structure since its function remains same through years of evolution. Random changes in the variable regions occur time to time and reflect to evolutionary relationship of organisms.

There are several molecular biology approaches in the studies on microbial ecology of the anaerobic reactors, a summary is given in Figure 2.7 and Table 2.6.

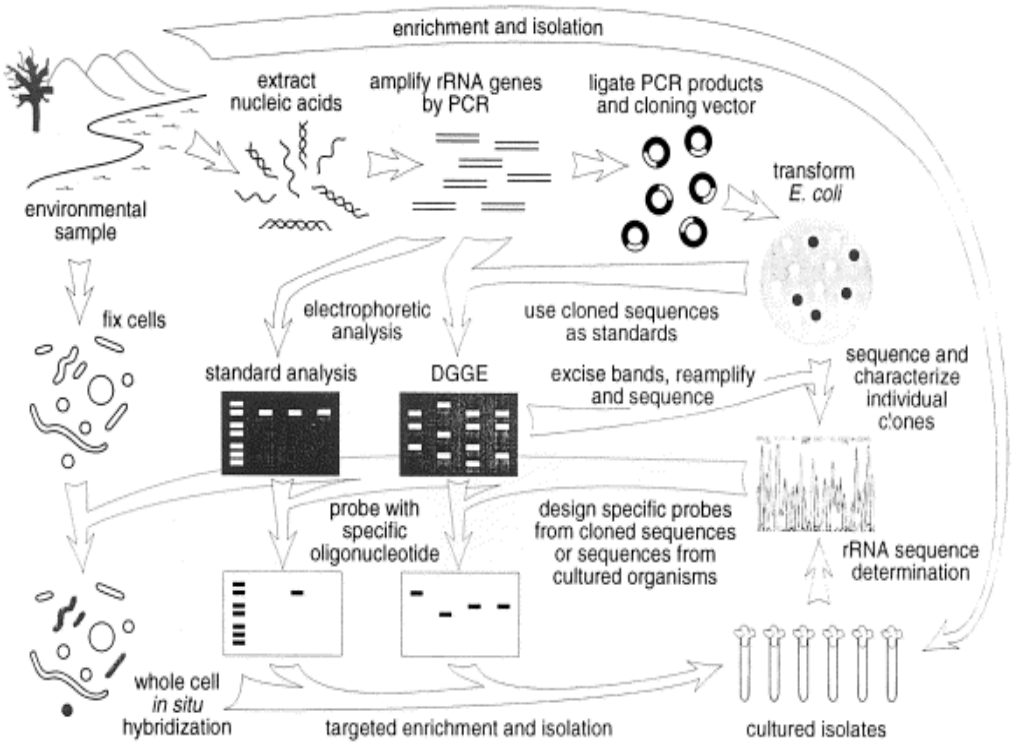


Figure 2.7. Summary of phylogenetic methodologies used in microbial ecology (Scow et al., 2004).

Table 2.6. Molecular biology applications in microbial ecology (Giraffa & Neviani, 2001).

	Taxonomic resolution	Applications to microbial ecology
Genetic fingerprinting of microbial communities		
DGGE/TGGE	Community members (genus/species level)	Dynamics between microbial populations in different natural environments
SSCP	Community members (genus/species level)	Mutation analysis; dynamics between microbial populations in different natural environments
T-RFLP	Community and population members (genus/species/strain level)	Strain identification; dynamics between and within microbial populations in soils, activated sludge, aquifer sand, termite gut
LH-PCR	Community members (genus/species level)	Dynamics between microbial populations in aquatic and soil microbial environments
PCR-ARDRA	Community members (species level)	Automated assessment of microbial diversity within communities of isolated microorganisms
RISA/ARISA-PCR	Community members (species level)	Estimation of microbial diversity and community composition in freshwater environments
AP-PCR	Population members (strain level)	Automated estimation of microbial diversity (typing) within lactic acid bacteria populations
AFLP	Community and population members (genus/species/strain level)	Automated estimation of microbial diversity within communities (species composition) and populations (typing) of various Gram positive and Gram negative bacteria
Competitive PCR	Community members (species level)	Detection of microbial cells into the VNC state in freshwater samples
Fluorescence in situ techniques		
Fluorescence in situ hybridization	Community members (species level)	Detection of viable cells within bacterial communities from environmental samples or food ecosystems
Fluorescence in situ PCR	Community members (species level)	Detection of viable, slow growing cells within bacterial communities, particularly pathogens in clinical specimens

2.4.2. Polymerase Chain Reaction (PCR)

Amplification of DNA segments via Polymerase Chain Reaction (PCR) using thermostable DNA polymerase was one of the most important advancement in molecular biology and opens wide range of alternatives of usage DNA in the field of environmental microbiology (Saiki et al., 1992).

PCR is used to amplify specific regions of a DNA strand. This can be a single gene, just a part of a gene, or a non-coding sequence. PCR process mainly based on three steps: Denaturation, annealing, and extension. In denaturation step double stranded DNA templates melted and separated by high temperature. In annealing step the reaction temperature is lowered so that the primers can attach to the single-stranded DNA template. Then temperature is increased again to a level (72 °C mostly) in which Taq polymerase can elongate the chain by adding nucleotides (dNTPs). This cycle of binding of primer and elongation and then disassociation repeated 30-40 times to recover enough DNA segment of interest. The addressed sequence amplified in order of 2 (2^n where n is the cycle number). The resulted product will be run on an agarose gel to monitor efficiency of the PCR. Mostly Ethidium Bromide (EtBr) is used to stain DNA which renders DNA visible under UV light.

Although the general steps and ingredients are well defined, there will be small corrections or changes according the purpose of PCR or products planned to have. The changes can be made in enzyme concentration, dNTP concentration, magnesium concentration, annealing and extension temperatures and times, cycle number and other reaction components.

PCR is one of the most important tools in molecular techniques. It is very powerful but without doubt it has also some limitations. First of all, DNA polymerase is not 100% trustworthy in transcribing DNA. Approximately 0.02-0.3% incorrect nucleotides are incorporated during amplification (Bej et al., 1991). The contamination present in template like humic acids, phenolic compounds or chelating agents will decrease efficiency and fidelity of Taq polymerase. To overcome this problem the DNA purification methods were developed. Due to processive characteristics of Taq polymerase, the depletion of nucleotides may increase the error rate. Primer dimer formation is possible when primers complement each other at 3’ end (Bej et al., 1991). Creation of recombinant or chimeric products is another problem. This problem mostly arises when target sequence of primers was shared in other DNAs other than template. Mostly mixed culture DNA like environmental sample may create chimeric sequences of different species (Amann et al., 1995).

Most common problem regarding PCR comes from its power to amplify DNA. Sensitivity of PCR is so high even a very small amount of DNA (a single copy in theory) out of the sample DNA can be detected and amplified by Taq polymerase. An extreme sterilization and care needed in performing PCR. A negative control without a DNA template or DNaseI treatment of reagents can be done to prevent contamination caused by a foreign DNA (Schmidt et al., 1991).

Primer selection of PCR can produce DNA sequences at different taxonomic levels (strain, genus, species etc.). These sequences may belong to same organism or mixed culture of organisms. With the help of some molecular techniques, these specific sequences reveal secrets of mixed cultures or relation of microorganisms. In some studies different techniques were used to analyze same data. Although results are generally similar, some methods are less efficient in specific situations (Moeseneder et al., 1999; Casamayor et al., 2002; Nikolcheva et al., 2003; Dorigo et al., 2004). Single-strand conformation polymorphism (SSCP), Terminal-restriction Fragment Length Polymorphism (T-RFLP), Ribosomal Intergenic Spacer Analysis (RISA), Automated Ribosomal Spacer Analysis (ARISA) and Denaturing Gradient Gel Electrophoresis (DGGE)/Temperature Gradient Gel Electrophoresis (TGGE) are most common PCR-based methods used in Microbial Ecology.

There are a number of techniques used in microbial ecology which are not integrated with the PCR, thus the problems and biases of the PCR are overcome. Fluorescent *in situ* hybridization and DNA re-association analysis are main non-PCR based methods. However, it is not possible to amplify gene fragments as fast as PCR based techniques.

2.4.3. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) have been studied for almost 17 years in microbial ecology. In spite the principle is similar to SSCP and TGGE, DGGE becomes much effective, easy and fast in application. In DGGE, PCR amplified gene sequences with same length are run in denaturing gradient polyacrylamide gel and separated by its melting domain, literally

according to sequence (Myers et al., 1987). Double stranded DNA will melt in discrete segments called melting points due to increasing denaturant concentration. Each melting point is sequence specific therefore each melting and separation of double strand occurs in specific melting temperature (Tm). As the DNA partially melted at the melting point, branched molecule decreased in mobility and separated from other DNA molecules with different melting points. DGGE exploits the fact that DNA molecules that have the same length, but differ at least by one nucleotide, can be separated by electrophoresis through a linear gradient of increasing chemical denaturants of urea and formamide.

DGGE and TGGE were introduced to environmental sciences by Muyzer's studies (Muyzer et al., 1993). It becomes a routine technique to monitor microbial diversity and their dynamics. The technique becomes a highly used technique in environmental microbiology after its introduction. Rapid and reliable results favor it and versatility of the technique makes it possible of its usage in a wide range area

DGGE can be used to determine the genetic diversity of a microbial community without identifying its individuals. It can be used to compare different communities like soil samples, bacterial and archaeal communities (Heuer and Smalla, 1997; Ovreas et al., 1997).

In some cases, microbial ecologists often require to have samples spanning long time periods. As cloning is not suitable to use in this kind of study, DGGE becomes a savior for the scientists. With DGGE, different samples taken at different times can be analyzed and compared in one gel. The simultaneous analysis makes it a powerful tool to analyze microbial community changes over time (Çetecioğlu et al., 2009). DGGE is one of the commonly used techniques to screen clone libraries. Rapid and reliable results of DGGE decreases the amount needed to perform clone libraries (Kowalchuk et al., 1997).

DGGE is well known and abundantly used technique in environmental microbiology. It is very powerful and has usage in different areas. But like all techniques it has limitations. Many limitations may be avoided by careful planning and performing of experiments but some of them are inevitable.

The band excision is a powerful feature of DGGE but it has some difficulties since mostly a band will consist of 150-200 bp DNA which is rather short for a phylogenetic analysis. Co-migration of bands also will give a poor identification in the sequencing. This problem may be overcome by having a clone library screening. It is then combines both techniques' powerful aspects. Another problem with excision is distance of two bands. In some cases bands are too close; a proper excision of bands cannot be made. Also during excision step UV may affect DNA and in re-amplification it may create ambiguous sequences.

The DGGE has biases also because it is a PCR dependent technique. DGGE is being negatively affected by biases of PCR like fidelity error of polymerase or chimeric products. Therefore it has been accepted that DGGE is a semi quantitative method since number of bands and intensities may be affected by PCR.

The choice of the primer set and the optimization of the gel running conditions before the technique can be used to screen for sequence polymorphism of a particular gene are the main limitations and the difficulty of comparing patterns across gels, when these patterns include numerous bands. This implies that multiple gels and different combinations of samples are required if numerous samples are being investigated (Muyzer et al., 1993).

2.4.4. Molecular Cloning, Sequencing and Phylogenetic Analysis

Molecular cloning is a primary genetic engineering procedure that any genome analysis is possible with cloning. Using molecular cloning, large quantities of genes or chromosomal fragments can be isolated in pure form (Madigan et al., 2002). Also, cloning can be used to identify the organisms in an environmental sample. The DNA fragments can be produced after digestion with restriction enzymes of the DNA extracted from a sample (i.e., shotgun cloning), or after PCR or RT-PCR (if RNA is the template) (Hofman-Bang et al., 2003). 16S rRNA clone library is an important approach in microbial diversity studies (Giovannoni et al., 1990).

Cloning PCR products is a rapid and convenient application but has biases (Ward et al., 1992). Compared to cloning after PCR, shotgun cloning introduces less bias and produces clones of multiple genes at the same time (Pace, 1997). Additionally, different rRNA gene fragments may be cloned with different efficiencies. This is also time consuming and labor-intensive for the study of the vertical structure of communities in multiple sample analysis.

2.5. Aim of the Study

It has been pointed out that green energy production has gained interest all over the world including Turkey. Manure is among the most popular substrates used for the production of biogas in which inhibitory substances like veterinary antibiotics are commonly detected. Although effect of these substances on biogas production has been studied, effect on microorganisms take place in the process is unknown. This study aims to evaluate the effect of OTC, a commonly used veterinary antibiotic, on microbial community structure and biogas production.

3. MATERIALS AND METHODS

There were two sets of microcosms operated during this study. In the first one, specific amounts of OTC were added externally to the manure to be used as substrate. In the second one, OTC was injected into a cow and manures of the cow were sampled for 20 days. OTC concentrations in these samples were measured and microcosms were set up using these manures selected by their OTC concentrations.

3.1. Serum Bottle Tests

3.1.1. Manure Sampling and Animal Medication

A female, Holstein race, 3.5 years old, 440 kg body mass dairy cow was kept in a pen at the Istanbul University Veterinary Faculty Barn. The manure in rectum was collected and stored at 4 °C until later use as the "unmedicated manure". The dairy cow was then medicated once with 50 mL Oxytetracyline injection solution (20 mg/kg). This is a standard dosage in veterinary practice. Equal doses were injected to right and left body between *musculus semitendinosus* and *musculus semimembranosus* muscles. Manure was collected from rectum every 24 hours for 20 days and used throughout the experiments as "medicated manure".

3.1.2. Manure and Seed Characteristics

Characteristics of the manure samples prior to slurry preparation are given in Table 3.1. Seed sludge with TS and TVS concentrations 24.2 g/L and 17.7 g/L, respectively, was taken from an anaerobic digester using cattle manure as substrate.

Table 3.1. Manure characteristics.

Sampling Day	TS, %	TVS, %	TKN, mg/kg	Alkalinity, $mgCaCO_3/L$	Total*** C/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

*Used as control in experiments that OTC is added externally to the manure. **Used as control in experiments that medicated manure is used as substrate. *** 95% of total carbon was organic C and 90% of total nitrogen was organic N.

3.1.3. Microcosms Set-up

Manure samples were diluted with tap water and mixed with the seed sludge (1:4) to a final TS concentration of 5%. Prior to microcosm set-up, pH was set to 6.8. 40 mL slurry was added into 120 mL serum bottles (Figure 3.1). OTC concentrations of 50, 100 and 200 mg/L were added into nonmedicated slurries. In the second part of the experiments, medicated manure were collected on 1^{st}, 2^{nd}, 3^{rd}, 5^{th}, 10^{th}, 15^{th} and 20^{th} day and were used in microcosm test. All samples and negative control microcosms (for each set) containing no oxytetracycline were set-up in triplicate. Microcosms were flushed with nitrogen gas for 3 minutes to obtain anaerobic conditions. After that, microcosms were incubated on a shaker in a warm room at 100 rpm (37.1 ± 1.0 °C). Digestion time for first part and second part of this study were 60 and 30 days, respectively.

Figure 3.1. 120 mL serum bottle with 40 mL active volume used for manure slurry.

3.2. Chemical Analyses and Analytical Techniques

VFA, alkalinity, TKN and TS/TVS measurements were carried out according to American Public Health association APHA, 2005. Gas pressures were measured with a manometer (HACH PM-9107) for every 5 days. Gas compositions were determined using Gas Chromatograph HP Agilent 6850 with a thermal conductivity detector and HP Plot Q Column (30 m, 530 μm). Methane and biogas productions were calculated and given as the volume in ambient conditions (1 atm, 20 °C). VFA measurements were carried out in a Perkin Elmer Gas Chromatograph (Clarus 600) with an FID detector and Elite-FFAP column (30 m, 0.32 mm).

Oxytetracycline was commercially supplied (Agro Chemicals, Spain). All the liquid chemicals used in this study were HPLC grade except acetic acid glacial and oxalic acid. Double distilled water was used through the analysis. The 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 column used in this study was Inertsil ODS-3 HPLC column, (25 cm x 4.6 mm). An autosampler, SIL-10 AD was used for injection. The injection volume was 20 μl. Degas of the solvents was achieved by sonication in a transonic ultrasonic bath (ELMA D-78224, Singen/Htw) prior to use.

Results were analyzed 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 achieved, standards and samples were analyzed.

Stock standard solution of OTC was prepared by dissolving 100 mg of OTC in HPLC grade methanol and stored at -20 °C. A total of five working standard solutions were prepared in methanol at concentrations of 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, and analyzed within 2 days. In order to plot a calibration curve, serial dilutions of OTC standard solution prepared in 100% methanol, were analyzed by HPLC.

Prior to the extraction of samples, extraction efficiency was determined. 5 g nonmedicated 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.

Manure was extracted with a method modified from a previous study (Yuan et al., 2010). 5 g wet manure was put into 50 mL polycarbonate centrifuge tubes with 0.5 g oxalic acid ($C_2O_4H_2 \bullet 2H_2O$), 4 mL acetic acid and 7.5 mL of 90% methanol and shaken at 100 rpm 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 volumetric curve 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.

3.3. Molecular Analyses

3.3.1. DNA Extraction

After GC measurements, microcosms were sacrificed and DNA was extracted using FastDNA® SPIN Kit for Soil (Q-Biogene, Bio 101 Thermo Electron Corporation, Belgium). Approximately 0.5 g sample from manure slurries 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, Belgium) 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 Eppendorf tubes and 250 µl PPS reagent was added. To mix the composition, tubes were shaken by hands for 30 seconds. After mixing, the tubes were centrifuged again at 14000xg for 5 minutes to precipitate the pellet. 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, Japan.

3.3.2. Polymerase Chain Reaction (PCR)

Amplification of 16S rDNA gene sequences was performed by PCR using archaeal and bacterial specific primers. Bac8f-Bac1541r and Arch07f-Arch1384r primers were used

for the amplification 16S rDNA of bacteria and Archaea respectively. Extracted gDNAs were used as a template for these primers. Bac341f-Bac534r and Arch344f-Univ522r primers were used to amplify V3 region of 16S rDNA (approximately 200 bp long) of bacteria and archaea, respectively. Primers used in the molecular analyses were shown in Table 3.2 and their sequences were given in Table 3.3.

Table 3.2. Bacterial and archaeal oligonucleotide primers used for PCR amplification.

Primer	**Experimental Stage**	**Annealing Temperature, ^{0}C**	**Position**	**Reference**
Bact341f-GC	DGGE	55	341-357	Muyzer et al., 1993
Bact534r	DGGE	55	534-518	Muyzer et al., 1993
Bact8f	First round of nested PCR, sequence PCR, cloning	55	8-27	Edwards et al., 1988
Bact1541r	First round of nested PCR, cloning	55	1541-1522	Edwards et al., 1988
Arch07f	First round of nested PCR, cloning	52	07-24	Lueders et al., 2004
Arch1384r	First round of nested PCR	52	1384-1368	Lueders et al., 2004
Arch344f-GC	DGGE	53	344-358	Raskin et al., 1994
Univ522r	DGGE, sequence PCR	53	522-504	Amann et al., 1995
Arch855	Cloning	53	855-836	Shinzato et al., 1999

PCR reactions were performed in a 30 µl (total volume) mixture containing 0.6 µM forward primer, 0.6 µM reverse primer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1U of Taq polymerase enzyme and the buffer supplied with the enzyme (Fermentas Life Sciences), and 0.6 µl of template. Amplification was performed with a thermal cycler (TECHNE-TC 412). Conditions are given in Table 3.4. Products of all reactions were screened for the amplification of correct band size. All PCR products were 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). Gels were visualized by using a gel documentation system, Mitsubishi 91.

Table 3.3. Primer sequences used in 16S rDNA amplification.

Primer	Sequence (5'-3')	Reference
Bact341f-GC	**GC*** GCC TAC GGG AGG CAG CAG	Muyzer et al., 1993
Bact534r	ATT ACC GCG GCT GCT GG	Muyzer et al., 1993
Bact8f	AGA GTT TGA TCC TGG CTC AG	Edwards et al., 1988
Bact1541r	AAG GAG GTG ATC CAG CCG CA	Edwards et al., 1988
Arch07f	TTCYGGTTGATCCYGCC	Lueders et al., 2004
Arch1384r	CGGTGTGTGCAAGGAGCA	Lueders et al., 2004
Arch344f-GC	**GC*** GAC GGG GHG CAG CAG GCG CGA	Raskin et al., 1994
Univ522r	GWA TTA CCG CGG CKG CTG	Amann et al., 1995

***GC**: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG

Table 3.4. PCR conditions used in the study.

Primers	Denaturation	Annealing	Elongation	# of Cycles
Bact8f-Bact1541r	94 °C 45 sec.	55 °C 45 sec.	72 °C 60 sec.	30
Vf-Vr	94 °C 45 sec.	55 °C 45 sec.	72 °C 60 sec.	30
M13f-M13r	94 °C 45 sec.	55 °C 45 sec.	72 °C 60 sec.	30
Arch07f-Arch1384r	94 °C 30 sec.	40 °C 30 sec.	72 °C 60 sec.	35
Arch344f-GC-Univ522r	94 °C 30 sec	53 °C 30 sec.	72 °C 60 sec.	35

3.3.3. Denaturing Gradient Gel Electrophoresis (DGGE)

Acrylamide solution (30%), deionised formamide, urea and molecular biology grade ammoniumpersulfate were commercially supplied (Applichem, Germany). Both samples and the positive clones were run on an Ingeny phorU DGGE system (the Netherlands). The first step was the assembly of the perpendicular gradient gel sandwich. The thickness of the sandwich was established by using between two glass plates. Before assembly, glass plates were cleaned carefully with 70% EtOH to avoid any particle matter which may affect the gel. The position of spacers were checked to avoid any leakage and glass plate sandwich then placed on the casting stand. The next step was preparation of the denaturing gradient gel. 10% (w/v) acrylamide 40% denaturant solution was prepared by mixing 83 ml of 30% acrylamide with 5 ml 50xTAE (2.0 M Tris, 50 mM EDTA, and 1.0

M acetic acid) and 40 ml formamide and 42 g urea. 70% of denaturant concentration was reached by adding 70 ml formamide and 73.5 g urea to 83 ml of 30% acrylamide and 5 ml 50xTAE (2.0 M Tris, 50 mM EDTA, and 1.0 M acetic acid). Into both solutions, distilled water was added up to 250 mL. After solutions were prepared, they were filtered with 0.45 μm filter and sonicated for 15 minutes. The bottles were wrapped with foil paper to avoid sunlight and stored at 4 °C for further uses. Into two beakers, 25 mL of 10% (w/v) acrylamide solutions containing 40% and 70% denaturants were poured. To both solutions, 75 μl freshly prepared 20% ammonium per sulfate (APS) and 7.5 μl TEMED was added and immediately transferred to gradient forming system. With the gradient forming system and a pump, solutions were transferred to the form gel sandwich. After polymerization, a stacking solution (6-10 mL) excluding denaturants was mixed with APS and TEMED and added over the polymerized gel.

Electrophoresis tank was filled with 1xTAE until marked level and temperature was set to 65 °C. Sample loading step was started with preparation of samples. 4 μl of loading dye was mixed with 8 μL of PCR product to be run. Polymerized gel sandwiches placed to the core and then the core was inserted into the preheated tank. The comb was removed and wells were washed with 1xTAE buffer to avoid any early denaturation due to presence of denaturants in wells. The samples were carefully loaded into the wells. DGGE was conducted at a constant voltage of 100 V, 63-68 mAmp at 60 °C for 17 hours in 1xTAE containing electrophoresis tank.

The last step was staining and visualizing gels. The core was taken from the tank and gel sandwiches were separated from it. Glass plates were disassembled and the direction of gel was marked with a cut on the upper left corner. 50 μL of 1:100000 diluted SYBR Gold DNA staining dye was added to 500 mL 1xTAE washing buffer and gels were incubated for 15 minutes. Gels were distained with distilled water to remove background impurity. Gels were visualized by using a gel documentation system, Mitsubishi 91 (Japan).

For diversity analysis, DGGE images were converted, normalized and analyzed by using the Bionumerics 5.0 Software (Applied Maths, Belgium). Similarities between tracks

were calculated by using the Dice coefficient (S_D) (unweighted data based on band presence or absence) and UPGMA clustering. For analysis using Dice coefficient 0.7% optimization and 0.5% band position tolerance was applied. This was the minimum tolerance at which all marker lanes clustered at 100%. For intensity analysis, samples were clustered depending on band weights by using Pearson coefficient and UPGMA clustering.

3.3.4. Cloning

Bact8f-Bact1541r amplified bacterial PCR products and Arch344f-Arch855r amplified archaeal PCR products were cloned using TOPO TA Clonning® Kit (Invitrogen, USA). For this, 3 µL PCR product, 1µL salt solution, 1 µL TOPO vector and 1 µL sterile water were mixed and incubated at room temperature for 20 minutes. After incubation, 2 µL of mixture were transferred to One Shot Reaction Tube thawed on ice. This new mixture was incubated for 30 minutes on ice. After that, for 30 seconds at 42 °C heatshock was applied and immediately transferred on ice and 300 µL SOC medium was added on it. After incubation samples were shaken for 60 mins at 37 °C. Then, solutions were spread to 50 µg/mL Kanamycin plates. 100 µL solution was spread to each plate and incubated at 37 °C for 16 h.

After incubation, grown colonies were picked using sterile pipette tips and put into 50 µL sterile TE containing 200 µL Eppendorf tubes. Later, mixtures were boiled at 95 °C for 5 minutes and later frozen at -20 °C overnight. Next day, products were PCR amplified with M13f and M13r primers. Later, Bact8f-Bact1541r primers for bacterial colonies and Arch344f-Arch855r primers for archaeal colonies used in PCR. These products were used in sequence analysis.

3.3.5. Sequence Analysis

Clustered clone groups were taken to sequence analysis and matched with bands in the samples. Sequence PCR was reacted using 1 µL template, 0.3 µL primer (pA for Bacteria and Arch 855r for Archaea) 4.7 µL sterile water, 2 µL Big Dye Reaction mix and 2 µL Big Dye Reaction buffer. Products of PCR were mixed with 2 µL 3 M Sodium Acetate and 50 µL 95% ice cold ethanol. The mixtures were incubated for 30 minutes on

ice and centrifuged for 30 minutes at 14000 rpm. Supernatants were discarded and pellets were resuspended in 70% cold ethanol and centrifuged for 30 minutes at 14000 rpm. Later, supernatants were discarded and pellets were dried at 95 °C for 5 minutes. Pellets were resuspended in 20 µL hi-di formamide and denatured at 95 °C for 3 minutes. After that, samples were immediately transferred on ice and loaded to sequencer (A 3130 Sequencer, Abi Prism, USA) at the same day and sequenced automatically.

Outputs of the sequencer were analyzed by DNA Baser software and nucleotide sequence data was exported to Fasta format. Similarity search was done in EMBL environmental and prokaryote databases of European Bioinformatics Institute.

3.4. Statistical Analyses

Using PASW (Predictive Analytics Software) Statistics 18 (Polar Engineering and Consulting, U.S.A) t-test and correlations were calculated. DGGE gel pictures were analyzed with Bionumerics 6.1 (Applied Maths, Belgium) and outputs of Bionumerics matrixes were analyzed in MiniTab 14 (USA) for PCA analysis and Canoco 4.5 (Biometris, The Netherlands) for Canonical Components Analysis to determine the interactions between community, bands, OTC concentration and biogas production.

4. RESULTS AND DISCUSSION

During this study, 2 microcosm sets were operated. In the first part, OTC was externally added to the manure slurries in three concentrations of 50, 100 and 200 mg/L. Manure slurries were digested for prolonged 60 days to determine inhibition type. In the second part, 20 mg/kg animal weight OTC was injected to a cow, OTC medicated manure samples were collected for 20 days and used as substrates in microcosm tests. According to results of the first part, digestion time of the second part was chosen as 30 days. Both microcosm sets were operated at mesophilic conditions (37.1±1.0 °C) with 1.4 kgTVS/m^3d OLR and shaken at 100 rpm.

4.1. Effects of Externally Added OTC

4.1.1. Biogas Production Results

Biogas productions (Figure 4.2) were calculated using gas pressure data (Figure 4.1) and GC results. 315 mL biogas was produced in 30 days in the control bottles. After 30 days, there was no significant biogas production observed in control bottles. Inhibition in biogas production at the end of 30 days were 41%, 57% and 61% for the microcosms containing 50 mg/L, 100 mg/L and 200 mg/L OTC, respectively (Figure 4.3). Biogas production was observed in OTC present bottles until day 60. Inhibition in biogas production after 60 days digestion was 4%, 35% and 37% for the microcosms containing 50 mg/L, 100 mg/L and 200 mg/L, respectively. Figure 4.2 shows the behavior of the slurry microcosms referring to retardation in biogas production for 50 mg/L OTC concentration. In OTC present microcosms, substrate conversion was slower than in the control microcosms, thus, digestion lasted longer. In manure biogas digester systems, 60 days of digestion is too long to be applied. Also, biogas production of the control bottles stopped after 30 days. So, the biogas production after 30 days of digestion should be considered important

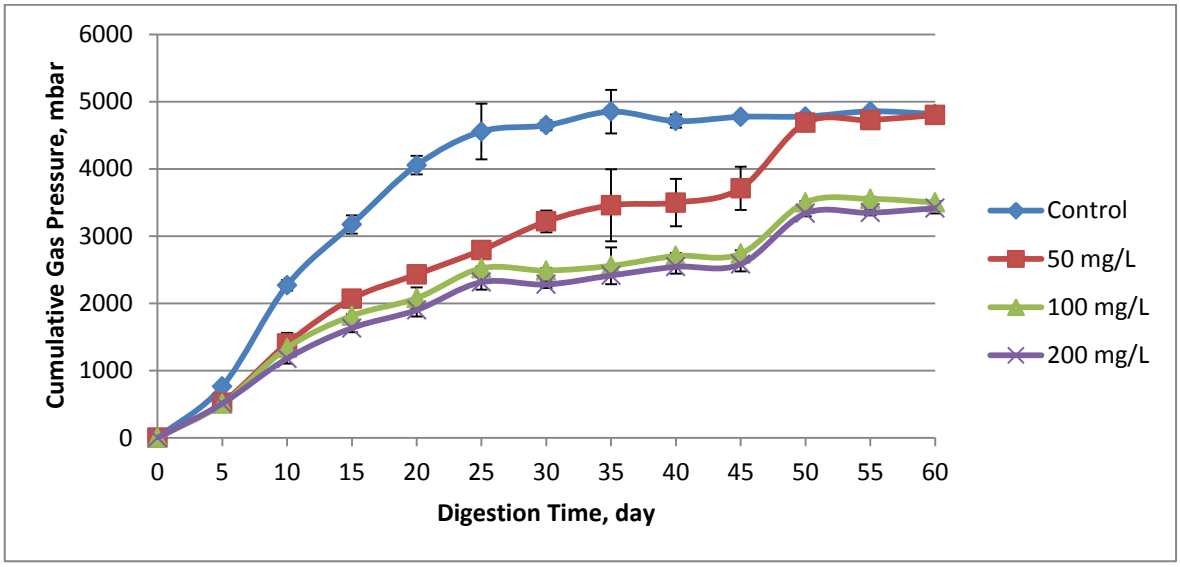


Figure 4.1. Cumulative gas pressures during externally OTC added microcosm tests.

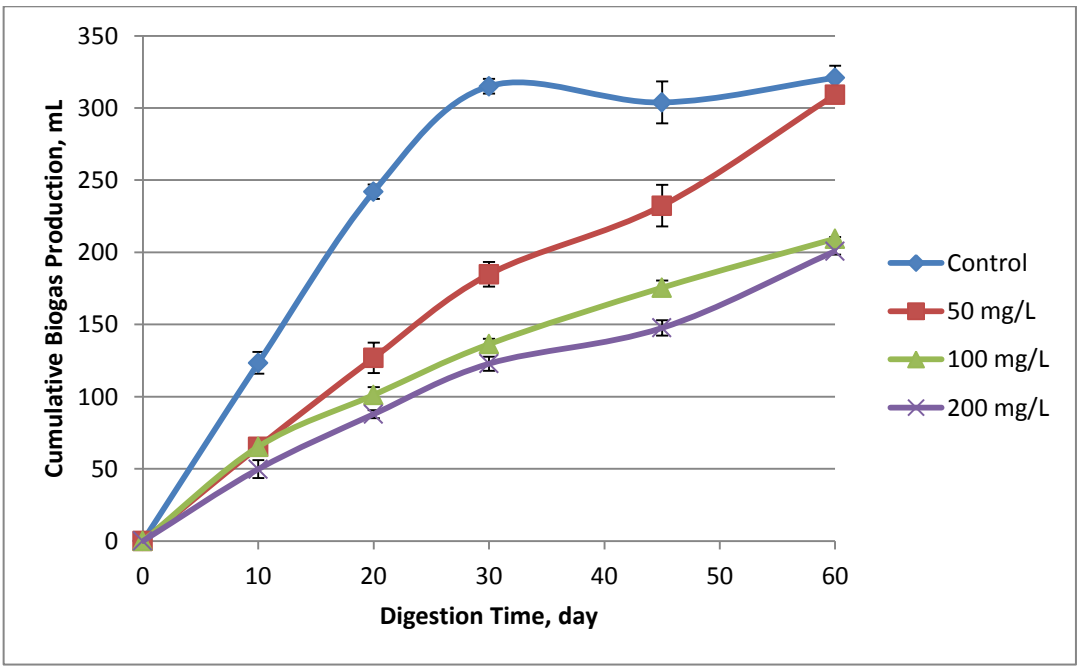


Figure 4.2. Cumulative biogas productions during externally OTC added microcosm tests.

There was a very large negative correlation (r=-0.714) observed between OTC concentration and biogas production ($p<0.01$). Hereby, effect of OTC was found extremely inhibitory to the microorganisms taking role in biogas production. The differences in biogas productions for all OTC concentrations after 30 days were significant. For the

samples digested longer than 30 days, inhibitions in biogas production decreased and OTC present microcosms continued to produce biogas until day 60. At the end of 60 days, biogas productions of the control and 50 mg/L OTC containing microcosms were almost equal (4% inhibition). Biogas volume of the 100 mg/L and 200 mg/L OTC containing samples were significantly different than each other, control and 50 mg/L ($p<0.05$). Inhibitions in biogas productions for these two OTC concentrations were clearly obvious. IC_{50} value of Oxytetracycline in biogas production in 30 days digestion of cattle manure slurry can be calculated as 68 (±0.5) mg/L.

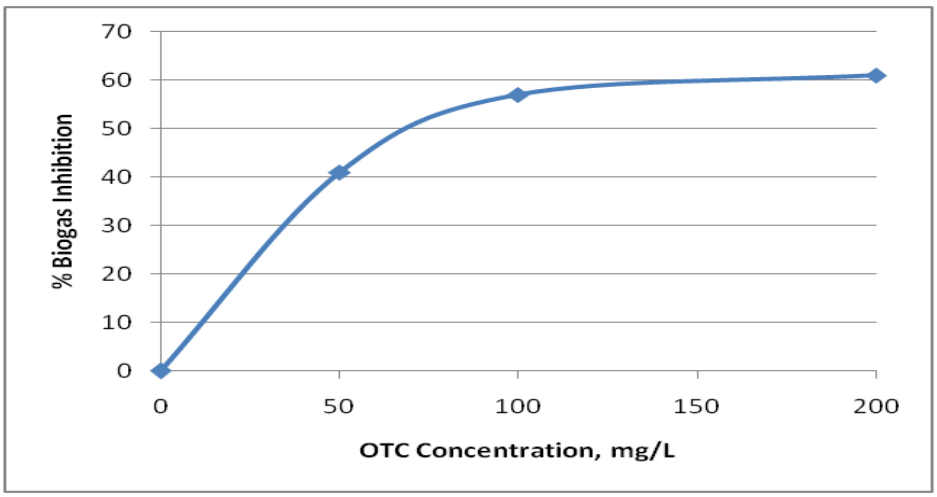


Figure 4.3. Biogas inhibition percentage with OTC concentration.

Quality of the biogas produced is related with methane content. When N_2 coming from the flushing was ignored, methane percentage in the biogas for all samples were 58% ±5% (Appendix C). No significant effect was observed in the inhibition of methane percentage of biogas ($p<0.05$, $n=60$). Thus, methane production shows the similar trend with biogas production except peak point. Methane production reached its maximum value in control microcosms slightly after 20^{th} day. Inhibitions in methane production in 20 days digestion for 50, 100 and 200 mg/L OTC concentrations were 49%, 55% and 65%, respectively. Control microcosms produced a small volume of methane after 20 days. Further digestion for methane production only resulted in the OTC present microcosms. Thus, inhibitions in methane productions were decreased until 60^{th} day. Methane production plot was given in Figure 4.4.

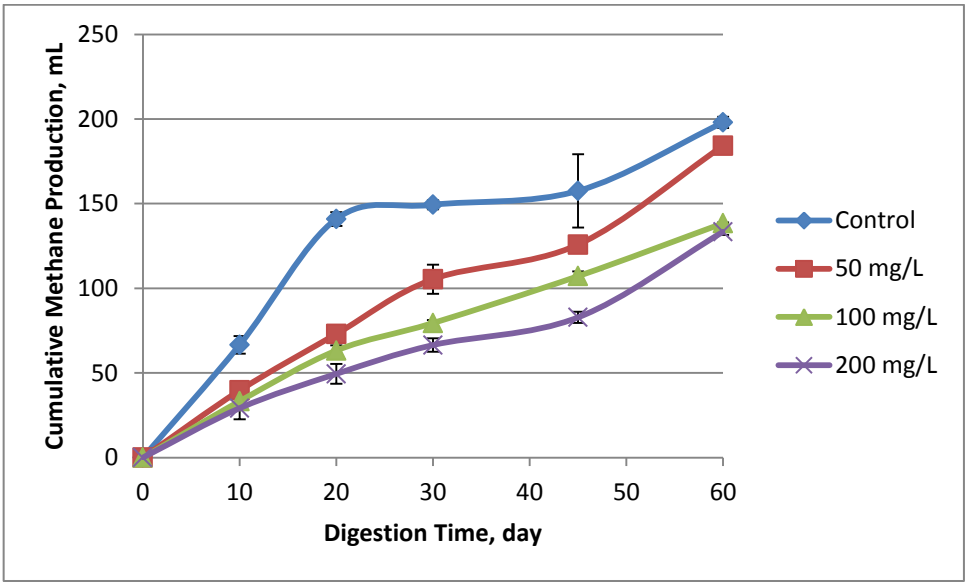


Figure 4.4. Cumulative methane productions during externally OTC added microcosm tests.

There was a large negative correlation between OTC concentration and both carbon dioxide (r=-0.576) and methane (r=0.452) production ($p<0.01$). Carbon dioxide production was much correlated than methane production with OTC concentration. This can be because of that OTC inhibited more bacterial cells than methanogenic *Archaea*.

Biogas and methane yields were given in Table 4.1.

Table 4.1. Biogas and methane yields during externally OTC added microcosm tests.

Digestion Time	**20 Days**		**30 Days**		**60 Days**	
Yield, L/kgTVS	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 mg/L OTC	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 mg/L OTC	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 mg/L OTC	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. Comparative table of similar studies on biogas production 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.

In a previous study, a continuous biogas digester fed with cow manure had 100 L/kgTVS methane yield at 50 days HRT at 25 °C which is similar to result of this study. The slight difference can be caused by the different reactor type, temperature and seed. In the same study, llama manure and sheep manure digesters reached 90 and 120 L/kgTVS methane yield. The highest methane yield was observed as 140 L/kgVS in the digestion of llama manure: sheep manure mixture (1:1) (Alvarez and Lidden, 2009). 208-268 NL/kgTVS biogas yield and 125-166 NL/kgTVS methane yield were reported in a

mesophilic digester (Amon et al., 2007). Comparing to the study of Arıkan et al., lower biogas was reported in this study (2006). Lower yield was probably caused by the low OLR used in this study, which was 1.4 kgTVS/m^3d. In 2009, 2.0 kgTVS/m^3d OLR was found as optimum for biogas production from manure (Alvarez and Lidden). Higher OLR could increase the methane yield, however, in higher OLRs, indications of instability is common and inhibitory compounds can cause system failure. Results of similar studies were given in Table 4.2 and 4.3.

Table 4.3. Summary of biogas reduction in manure digestion assays in the presence of oxytetracycline.

Manure Type	Antibiotic Type	Medication Type	Concentration, mg/L	Inhibition %	Reference
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
Cow	OTC	Intramuscular	1.0-3.3	50-60	This study
Cow	OTC	Intramuscular	0.2	27	This study

The inhibition in methane production in pig manure in a study in which OTC and CTC were tested together in equal concentrations, 10 mg/L, 50 mg/L and 100 mg/L resulted in 45.2%, 56.5% and 64.1% methane production inhibition, respectively (Alvarez et al., 2010). This was a batch test for 30 days. Comparing to result of this study, inhibitions in Alvarez et al.'s study were higher, showing the effect of extra antibiotic, chlortetracycline. Lower OTC concentrations were also studied. 1, 5 and 25 mg/L OTC resulted in 2%, 5% and 7% methane production decrease (Loftin et al., 2005). In contrast, OTC effect was reported to be not significant in a study with pig waste slurry (Lallai et al., 2002). The concentrations of OTC were 125 and 250 mg/L. This is contrary to our results because in this study, even 50 mg/L OTC inhibited biogas production by 41%. The same study of Lallai et al., amoxicillin had a slight effect on methane production in concentrations of 60 and 120 mg/L. Significant effect was reported with the 80 and 160 mg/L thiamphenicol present digestion.

4.1.2. VFA Results

Acetic acid concentrations in all the reactors were between 20 and 50 mg/L. 30th day sample of 200 mg/L OTC added microcosm and 45th day of 100 mg/L OTC added microcosm had higher acetic acid concentrations. Propionic acid concentrations were found about 500 mg/L in all OTC present reactors. In the control reactors, no propionic acid was found. Propionic acid accumulation was clearly observed in all OTC added reactors. Until 60th day, all the propionic acid was consumed in the OTC added reactors. Even propionic acid accumulated samples did not stopped producing biogas, and all the accumulated propionic acid were consumed. Higher propionic acid concentrations would have resulted in permanent failure of digesters. No isobutryic acid was found in the control reactors. For 50 mg/L OTC containing reactor, isobutyric acid concentrations were higher in 20th day however decreased and finished between 30th and 45th days. 100 mg/L and 200 mg/L OTC present reactors showed a similar trend in isobutyric acid concentration between 20th and 45th day. A slight increase was observed until 45th day. After 45th day, all the isobutryic acid in the 100 mg/L OTC added reactors were consumed, however, remained constant in the 200 mg/L OTC added reactors. Here, 500 mg/L isobutrycic acid inhibited biogas production for a period. Later, accumulated isobutyric acid was consumed. No isovaleric acid was found in the control reactors. 50 and 100 mg/L OTC present reactors contained about 60 mg/L isovaleric acid in the 20th day and all the isovaleric acid consumed until 60th day. 200 mg/L OTC present reactors had 40-60 mg/L isovaleric acid any time during digestion. There was no isocaproic acid in none of the reactors. Specific VFA concentrations measured in digesters were given in Table 4.4.

Table 4.4. Volatile fatty acid concentrations during externally OTC added microcosm tests.

OTC Concentration, mg/L	**Digestion Time, day**	**Volatile Fatty Acids Concentration, mg/L**			
		Acetic	Propionic	Isobutyric	Isovaleric
Control	20	20	0	0	0
Control	30	20	0	0	0
Control	45	26	0	0	0
Control	60	23	0	0	0
50	20	24	509	57	58
50	30	37	424	11	41
50	45	32	494	0	14
50	60	29	0	0	0
100	20	26	488	38	63
100	30	28	402	46	55
100	45	49	292	56	32
100	60	42	0	0	0
200	20	31	493	38	38
200	30	47	503	36	51
200	45	34	516	62	65
200	60	36	12	64	34

There was not a significant correlation between biogas production and acetic acid concentration. Biogas production was inhibited by propionic acid (r=-0.819) and isobutyric acid (r=-0.734) concentrations. Isovaleric acid was positively correlated (r=0.897) with biogas production ($p<0.01$). According to Pearson's correlations, biogas production was mostly inhibited by propionic acid. In literature, propionic acid fermentation was related with no specific biogas production (Dinopolou et al., 1988). Secondly, isobutyric acid had also significantly inhibited the biogas production. However, these VFA concentrations were not above the limit that causes failure of the system. Retardation in biogas production can be explained by moderate VFA accumulation.

Methane productions were related with volatile fatty acids accumulation similar to biogas production. The differences were that methane production inhibition was less correlated with acetic acid (r=-0.305) and isobutyric acid (r=-0.679), and higher correlated with propionic acid (r=-0.865) comparing to biogas production correlations with VFA accumulations. Significance value was lower than p=0.01.

4.1.3. Community Fingerprinting with DGGE

Community fingerprints were analyzed with Bionumerics software. In the dendograms, OTC refers to the first part of experiments, the first number refers to OTC concentration in the microcosm and the second number refers to digestion time.

In bacterial community, according to presence/absence of the bands, for control experiments, first 20 days were close to each other however not close to the days 30-60. Differences depending on the OTC concentration were also observed. Accoring to the band intensities (pearson coefficient), it was observed that pattern changed with OTC concentration much than digestion time. Thus, different OTC concentrations resulted in different clusters of community. The similarities are given in dendograms in Figure 4.5 and 4.6 and similarity matrixes in Appendix A.

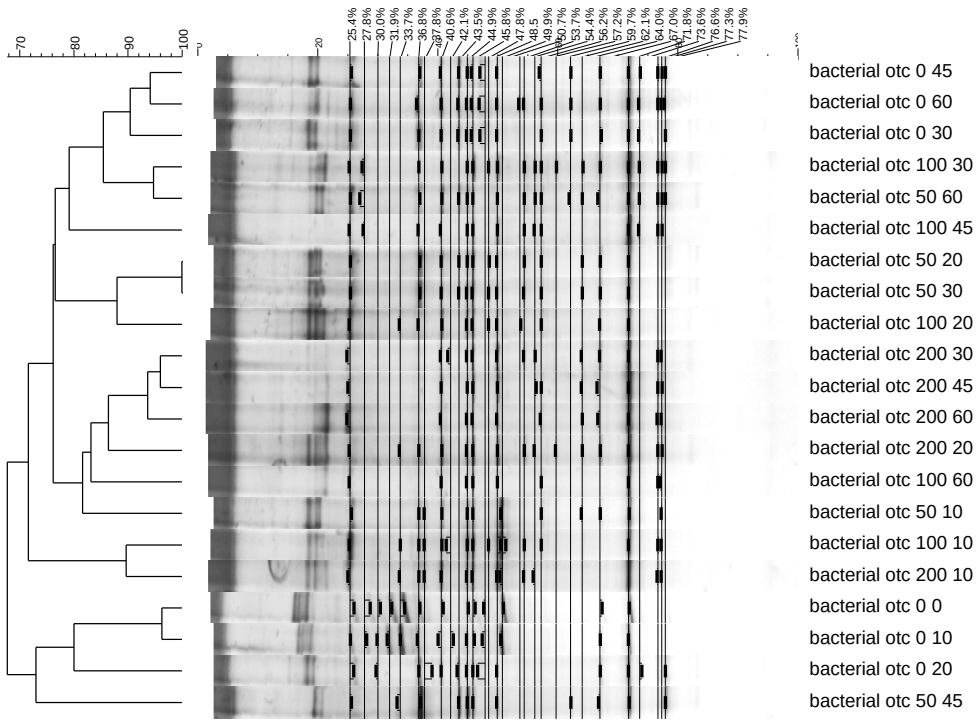


Figure 4.5. Bacterial community dendogram according to band presence/absence.

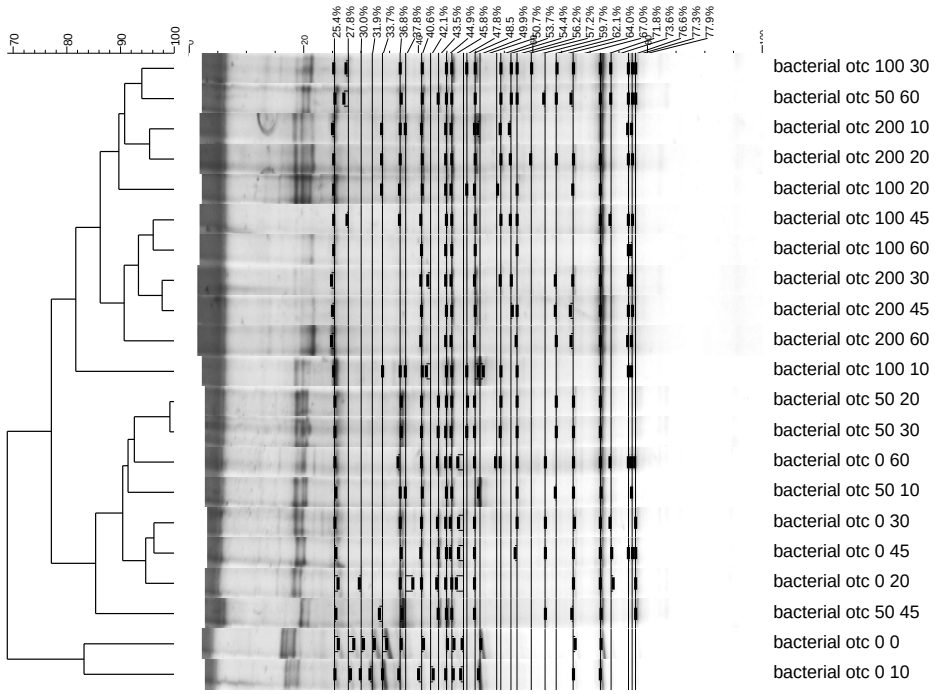


Figure 4.6. Bacterial community dendogram according to band intensities.

Results of PCA analysis of bacterial samples showed that the first two components should be chosen. 1st and 2nd components had 63.8% and 11.6% coverage of the matrix. Using the 2 components, 75.4% of the matrix could be narrowed for correlation analyses. The first component was not correlated with biogas production, however, the second component showed a large correlation with biogas production ($r=0.502$, $p<0.05$). The PCA similarity analyses showed a similarity with Pearson analysis of Bionumerics. Since it could be confusing to show all the samples on a plot, 30th day samples were given in Figure 4.7. According to this, bacterial community in control was similar to 50 mg/L OTC. Also, bacterial communities in 100 mg/L and 200 mg/L OTC added microcosms were very similar.

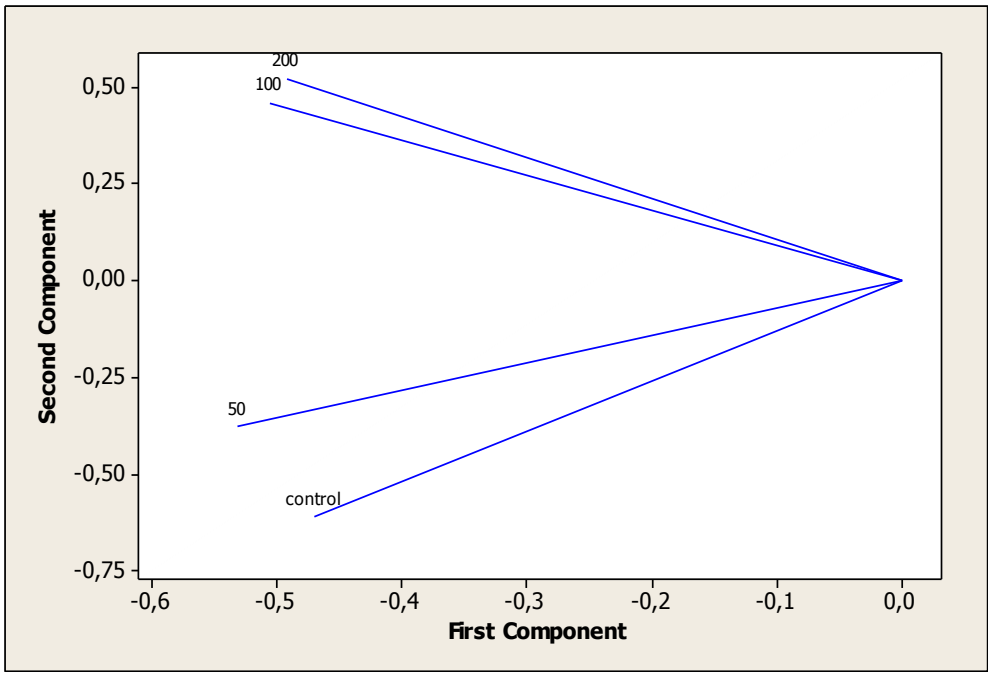


Figure 4.7. Similarity plot of PCA for bacterial communities.

Archaeal community dendogram according to presence/absence of the bands were given in Figure 4.8. Archaeal community structures for control and 50 mg/L OTC present microcosms were found similar. Also, 100 mg/L and 200 mg/L OTC including communities were found close. The similarities are given in dendogram in Figure 4.8 and similarity matrix in Appendix A.

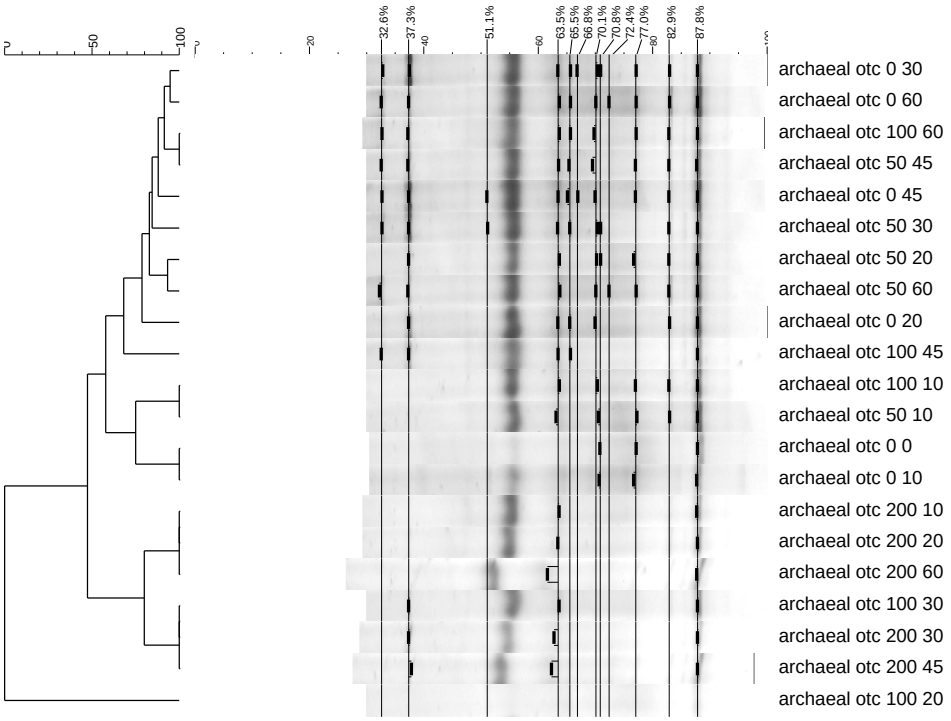


Figure 4.8. Archaeal community dendogram according to band presence/absence.

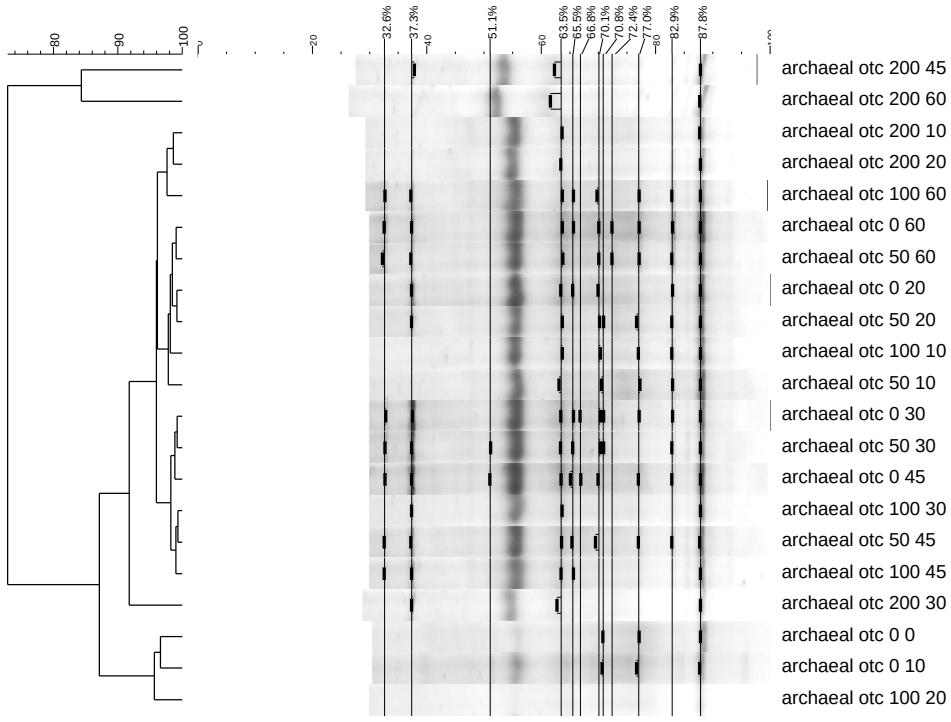


Figure 4.9. Archaeal community dendogram according to band intensities.

Band intensity based clusters are given in Figure 4.9. Communities of 200 mg/L OTC containing digesters were different than others. However, control, 50 mg/L and 100 mg/L OTC containing microcosms show similar community structure. Similarity matrix is given in Appendix. Band presence/absence based clustering in archaeal communities were found compatible with biogas data, contrastly, in analysing bacterial communities, intensity based clustering gave better results. This can be explained by the lower growth rate of archaeal cells as reported in a previous study (Öz, 2008).

First 3 components of PCA were selected according to results. Reference percentages for the first 3 components were 67.6%, 14.0% and 6.6%, respectively. Thus, 88.2% of the community structure could be analyzed. Similarity plot of archaeal communities was similar to bacterial plot (Figure 4.10). First component was found to be correlated with OTC concentration (r=-0.491). Second component was correlated with biogas production (r=0.582) and methane production (r=0.513). 3rd component was correlated with OTC concentration (r=0.502), biogas production (r=0.513), methane production (r=0.574), propionic acid concentration (r=-0.686) and isobutryic acid concentration (r=-0.715) ($p<0.05$).

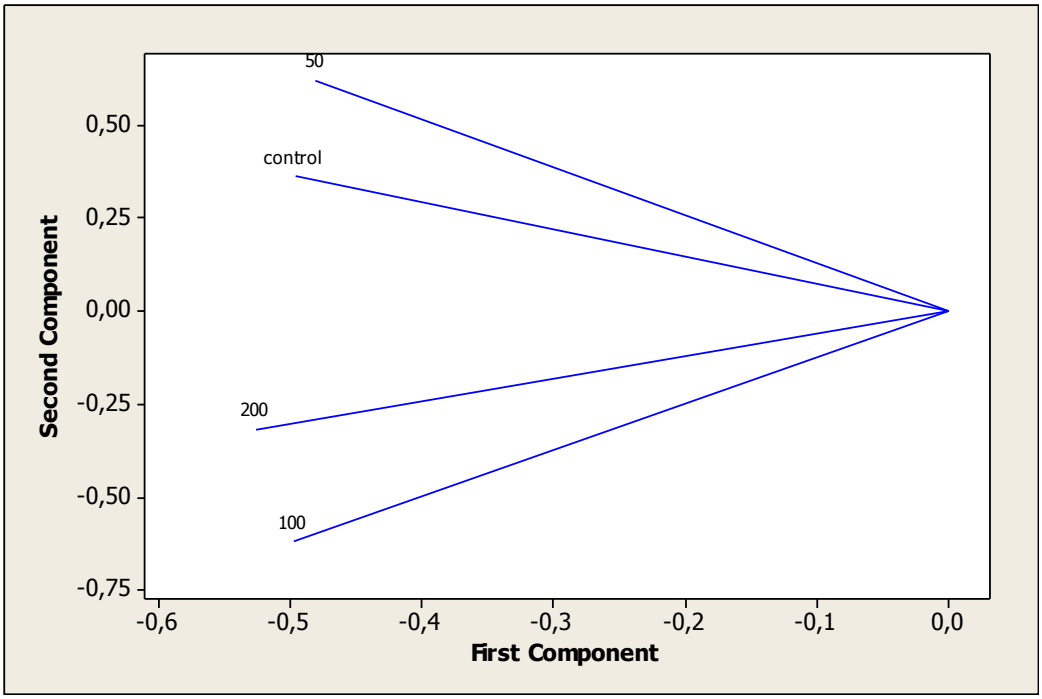


Figure 4.10. Similarity plot of PCA for archaeal communities.

4.1.4. Cloning and Sequencing Results

192 bacterial clones were screened with DGGE and total 18 groups were formed. A plot was given in Figure 4.11 in order to show the screening was sufficient.

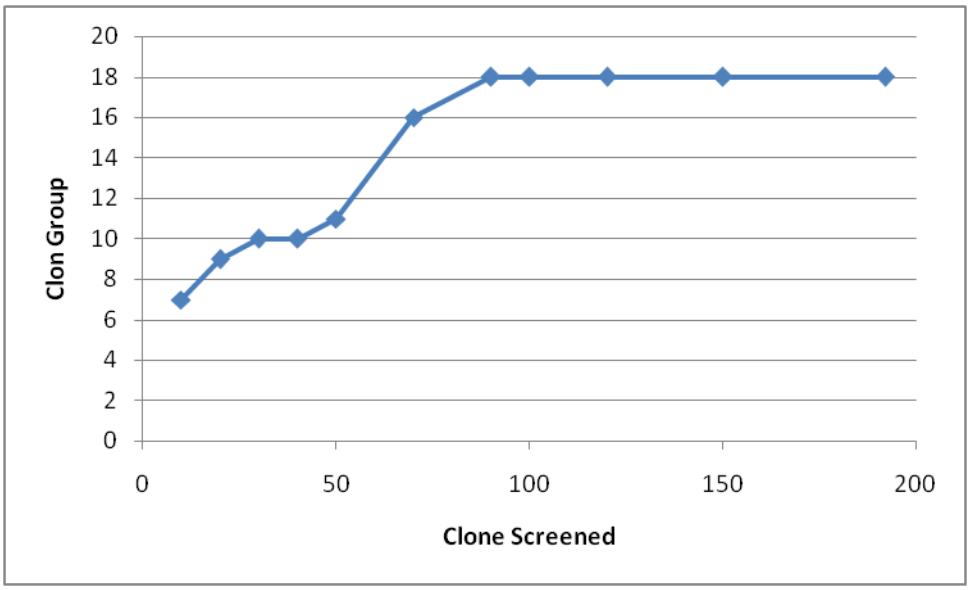


Figure 4.11. Screened clone numbers for bacterial cloning.

These bacterial groups were given in Figure 4.12 and 4.13. The dominant bacterial clone groups belong to *Bacillales* bacterium (31%) and *Clostridium lituseburense* (24%). 33% of the clones were *Clostridiales* and 49% of the clones were *Bacillales*.

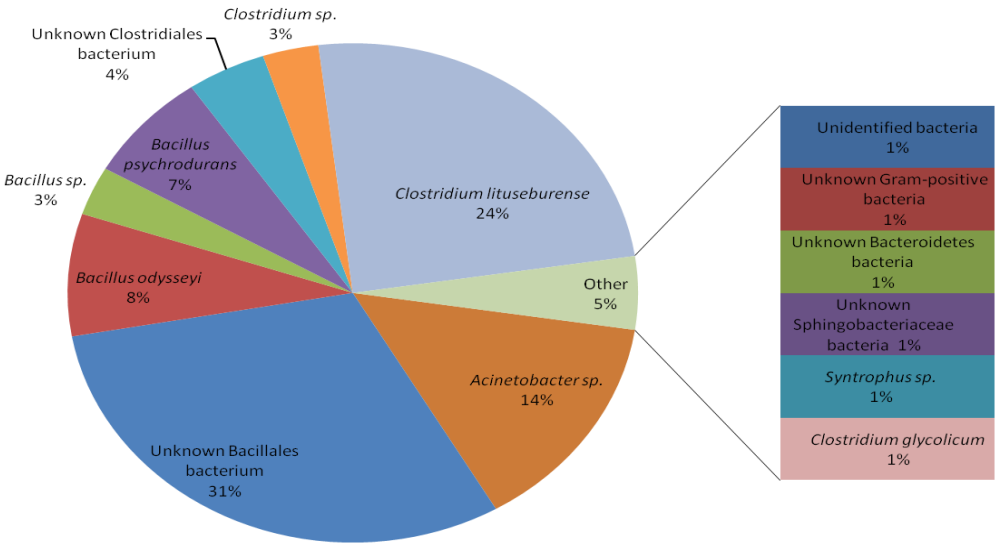


Figure 4.12. Bacterial clone groups according to sequence analyses.

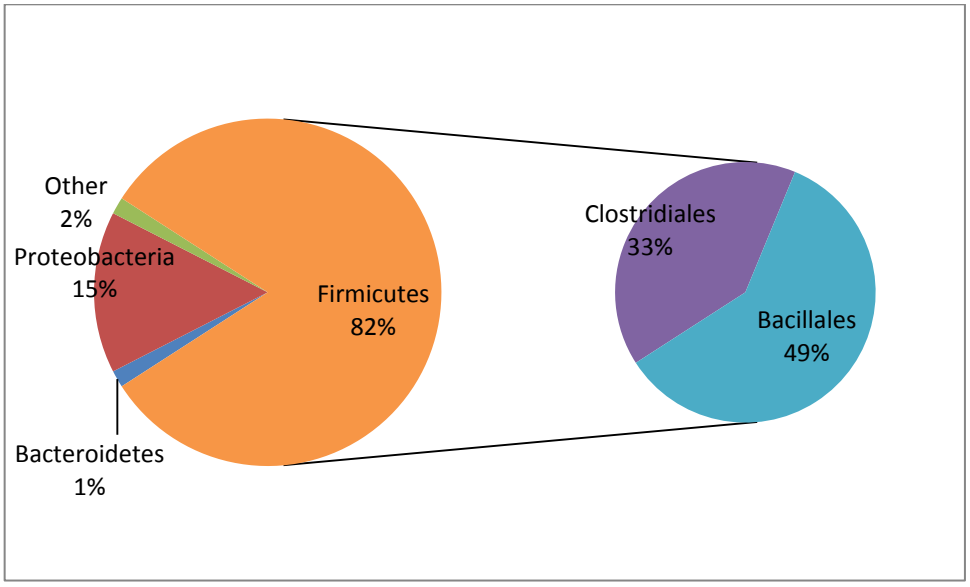


Figure 4.13. Bacterial clone groups in phylum degree.

The clones were compared with the samples' DGGE bands. Using band intensities, both linear correlation and canonical correspondance analysis were applied to understand the relations of species by digestion time and OTC concentration. A plot is given in Figure 4.14.

The most sensitive clones to OTC according to linear correlation were found as Bacillales (r=-0.571), *Bacillus psychrodurans* (r=-0.729), Clostridium (r=-0.636), *Clostridium lituseburense* (r=-0,509), *Clostridium glycolicum* (r=0.481), Clostridiales (r=0.584) and *Sphingobacteriacaea* (r=0.560). It was found that *Bacillales* were affected negatively by OTC while some *Clostridiales* were affected positively. Especially, *Clostridium glycolicum* was found tolerant to OTC. The reason of the increase might be that some *Clostridium sp.* cannot compete with other microorganisms (Baron, 1996). When other microorganisms such as *Bacillales* bacteria were inhibited with the antibiotic, *Clostridum glycolicum* may have growed. *Clostridum glycolicum* is known as its tolerance to environmental conditions, (Kuesel et al., 2001). According to CCA results, most sensitive clones to OTC were found as an unknown *Bacillus sp.* and *Clostridium sp. Clostridiales, Sphingobacteriaceae, C. glycolium* and unknown *Eubacteria* clones were found correlated with longer digestion time (Figure 4.14).

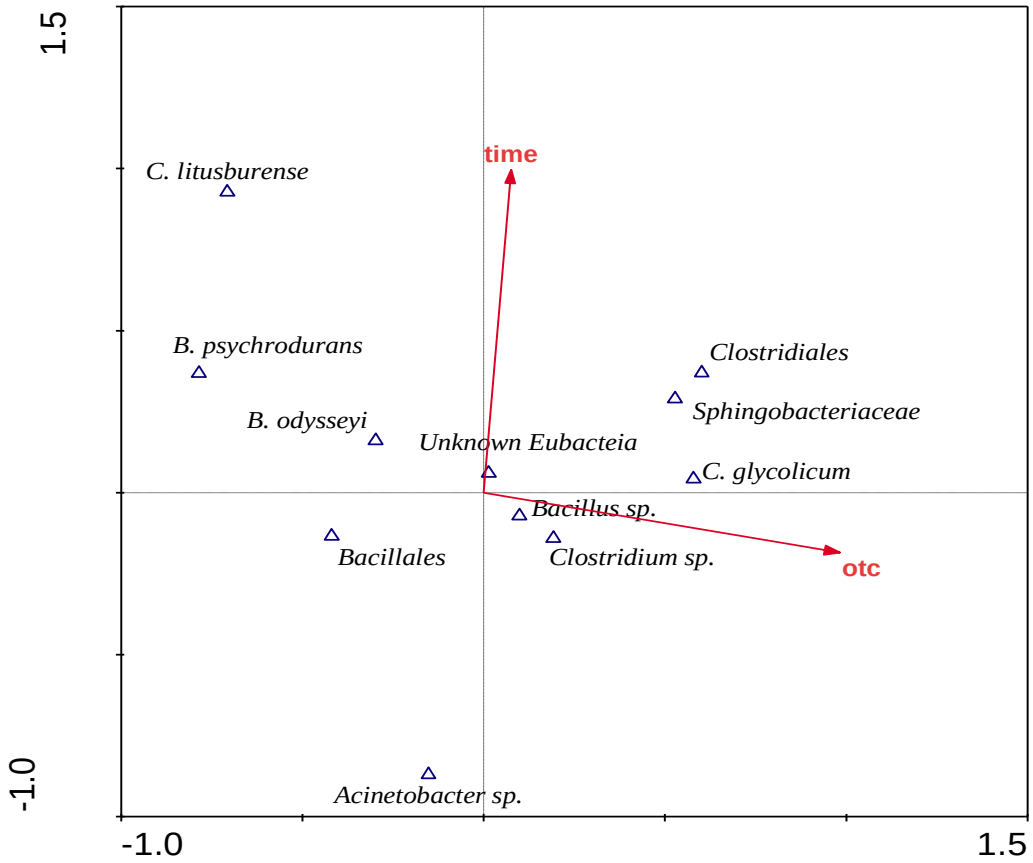


Figure 4.14. Canonical correspondence analysis result of bacterial species vs. environmental factors.

One of the *Bacillus sp.* clone had a 85% similarity with a clone found in African elephant (*Loxodonta africana*) feces, in a previous study in which mammal gut microbial community composition and structure was studied to investigate the relationship with the mammalian phylogeny and diet (Ley et al., 2008). In that study, 17 phyla were detected. 65.7% of the sequences were belonging to *Firmicutes* and 16.2% of the sequences were *Bacteroidetes*. *Proteobacteria* and *Acinetobacteria* were also found. This explains the dominance of *Firmicutes* in this study. Another *Bacillus sp.* clone was 94% similar to one in feces sample of *Bos taurus* (EM_ENV:GQ448631). *Bacillus psychrodurans* clone was similar to a clone in rumen sample in a previous study (EM_ENV:FJ172868). *B. psychrodurans* is a phychrotolerant, gram positive species with ornitine as diamino acid in the peptide side chain of the cell wall. They rarely produce spores. Anaerobic growth was reported to be only in the KNO_3 present environments. Temperature range for *B. psychrodurans* is 0-35 °C. In the digesters at least 100 mg/L OTC was found, no *B. psychrodurans* was found. It may be caused by the multi-inhibitor effect which temperature was also inhibiting the growth. Only weak acid production by *B. psychrodurans* was reported (Abd El-Rahman et al., 2002). *Bacillales* clones were 96% similar to a bacterial sequence in mosquito midgut (Lindh et al., 2005). *Clostridum sp.* clone was highly similar (92%) to the clone isolated from a long-term biogas completely stirred tank reactor (EM_ENV:FN985608). *Clostridium* was found to be dominant in low OLR in one-stage anaerobic digestion (Rincon et al., 2008). Most of the *Clostridium sp.* clones were in a high similarity with a clone isolated from reticulated giraffe (*Giraffa camelopardais*) feces. Another similar clone was isolated from dog duedonum (Suchodolski et al., 2008).

217 archaeal clones were screened with DGGE. These clones clustered 8 different groups. These groups were given in Figure 4.15.

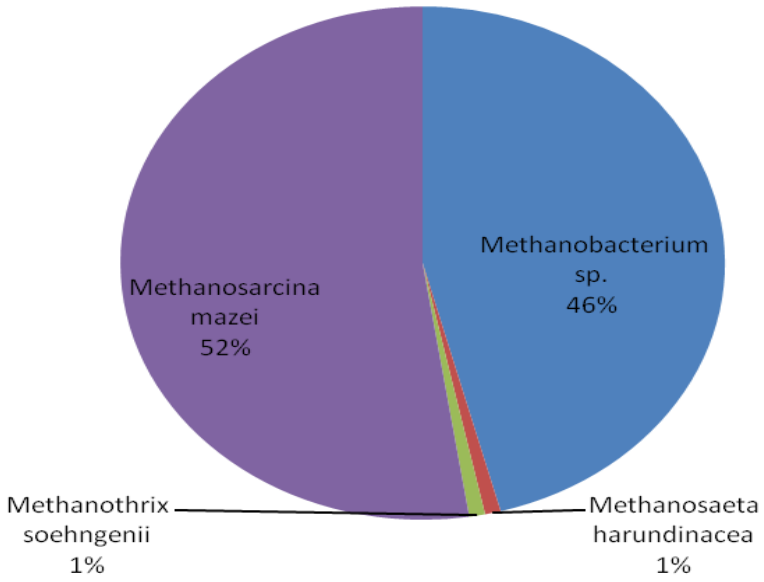


Figure 4.15. Archaeal clone groups according to sequence analyses.

All the archaeal clones were significantly negatively affected with OTC concentration according to linear correlation analysis ($p<0.05$). Biogas and methane productions were found correlated very largely with *Methanobacterium spp.* and largely with *Methanosarcina mazei*. Band intensities did not show a significant change with time. This can be explained by the slower grow rates of archaeal cells. Linear correlation table was given in Table 4.5 and canonical correspondence analysis was given in Figure 4.16.

Table 4.5. Correlation of species vs. environmental factors.

Clone	*Methanobacterium sp.*	*Methanobacterium sp.*	*M. harundinacea*	*M. soehgenii*	*M. mazei*
OTC	-0.545**	-0.604*	-0.746*	-0.682*	-0.809*
Biogas	0.750*	0.711*	N.S.	N.S.	0.543**
Methane	0.651*	0.665*	N.S.	N.S.	0.514**

*p<0.01 **p<0.05 N.S: Not significant

In anaerobic digesters, *Methanosaeta* and *Methanosarcina* were observed as the acetoclastic methanogens. It is reported that in seed sludge of solid wastes and biosolid digesters, *Methanosaeta* species was the most abundant, however, in biogas CSTRs which cattle manure was digested, the only recognized acetate utilizing methanogen was observed to be *Methanosarcina*-related microorganisms, independent of whether the reactors were

fed on cattle manure with or without co-substrates (Schmidt et al., 2000). Acetate utilizing methanogens with thin filaments seemed to be more sensitive to ammonia concentrations than hydrogenotrohic methanogens growing as rods or *Methanosarcinaceae* with thick clumps. Thus, in manure digesters (especially swine) *Methanosaeta* was not observed (Schmidt et al., 2000). *Methanosarcina mazei* are irregular cocci forming cysts and packets. This morphology brings advantage to *Methanosarcina mazei* compared to *Methanosaeta spp*. It is also claimed that the acetate oxidation to H_2/CO_2 following methane production is the dominant pathway reacted by hydrogenotrophic methanogens (Karakashev et al., 2006). As described in the theoretical background section, there are two mechanisms for methane formation from acetate. One of them is aceticlastic pathway, in which *Methanosaetaceae* and *Methanosarcinaceae* took role in the conversion (Ferry, 1993). *Methanosarcina* species are obligate anaerobic archaea and indispensable members of anaerobic food chains. (Zinder, 1993). *M. barkeri* and *M. mazei* can use H_2+CO_2, acetate, methanol and methylamines as substrate. *Methanosarcina* usually coexist with fermentative bacteria which produce acetate from various carbohydrates or trimethylamine from betaine or choline (Hippe et al., 1979). Generally *Methanosarcinaceae* have a higher acetate threshold but a higher growth rate and yield than *Methanosaetaceae*. The other pathway is a 2-step reaction in which acetate is first oxidized to H_2 and CO_2, and later to CH_4. This reaction is performed by acetate-oxidizing bacteria (often *Clostridium spp.*) in a syntropic association with hydrogenotrophic methanogens (often *Methanomicrobiales* or *Methanobacteriales*) (Hattori et al., 2000; Schnurrer et al., 1997). This explains the dominance of *Clostridium sp.* and *Methanobacteriales sp.* In the digesters, *Methanomicrobiales spp.* was not found. *Methanomicrobiales* was reported to be in a syntrophic relation with *Methanosarcinales*, and found dominantly in the leachate of municipal solid waste landfills (Huang et al., 2003). However, in the manure digester, *Methanobacteriales* was reported to be present rather than *Methanomicrobiales*. This might be due to the morphology of microorganisms. *Methanomicrobiales* are short rods and *Methanobacteriales* are long rods to filaments (Demirel and Scherer, 2008). This morphology can bring advantage to *Methanobacteriales* in high ammonia concentration which is regular for manure. In addition, *Methanosaeta spp.* levels were higher in bioreactors fed with granular sludge than in those with flocculent sludge (Zheng and Raskin, 2000).

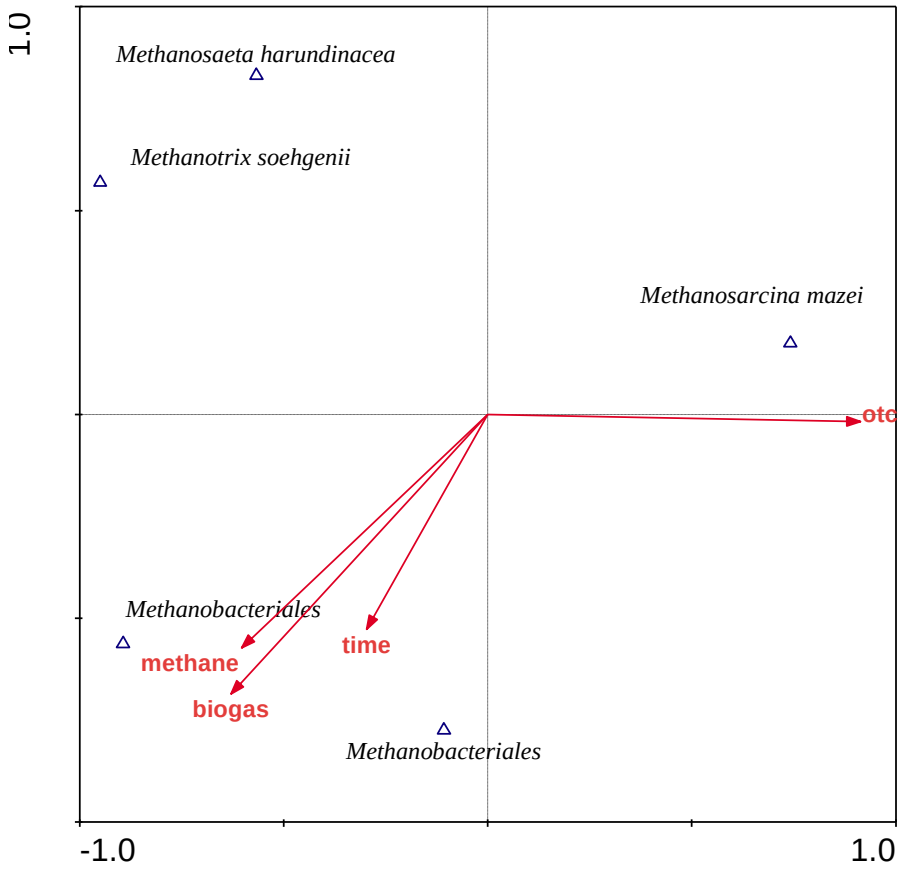


Figure 4.16. Canonical correspondence analysis result of archaeal species vs. environmental factors.

Result of Pearson correlation and CCA were found similar except for *M. mazei*. In studying relationships between species and environmental variables, CCA was suggested because of unimodal relationships (Zuur et al., 2007). In this case, CCA results should be taken into account. Thus, even *Methanosarcina mazei* was found dominant in clones, methane was converted mostly by *Methanobacteriales* in a syntrophic relationship with *Clostridum sp.*

4.2. Effects of OTC in Medicated Cow Manure

In manure digesters, not only OTC itself, but mostly its metabolites were reported to be inhibitors (Fedler and Day, 1985). These metabolites are produced in the digestion track of the animal. A cow was medicated intramuscularly with OTC and the manures were sampled for 20 days to determine the inhibitory effects of both OTC and metabolites,.

OTC concentrations of manures were determined and these manures were used in microcosm tests.

4.2.1. Results of OTC Measurements

HPLC measurements showed that oxytetracycline was excreted in manure in 12 days. After 12th day, OTC concentrations in the manures were below the detectable limit. The sample which collected on day 1 had the highest OTC concentration, and the concentrations decreased till day 12 except day 4 (78-92 recovery rate). In Figure 4.17, results of OTC measurements were given.

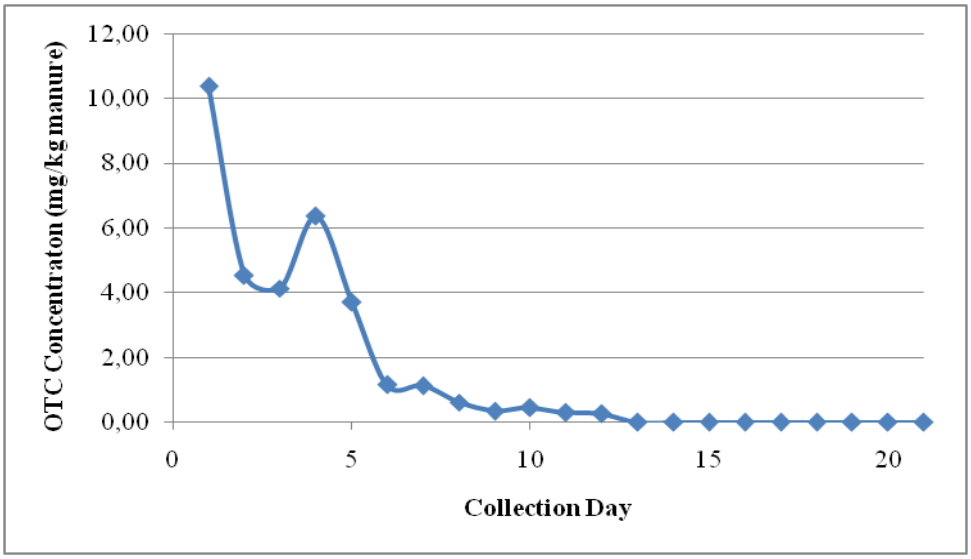


Figure 4.17. Measured OTC concentrations in manure.

In the studies on the effect of tetracyclines on anaerobic digestion, generally oral administration of the drug used, which is common for growth promoter applications. Especially in USA, tetracyclines are still being used for non-therapeutic purposes and oral administration of the drug is common. However, in EU countries, growth promoter use of antibiotics was banned and only medical use is legal. In this study, oxytetracycline was intramuscularly injected to a cow, which is the general medication practice and about 10% of the OTC injected into cow was found in the manure. The amount of OTC in manure depends on the way and load of administration. About 10 mg/kg OTC was detected in a 5 fold diluted manure slurry of an oral medicated calf (Arıkan et al., 2006). 871 mg/kg OTC

was reported in swine manure, which is nearly 80 times higher than the amount detected in this study in which OTC was given in the feed. In another study, 5.88 mg/kg CTC was detected in manure samples collected from different farming areas. In previous studies, tetracyclines were reported to be present at a wide concentration range in manure, differing from 0.1 to 173 mg/kg (Hamscher et al., 2003; Jakobsen et al., 2004). The differences are likely due to mostly administration of the drug and also sampling and storage conditions, the diet, general health of the animal and type of the animal.

4.2.2. Biogas Production Results

Biogas productions were calculated using gas pressure data in Figure 4.18. 255 mL biogas was produced in 30 days in control bottles. At the end of 30 days digestion, inhibitions were 46%, 58%, 57%, 51% and 21% in the manures collected on day 1, 2, 3, 5 and 10, respectively. Biogas productions of the digesters of day 15 and 20 were not significantly different than of the control microcosms. Sample collection time was found very important in biogas efficiency. Mostly, inhibited samples had stable increasing gas pressures which again reflected a retardation in biogas production. Further digestion over 30 days might have resulted in higher biogas production in these digesters. Biogas productions were given in Figure 4.19.

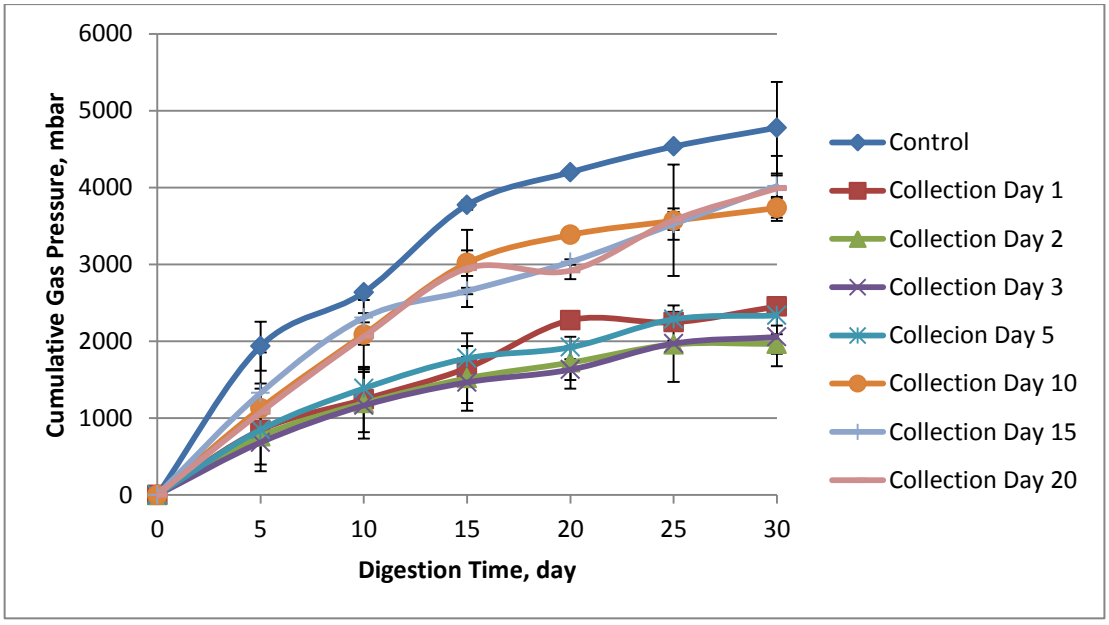


Figure 4.18. Cumulative gas pressures during medicated OTC used microcosm tests.

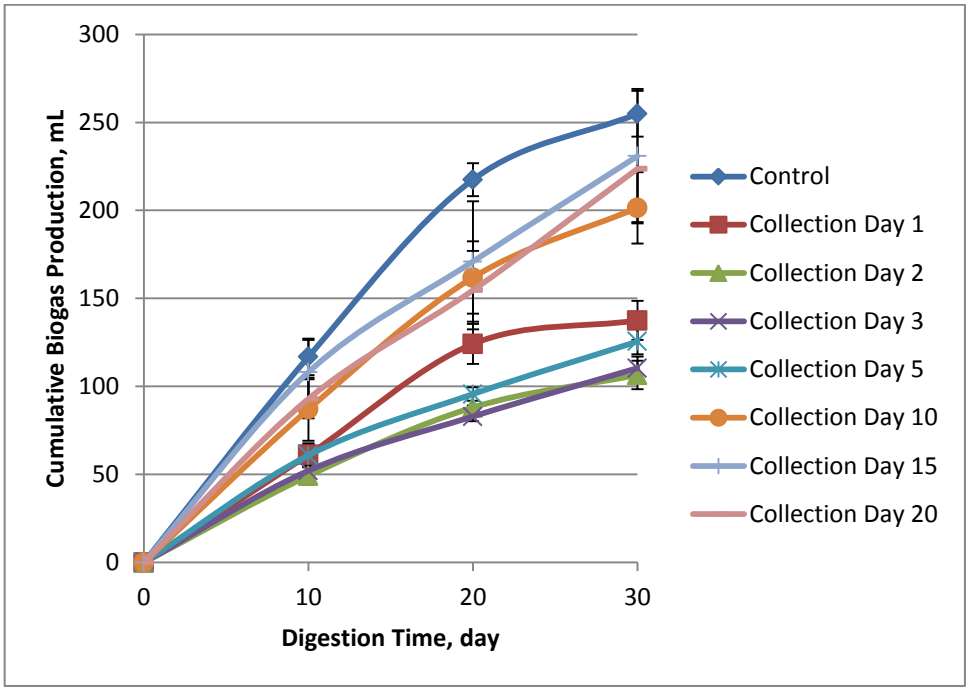


Figure 4.19. Cumulative biogas productions during medicated OTC used microcosm tests.

The methane percentages in the biogas for all samples were 58±5 for all samples (Appendix C). There was not a correlation between manure collection day and methane content of the biogas. Methane production showed a similar trend with biogas production, however, methane production stopped after 20 days (Figure 4.20).

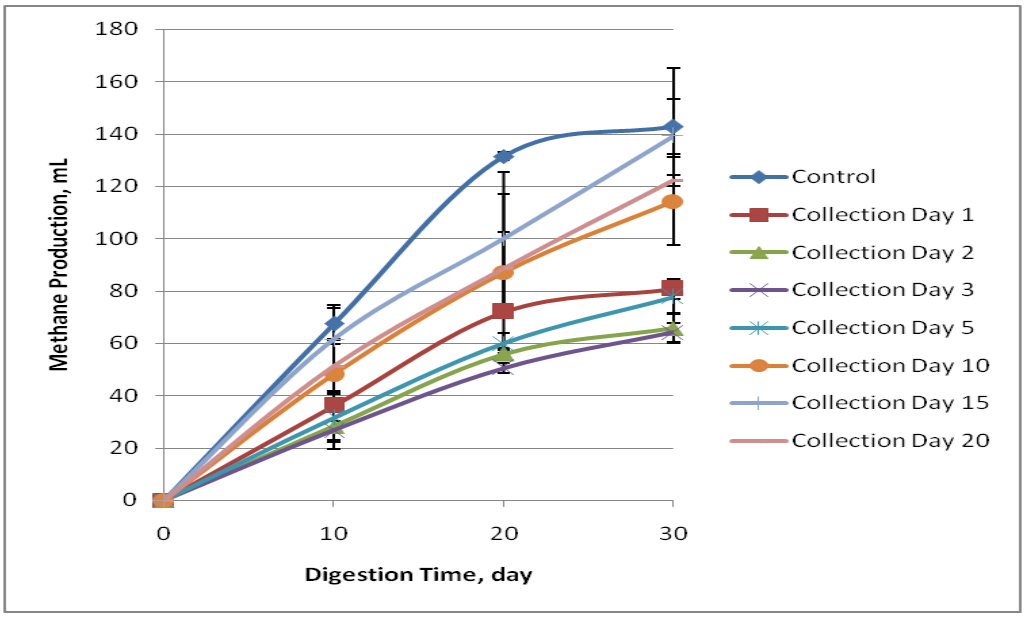
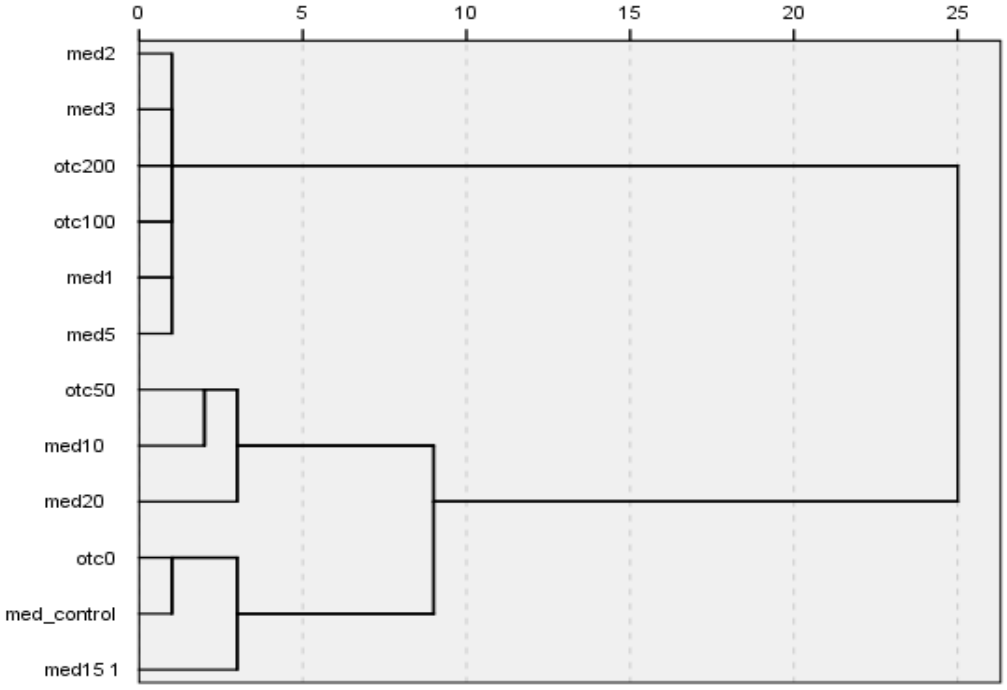


Figure 4.20. Cumulative methane productions during medicated OTC used microcosm tests.

For the first 5 days, inhibitions in biogas productions were in ranges 50-60% and not significantly different than each other. Inhibition in biogas production for manure collected on day 10 was 23%. Control, day 15 and 20 manures produced highest biogas ($p<0.05$). Biogas and methane yields were given in Table 4.6. Methane yields of the control digesters were similar than to the values in the first part of experiment, in which OTC was externally added.

Table 4.6. OTC concentrations (mg/L) with biogas and methane yields (L/kgTVS) during medicated OTC used microcosm tests.

		20 Days Digestion		30 Days Digestion	
Manure Collection Day	**OTC Concentration**	**Biogas Yield**	**Methane Yield**	**Biogas Yield**	**Methane Yield**
Control	0.0	132.43±5.72	80.07±0.92	155.22±7.91	86.96±6.41
1	3.3	70.46±6.46	40.83±0.56	78.04±6.29	45.82±2.13
2	1.4	51.73±0.68	32.85±1.26	62.57±4.75	38.62±3.24
3	1.4	48.32±1.54	29.45±1.12	64.30±3.88	37.35±2.16
5	1.0	54.10±2.17	33.96±2.16	71.00±4.09	43.93±3.53
10	0.2	95.67±12.12	51.52±17.68	119.08±11.97	67.53±10.02
15	0.0	103.08±20.61	60.38±15.27	139.17±23.01	83.93±15.67
20	0.0	94.66±13.65	54.19±8.58	136.85±18.56	74.77±1.23



otc: result of part 1, which otc was externally added, number refers to concentration, **med:** result of part 2, which manure of otc medicated cow was used, number refers to collection day.

Figure 4.21. Dendogram showing similarity of methane yields obtained from two different microcosm tests.

A dendogram in Figure 4.21 was plot for the comparison of methane yields. There, medicated manures which were collected in the first 5 days were similar to 100 and 200 mg/L OTC added manures by means of methane yield. The control microcosms of the two experiments were also similar to each other. In a previous study, 27% methane inhibition was reported in which OTC was given to calves by feeding medication and found in the slurry in concentration of 3.1 mg/L (Arıkan et al., 2006). In this study, 1.0-3.3 mg/L OTC resulted in 50-60% decrease in biogas production. A comparative table for similar studies was given in Table 4.3. Result of this and previous studies showed that smaller antibiotic concentrations in medicated manure can result in higher biogas inhibitions comparing to the external addition of antibiotic to the manure. This supports the idea that metabolites of OTC produced in the animal are the main inhibitors (Fedler and Day, 1985).

4.2.3. VFA Results

Table 4.7. Volatile fatty acid concentrations (mg/L) during medicated OTC used microcosm tests.

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 concentrations in all reactors were less than 56 mg/L. These concentrations were not reported as inhibitory in previous studies. At the end of 30 days

digestion, propionic acid was observed only in microcosm digesting manure collected on day 3. The highest inhibition in biogas production was in that sample which can be related with VFA accumulation inhibition. Almost in all the samples, no isobutyric, isovaleric or heptanoic acid was measured. For each sampling day of manures used as substrates, all specific VFA measurements were given in Table 4.6. Comparing to the first part, VFA concentrations were lower but inhibitions in biogas productions were higher. According to this, metabolites of the OTC might have inhibited the microorganisms taking part in methanogenesis, rather than acetogenesis.

4.2.4. Community Fingerprinting with DGGE

In the dendograms, bacterial and archaeal communities were labeled. OMG abbreviation indicates to the medicated manure digestion, first number indicates sample collection day and the last number indicates digestion time.

Initial bacterial communities in the samples were compared with Bionumerics. According to band presence (Figure 4.22), there was not a clustering depending on sample collection time. Pearson correlation results (Figure 4.23) showed that community in control microcosm was close to 3rd day sampled microcosm. 15th and 20th day samples were also close to each other. On the 10th day of digestion, bacterial community similarities were similar to gas production results especially in band intensity based UPGMA clustering.

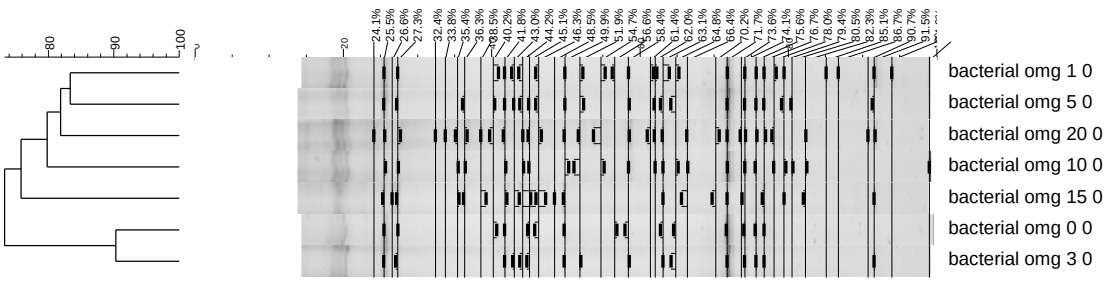


Figure 4.22. Initial bacterial communities in slurries according to presence/absence of the bands.

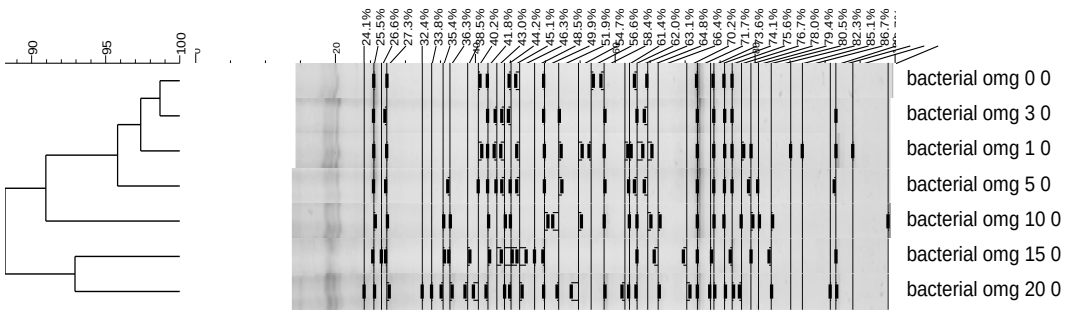


Figure 4.23. Initial bacterial communities in slurries according to band intensities.

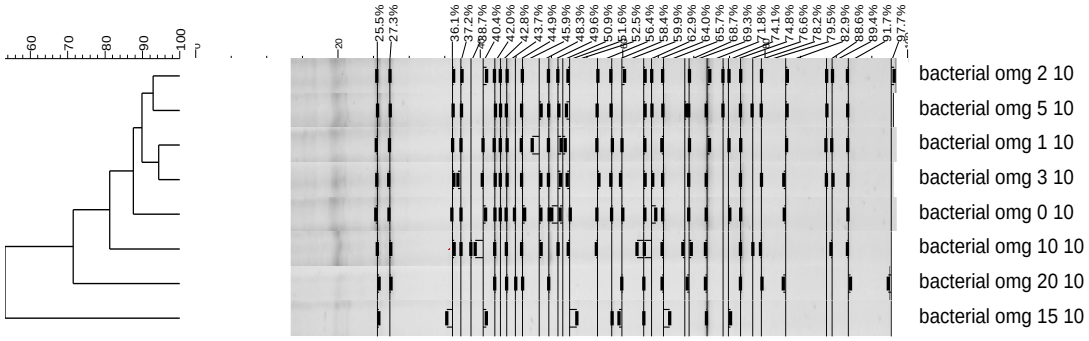


Figure 4.24. Bacterial communities in slurries according to presence/absence of the bands after 10 days digestion.

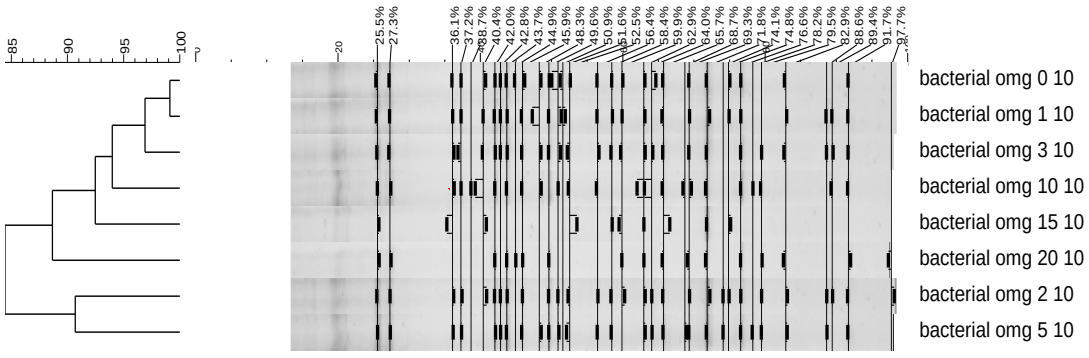


Figure 4.25. Bacterial communities in slurries according to band intensities after 10 days digestion.

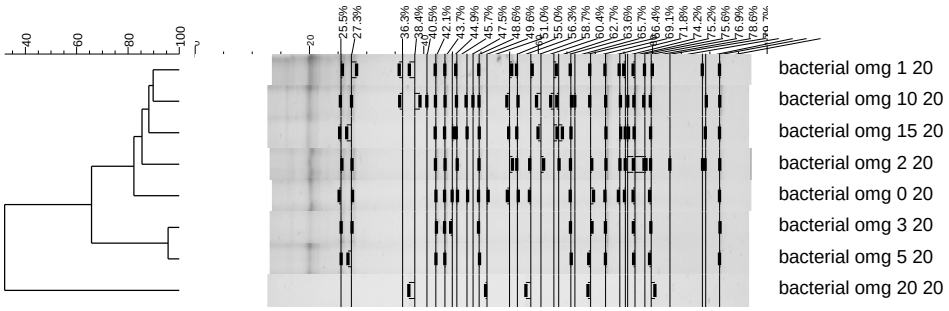


Figure 4.26. Bacterial communities in slurries according to presence/absence of the bands after 20 days digestion.

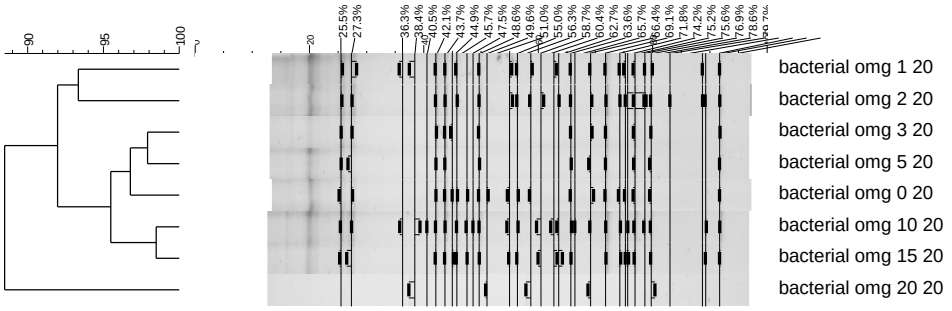


Figure 4.27. Bacterial communities in slurries according to band intensities after 20 days digestion.

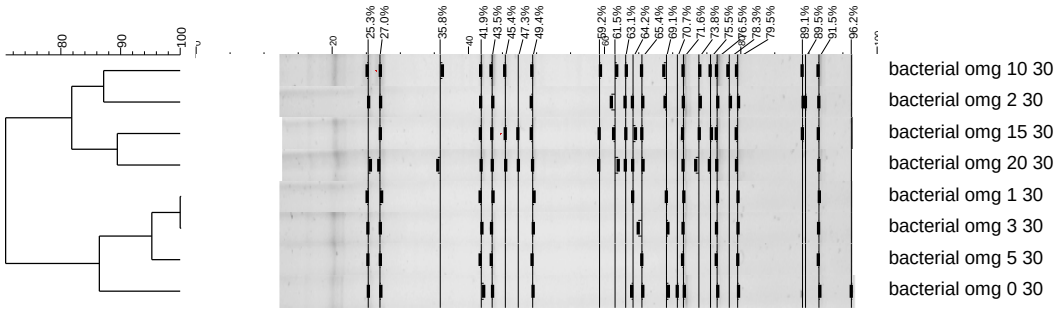


Figure 4.28. Bacterial communities in slurries according to presence/absence of the bands after 30 days digestion.

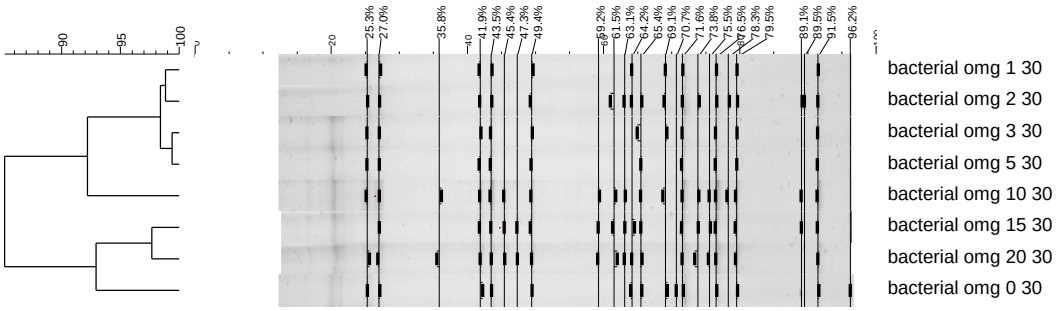


Figure 4.29. Bacterial communities in slurries according to band intensities after 30 days digestion.

After 10 days digestion, microbial community structures were grouped similarly to biogas production efficiencies. In band presence based clustering (Figure 4.24, 4.26 and 4.28), it was not clear. Especially band intensity based clustering was much likely to refer digester efficiency (Figure 4.25, 4.27 and 4.29). In bacterial samples, band intensity based analyses were suggested before (Öz, 2008). PCA analysis of 30th day samples showed the same clustering (Figure 4.30) with Bionumeric data (Appendix B). Community in control sample was different than all others. 20th day sampled and 15th day sampled manures had a close community structure to each other. The first 5 days sampled manures, after 30 day digestion of slurry, clustered closely. In Figure 4.30, 1st and 2nd components were given in a plot.

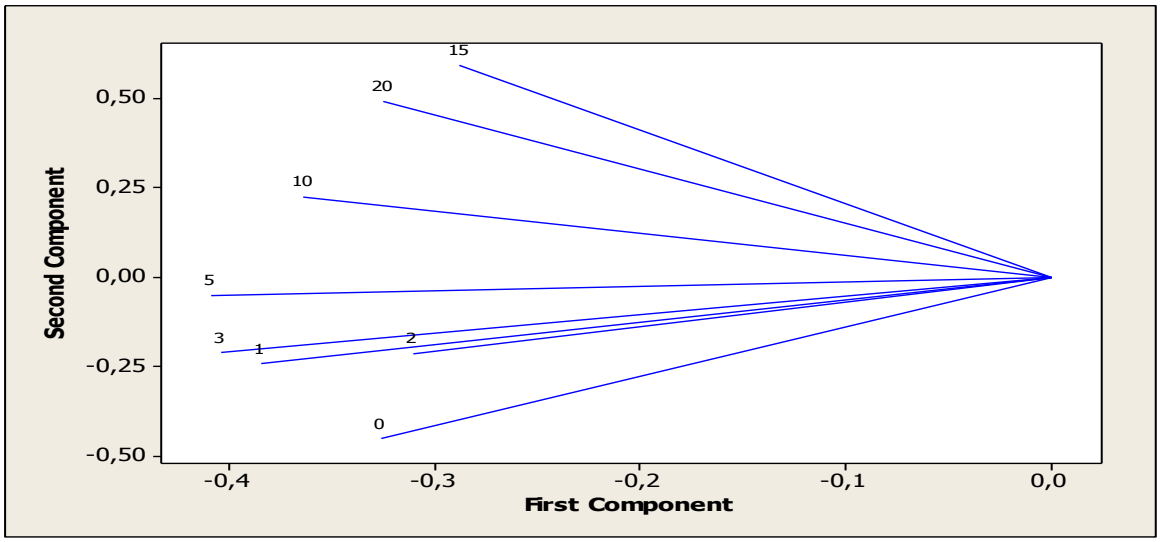


Figure 4.30. PCA plot of bacterial samples in medicated manure test.

Comparing DGGE bands with clone libraries showed clones were not significantly changing with the sampling time. Since sampling time did not only relate with decreasing OTC, linear correlations were narrow comparing to the experiments in which OTC was externally added to manure.

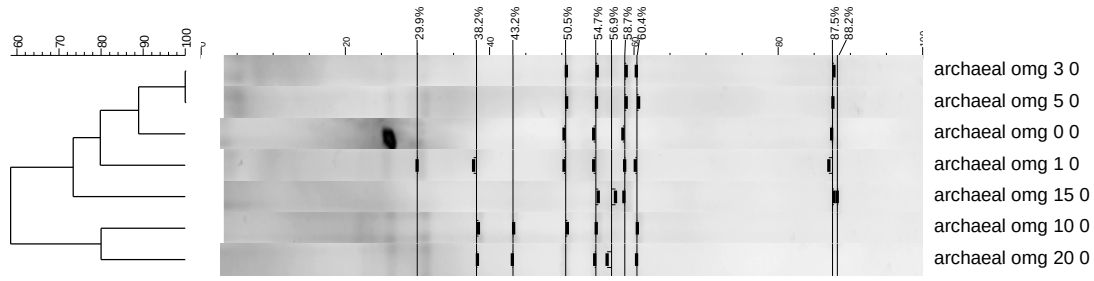


Figure 4.31. Initial archaeal communities in slurries according to presence/absence of the bands.

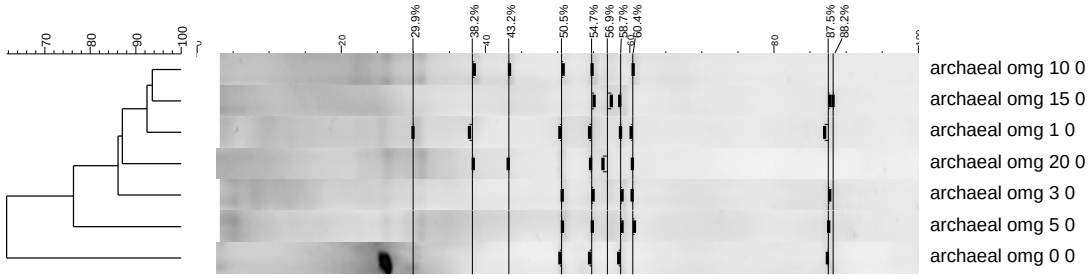


Figure 4.32. Initial archaeal communities in slurries according to band intensities.

According to presence absence analyses of initial archaeal communities, the first five days were closer to the control (Figure 4.31). The samples of day 10, 15 and 20 of collection were different than the control. Similarity between control and 20th day collected sample was only 44%. Band intensity based similarities were not compatible with presence/absence data (Figure 4.32). Initial control community was closer to 1st, 10th, 15th and 20th day samples (66-77%). 3rd and 5th day samples were found to have a very distinct archaeal community structure.

After 10 days of digestion, 1st day and 10th day collected samples changed their profiles (Figure 4.33). Other samples provided their similarities. Results of band intensities showed that control digester had a different archaeal community than medicated manure fed samples (Figure 4.34). In the 20th day of digestion, band presence data was not similar to biogas productions (Figure 4.35). However, band intensity similarities were much closer to biogas productions (Figure 4.36). 10th day sampled manure microcosm community became closer to the one of control sample. Also 15th and 20th day manure microcosms were closer to them. The highest different community to control was 3rd day sampled manure microcosm's community. The profile in 20th day reflected the changes in biogas production.

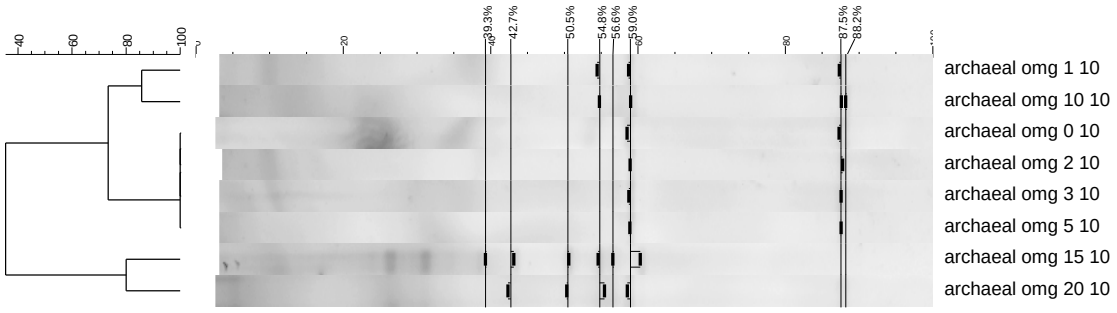


Figure 4.33. Archaeal communities in slurries according to presence/absence of the bands after 10 days digestion.

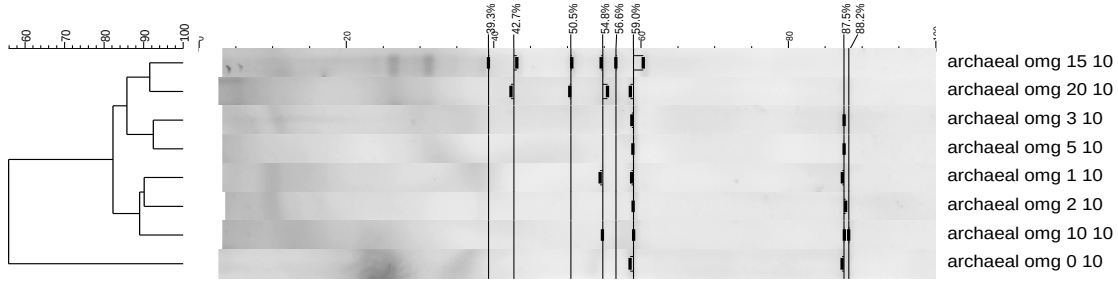


Figure 4.34. Archaeal communities in slurries according to band intensities after 10 days digestion.

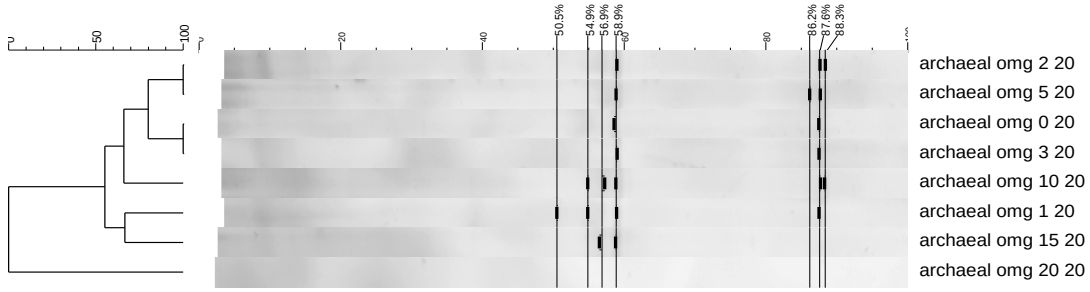


Figure 4.35. Archaeal communities in slurries according to presence/absence of the bands after 20 days digestion.

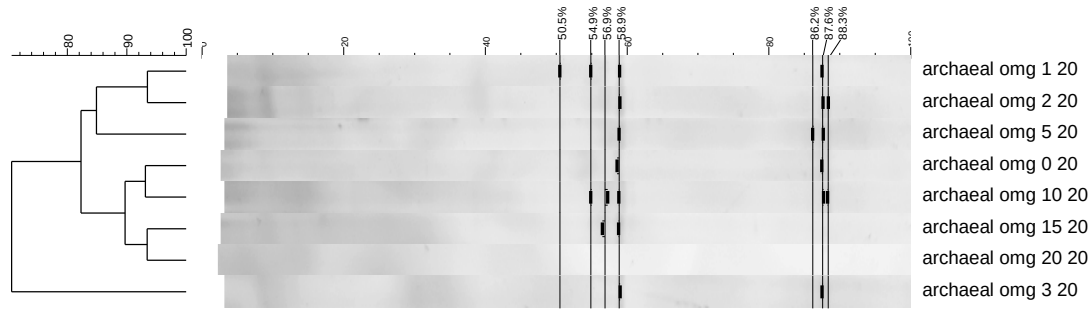


Figure 4.36. Archaeal communities in slurries according to band intensities after 20 days digestion.

At the end of 30 days digestion (Figure 4.37 and 4.38), control microcosm community was close to all samples except 3rd day sampled manure microcosm community. Comparing to biogas and methane production results, community in 20th day was much closer than community in 30th day.

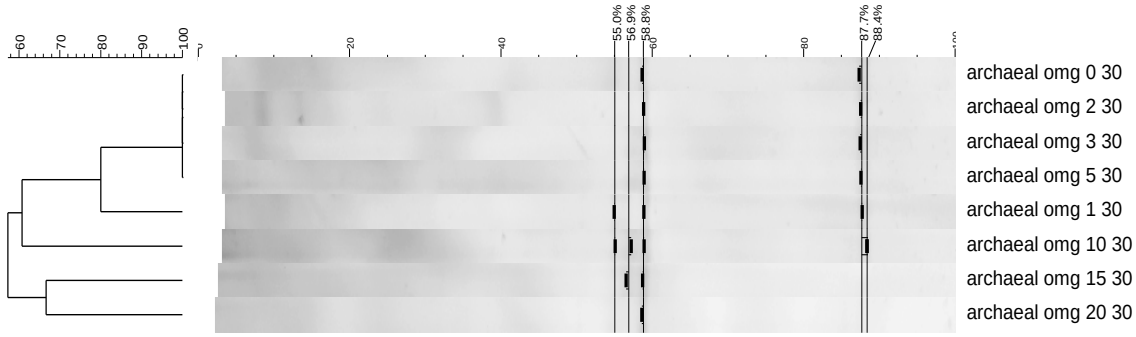


Figure 4.37. Archaeal communities in slurries according to presence/absence of the bands after 30 days digestion.

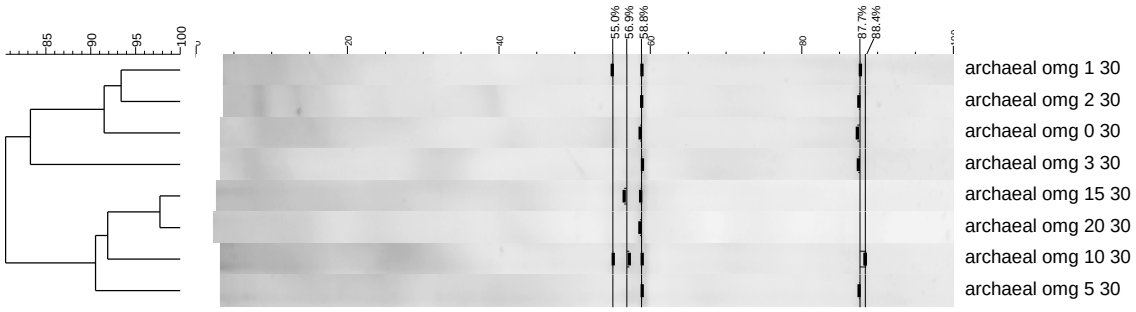


Figure 4.38. Archaeal communities in slurries according to band intensities after 30 days digestion.

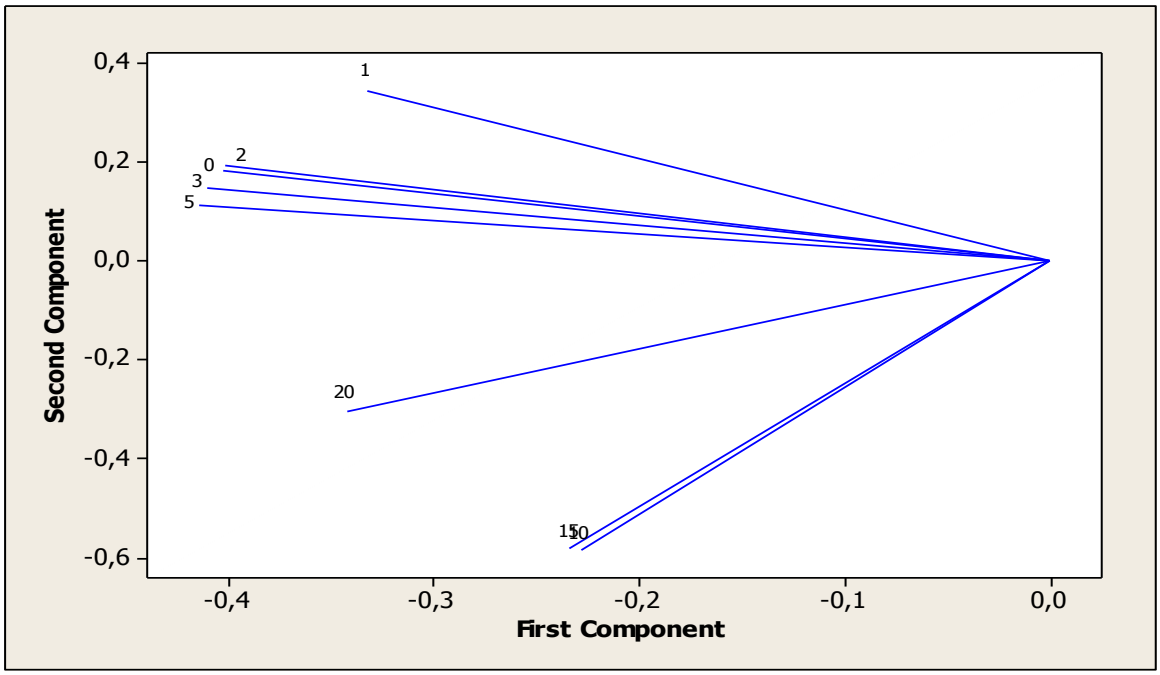


Figure 4.39. PCA plot of archaeal samples in medicated manure test.

Archaeal communities were analyzed with PCA (Figure 4.39). According to test result, the first 2 components were chosen. First component reflected 70.3% of the whole matrix and second component added 14.6% on it. Thus, 85% of the matrix could be represented in the correlations. First and second components showed a very large correlation with the sampling day with Pearson's r=0.783 and r=0.792, respectively. This shows that archaeal community was affected both by oxytetracycline and its metabolites. It was reported that majority of the antibiotics caused inhibition only in bacteria, however, protein synthesis inhibitors caused a complete inhibition in acetoclastic methanogenic *archaea* (Sanz et al., 1996). Since OTC is a protein synthesis inhibitor, both bacterial and archaeal communities were clustered depending on OTC and metabolites concentrations.

Taking results of the two parts into account, a scenario on the activity of OTC with and without metabolites can be suggested. When 50 mg/L OTC was externally added to the microcosms, bacterial community similarities to the control microcosms were relatively small (92%) than archaeal community similarities (99%). Because of that, both VFA production and VFA conversion to acetate were inhibited, resulted in a slight VFA accumulation. In the second part, where OTC medicated manure was used including its metabolites, bacterial communities in the highest biogas inhibition observed microcosms

(first 5 days) were similar to ones in control microcosms (93-97%). Thus, hydrolysis, acidogenesis and acetogenesis were not inhibited. VFA production and conversions were reacted well. The reason that bacterial species were not inhibited as in the first part could be the low concentration of OTC (1.0-3.3 mg/L). On the other hand, archaeal communities were found 73-87% similar to community in control microcosms. Different from the first part, in the second part, metanogenesis was inhibited. This can be explained by the activity of metabolites on methanogenic *Archaea*. As a result of this, VFA accumulation was not observed in inhibited microcosms of the second part, however methane inhibitions were much higher than the first part.

5. CONCLUSIONS

The major objective of this study was to determine inhibitory effect of OTC on biogas production, biogas quality and microbial communities in the biogas digesters using cow manure as substrate.

Methane yields in microcosms excluding OTC were 87-90 L/kgTVS. Inhibitions in biogas productions in 50, 100 and 200 mg/L externally OTC added microcosms were 41%, 57% and 61%, respectively. Retardation in biogas production for 50 mg/L OTC concentration was observed up to 60 days. Using medicated manures collected on the first five days, 50-60% inhibitions were observed in biogas yields. Even OTC concentrations in slurries were lower (1.0-3.3 mg/L) than the first part, biogas inhibitions were much disticy in the second part.

In bacteria *Firmicutes*, in archaea *Methanobacterium sp.* and *Methanosarcina mazei* were dominated. Almost all bacterial and archaeal species were inhibited with OTC. Comparing whole communities, external OTC addition resulted in much significant differences in bacterial community structure than archaeal community structure. In contrast, using manures of a cow medicated with OTC distinctly changed archaeal community than bacterial community.

Taking all the results mentioned above into account, high concentrations of OTC inhibited bacterial species in hydrolysis, acidogenesis and acetogenesis resulting in a slight VFA accumulation. Since OTC concentrations in manures of a medicated cow were much lower, bacterial species were not inhibited. The main inhibition observed on the metanogenic *Archaea* could probably due to the metabolites produced in the digestive track of the animal.

6. RECOMMENDATIONS

One of the main objectives of this study was to determine inhibitory effect of oxytetracyline on microbial communities using denaturing gradient gel electrophoresis of 16S rDNAs in manure slurry microcosms. A future work is needed to investigate the inhibition in rRNA level for understanding the inhibition in activity of microorganisms. Since OTC is a protein synthesis inhibitor, rRNA work is strongly necessary and quantification of cDNAs in QPCR using genus specific primers of *Methanomicrobiales* and *Methanosarcinales* may result in better understanding of inhibition on methanogenic archaea.

In the study, lower OTC concentrations including metabolites resulted in higher inhibitions in biogas production than higher concentrations of OTC without metabolites. In a future study, metabolites will be measured as given in TUBITAK Project (109Y275) and the effects of metabolites will be determined.

In this study, digesters were 120 mL microcosm bottles. In larger volumes, effect of OTC should be studied for better representation of real scenario. Also, measurement of daily biogas production and methane content would give better results than cumulative measurements.

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APPENDIX A: COMMUNITY SIMILARITY MATRIXES PART 1

Table A.1. Bacterial community similarity matrix according to band presence/absence.

	OTC	0	0	0	0	0	0	50	50	50	50	50	100	100	100	100	100	200	200	200	200	200
OTC	Time	0	10	20	30	45	60	10	20	30	45	60	10	20	30	45	60	10	20	30	45	60
0	0	100.00																				
0	10	96.30	100.00																			
0	20	81.49	78.57	100.00																		
0	30	66.67	71.43	85.72	100.00																	
0	45	62.07	66.67	80.01	93.33	100.00																
0	60	58.07	62.50	75.00	87.50	94.12	100.00															
50	10	72.00	69.23	76.93	84.62	78.57	73.33	100.00														
50	20	69.23	74.08	74.08	88.89	82.76	83.87	80.01	100.00													
50	30	69.23	74.08	74.08	88.89	82.76	83.87	80.01	100.00	100.00												
50	45	75.00	72.00	72.00	80.01	74.08	68.97	78.26	75.00	75.00	100.00											
50	60	62.50	60.61	66.67	78.79	85.72	91.89	70.97	75.00	75.00	66.67	100.00										
100	10	68.97	66.67	73.33	73.33	75.00	82.35	71.43	75.87	75.87	66.67	68.57	100.00									
100	20	80.01	76.93	69.23	76.93	71.43	73.33	75.00	88.00	88.00	78.26	70.97	78.57	100.00								
100	30	62.50	60.61	66.67	78.79	85.72	91.89	70.97	75.00	75.00	66.67	94.74	68.57	70.97	100.00							
100	45	59.26	57.15	71.43	71.43	73.33	81.25	69.23	66.67	66.67	56.01	84.85	73.33	69.23	84.85	100.00						
100	60	54.55	52.17	69.57	69.57	72.00	66.67	76.19	63.64	63.64	60.00	64.29	72.00	66.67	64.29	78.26	100.00					
200	10	69.23	59.26	66.67	59.26	62.07	64.52	72.00	69.23	69.23	58.33	62.50	89.66	80.01	68.75	74.08	72.73	100.00				
200	20	64.29	62.07	68.97	75.87	77.42	84.85	81.49	78.57	78.57	76.93	82.35	77.42	81.49	82.35	82.76	75.00	78.57	100.00			
200	30	53.85	59.26	74.08	81.49	82.76	83.87	80.01	84.62	84.62	75.00	81.25	75.87	72.00	75.00	74.08	81.82	69.23	85.72	100.00		
200	45	56.01	53.85	69.23	76.93	78.57	80.01	83.33	80.01	80.01	69.57	77.42	71.43	75.00	77.42	76.93	85.72	72.00	88.89	96.00	100.00	
200	60	58.33	56.01	72.00	80.01	81.49	75.87	86.96	75.00	75.00	72.73	73.33	66.67	69.57	73.33	72.00	90.01	66.67	84.62	91.67	95.65	100.00

Table A.2. Bacterial community similarity matrix according to band intensities.

	OTC	0	0	0	0	0	0	50	50	50	50	50	100	100	100	100	100	200	200	200	200	200
OTC	Time	0	10	20	30	45	60	10	20	30	45	60	10	20	30	45	60	10	20	30	45	60
0	0	100.00																				
0	10	83.19	100.00																			
0	20	77.09	81.95	100.00																		
0	30	72.44	81.21	94.53	100.00																	
0	45	68.19	77.62	94.86	96.25	100.00																
0	60	65.43	73.67	90.91	90.39	91.84	100.00															
50	10	73.89	84.17	88.91	86.72	87.33	89.95	100.00														
50	20	73.43	78.63	91.85	92.02	90.24	92.35	92.32	100.00													
50	30	72.81	78.68	92.17	91.86	90.57	92.87	91.92	99.24	100.00												
50	45	64.67	69.84	85.83	82.76	82.89	83.45	81.75	90.36	90.55	100.00											
50	60	65.60	67.28	81.97	80.30	82.52	90.61	84.35	87.20	87.70	81.89	100.00										
100	10	68.87	74.59	74.98	77.16	75.13	81.69	90.19	83.75	83.74	75.05	86.88	100.00									
100	20	72.51	73.85	84.03	85.23	84.26	86.55	87.65	91.60	91.79	77.63	91.52	87.24	100.00								
100	30	61.19	63.59	77.46	73.45	77.21	84.57	81.47	80.71	81.70	74.61	94.01	82.70	87.71	100.00							
100	45	58.39	56.92	70.99	64.46	70.67	77.25	74.81	70.32	70.29	68.67	87.39	76.67	74.47	89.31	100.00						
100	60	64.11	61.62	74.63	69.23	74.39	78.89	77.50	75.07	75.45	71.83	90.17	79.79	81.63	88.06	96.13	100.00					
200	10	72.61	72.45	78.79	76.60	78.79	82.31	88.59	83.22	82.85	75.48	90.15	90.03	88.37	89.49	90.15	92.15	100.00				
200	20	71.29	70.16	80.81	78.04	79.67	83.05	84.21	84.65	84.45	74.95	91.11	83.10	91.24	92.37	89.71	92.25	95.45	100.00			
200	30	58.91	57.57	69.86	64.03	69.43	74.27	74.07	69.79	69.93	66.29	83.98	76.01	75.32	86.21	94.67	93.59	88.13	89.39	100.00		
200	45	56.74	55.91	68.40	63.36	68.27	75.44	73.17	69.10	69.01	65.51	85.01	75.93	74.21	86.99	94.23	90.93	87.75	88.67	97.81	100.00	
200	60	60.13	58.71	70.12	68.17	70.35	77.64	73.53	73.24	74.19	66.09	87.49	78.41	82.55	86.26	86.99	89.59	87.11	91.10	92.35	93.89	100.00

Table A.3. Archaeal community similarity matrix according to band presence/absence.

	OTC	0	0	0	0	0	0	50	50	50	50	50	100	100	100	100	100	200	200	200	200	200
OTC	Time	0	10	20	30	45	60	10	20	30	45	60	10	20	30	45	60	10	20	30	45	60
0	0	100																				
0	10	100	100																			
0	20	44.45	44.45	100																		
0	30	46.15	46.15	75	100																	
0	45	46.15	46.15	75	90.01	100																
0	60	50	50	80.01	94.74	84.21	100															
50	10	75	75	72.73	66.67	66.67	71.43	100														
50	20	60	60	76.93	82.35	70.59	87.5	83.33	100													
50	30	33.33	33.33	80.01	84.21	84.21	88.89	57.15	75	100												
50	45	54.55	54.55	85.72	88.89	88.89	94.12	76.93	80.01	82.35	100											
50	60	54.55	54.55	71.43	88.89	77.78	94.12	76.93	93.33	82.35	87.5	100										
100	10	75	75	72.73	66.67	66.67	71.43	100	83.33	57.15	76.93	76.93	100									
100	20	0	0	0	0	0	0	0	0	0	0	0	0	100								
100	30	33.33	33.33	66.67	46.15	46.15	50	50	60	50	54.55	54.55	50	0	100							
100	45	25	25	72.73	66.67	66.67	71.43	40	50	71.43	76.93	61.54	40	0	75	100						
100	60	54.55	54.55	85.72	88.89	88.89	94.12	76.93	80.01	82.35	100	87.5	76.93	0	54.55	76.93	100					
200	10	40	40	50	33.33	33.33	36.37	57.15	44.45	36.37	40	40	57.15	0	80.01	57.15	40	100				
200	20	40	40	50	33.33	33.33	36.37	57.15	44.45	36.37	40	40	57.15	0	80.01	57.15	40	100	100			
200	30	33.33	33.33	66.67	46.15	46.15	50	50	60	50	54.55	54.55	50	0	100	75	54.55	80.01	80.01	100		
200	45	33.33	33.33	66.67	46.15	46.15	50	50	60	50	54.55	54.55	50	0	100	75	54.55	80.01	80.01	100	100	
200	60	40	40	50	33.33	33.33	36.37	57.15	44.45	36.37	40	40	57.15	0	80.01	57.15	40	100	100	80.01	80.01	100

Table A.4. Archaeal community similarity matrix according to band intensities.

	OTC	0	0	0	0	0	0	50	50	50	50	50	100	100	100	100	100	200	200	200	200	200
OTC	Time	0	10	20	30	45	60	10	20	30	45	60	10	20	30	45	60	10	20	30	45	60
0	0	100.00																				
0	10	96.61	100.00																			
0	20	87.61	92.99	100.00																		
0	30	82.13	88.59	97.59	100.00																	
0	45	84.81	90.59	98.84	99.13	100.00																
0	60	90.61	94.17	97.71	93.17	95.88	100.00															
50	10	91.20	93.99	97.09	91.05	94.01	98.14	100.00														
50	20	90.46	94.39	99.17	95.66	97.47	98.29	98.55	100.00													
50	30	81.10	88.25	97.47	99.21	98.59	93.06	91.58	96.07	100.00												
50	45	85.01	91.29	99.13	97.95	98.85	96.27	95.43	98.43	98.80	100.00											
50	60	88.53	93.61	98.89	95.15	97.29	99.07	97.57	98.99	95.61	98.48	100.00										
100	10	85.98	91.36	98.37	93.33	95.75	97.20	97.72	98.35	93.81	97.47	98.60	100.00									
100	20	95.95	95.39	85.80	84.68	85.09	86.67	85.49	87.94	84.06	85.29	86.22	81.69	100.00								
100	30	85.95	91.91	98.97	96.89	98.02	96.30	96.43	98.76	97.77	99.33	98.24	98.20	85.65	100.00							
100	45	82.71	89.52	97.70	98.25	98.30	93.65	92.97	96.87	99.19	99.04	96.31	95.29	85.17	98.95	100.00						
100	60	85.42	91.19	97.19	95.35	96.47	96.40	95.01	97.19	96.27	97.65	97.73	96.09	85.21	97.55	97.11	100.00					
200	10	82.39	89.79	96.51	92.94	94.37	94.38	94.97	96.31	94.33	96.73	96.70	97.69	80.45	97.51	95.98	97.80	100.00				
200	20	83.09	89.47	96.44	93.98	94.92	94.16	93.63	95.98	94.66	96.56	96.59	97.37	81.94	97.19	96.00	97.45	98.61	100.00			
200	30	80.51	86.59	91.82	93.89	92.61	87.44	85.85	90.83	93.35	92.75	90.43	89.53	85.17	92.52	93.89	93.85	92.73	94.89	100.00		
200	45	69.50	73.00	76.28	76.23	76.31	74.08	71.25	75.53	75.68	76.94	76.57	76.27	72.77	77.73	78.53	79.73	79.54	83.03	85.99	100.00	
200	60	69.45	69.63	67.26	67.64	67.47	66.89	64.41	68.12	67.01	67.38	67.87	65.60	72.07	69.35	70.78	72.63	69.04	72.57	77.67	84.31	100.00

APPENDIX B: COMMUNITY SIMILARITY MATRIXES PART 2

Table B.1. Initial bacterial community similarity matrix according to presence/absence of the bands.

Sampling Day	0	1	3	5	10	15	20
0	100.00						
1	73.17	100.00					
3	90.33	76.19	100.00				
5	75.68	83.33	84.21	100.00			
10	73.69	81.63	71.80	80.01	100.00		
15	70.27	66.67	78.95	81.82	75.56	100.00	
20	60.87	80.71	68.09	83.02	77.78	79.25	100.00

Table B.2. Initial bacterial community similarity matrix according to band intensities.

Sampling Day	0	1	3	5	10	15	20
0	100.00						
1	96.51	100.00					
3	98.66	98.20	100.00				
5	96.33	94.25	96.80	100.00			
10	90.63	93.17	88.94	91.05	100.00		
15	88.22	91.19	91.08	90.82	85.20	100.00	
20	83.65	88.33	88.17	88.78	86.44	92.92	100.00

Table B.3. Bacterial community similarity matrix according to presence/absence of the bands t=10.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	90.57	100.00						
2	85.72	90.91	100.00					
3	88.89	94.34	92.86	100.00				
5	85.19	86.79	92.86	88.89	100.00			
10	76.93	82.35	81.49	80.77	84.62	100.00		
15	54.06	55.56	51.29	54.06	54.06	51.43	100.00	
20	68.19	69.77	73.91	72.73	72.73	71.43	51.85	100.00

Table B.4. Bacterial community similarity matrix according to band intensities, t=10.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	99.12	100.00						
2	88.54	88.65	100.00					
3	97.05	96.77	82.59	100.00				
5	89.45	88.85	90.69	91.37	100.00			
10	93.19	92.97	78.73	95.77	84.56	100.00		
15	92.53	92.69	76.99	91.73	81.80	92.91	100.00	
20	87.20	87.52	72.45	87.43	89.12	91.23	89.89	100.00

Table B.5. Bacterial community similarity matrix according to presence/absence of the bands t=20.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	80.95	100.00						
2	77.27	86.96	100.00					
3	75.00	70.59	66.67	100.00				
5	70.97	66.67	62.86	95.65	100.00			
10	85.11	89.80	82.35	61.54	57.90	100.00		
15	85.72	86.37	86.96	64.71	60.61	89.80	100.00	
20	32.01	37.04	27.59	35.30	37.50	31.25	22.23	100.00

Table B.6. Bacterial community similarity matrix according to band intensities, t=20.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	95.47	100.00						
2	93.43	93.35	100.00					
3	95.92	90.53	90.30	100.00				
5	97.63	93.86	90.95	97.91	100.00			
10	93.41	90.96	91.81	95.63	95.72	100.00		
15	94.86	90.56	91.75	96.42	96.92	98.47	100.00	
20	89.66	80.31	83.25	91.67	91.73	88.58	94.13	100.00

Table B.7. Bacterial community similarity matrix according to presence/absence of the bands t=30.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	88.00	100.00						
2	81.25	75.87	100.00					
3	88.00	100.00	75.87	100.00				
5	83.33	95.24	71.43	95.24	100.00			
10	74.29	68.75	87.18	68.75	64.52	100.00		
15	66.67	66.67	81.09	66.67	62.07	80.01	100.00	
20	72.73	73.33	81.09	73.33	68.97	85.00	89.47	100.00

Table B.8. Bacterial communiyt similarity matrix according to band intensities, t=30.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	82.73	100.00						
2	81.49	98.83	100.00					
3	82.27	98.89	98.13	100.00				
5	82.39	98.75	97.89	99.40	100.00			
10	78.21	90.06	92.15	92.88	93.70	100.00		
15	92.27	89.67	88.82	90.21	90.99	89.54	100.00	
20	93.61	84.07	83.47	84.52	84.79	84.09	97.67	100.00

Table B.9. Initial archaeal community similarity matrix according to presence/absence of the bands.

Sampling Day	0	1	3	5	10	15	20
0	100.00						
1	72.73	100.00					
3	88.89	83.33	100.00				
5	88.89	83.33	100.00	100.00			
10	66.67	66.67	60.00	60.00	100.00		
15	66.67	66.67	80.01	80.01	40.00	100.00	
20	44.45	66.67	60.00	60.00	80.01	60.00	100.00

Table B.10. Initial archaeal community similarity matrix according to band intensities.

Sampling Day	0	1	3	5	10	15	20
0	100.00						
1	68.16	100.00					
3	49.28	91.37	100.00				
5	40.38	77.11	79.48	100.00			
10	77.69	91.94	79.28	65.05	100.00		
15	66.57	92.97	89.49	73.03	93.63	100.00	
20	67.26	89.95	84.21	86.88	85.92	85.33	100.00

Table B.11. Archaeal community similarity matrix according to presence/absence of the bands t=10.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	80.01	100.00						
2	100.00	80.01	100.00					
3	100.00	80.01	100.00	100.00				
5	100.00	80.01	100.00	100.00	100.00			
10	66.67	85.72	66.67	66.67	66.67	100.00		
15	25.00	44.45	25.00	25.00	25.00	40.00	100.00	
20	33.33	57.15	33.33	33.33	33.33	50.00	80.01	100.00

Table B.12. Archaeal community similarity matrix according to band intensities, t=10.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	51.75	100.00						
2	37.73	90.13	100.00					
3	58.13	90.33	84.00	100.00				
5	37.50	80.21	82.37	92.46	100.00			
10	63.14	89.17	88.77	84.55	78.77	100.00		
15	76.98	79.45	67.54	86.00	77.01	83.63	100.00	
20	65.57	83.82	80.79	91.81	88.36	91.15	91.51	100.00

Table B.13. Archaeal community similarity matrix according to presence/absence of the bands t=20.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	66.67	100.00						
2	80.01	57.15	100.00					
3	100.00	66.67	80.01	100.00				
5	80.01	57.15	100.00	80.01	100.00			
10	57.15	66.67	75.00	57.15	75.00	100.00		
15	50.00	66.67	40.00	50.00	40.00	57.15	100.00	
20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00

Table B.14. Archaeal community similarity matrix according to band intensities, t=20.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	83.96	100.00						
2	84.67	93.45	100.00					
3	73.38	72.16	71.37	100.00				
5	86.59	79.15	90.63	71.37	100.00			
10	93.13	91.75	91.17	74.11	85.40	100.00		
15	90.77	83.11	79.37	67.93	76.03	93.08	100.00	
20	85.25	83.00	76.85	63.76	65.30	89.82	93.43	100.00

Table B.15. Archaeal community similarity matrix according to presence/absence of the bands t=30.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	80.01	100.00						
2	100.00	80.01	100.00					
3	100.00	80.01	100.00	100.00				
5	100.00	80.01	100.00	100.00	100.00			
10	57.15	75.00	57.15	57.15	57.15	100.00		
15	50.00	80.01	50.00	50.00	50.00	57.15	100.00	
20	66.67	50.00	66.67	66.67	66.67	33.33	66.67	100.00

Table B.16. Archaeal community similarity matrix according to band intensities, t=30.

Sampling Day	0	1	2	3	5	10	15	20
0	100.00							
1	93.07	100.00						
2	89.95	93.41	100.00					
3	77.41	85.69	86.69	100.00				
5	82.31	74.67	82.10	68.77	100.00			
10	85.33	81.89	84.93	80.30	90.19	100.00		
15	87.28	79.19	84.29	76.39	94.04	93.77	100.00	
20	86.37	76.93	83.20	73.71	87.35	90.05	97.73	100.00

APPENDIX C: METHANE PERCENTAGES IN BIOGAS

Table C.1. Methane percentages during externally OTC added microcosm tests.

OTC Concentration, mg/L	Digestion Time, day	Methane %
0	10	54
0	20	58
0	30	47
0	45	52
0	60	62
50	10	61
50	20	58
50	30	57
50	45	54
50	60	60
100	10	51
100	20	62
100	30	58
100	45	61
100	60	66
200	10	59
200	20	56
200	30	54
200	45	56
200	60	66

Table C.2. Methane percentages during medicated manure used microcosm tests.

Collection Day	Digestion Time, day	Methane, %
0	10	58
1	10	59
2	10	57
3	10	51
5	10	51
10	10	55
15	10	57
20	10	55
0	20	61
1	20	58
2	20	64
3	20	61
5	20	63
10	20	53
15	20	58
20	20	57
0	30	56
1	30	59
2	30	62
3	30	58
5	30	62
10	30	57
15	30	60
20	30	55