BEHAVIOUR OF OXYTETRACYCLINE IN TWO-PHASE ANAEROBIC CATTLE MANURE DIGESTERS AND ITS EFFECTS ON MICROBIAL COMMUNITIES

by

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In loving memory of my grandmother,
Fevziye Atabay

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ABSTRACT

Antibiotics used in veterinary practice may have inhibitory effects on microorganisms in anaerobic digestion of manure for biogas production as a renewable energy. Since microbial community plays a key role in the digestion process, defining the effects of antibiotics on microbial diversity and activity can lead to improvements in better understanding of interactions between microbial communities and biochemical processes, thus achieving a more efficient system performance in terms of higher digestion capacity and biogas production. In this study, inhibitory effects and behaviour of commonly used veterinary antibiotic, oxytetracycline (OTC) on the system performance and biogas production in a two-phase anaerobic digestion of cattle manure was investigated, as well as degree of acidification of cattle manure was improved.

In the first part, different combinations of batch anaerobic digesters were set-up to investigate the optimum operational conditions for acidogenic phase. The digesters were operated at different pH ranges between 5.0 and 6.0; and at different % total volatile solids (TVS). pH of 5.5±0.1 and 6% TVS were found to be optimal due to higher production of VFA and acidification rate.

In the second part, a commercially available hydrolytic enzyme mixture was added in order to improve the acidification rate of the cattle manure in the acidogenic phase of a two-phase anaerobic digestion system. Better solubilization resulted in higher VFA productions which caused higher biogas productions than previously achieved.

In the third part, 2 seperate two-phase anaerobic cattle manure digesters, one operated with non-medicated manure and the other operated with medicated manure, and furthermore, 2 single-phase digesters were operated for comparison in semi-continuous mode for 60 days. SRT/HRT for two-phase and single-phase operations were 5 days for acidogenic digesters, 15 days for methanogenic digesters and 20 days for single-phase digesters. Organic loading rates were 6.25±0.15 kg TVS/m^3 day in two-phase digesters and 1.50±0.02 kg TVS/m^3 day in single-phase digesters. Biogas yields of non-medicated two-phase and single-phase manure digesters were almost the same, 299±26 and 289±25 L

biogas/kg-TVS added, respectively; however, non-medicated two-phase digester had higher methane yield as 173±15 L CH_4/kg-TVS added, which was 150±13 L CH_4/kg-TVS added in single-phase digester. In medicated manure digesters, OTC concentration of 3.11±0.12 mg/L caused 35% inhibition on biogas yield and 43% inhibition on methane yield in the two-phase digester. In single-phase digester, OTC concentration of 3.07±0.14 mg/L caused 47% and 52% inhibitions on biogas and methane production, respectively. Reduction in OTC concentrations could have been attributed to abiotic and biotic reduction mechanisms such as photodegradation, temperature, humidity, pH, dilution during feeding of digesters and other environmental conditions, binding to organics and microbial degradation. The results indicated that the two-phase anaerobic digestion performed more efficient in the presence of OTC with respect to the biogas and methane production. According to DGGE and FISH results, almost all bacterial and *Archaeal* species were negatively affected by OTC. During the digestion period, DGGE results showed that the bacterial diversity in acidogenic digesters and *Archaeal* diversity in methanogenic digesters were higher than single-phase digesters. Bacteria groups of *Firmicutes* and *Gammaproteobacteria*; and *Archaeal* groups of *Methanobacteriales* and *Methanosarcinales* were found as the most abundant microorganisms. FISH results indicated that the relation between digestion period and the activity of microorganisms were mostly effective on *Firmicutes* and *Gammaproteobacteria*, showing a decreasing trend towards the end of digestion. In methanogenic activity, OTC presence was comparatively more effective on *Methanosarcinales*. The activity of *Methanobacteriales* and *Methanosarcinales* showed a more stable trend compared to bacteria; however, the ratios of active methanogenic cells were higher in methanogenic digesters compared to single-phase digesters, both in the absence and presence of OTC.

ÖZET

Veteriner hekimliğinde kullanılan antibiyotikler, hayvan dışkısından biyogaz eldesi amaçlı işletilen havasız çürütme sistemlerinde rol alan mikroorganizmalar üzerinde inhibitör etkide bulunabilirler. Çürütme sürecinde mikrobiyal komünite önemli bir rol oynadığından, antibiyotiklerin mikrobiyal çeşitliliğe ve aktiviteye etkilerinin araştırılması, proses ve etkileşimlerin daha iyi anlaşılmasında ve geliştirilmesinde ilerlemelere yol açabilir. Bu çalışmada, yaygın bir antibiyotik olan oksitetrasiklinin (OTC), iki fazlı havasız çürütücülerde sistem performansına, biyogaz üretimine ve mikrobiyal komüniteye etkileri incelenmiş, aynı zamanda sistem içinde hayvan dışkısının asidifikasyonuna dair iyileştirmeler çalışılmıştır.

İlk bölümde, en uygun işletme parametrelerini bulabilmek adına, kesikli çürütücüler birbirinden farklı koşullar altında kurulmuştur. Çürütücüler, 5.0 ile 6.0 arasında değişen pH aralıklarında ve farklı toplam uçucu katı oranlarında işletilmiştir. Yüksek uçuğu yağ asidi üretimi ve asidifikasyon verimi sebebiyle pH = 5.5±0.1 ve toplam uçucu katı oranı 6% en uygun işletme koşulları olarak belirlenmiştir.

İkinci bölümde, iki fazlı havasız çürütücü sisteminin asidojenik fazında hayvan dışkısının asidifikasyon verimini artırmak için, ticari olarak bulunan hidrolitik enzim karışımı sisteme ilave edilmiştir; bununla beraber uçucu yağ asidi üretiminde ve takiben biyogaz üretiminde öncesine gore artışlar elde edilmiştir.

Üçüncü bölümde, OTC içeren ve içermeyen gübrenin substrat olarak kullanıldığı farklı 2 adet iki fazlı havasız çürütücü sistemi ve bunların kontrolü olarak 2 adet tek fazlı çürütücü karşılaştırmak amacıyla yarı sürekli olarak 60 gün boyunca işletilmiştir. Çamur yaşı/hidrolik bekletme süreleri asidojenik reaktörler için 5 gün, metanojenik reaktörler için 15 gün ve tek faz reaktörler için 20 gün olarak belirlenmiştir. İki fazlı ve tek fazlı çürütücülerdeki organik yüklemeler ise sırasıyla 6.25±0.15 kg UKM/m^3-gün ve 1.50±0.02 kg UKM/m^3-gün olarak hesaplanmıştır. OTC içermeyen iki fazlı ve tek fazlı çürütücülerin biyogaz verimliliği sırasıyla 299±26 ve 289±25 L biyogaz/kg UKM olup neredeyse aynı oranlardadır; ancak, OTC içermeyen tek fazlı çürütücüde metan verimliliği 150±13 L

CH_4/kg TVS iken iki fazlı çürütücüde 173±15 L CH_4/kg UKM elde edilmiştir. OTC içeren çürütücülerden iki fazla çürütücüde, dışkıdaki 3.11±0.12 mg/L OTC konsantrasyonu biyogaz üretiminde %35 ve methan üretiminde %43 inhibisyona yol açmıştır. Tek fazlı çürütücüde ise, 3.07±0.14 mg/L OTC konsantrasyonu biyogaz verimliliğinde 47% ve metan verimliliğinde %52 inhibisyona sebep olmuştur. OTC konsantrasyonlarındaki azalmalar fotodegradasyon, sıcaklık, nem, pH, çürütücülerin beslenme sırasındaki seyrelmeler vb. çevresel koşullar, organic maddeye bağlanma ve de mikrobiyal degradasyon gibi abiyotik ve biyotik mekanizmalardan kaynaklanmış olabilir. Sonuçlar ele alındığında, OTC içeren dışkıyla işletilen havasız çürütme sistemlerinde, iki fazlı işletmenin tek faza oranla biyogaz ve metan üretimi açısından daha verimli olduğu bulunmuştur. DGGE ve FISH sonuçlarından, neredeyse bütün bacteri ve Arkeal türlerin OTC'den olumsuz etkilendiği çıkarılmıştır. DGGE sonuçlarında, asidojenik çürütücülerdeki bakteri çeşitliliğinin ve metanojenik çürütücülerdeki Arkeal çeşitliliğin, tek fazlı çürütücülerden fazla olduğu görülmüştür. Bakteriler arasında *Firmicutes* ve *Gammaproteobacteria* gruplarının ve Arkealar arasında *Methanobacteriales* ve *Methanosarcinales* gruplarının çoğunlukta olduğu tespit edilmiştir. FISH sonuçlarında ise, çürüme zamanının sonuna doğru aktiviteleri azaldığından, çürüme zamanının daha çok *Firmicutes* ve *Gammaproteobacteria* üzerinde etkili olduğu gözlemlenmiştir. Metajonik aktivitede *Methanosarcinales*, OTC varlığından nispeten daha olumsuz etkilenmiştir. *Methanobacteriales* ve *Methanosarcinales* gruplarının aktivitelerinin zaman içinde bakterilere göre daha istikrarlı olduğu ve hem OTC varlığında hem de yokluğunda, bu grupların aktivitelerinin metanojenik çürütücülerde tek fazlı çürütücülere oranla daha yüksek olduğu görülmüştür.

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Symbol	Explanation	Units
AD	Anaerobic Digestion	
APS	Ammonium Persulfate	
ATP	Adenosine Triphosphate	
COD	Chemical Oxygen Demand	mg/L
CSTR	Continuous Stirred Tank Reactor	
CTC	Chlorotetracycline	
DABCO	1,4-diazabicyclo[2.2.2]octane	
DGGE	Denaturing Gradient Gel Electrophoresis	
DNA	Deoxyribonucleic Acid	
dNTP	deoxynucleoside triphosphate	
EDTA	Ethylenediamine Tetraacetic Acid	
EOTC	4-epi-Oxytetracycline	
EtBr	Ethidium Bromide	
FISH	Fluorescent *in situ* Hybridization	
GC	Gas Chromatography	
GC-MS	Gas Chromatography-Mass Spectrophotometry	
gDNA	Genomic DNA	
DAPI	4,6-diamine phenylindol	
HPLC	High Pressure Liquid Chromatography	
HRT	Hydraulic Retention Time	day
NRB	Nitrate Reducing Bacteria	
OLR	Organic Loading Rate	g TVS/L-day
OTC	Oxytetracycline	
PBS	Phosphate Buffered Saline	
PCR	Polymerase Chain Reaction	
PMP	Potential Methane Production	
PFA	Paraformaldehyde	
RNA	Ribonucleic Acid	

rDNA	Ribosomal DNA	
rRNA	Ribosomal RNA	
Q-PCR	Real Time PCR (Quantitative PCR)	
sCOD	Soluble Chemical Oxygen Demand	
SDS	Sodiumdodecylsulfate	
SMA	Specific Methane Activity	
SRB	Sulphate Reducing Bacteria	
SRT	Sludge Retention Time	day
SS	Suspended Solids	mg/L
SSU	Small Subunit	
TAE	Tris-Acetic Acid-EDTA	
TC	Tetracycline	
TEMED	Tetra Methyl Ethylene Diamine	
TGGE	Thermal Gradient Gel Electrophoresis	
TPAD	Two-phase Anaerobic Digestion	
TS	Total Solid	mg/L
TVS	Total Volatile Solid	mg/L
UV	Ultraviolet	
VFA	Volatile Fatty Acids	mg/L

1. INTRODUCTION

Every year, millions of tons of wastes are generated from municipal, industrial and agricultural sources. Animal manure, generated from livestock industries and agricultural activites, have been identified as a major source of environmental pollution. The most common problems of untreated animal manure are odor, methane and ammonia emissions, and the release of nutriens and pathogens that may harm human health. The potential use of manure as an environmentally safe energy source and the environmental benefits made anaerobic digestion of manure a significant process.

Veterinary antibiotics that are used in animal husbandries become a problem in anaerobic digestion systems of animal manure. These antibiotics are commonly used to prevent infections, treat diseases as well as growth prometers (Kemper, 2008). According to Animal Health Institute, antibiotics used for animal feeding have increased from 91 mg in 1950 to 9900 mg (including 3000 mg of tetracyclines) in 2004 (Álvarez et al., 2010). These compounds can be metabolized through different pathways when they are ingested by animals. They are eventually excreted, maintaining the same chemical structure or as metabolites transformed into epimers or isomers (Kemper, 2008). Oxytetracycline (OTC) is one of the most common antibiotics that are used in animals due to its broad range of activity and low cost. OTC is administered to livestock animals, including cattle, swine, poultry and fish, to promote growth and for prophylactic and therapeutic treatment (Álvarez et al., 2010). OTC, its degradation products and epimers (such as 4-epi-oxytetracycline, α-apo-oxytetracycline or β-apo-oxytetracycline) are strongly adsorbed in manure since they create complexes with metal ions, humic acids, proteins and other organic matters in the manure (Loke et al., 2002, 2003). The overuse of antibiotics in livestocks cause high concentrations of antibiotics and their metabolites in manure that could be easily released into the environment (Álvarez et al., 2010). The presence of antibiotics or antibiotic metabolites in manure can inhibit the digestion activity of anaerobic microorganisms (Arikan et al., 2006; Arikan, 2008).

Acidogenic and methanogenic microorganisms are different in physiological and nutritional requirements, growth kinetics, and sensivity to environmental conditions, a two-

phase system has the advantage of phase seperation, using seperate units for acidogenesis and methanogenesis, in order to optimize environmental conditions for each phase (Azbar and Speece, 2001; Babel et al., 2004). It leads to the production of the most suitable acid metabolites for the methanogens and consequently an increase in the rate of substrate of turnover, which may allow a reduction in total reactor volume and also higher methane production rate. Proper control of acidification-phase increases the stability of the process due to the prevention of organic and hydraulic overloadings and the build-up of toxic materials for methanogenic bacteria (Ince, 1998; Demirer and Chen, 2005; Panichnumsin et al., 2010).

The failure of many anaerobic digesters to operate reliably has lead to the need for more information on the biological aspects of the anaerobic digestion ecosystem (Godon et al., 1997). Recent developments with the integration of microbial ecology and molecular biology have involved and provided a new sight into the interrelations between the microorganisms and their environment in bioreactors. Understanding the microbial ecology in anaerobic reactor systems require identification and classification of microorganisms, quantification of microbial abundance and quantification and identification of activity. Recently, the microbial ecology of anaerobic digestion systems has been investigated in detail by using Ribosomal RNA (rRNA) and ribosomal DNA (rDNA) based molecular techniques such as Fluorescence is situ Hybridization, Real-time PCR and Denaturing Gradient Gel Electrophoresis (DGGE) (Hofman-Bang et al., 2003).

Microbial characterization of the anaerobic manure digestion systems in changing oxytetracycline environments is crucial. Since the fuel based energy sources are running out, achieving higher biogas production and increasing the overall digestion system efficiency are very important. This study may assist to researchers dealing with anaerobic digestion of manure in improving the operational conditions as well as determining microbial community dynamics and inhibitory effects of oxytetracycline in the digestion process.

2. LITERATURE REVIEW

2.1. Fundamentals of Anaerobic Digestion

Today's waste management policies highlight the main trends that reduce the stream of waste going to landfills and recycle the organic material and the plant nutrients back to soil. Since the waste problem is increasing day by day, its recirculation is gaining more and more attention. Anaerobic digestion is one of these trends to achieve this goal and furthermore, reduce energy consumption and even produce energy, which is the major importance to the global environment. Back in the days, anaerobic digestion was mostly implemented for the stabilization of sewage sludge. However, during the past years, anaerobic digestion processes have been expanded to lay emphasis on treatment and energy recovery from many other types of wastes including household wastes, animal wastes, organic industrial wastes, domestic and industrial wastewater (Ahring, 2003).

Anaerobic digestion is the biodegradation and stabilization of organic materials in the absence of oxygen by microbial organisms and leads to the formation of biogas, mixture of cardon dioxide and methane, and microbial biomass. The production of biogas through anaerobic digestion is considered as one of the best ways to produce renewable energy since it is environment-friendly and economically beneficial. As one of the most efficient waste and wastewater treatment technologies, anaerobic digestion offers other various advantages such as low sludge production, low energy requirement and possible energy recovery. Compared to mesophilic digestion, thermophilic anaerobic digestion has several additional benefits including a high degree of waste stabilization, greater destruction of pathogens and improved post-treatment sludge dewatering. Despite all these benefits, poor operational stability prevents anaerobic digestion to be widely applied (Chen et al., 2008).

2.1.1. Process Biochemistry

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 specifities (Hutnan et al., 1999). To date, several models have been developed to explain 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).

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_4 in the last phase (Liu et al., 2002).

As shown in Figure 2.1., Harper and Pohland (1986) explained the overall process in nine steps as follows:

i. Hydrolysis of organic polymers to intermediate organic monomers,
ii. Fermentation of organic monomers,
iii. Oxidation of propionic and butyric acids and alcohols by obligate H2 producing acetogens,
iv. Acetogenic respiration of bicarbonate by homoacetogens,
v. Oxidation of propionic and butyric acids and alcohols by sulphate reducing bacteria (SRB) and nitrate reducing bacteria (NRB),
vi. Oxidation of acetic acid by SRB and NRB,
vii. Oxidation of hydrogen by SRB and NRB,
viii. Acetoclastic methane formation,
ix. Methanogenic respiration of bicarbonate.

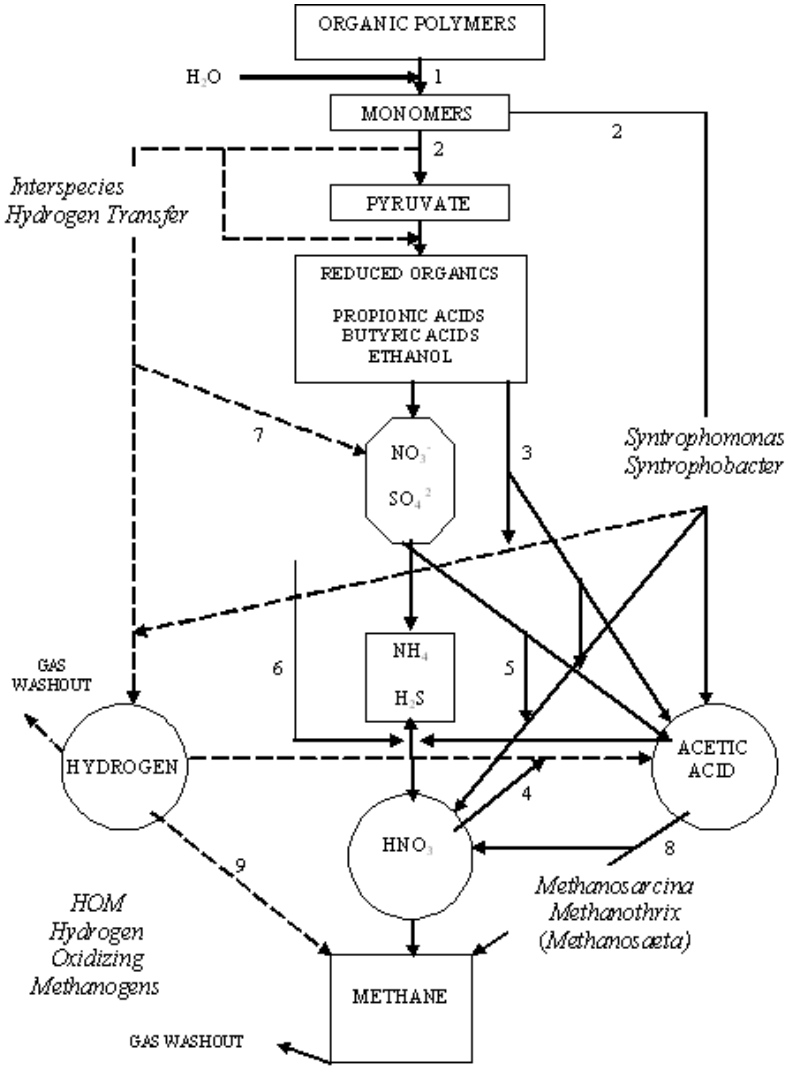

Figure 2.1. Substrate conversion patterns associated with the anaerobic digestion (Harper and Pohland, 1986).

During the degradation of carbohydrates, proteins and lipids, volatile fatty acids are formed as intermediate products. The short-chain volatile acids such as formic, acetic, propionic, butyric, valeric and isovaleric acids are the most important components resulting from the biochemical decomposition of the organic material. Acetic and propionic acids, precursors of methane formation, are the most important ones among them (Chernicharo, 2007).

Acetic acid is the most abundant intermediate acid, formed from all the organic compounds. Propionic acid results mainly from the fermentation of the carbohydrates and

proteins, and about 30% of the organic compounds are converted into propionic acid before they are finally converted into methane. Some of the conversion reactions of the products from fermentative bacteria into acetate, hydrogen and cardon dioxide are shown in Table 2.1.

Table 2.1. Some important oxi-reduction reactions in anaerobic digestion (Chernicharo, 2007).

Nr	**Oxidation reactions (electron donors)**	**ΔG_o (kJ/mole)**
1 Propiante → acetate	$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	+ 76.1
2 Butyrate → acetate	$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$	+ 48.1
3 Ethanol → acetate	$CH_3CH_2OH + H_20 \rightarrow CH_3COO^- + H^+ + 2H_2$	+ 9.6
4 Lactate → acetate	$CH_3CHOHCOO^- + 2H_2 \rightarrow CH_3COO^- + HCO_3^- + H^+ + 2H_2$	- 4.2
	Reduction reactions (electron acceptors)	
5 Bicarbonate → acetate	$2HCO_3^- + 4H_2 + H^+ \rightarrow CH_3COO^- + 4H_2O$	- 104.6
6 Bicarbonate → methane	$HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + 3H_2O$	- 135.6
7 Sulfate → sulfide	$SO4_2^- + 4H_2 + H^+ \rightarrow HS^- + 4H_2O$	- 151.9

The main components of biogas are methane and carbon dioxide with smaller amounts of hydrogen sulfide and ammonia. The overall biogas composition produced during the anaerobic digestion varies according to the environmental conditions in the reactor. The composition changes rapidly during the start-up period of the system and also when there is an inhibition in the digestion process. However, the ratio between carbon dioxide and methane can vary substantially, depending on the characteristics of the organic compound to be degraded (Chernicharo, 2007).

Table 2.2. Maximal Gas Yields and Theoretical Methane Contents of Different Substrates (Baserga, 1998).

Substrate	**Biogas (NM^3 / t TS)**	**CH_4 (%)**	**CO_2 (%)**
Carbohydrates	790-800	50	50
Raw protein	700	70-71	29-30
Raw fat	1200-1250	67-68	32-33

2.1.2. Process Microbiology

Anaerobic digestion is generally considered as a two-phase process, but it can be subdivided into various metabolic pathways, with the participitation of several microbial groups, showing different physiological behaviour (Chernicharo, 2007).

The first phase in the anaerobic digestion is the hydrolysis of complex particulate material (polymers) into smaller dissolved materials, which can be penetrate through the cell membranes of the fermentative bacteria. Hydrolatic fermentative bacteria convert these particulate materials into dissolved materials by the action of exoenzymes excreted. Generally, the hydrolysis of polymers occurs slowly in anaerobic conditions and many factors such as temperature, substrate composition, pH, residence time of the substrate in the reactor, concentration of products from hydrolysis affect the degree and rate at which substrate are hydrolysed (Letting et al., 1996). It was stated that *Clostridium* is responsible for the degradation of compounds containing cellulose and starch, while *Bacillus* play role in the degradation of proteins and fats (Noike et al., 1985; Lema et al., 1991). The types of hydrolytic microorganisms are reported namely as, the cellulytic (*Clostridium thermocellum*), proteoytic (*Clostridium bifermentas, Peptococcus*), lipolytic (genera of clostridia and micrococci) and aminolytic (*Clostridium butyricum, Bacillus subtilis*) bacteria (Payton and Haddock, 1986).

The fermentative bacteria metabolise the soluble products from the hydrolysis phase inside their cells and convert them into several simpler compounds, which are then excreted by the cells. The compounds produced include volatile fatty acids, alcohols, lactic acid, carbon dioxide, hydrogen, ammonia and hydrogen sulfide, besides new cells (Chernicharo, 2007).

Acidogenesis is carried out by a wide and diverse group of fermentative bacteria. Main species belong to the clostridia group, which comprises anaerobic species that can survive in very adverse environments, and the family *Bacteroidaceaea*, which participates in the degradation of sugars and amino acids (Chernicharo, 2007). Single amino acids are converted by *Clostridia, Mycoplasmas and Streptococci*; meanwhile, butyric acid, butanol,

acetone and isopropanol are generally produced by the bacteria of the genera *Clostridium* and *Butyribacterium*.

Acetogenic bacteria are part of an intermediate metabolic group which produces substrate for methanogenic microorganisms. They convert the products generated in the acidogenesis phase into a substrate appropriate, such as acetic acid, hydrogen and carbon dioxide, for the methanogenic microorganisms. A significant amount of hydrogen is formed during the formation of acetic and propionic acids, which cause the pH in aqueous medium to decrease. This produced hydrogen is consumed in two ways: (i) through the methanogenic microorganisms that use hydrogen and carbon dioxide to produce methane, and (ii) through the formation of organic acids like propionic and butyric, which are formed through the reaction among hydrogen, carbon dioxide and acetic acid (Chernicharo, 2007).

Acetic acid producing bacteria are *Methanobacterium bryantii*, *Desulfovibrio Syntrophobacter wolinii*, *Syntrophomonas wofei* and *Syntrophus buswellii* (Gujer et al., 1983; Stronach et al., 1986; Malina et al., 1992).

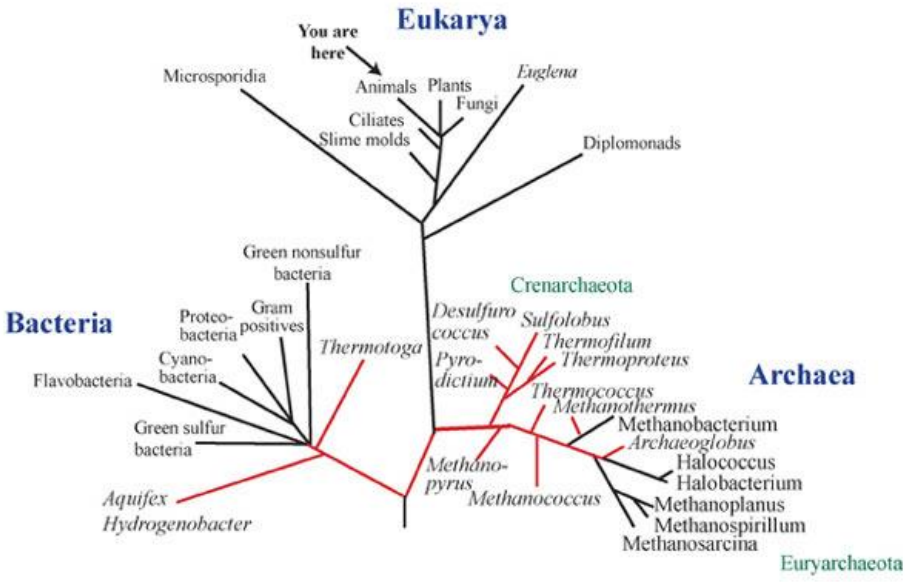

Figure 2.2. Universal phylogenetic tree (Woose et al., 1990).

Methanogenesis is the final step in the overall anaerobic digestion process of organic compounds into methane and carbon dioxide driven by methanogenic *Archaea*. All methanogens are strictly anaerobic *Archaea* belonging to *Euryarchaeota*. Methanogens are classified into five orders within kingdom *Archaeobacteria*: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales* and *Methanopyrales* (Figure 2.3).

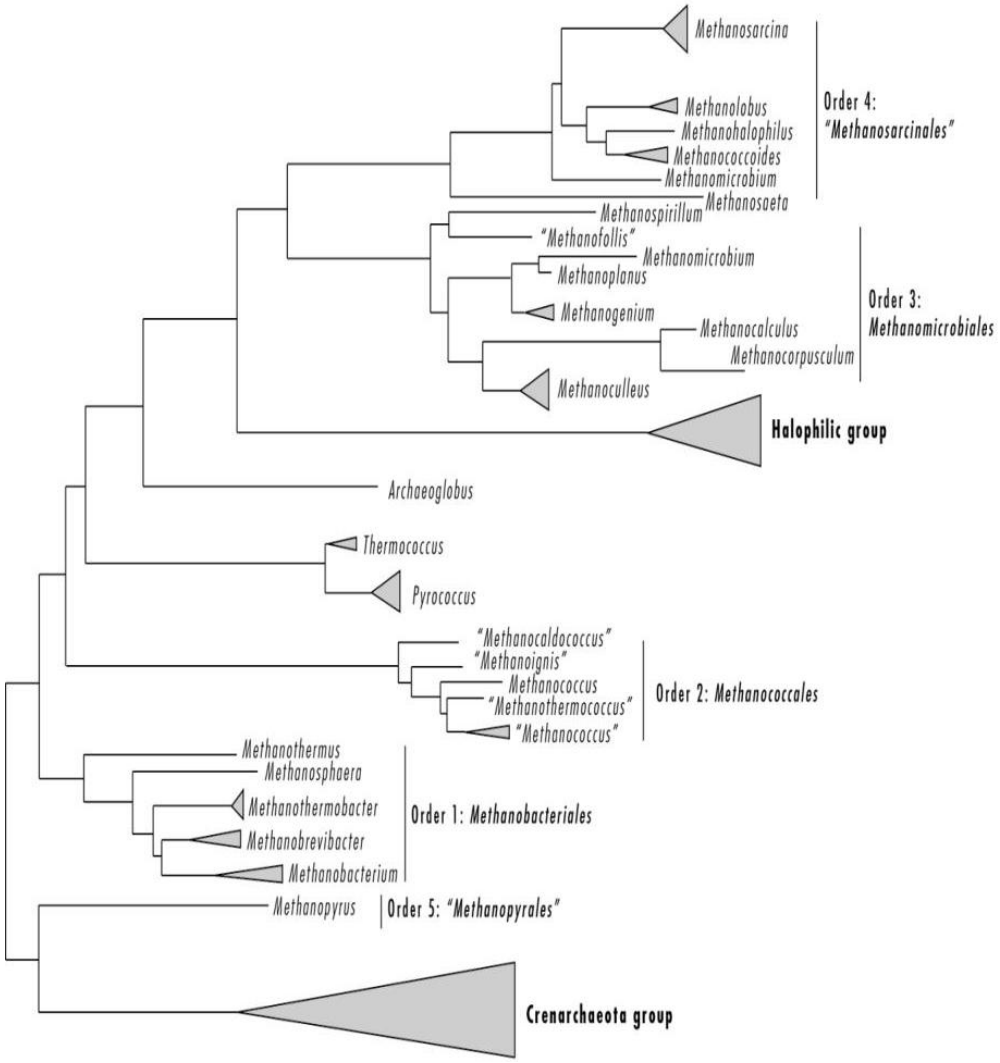

Figure 2.3. Phylogeny of methanogens, domain Archaea (Garcia et al., 2000).

Methanogens can only use a limited number of substrates, comprising acetic acid, hydrogen/carbon dioxide, formic acid, methanol, methylamines and carbon monoxide. Methanogens are divived into two main groups according to their affinity for these

substrates, one that forms methane using acetic acid or methanol, and the other one that produces methane from hydrogen and carbon dioxide:

- acetate-using microorganisms (acetoclastic methanogens)
- hydrogen-using microorganisms (hydrogenotrophic methanogens)

Acetoclastic methanogens are only a few of the methanogenic species that are capable of forming methane from acetate, which usually dominate the anaerobic digestion systems. They are responsible for about 60 to 70% of all methane production. Two genera utilize acetate to produce methane: *Methanosarcina* prevails above 10^{-3} M methane, while *Methanosaeta* prevails below this acetate level (Zinder, 1993). *Methanosaeta* usually have lower yields and is more sensitive to pH changes, comparing to *Methanosarcina* (Schmidt and Arhing, 1996). While *Methanosarcina* has a greater growth rate, *Methanosaeta* needs longer solids retention time. *Methanosaeta* genus is characterized by exclusive use of acetate and having a higher affinity with it than *Methanosarcina* genus. *Methanosarcina* genus are considered as the most versatile ones among the methanogenic microorganisms, since they can also use hydrogen and methylamines (Soubes, 1994).

$$C^*H_3COOH \rightarrow C^*H_4 + CO_2 \quad (2.1)$$

(Microbial group involved: acetoclastic methanogenic microorganisms)

Hydrogenotrophic methanogens are capable of producing methane from hydrogen and carbon dioxide. *Methanobacterium, Methanospirillum* and *Methanobrevibacter* are the genera more frequently isolated in anaerobic reactors (Chernicharo, 2007).

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

(Microbial group involved: hydrogenotrophic methanogenic microorganisms)

Table 2.3. Characteristics of methanogenic *Archaea* (Madigan et al., 2002).

Order	Morphology	Substrate for methanogenesis
Methanobacteriales		
Methanobacterium	Long rods	$H_2 + CO_2$, formate
Methanobrevibacter	Short rods	$H_2 + CO_2$, formate
Methanosphaera	Cocci	Methanol + H_2
Methanothermus	Rods	$H_2 + CO_2$, can also reduce S^0; hyperthermophile
Methanococcales		
Methanococcus	Irregular cocci	$H_2 + CO_2$, formate, pyruvate + CO_2
Methanomicrobiales		
Methanomicrobium	Short rods	$H_2 + CO_2$, formate
Methanogenium	Irregular cocci	$H_2 + CO_2$, formate
Methanospirillum	Spirilla	$H_2 + CO_2$, formate
Methanoplanus	Plate-shaped cells	$H_2 + CO_2$, formate
Methanocorpusculum	Irregular cocci	$H_2 + CO_2$, formate, alcohols
Methanoculleus		$H_2 + CO_2$, formate, alcohols
Methanosarcinales		
Methanosarcin**a**	Large irregular cocci in packets	$H_2 + CO_2$, methanol, methylamines, acetate
Methanolobus	Irregular cocci in aggregates	Methanol, methylamines
Methanohalobium	Irregular cocci	Methanol, methylamines; halophilic
Methanococcoides	Irregular cocci	Methanol, methylamines
Methanohalophilus	Irregular cocci	Methanol, methylamines, methyl sulfides; halophilic
Methanosaeta	Long rods to filaments	Acetate
Methanopyrales		
Methanopyrus	Rods in chains	CO_2, hyperthermophile, growth rate at 100^oC

In anaerobic digestion systems, sulfate or sulfite can be used by sulfate-reducing bacteria (SRB) as acceptors of electrons released during the oxidation of organic materials, The metabolism of SRB is very important because of their end-product, hydrogen sulfide (Lettinga et al., 1996). SRB group species are considered a very versatile group of microorganisms that can use a wide range of substrate, including the whole chain of volatile fatty acids, several aromatic acids, hydrogen, methanol, ethanol, glycerol, sugars, amino acids and several phenol compounds (Chernicharo, 2007). Two major group of SRB

can be distinguished: (i) the one that is able to oxidize incompletely its substrates to acetate, like the genera *Desulfobulbus sp*. and *Desulfomonas sp.*, and most of the species of the genera *Desulfofotomaculum* and *Defulfofovibrio* belong to this group; and (ii) the other group that is able to oxidize its organic substrates, including acetate, to carbon dioxide, including the genera *Desulfobacter, Desulfococcus, Desulfosarcina, Desulfobacterium and Desulfonema* (Chernicharo, 2007).

2.1.3. Environmental and Operational Factors Affecting Anaerobic Digestion

Among the many factors affecting the anaerobic digestion process, temperature is an important one. It affects the system in several ways including solubility of substrates, substrate removal rate, ionization equilibrium and other constants such as specific growth rate, decay biomass yield and half saturation constant. It is proven that anaerobic processes are highly sensitive to the temperature variations. Especially, methane conversion of acetate to CH_4 is known as more sensitive to temperature than the acetate forming process (Stover et al., 1994). Anaerobic digestion can be processed under psychrophilic (<25^{o}C), mesophilic (25-40^{o}C) and thermophilic (>45^{o}C) conditions. Thermophilic digestion processes offer many advantages such as higher metabolic rates, higher specific growth rates and higher destruction of pathogens (El-Mashad et al., 2004). Under thermophilic conditions, since the growth rate of anaerobic microorganisms is higher; process is faster and more efficient. Under optimal operating conditions, a thermophilic reactor can be fed with higher organic loading rates at lower hydrolic retention times than mesophilic reactors; however most of the anaerobic digestion plants are processed under mesophilic conditions, mostly between 35-42^{o}C, due to lower stability and higher susceptibility to changes in environmental and operational conditios of thermophilic treatment systems. Mesophilic microflora is able to tolerate temperature fluctuations within ±3ºC without considerable reductions in methane production (Weiland, 2010).

Hydraulic retention time (HRT) represents the average time the substrate remains in a digester. It is defined as the reactor working volume divided by the mean volume flowrate. If the HRT is too short the organic material will not be completely degraded resulting in low gas yields and possible inhibition of the process. Short retention time can also result in washout of the methanogens if the retention time is shorter than their rate of

multiplication. The optimum choice of HRT is mostly dependent on the temperature and the type of material being digested. The retention times of mesophilic and thermophilic digesters range between 10-30 days (Yılmaz, 2007).

Solids retention time (SRT) 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 (Metcalf and Eddy, 2003).

Organic loading rate (OLR) describes the amount of organic material (expressed as chemical oxygen demand-COD or volatile solids-VS) which is fed daily per m^3 of digester working volume. If there is an excess of easy degradable nutrients fed to the digester, the process may be affected, because in the acidification phase, there would be more end products than the second step can utilize. Such an overload leads to a drop in the pH-value and inhibition of the methanogenic activity. Some of the degradation steps will not yield energy unless their products are efficiently removed by the next group of microorganisms (Yılmaz, 2007).

Another important parameter affecting digestion performance is pH. It affects the solubility of substances and the reaction behaviour of microorganisms. As a result, the digestion performance is directly in a relation with pH. In single-phase reactors, methanogenic pH requirements are taken into account. Most methanogens function in a pH between 6.5 and 7.5. 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 methanogens while acid forming bacteria can live at this pH and keep producing volatile fatty acids despite low pH, therefore making the environmental conditions worse (Pohland and Suidan, 1987). Acidogenic bacteria 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).

For the growth and survival of the existing groups of microorganisms in anaerobic digesters, certain macro and micronutrients are essential. Macronutrients are carbon, nitrogen and phosphorus whereas iron, nickel, cobalt, selenium, molybdenum, and tungsten can be pronounced among the micronutrients. Nickel is generally required for all methanogens and takes place in the synthesis of cofactor F430. For optimal biomass production, cobalt is required for the build up of cell component corrinoid factor III. The amount of micronutrients neccesary for the process is very low, changing between 0.05 and 0.06 mg/L. Iron may be an exception required in concentrations between 1 and 10 mg/L (Bischoff, 2009). Utilization of manure as a substrate decreases the need for micronutrients however, it has been shown that addition of micronutrients always enhance the performance of anerobic digester (Preißler et al., 2009).

Mixing is a very important parameter in anaerobic digesters, especially operating with particulate substrates like manure. It allows the complete contact between the reactor contents and the biomass. It also reduces the possible inhibitory effects of local VFA accumulations and other digestion products. Mixing can be accomplished by mechanical mixers, biogas recirculation, or by slurry recirculation. In lab-scale anaerobic digesters, mixing can vary between 20-100 rpm (Wu et al., 2010). Mixing in high rpms is difficult to be obtained in full scale digesters; therefore, it 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).

The design of a digester is strongly influenced by the the composition, homogeneity and the dry matter content of the waste fed to it. For agricultural substrates and wastes, rich in solid material, the high-rate reactors are not suitable because granule formation is hindered and packed beds will clog immediately. Livestock manures, which

are the predominant waste material in agricultural anaerobic digestion, are heterogeneous materials with total solid concentrations varying between 2% and 10%. The required digester is therefore a simple continuously stirred tank reactor (CSTR) with the provision for co-digestion (Yılmaz, 2007).

Anaerobic digestion systems can be operated in batch-wise, semi-continuous or continuous mode. In a batch system, biomass is added to the reactor at the start of the process with an inoculum. The reactor is then sealed for the duration of the process. In semi-continuous digesters, quantities of waste are periodically added and removed to a digester leading to a *de facto* semi-continuous system. The most common type of medium and large-scale anaerobic digestion system is the continuous-flow tank reactor. The raw waste is fed regularly into a digester, displacing an equal volume of digested material. The working volume in the digester remains constant. Meanwhile, the digester content is homogenized regularly. Most of the smaller systems are fed once or twice a day, but the frequency of input is increased with the use of easily fermentable substrates.

Inhibitory substances are often found to be the main reason for the upset and failure of anaerobic reactors since they exist in substantial concentrations in wastewaters and sludges. A wide variety of substances have been reported as inhibitors to the anaerobic digestion processes. A material may be called as inhibitory when it causes an adverse shift in the microbial population or inhibition of bacterial growth. A decrease of the steady-state rate of methane gas production and accumulation of organic acids are usually the main indicators for inhibition (Chen et al., 2008). An inhibition may affect all groups of microorganisms involved in process but generally methanogenesis is the most sensitive step to inhibitory or toxic material (Speece and Parkin, 1983).

Ammonia is produced by the break down of nitrogenous compounds like urea or proteins (Kayhanian, 1999). Various mechanisms of ammonia inhibition have been proposed through the years including; a change in the intracellular pH, increase of maintenance energy requirement, and inhibition of a specific enzyme reaction (Whittmann et al., 1995). Inorganic nitrogen is found in the forms of ammonuim (NH_4^+) and free ammonia (NH_3), in anaerobic digesters. Methanogens are the least resistant group of microorganisms to ammonia inhibition (Kayhanian, 1994). 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 pH values. Ammonia inhibition in anaerobic digestion systems is controlled by concentration, pH, temperature, acclimation and presence of other ions (Chen et al., 2008).

Table 2.4. Effects of free ammonia on anaerobic processes (McCarty, 1964).

Concentration (as N, mg/L)	**Effect**
50-200	Beneficial
200-1000	No adverse effect
1500-3000	Inhibitor for pH > 7.4 to 7.6
Above 3000	Toxic

Sulfate is a common constituent of many industrial wastewaters (O'Flaherty et al., 1998). In anaerobic reactors, sulfate is reduced to sulfide by the sulfate reducing bacteria (SRB) (Koster et al., 1986; Hilton and Oleszkiewicz, 1988). H_2S is the toxic form of sulfide because it can penetrate into cells. Once inside the cytoplasm, H_2S may be inhibitory by denaturing native proteins through the formation of sulfide and disulfide cross-links between polypeptide chains (Conn et al., 1987). 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).

The balanced activity between mixed microbial populations can be easily disturbed by different factors triggering a rapid increase in the concentration of volatile fatty acids (VFA) with a consequent decrease in methane production (Ahring and Westermann, 1983). Methanogens are the most sensitive group to such situations, converting acetate and carbon dioxide into methane. VFA, the principle intermediates in anaerobic digestion mechanisms, tend to accumulate in digesters for various reasons, for example, by a substrate overload. Such an accumulation, in which VFA production is higher than the VFA consumption, can cause an inhibition of methane production. Except for acetic acid, which acetoclastic methanogens degrade directly to methane, the VFA, especially propionic and butyric, must be first degraded by obligate hydrogen-producing acetogenic

bacteria to acetate, hydrogen and carbon dioxide, which are then utilized by methanogens (Dogan et al., 2005). 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).

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

Heavy metals such as chromium, iron, cobalt, copper, zinc, cadmium and nickel can be present in significant concentrations in municipal sewage and sludge and unlike many other toxic substances, they are not biodegradable and can accumulate to potentially toxic concetrations in time (Jin et al., 1998). Heavy metal ions inhibit metabolisms of microorganisms and inactivate their certain enzymes; however, trace amounts of heavy metals are essential for the microorganism activity (Chen et al., 2008).

Organic chemicals are another example for the inhibiton in anaerobic digestion systems. They are poorly soluble in water and adsorbed to the surfaces of solids, accumulating to high levels which cause the membranes of bacteria to swell and leak, disrupting ion gradients and finally causing cell lysis (Heipieper et al., 1994; Sikkema et al., 1994). There are many parameters affectting organic chemical inhibition such as toxicant concentration, biomass concentration, toxicant exposure time, sludge age, feeding, acclimation and temperature. The inhibition concentration ranges are mostly depending on the organic chemical type (Yang and Speece, 1986).

2.2. Anaerobic Digestion of Animal Manure

Each year, millions of tons of wastes are generated from agricultural, municipal and industrial sources. Animal manure, generated from livestock industries and agricultural activities, have been identified as a major source of environmental pollution. In the EU-27

alone, more than 1500 million tons of animal manure is produced every year and European agriculture handles more than 65% of livestock manure as slurry, liquid mixture of feces, urine, water and bedding material (Menzi, 2002). These large amounts of animal manure and slurries produced today represent a great pollution risk with a potential negative impact on the environment, if they are not managed properly (Holm-Nielsen et al., 2009).

Animal manure becomes a major source of air and water pollution, when untreated or poorly managed. Some of the major problems include nutrient leaching, mainly nitrogen and phosphorous, ammonia evaporation and pathogen contamination. The animal production sector causes 18% of the overall greenhouse gas emissions, measured in CO_2 equivalent and for 37% of the anthropogenic methane, which has 23 times the global warming potential of CO_2. Furthermore, 64% of anthropogenic ammonia emission and 65% of anthropogenic nitrous oxide originates from the animal production sector all over the world (Steinfeld et al., 2006).

To date, many alternatives have been applied to treat and dispose animal manure. Pond systems (Wang et al., 1996), composting (Tiqua and Tam, 1998; Guerra Rodriquez et al., 2001), land application (Sommer and Hutchings, 2001; Araji et al., 2001), constructed wetlands (Knight et al., 2000; Clarke and Baldwin, 2002), anaerobic treatment (Lo and Liao, 1984; Wen et al., 2007; Alvarez and Giden, 2009) are examples of these techniques. The researches show that anaerobic digestion offers the best solution in terms of pollution reduction and energy production, which also improves the fertilize value of the manure.

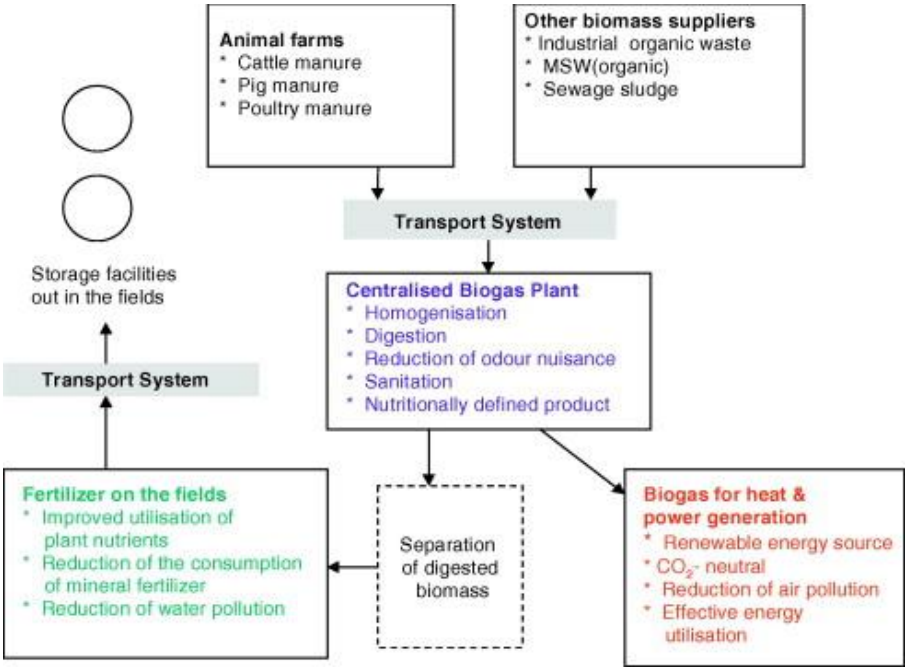

Figure 2.4. The main streams of integrated concept of centralized co-digestion plant (Holm-Nielsen and Al Seadi, 2004).

Anaerobic digestion of animal manure has the general aim to convert organic residues into two valuable products: biogas, a renewable fuel further to produce heat, electricity or as vehicle fuel and fertilizer to be used in agriculture. Concentrated fertilizers, fiber products and clean water can also be gained from further refining of digestate (Holm-Nielsen et al., 2009). In Turkey alone, there are an estimated of over 13 million of cattles. As the calorific value of biogas is about 6 kWh/m^3, this process would save a great amount of fuel per year. This amount of animal wastes produces 11 million-ton of dry solid per year (Başçetinçelik et al., 2005). Several factors affect the properties of dairy cattle manure, including the digestibility and protein and fiber contents of the feed, and the animal's age, environment, and productivity.

Table 2.5. Production and characteristics of dairy cattle manure (Midwest Plan Service, 1993).

Animal size (kg)	**Total manure production (kg/d)**	**Water (%)**	**Density (kg/m^3)**	**TS (kg/d)**	**VS (kg/d)**	**BOD_5 (kg/d)**	**Nutrient content (kg/d)**		
							N	**P**	**K**
68	5.9	87.3	994	0.8	0.7	0.12	0.03	0.006	0.019
114	10.0	—	—	1.4	1.1	0.20	0.04	0.008	0.034
227	19.5	—	—	2.7	2.3	0.39	0.10	0.018	0.064
454	39.0	—	—	5.4	4.5	0.77	0.19	0.034	0.128
636	54.5	—	—	7.6	6.4	1.08	0.27	0.048	0.181

Table 2.6. Performance data for different anaerobic reactors treating dairy or cattle manure (Yılmaz, 2007).

Reactor configuration	**OLR (g VS / L.day)**	**HRT (days)**	**Temperature (^oC)**	**CH_4 (ml / g VS.d)**	**VS reduction (%)**	**Reference**
CSTR	3.3	18	35	260	52	Varel et al., 1980
CSTR	5	12	35	235	55	Varel et al., 1980
CSTR	6.7	9	35	218	52	Varel et al., 1980
CSTR	10	6	35	160	50	Varel et al., 1980
CSTR	2	16.2	35	270	50-63	Karim et al., 2005
CSTR	3	15	37	224	37	Mladenovska et al., 2003
Plug flow	9	15	35	78	24	Hills and Mehlschau, 1984
CSTR	11.6	15.1	35	90	25	Hill, 1980
CSTR	3	15	55	241	43	Nielsen et al., 2004
TPAD	3	3+12	65+55	260	47	Nielsen et al., 2004
CSTR	0.91	20	36	65	20	Quasm et al., 1984
Two-phase CSTR	2	10	35	65	68	Demirer and Chen, 2005
Two-phase CSTR	6.3	10	35	112	33-40	Demirer and Chen, 2005
CSTR	2	20	35	130	48-50	Demirer and Chen, 2005
CSTR	6.3	20	35	135	42-52	Demirer and Chen, 2005
CSTR	2.79	25	35	250	38.3	Singh et al., 1988
TPAD	2.84	4+10	58+38	250	39	Harikishan and Sung, 2003
TPAD	4.5	4+10	58+38	240	40	Harikishan and Sung, 2003
CSTR	3	13	40	210		Mackie and Bryant, 1995
CSTR	2.90	10	30	133		Lo et al., 1984
Batch	-	-	35	148		Moller et al., 2004

Serious health risks occur due to the wide use of veterinary pharmaceuticals not only in the emergence and spread of resistant bacteria, but also in other human, animal and environments. Just like in human, antibiotics are used to treat and prevent disease in veterinary medicine. Antibiotics are defined as naturally occurring, semi-synthetic and synthetic compounds with antimicrobial activity that can be applied parentally, orally or topically. Besides the indirect impact on health via resistant microorganisms, antibiotics are a matter of concern because of their direct organic damage and influences on the biotic environment (Kemper, 2008).

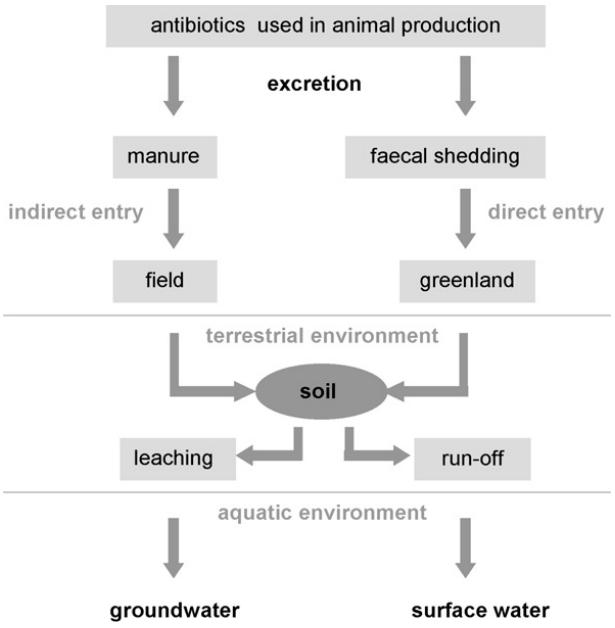

Figure 2.5. Estimated exposure pathways of veterinary antibiotics in the environment (Kemper, 2008).

The development of antibiotic resistant bacteria strains is the main interest regarding to the application of antibiotics in human and animal treatment. Especially, veterinary antibiotics applied to food animals enhances the selection for strains resistant to antibiotics used in human medicine. Transmission of these strains might occur via direct contact with animals or via the food-chain to the consumers. Since antibiotic resistance protects antibiotic-producing organisms from their own products, and also other susceptible organisms from competitive attack, it is cult as antibiotics themselves (Kemper, 2008).

Although EU regulations have restricted the use of antibiotics as growth promoters, they are still used in farms for mass treatment for periods of at least 5 days (Liguoro et al., 2003). In the United States, according to Animal Health Institute, antibiotics used for animal feeding have increased from 91 mg in 1950 to 9900 mg (including 3000 mg of tetracyclines) in 2004, 60-80% of which were used for non-therapeutic purposes (Alvarez et al,, 2010).

Table 2.7. Resistance in zoonatic bacteria (Kemper, 2008).

Species	**Clinical disease in humans**	**Possible resistance against**	**Literature**
Escherichia coli	Diarrhoea, urinary tract infections, septicaemia	B-Lactams Tetracyclines Streptomycin/spectinomycin Sulphonamides Cimethoprim Chinolones Chloramphenicoles Gentamycin/kanamycin/ neo-mycin	Angulo et al., 2004; Bundesinstitut für Risikobewertung, 2004.
Salmonella spp.	Diarrhoea	B-Lactams Tetracyclines Streptomycin/spectinomycin Sulphonamides Cimethoprim Chinolones Chloramphenicoles Gentamycin/kanamycin/ neo-mycin	Angulo et al, 2004; Hensel and Helmuth, 2005; Davis et al., 2007.
Campylobacter spp.	Diarrhoea, neuronal damages as sequels	Ciprofloxacin Tetracyclines Doxycylines Erythromycin Trimethoprim Sulphamethoxazole	Luber et al., 2003; Angulo et al., 2004; Bae et al., 2005; Senok et al., 2007.

All growth promoters have been banned from European agriculture since 2006 and therefore have not been taken into consideration in the following reflections. Individual antibiotic treatment seems practical for cattle and swine, but for poultry, antibiotics are applied orally. For instance, antimicrobials are generally used for treatment of mastisis in cattle and of respiratory infections in calves (Kemper, 2008).

Antibiotics, which are bioactive substances, act highly effectively at low doses and are excreted after a short time of residence without being completely eliminated in animal organisms. Excretion rates depend on the substance, the excreting species, the mode of application and time after administration. It has been shown that excretion rates differ between 40-90% for tetracyclines and sulphonamides. They are either excreted maintaining the same chemical structure or as metabolites that have been transformed into epimers or isomers (Kemper, 2008).

Environmental factors such as physical-chemical properties, prevailing climatic conditions, and soil types all affect the antibiotic efficiency in the environment (Kemper, 2008). Veterinary antibiotics are excreted by the animals ending up in soils via grazing livestock or manure used as agricultural fertilizer (Jørgensen and Halling-Sørensen, 2000). Most antibiotics are adsorbed quickly and their antibiotic potency decreases by sorption and fixation which does not mean a complete elimination of the antimicrobial activity (Sengeløv et al., 2003). For example, although soil-bound tetracyclines and tysolin are tightly adsorbed by clay particles, they remain active and show antimicrobial effects that may influence the selection of antibiotic resistant bacteria throughout the terrestrial environment (Chander et al., 2005). For tetracyclines, distribution coefficients in manure are smaller than in soils (Loke et al., 2002). Besides that, they don't exist in significant amounts in soil after fertilization with liquid manure but persist and accumulate in the environment with time. They form complexes with double-charged cations, such as calcium, which is present in high concentrations in soil (Samuelsen et al., 1992).

Antibiotics used in animal husbandry, their metabolites or degradation products reach the aquatic environment by the application of manure/slurry to agricultural areas or by pasture-reared animals excreting directly on the land, followed by run-off, driftage and leaching into the deeper layers. Due to the hydrolysation of penicilins and the precipitation and accumulation of tetracyclines, they are not usually expected to be found in the aquatic environment (Kemper, 2008). In addition to the aquatic environments, resistant and multi-resistant bacteria have been detected in wastewater and sewage treatment plants, probably entering the food chain via sewage sludge used as fertilizer or wastewater serving for irrigation (Guardabassi et al., 1998; Witte, 1998; Feuerpfeil et al., 1999; Kümmerer, 2003).

Table 2.8. Tetracyclines in soil and water (Kemper, 2008).

	Compounds	**Concentration**	**Literature**
Soil (ng/kg)	Tetracycline	450,000-900,000	Winckler and Grafe, 2000
	Oxytetracycline	305,000	Boxall et al., 2005
	Chlortetracycline	39,000	Hamscher et al., 2005
Water (ng/l)	Tetracycline	400	Krapac et al., 2004
	Oxytetracycline	32000	Kay et al., 2005
	Chlortetracycline	0-690	Kolpin et al., 2002

Tetracyclines are much favored in veterinary medicine since they are active against a range of organisms such as chlamydia and myco-plasma, as well as a number of Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001). Tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) are commonly used in animal feeds to maintain health and improve growth efficiency worldwide because of their broad range of activity and low cost (Alvarez et al., 2010). These chemicals are characterized by a partially conjugated four-ring structure with a carboxyamide functional group (Mitscher, 1978) (Figure 2.6).

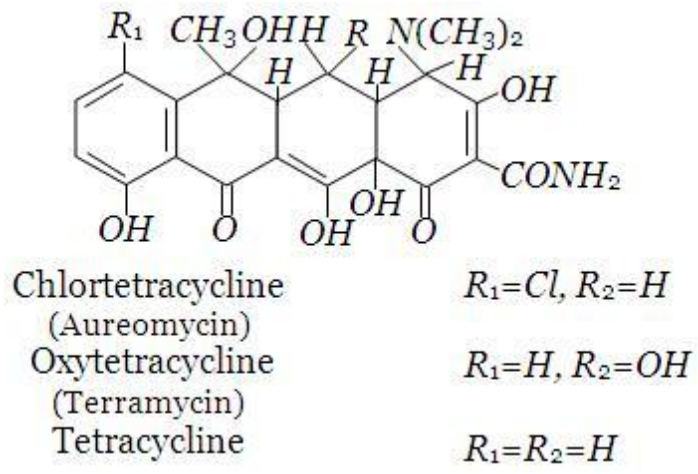

Figure 2.6. Molecular structure of Tetracyclines.

Oxytetracycline is a common antibiotic used for animals in livestocks (including poultry, cattle, swine and fish) for prophylactic and therapeutic treatment as well as a

growth promoter (Arikan, 2006). Degradation products of OTC are 4-epi-oxytetracycline (EOTC), α-apo-oxytetracycline (α-Apo-OTC), and β-apo-oxytetracycline (β-Apo-OTC).

Table 2.9. Important tetracyclines in human and animal medicine (Kemper, 2008).

Compounds	**Primary usage**	**Potential side effects**
Chlortetracycline	Cattle, pigs	Hepatotoxic
Oxytetracycline	Humans, cattle, sheep, pigs	
Tetracycline	Humans, horse, sheep, pigs	

The presence of antibiotics and/or antibitotic matebolites in manure can inhibit the microbial communities in anaerobic digesters (Arikan et al., 2006). Fate and inhibitory effect of oxytetracycline in anaerobic digestion processes have been a hot topic and studied for quite a long time (Sanz et al., 1996; Lallai et al., 2002, Liguoro et al., 2003; Arikan et al., 2006, Alvarez et al., 2010). Despite some adverse results, it can be said that oxytetracycline has negative effect on biogas and methane yield in digestion systems, mostly without causing complete system failure. These studies show that toxicity of OTC increases by the presence of its metabolites and that OTC itself is not a very competent inhibitor to cause any significant failures on the anaerobic digestion processes. The degree of the inhibition is highly dependable on environmental and operational parameters.

2.3. Two-phase Anaerobic Digestion

Conventional single phase anaerobic digestion of slurry is not effective for wastes containing high levels of solids (>10%), since they require the manure that is capable of being pumped, which itself needs a solid concentration of <10%. Correspondingly, this results in a significant increase in fluid and digester volume which causes increased capital and operating costs (Demirer and Chen, 2004). Failure or instability of single phase digesters have been reported widely, especially under high loading conditions (Fox and Pohland, 1994; Ghosh, 1995). These stability and control problems in conventional design applications have led researchers to search for new solutions.

To improve the process stability and efficiency, the concept of two-phase reactor thus proposed. A two-phase anaerobic digester is based upon the fact that the

environmental conditions belonging in most anaerobic digesters are not optimum for both fermentative and methanogenic microorganisms. Their different growth characteristics make it impossible to select a single set of digester that can maximize both acid and methane-forming microorganisms' growth (Ince, 1998). Since the acidogenic phase and methanogenic phase greatly differ in physiological and nutritional requirements, growth kinetics, and sensivity to environmental stresses, a two-phase configuration takes advantage of phase separation, using separate units for acidogenesis and methanogenesis, in order to optimize conditions for each phase (Azbar and Speece, 2001; Babel et al., 2004). Such two-phase anaerobic digestions are proposed as a way to optimize for the growth of each type of microorganisms in the separate reactors, specifically by growing the acetogenic bacteria at a lower pH (e.g., 5-6), and short hydraulic retention time (typically 1-2 days) in the first stage, while the slower growing methanogenic *Archaea* require a more neutral pH and longer hydraulic retention time (typically 10-20 days) (Cooney et al., 2007).

A detailed research was proposed first by Pohland and Ghosh (1971) to separate the two main groups of microorganisms physically into serial reactors to take the advantage of the differences in their growth kinetics. In order to achieve phase separation, several techniques have been conducted in the history; such as membrane separation (Fernandes, 1986), kinetic control (Ghosh and Pohland, 1974; Massey and Pohland, 1978; Cohen et al., 1979) and pH control (Pohland and Mancy, 1969). A combination of the last two techniques have been the most successful ones for the separation of acid and methane phases and have been applied in many studies and applications of anaerobic digestion systems (Ince, 1998).

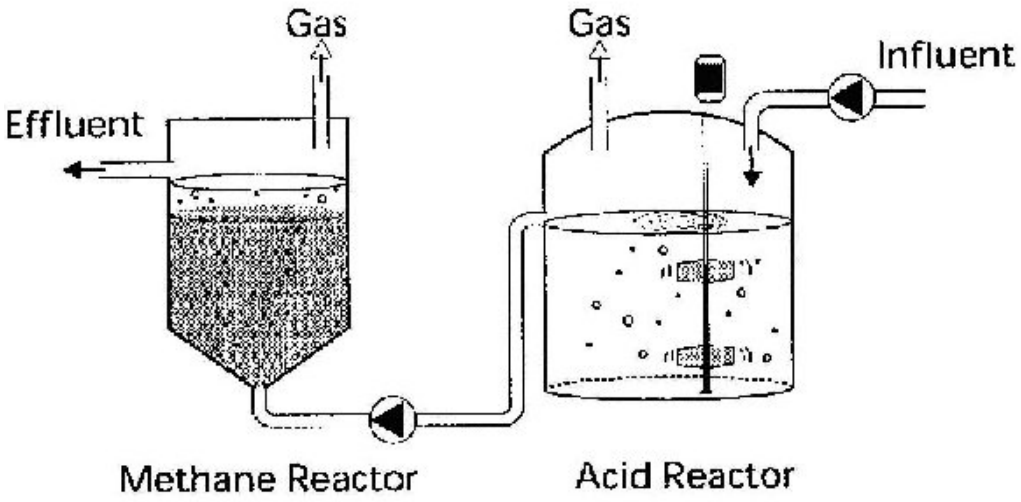

Figure 2.7. Schematic diagram of laboratory-scale two phase anaerobic digestion system (Fox and Pohland, 1994).

Many studies have showed that two-phase anaerobic digestion resulted at higher efficiencies and rates than those achieved by conventional single-stage CSTR digestion at mesophilic and also thermophilic temperatures, and at several levels of HRT, loading rate, and feed VS concentration. The analysis indicated that the two-phase process is less vulnerable to upsets due to unbalances in acidogenic-methanogenic fermentation and the accumulation of acids and prevalence of acidic pH. In contrast, in single-stage CSTR digestion, the rate of volatile acids production is higher than the volatile acids conversion rate at lower HRT's and higher loading rates; therefore, reliable system operation can be expected only at high HRT's, where the rates of acids production and conversion are balanced.

To date, a significant amount of literature has been published about the benefits of treating wastes in two-phase reactors (Massey and Pohland, 1978; Cohen et al., 1980; Verstraete et al., 1981; Ghosh and Henry, 1982; Fernandes, 1986; Zhang et al., 1991; Kasapgil *et a*l., 1995; Ince, 1998; Ince and Ince, 2000; Azbar and Speece, 2001; Demirel and Yenigün, 2002; Demirer and Chen; 2005; Cooney, *et a*l., 2007; Göblös et al., 2008; Yılmaz and Demirer, 2008; Li et al., 2010; Panichnumsin et al., 2010).

The two-phase configuration has several advantages over conventional single-phase anaerobic digestion systems such as:

- Optimization of the conditions for the hydraulic acidogenic group of bacteria as well as for the acetongenic-methanogenic group leads to the production of the most suitable acid metabolites for the methanogens and naturally an increase in the rate of substrate turnover. A two-phase system may provide a reduction in total reactor volume (Ince, 1998).
- An increased stability due to the more heterogeneous nature of the bacterial population results since the system insure against organic and hydraulic over loadings and fluctuations while the first phase acts as a metabolic buffer. Consequently, materials toxic to methanogens may be removed in the first phase (Zoetemeyer, 1982).
- Fast growing, acidogenic biomass/sludge may be washed out without the loss of slow growing methanogens (Cohen, 1982).
- When a waste with high solids-concentration is introduced to the first phase, it is liquefied along with acidification which leads to less liquid addition and thus, less energy requirements for heating, storing, etc. (Yılmaz and Demirer, 2008).
- It is possible to produce hydrogen during the first phase, and later on to produce methane during the second phase which makes two-phase anaerobic digestion processes an alternative to produce hydrogen and methane from various sources of biomass (Demirel et al., 2010).

Table 2.10. Applications of two-phase anerobic digestion processes (modified from Demirel et al., 2010).

Substrate type	**Application status**	**Digester types**	**Temp. (^oC)**	**HRT (days)**	**Methane productivity**	**Methane content (%)**	**Reference**
Sugar beet pulp	Lab-scale		35	4 (AR)* 8.9-13.3 (MR)$^+$	363 mL/g VS 280 mL/g COD	71.9	Hutnan et al., 2000
Food waste	Lab-scale	UASB (MR)	35±1		0.25 L/ g VS	68-70	Hai-Lou et al., 2002
Grass	Pilot-scale	Solid bed (AR) + Anaerobic filter (MR)			0.15 m^3/ g VS	71	Yu et al., 2003
Sewage sludge + OFMSW$^{\wedge\wedge}$	Lab-scale	CSTR (AR) + UASB (MR)	56 (for CSTR) 36 (for UASB)		0.024 dm^3/ g VSS added	>60	Sosnowski et al., 2003.
Fruit and vegetable waste	Lab-scale	ASBR	35	3 (AR) 10 (MR)	320 L/kg COD input	69-71	Bouallagui et al., 2004
Dairy manure	Lab-scale		36±2	2 (AR) 8 (MR)	0.10 L/ g VS added	60-67	Demirer and Chen, 2005
Dairy manure	Lab-scale		35±2	2 (AR) 8.6 (MR)	216 mL/ g VS	63-65	Yılmaz and Demirer, 2008
Cassava pulp + pig manure	Lab-scale	CSTR	37±1	2 (AR) 13 (MR)		65-67	Panichnumsin et al., 2010
Food waste + dairy manure	Lab-scale	CSTR	35±2	1 (AR) 12 (MR)		59-63	Li et al., 2010

*AR = acidification reactor, $^+$MR = methane reactor, $^{\wedge\wedge}$OFSMW = organic fraction of municipal solid waste

Applications of two-phase anaerobic digestion were conducted in the biogasification of: wastewater treatment sludge, organic fractions of municipal solid wastes, industrial wastes and sludge, olive mill solid waste and olive pomace, grass, coffee

pulp juice, food waste, cane–molasses alcohol stillage, spent tea leaves, brewery wastewater, dairy wastewater as well as some other studies focusing on improving reactor design, control and operational parameters.

Effective controls during start-up and steady-state periods are required for efficient full-scale application of these systems. This is because parameters such as temperature, pH, hydraulic retention time, substrate concentration, mixing and shear influence the number and the composition of the microbial populations. A well operated acid reactor should ideally contain few methanogens. Optimum conditions for acidification, lower pH and shorter HRT, retard methanogenic activity but do no eliminate all methanogens, which are sensitive to the operating conditions (Ince and Ince, 2000).

As mentioned above, anaerobic digestion involves a commensal interaction of the two general types of microorganisms, in which the methanogens feed on and remove the waste products (H_2 and acetic acid) of the acidogenic bacteria. Thus, the phase separation may not generally significantly accelerate or increase overall methane production (Cooney et al., 2007).

The operation and control of the two-phase system is also complicated since there is a need to adjust the conditions, such as pH, volatile fatty acids level or nutrients, of the effluent from acidogenic reactor before feeding to the methanogenic reactor. Some degradation products from acidogenesis such as long chain fatty acids can also inhibit the methanogens. Although the two-phase system can improve biodegradability of recalcitrant materials, its main disadvantage may be the fact that separation of acidogenic and methanogenic step can disrupt the synthropic relationship between bacteria and methanogens, which can lead to product inhibition in the acidogenic reactor (Boe and Angelidaki, 2009).

Generally, every organic material is well-advised for anaerobic digestion as long as the lignin, hemi-cellulose and cellulose fractions are small and both in conventional single-phase or two-phase anaerobic digestion systems; the initial hydrolysis of particulate organic matter to soluble substance is believed to be the rate-limiting step. Especially, the hydrolysis of cellulose and hemicellulose can be very problematic, when dealing with

agriculture wastes containing lignocellulosic material. Increasing the hydolysis rate is very important in order to achieve efficient biomass conversion (Romano et al., 2009).

Several physical methods known from other fields of preparing crops for material use and relying on mechanical or thermal treatment to destroy cell structures might be applicable to anaerobic digestion. These physical methods can also be combined with chemical treatment, such as acidifying or alkalizing. However, the effect of these methods depends on the biomass composition and operating conditions. All these alternative methods have their own advantages and disadvantages and more research is needed to optimize them prior to apply (Quiñones et al., 2012).

There is an increasing interest in using biological alternatives like enzymes to increase the system efficiency. Enzymes are naturally occurring compounds which are biodegradable and therefore environmental-friendly. One of the promising options seems to be the application of hydrolytic enzymes to the feedstock. Enzymatic hydrolysis leads to higher yields of monosaccharides, because cellulases catalyze only hydrolysis reactions without further sugar degradation reactions (Palmqvist and Hahn-Hägerdal, 2000).

The anaerobic digestion of lignocellulosic fractions of biogenic resources in agricultural biogas plants perform inefficiently. Generally, fibrous residues remain in a large amount in the digested effluent since the degradation of lignocellulosic fraction occurs very slowly due to the incrustation of the polysaccharide structure by lignin. A large part of the cellulose is in crystalline structure which is very hard to hydrolize. An almost complete digestion can be achieved after fermentation with a long HRT. For all the these reasons, the contribution of various enzyme activities is required for the degradation of such agricultural wastes (Ellenrieder et al., 2010).

Table 2.11. Fiber content in cattle manure (Pullin and Shehadeh, 1979).

Parameter	**Content in manure (% dry weight)**
Lignin	14-20
Crude fiber (not lignin)	30-40
Total fiber (crude fiber, lignin, hemicellulose)	60

There have been many studies suggesting that the addition of exogenous enzymes can improve the performance of anaerobic digestion system (Sonakya et al., 2001; Ayol, 2005; Romano et al., 2009; Ellenrieder et al., 2010; Yang et al., 2010; Luo et al., 2011; Quiñones et al., 2012). However, there are many factors that affect the enzyme activity such as the substrate, incubation time, system configuration and environmental conditions. Enzymes could be added into a single-phase anaerobic digester directly or could be used to pre-treat the biomass material prior to anaerobic digestion. In a two-phase anaerobic digestion system, enzymes could be added to the acidogenic phase where hydrolysis takes place, prior to biogasification (Romano et al., 2009).

2.4. Molecular Methods Used in Microbial Ecology of Anaerobic Digestion

The science of microbial ecology explores how microbial communities interact with each other and their surrounding environment. In this area of of microbiology, microbial activity and biodiversity are the two most important subjects (Gray and Head, 2008).

In the earlier applications of modern microbiology, the most common methods for identification of microorganisms was culture dependent methods, which were time consuming and lacked high sensivity. The main limitation of this method was cultivability of a small fraction of all microorganisms. Furthermore, culture dependent methods cause cultivation shift by favoring a normally not favorable microorganisms by changing competitions. Therefore, a microbial community cannot be cultured as a whole and cultured microorganisms do not reflect microbial community. The cultivable microorganisms make up 0.1-10% of all microorganisms on earth. (Muyzer et al., 1993; Amann, et al., 1995; Hugenholtz et al., 1998; Gouillou et al., 1999; Lim et al., 1999; Muyzer, 1999).

Despite the developments in the microscopy, direct microscopic analyses have many limitations in identifying microorganisms. The absence of distinguishing phenotypic characters, the small size of prokaryotic organisms and the fact that most of these organisms cannot be cultered are the most limiting factors within the evaluation of the biodiversity (Pace, 1997). A significant number of studies dealing with microbial diversity

during the last decades involve recognition of DNA and RNA based culture independent methods.

The culture independent methods can be divided into two categories, ones that include isolation and analysis of genetic material from environmental samples to detect which organisms are present and ones that include using nucleic acid based stains to microscopically visualize, numerate and identify microorganisms (Gray and Head, 2008). A scheme of culture independent methods approaches used in microbial ecology is shown on Figure 2.8.

Molecular phylogeny, which employs nucleic acid sequences to document the history of evolution, has provided a new basis for the direct identification and quantification of microorganisms. So far, in microbial ecology studies, ribosomal RNA (rRNA) and ribosomal DNA (rDNA) have been the most commonly used target nucleic acids. Especially, the rRNAs have become the most commonly used molecules for phylogenetic analyses since they are key elements of cells and homologous for all organisms, much conserved in overall structure and very abundant in most cells (Hofman-Bang et al., 2003).

A phylogenetic analysis provides the identification of a microorganism based on a molecular sequence, eliminating the need for cultivation. A sequence can be retrieved from an environmental sample, sequenced, and compared to already known sequences for identification of the interested organism (Hofman-Bang et al., 2003).

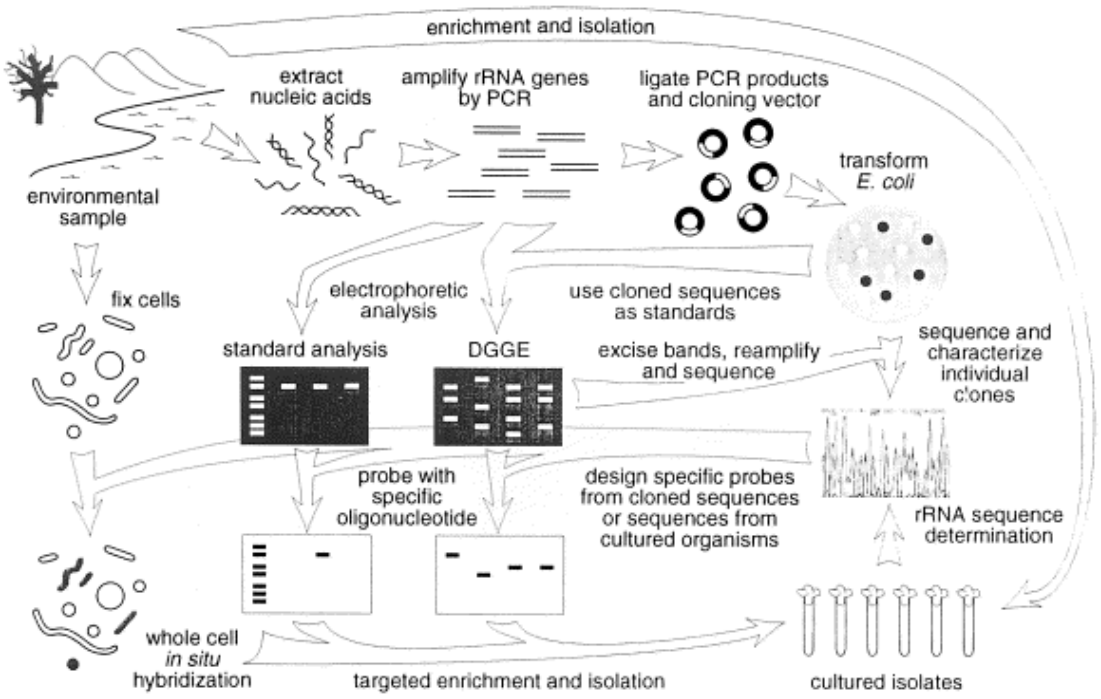

Figure 2.8. Summary of phylogenetic methodologies used in microbial ecology. (Scow et al., 2004).

Many researches have shown that microorganisms in anaerobic environments including bioreactors exhibit supreme biodiversity and their relationships and metabolic functions need to be clarified. Cultivation dependent methods lack of information since microorganisms living in anaerobic environments are hard to grow because of low growth rates, syntrophic interactions and unknown growth requirements (Hugenholtz et al., 1998). By the use of molecular methods, the gap between microbiologists and engineers in the field of anaerobic digestion tends to be bridged. Data obtained from molecular techniques can serve to model and optimize bioreactor systems.

Table 2.12. Molecular biology applications in microbial ecology (Giraffa and Neviani, 2001).

	Taxonomic resolution	**Applications to microbial ecology**
Genetic fingerprinting of microbial communitites		
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 (FISH)	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

2.4.1. 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 but of course it has 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 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.

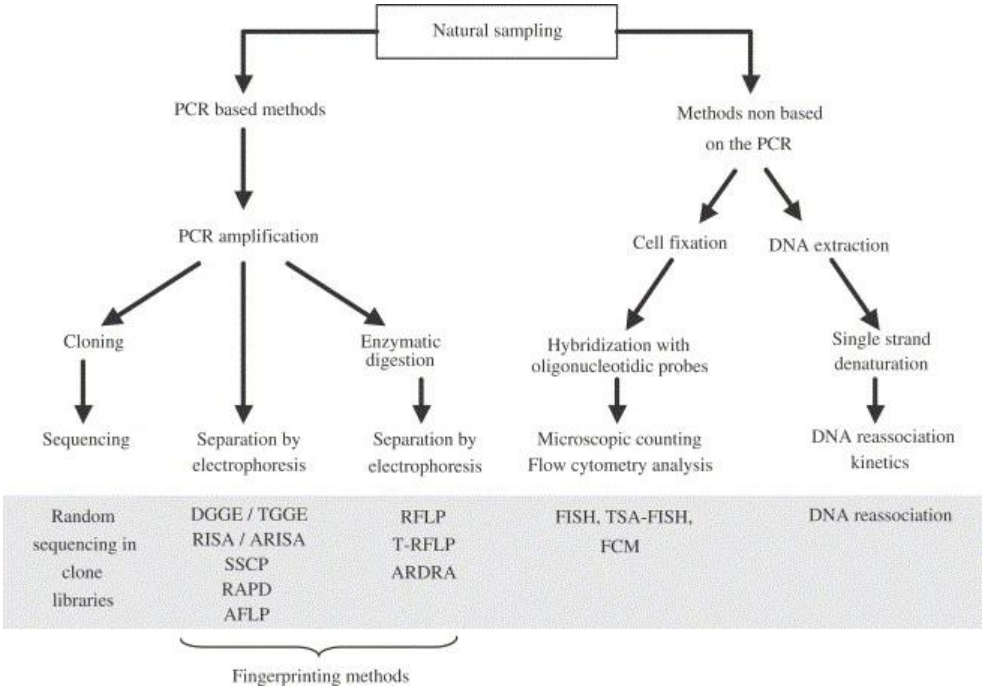

Figure 2.9. - PCR based/non-based molecular methods (Dorigo et al., 2005).

2.4.2. Denaturating Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) were introduced to environmental sciences by the studies of Muyzer (Muyzer et al., 1993). In spite the principle is similar to SSCP and TGGE, DGGE becomes much effective, easy and fast in application. Rapid and reliable results favor it and versatility of the technique makes it more usable in a wide range area.

DGGE provides the chance to determine the genetic diversity of a microbial community without identifying its individiuals. It can be used to compare any different communitites such as soil samples or bacterial and *Archaeal* communities (Heuer and Smalla, 1997; Ovreas et al., 1997). Different samples taken at different times can be analyzed and compared in one gel which makes DGGE a powerful tool to analyze microbial community changes over time (Çetecioğlu et al., 2000).

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. 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.

Despite many advantages, DGGE has its own limitations which may be avoided by carefully planing and performing but some of them are inevitable anyway. In complex microbial communities, DNA sequence information from excised gel bands may require cloning because of co-migration or poor separation of gel bands. Gel to gel variation and lesser sensivity are also limiting to ensure the detection of minör populations and subtle changes (Talbot et al., 2008). Moreover, the size of DGGE bands are less than 500bp, usually 150-200bp, so that the DNA sequence information obtained from gel bands is limited and therefore phylogenetic identification may be poor (Hugenholtz et al., 1998).

This method can be used to obtain qualitative and semi-quantitive estimations of biodiversity. DGGE pattern provides rapid information of the predominant species in the microbial community as well.

2.4.3. Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* hybridization is a microscopic analysis based method of already defined (at least its SSU rRNA gene sequence) of microorganisms by a fluorogenic oligonucleotide (or probe) targeting SSU rRNA molecules inside cells (Giovannoni et al., 1988; Amann et al., 1990). Microbial cells are first fixed with appropriate chemical fixatives and then hybridised with oligonucleotide probes under optimal conditions on a glass slide or in solutions. These specific probes are generally 15-25 nucleotides in length and are labelled at the 5’ end with a fluorescent dye. After washing steps, specifically stained cells are observed by epifluorescence microscopy or flowcytometry. The determination of composition and number of groups can be achieved by rRNA-targeted oligonucleotide probes without cultivation, directly in their natural environment.

Since the pioneering study of De Long et al. (1989), using rRNA gene fragments as phylogenetic stains, FISH technique has become a common tool for identification of microorganisms in environment samples (Amann et al., 2001).

The cellular rRNA content determines the signal intensity of cells hybridized with oligonucleotide probes. This allows a quantification of rRNA concentrations both in single cells and in the environment (Poulsen et al., 1993). After the evaluation of the methanogenic group composition in anaerobic digesters by oligonucleotide probe hybridization by Raskin et al. (1994), several studies including FISH results using the same oligonucleotides but different experimental conditions had been carried out (Merkel et al., 1999; Imachi et al., 2000; Tagawa et al., 2000; Upton et al., 2000; Wu et al, 2001). These probes are still accurate to target most of the defined phylogenetic groups of methanogenic *Archaea*.

FISH is an easy and fast technique which allows direct visualization of organisms without cultivation. It provides the possibility to detect active microorganisms in the sample. The fact that it does not require any DNA or RNA amplification is probably FISH's main advantage (Sanz and Kohling, 2006).

Despite the great advantages above, FISH technique has its own limitations just like the other molecular tools. Probably the most significant one is that, not all bacterial and *Archaeal* cells can be permeabilisied by oligonucletotide probes using standard fixation protocols (Amann et al., 1995). The other disadvantages include the need for prior knowledge of the related ecosystem and the microorganisms to be detected. Furthermore, the rRNA sequence of the studied microorganism must be known in case a particular microorganism wants to be detected (just in case the corresponding probe has not yet been published). In some cases, it is not always possible to design a specific probe for a certain group of microorganism which is another limitation for the application of FISH. Also, the design and assessing the optimum conditions for hybridization for a new probe is not easy and quantification of microorganisms can be time-consuming, subjective or complicated.

3. STATEMENT OF THE PROBLEM

Utilization of biogas as a renewable energy source is an emerging application around the world in the last decades. Accordingly, studies investigating this phenomenon have been gaining more and more importance, including the ones focusing on the effects of inhibitory compounds in anaerobic digestion processes, such as veterinary antibiotics. Although it is clear that microbiology of biogas production process is primarily related to the quality and quantity of the produced biogas, there is still lack of information on this side of the matter. This study aims to determine the behaviour and inhibitory effects of a commonly used veterinary compound, oxytetracycline, on the two-phase anaerobic digestion of cattle manure with evaluation and improvement of the digester performance and investigation of microbial population dynamics by molecular tools.

4. MATERIALS AND METHODS

4.1. Animal Medication and Manure Sampling

Fresh manure used during the study was obtained from the barn of Veterinary Faculty of Istanbul University, Istanbul, Turkey. OTC medicated manure was collected from the cattle that was medicated with 50 mL Oxytetracycline injection solution (20 mg/kg) under commercial name of Pentamycin LA 200 (Topkim, Turkey), injected equally into the left and right sides between *musculus semitendinosus* and *musculus semimembranosus* muscles. Following the medication, manure was collected from the rectum for 5 days. These OTC medicated manure samples were mixed equally and then used in the digesters in the three studies. Manure that was used as the control during the study was collected from non-medicated cattles. All manure samples were stored in containers at $+4^{o}$C prior to use.

4.2. Charactetistics of Manure and Seed Sludge

Characteristics of the manure samples prior to slurry preparation are given in Table 4.1. Seed sludge was obtained from an already operating lab-scale anaerobic cattle manure digester.

Table 4.1. Manure characteristics.

	Total Solids (%)	**Total Volatile Solids (%)**	**TVS/TS (%)**	**Total C/N**
Medicated manure	13.1	11.3	86	27.3:1
Non-medicated manure	14.0	11.9	85	24.3:1

The fresh manure was approximately one-fold diluted with tap water to set the desired % TS and TVS, prior to feeding into the digesters and in all steps; the digesters were seeded with a ratio 1:4 (v:v). Nitrogen gas was flushed into the digesters for 5 minutes before the operation to maintain the anaerobic conditions.

4.3. Experimental Design and Digester Operation

This study mainly consists of 3 different parts: (i) Optimization for acidification of cattle manure, (ii) improvement in acidification of cattle manure due to enzyme addition and (iii) comparison of single and two-phase anaerobic digestion of cattle manure in presence of OTC. Therefore, 3 different sets of digester set ups were utilized.

4.3.1. Optimization of Operational Conditions for Acidification of Cattle Manure

Different sets of digesters were operated at 37±1^{o}C and continuously stirred at 100 rpm in an incubator shaker.

First of all, 2 digesters were operated with non-medicated and medicated manure in 3 sets at different pH values, pH=5.0 – 5.5 – 6.0, for 7 days. Twice a day, samples were taken from the digesters for VFA analysis and pH was controlled. After the determination of optimum pH and digestion time, 3 digesters were operated with blank manure at different TVS concentrations at pH=5.5±0.1 to see the effect of organic loading. Samples were taken from the digesters once or twice a day for VFA analysis.

The degree of acidification was calculated using the percentage of the initial substrate concentration converted to VFA. The initial substrate concentration (*S*i) was measured in mg total COD/L and the quantity of VFA was converted to the theoretical equivalent in mg COD/L (*S*p), using the COD equivalents for each VFA. The following formula was used to express the degree of acidification in this work:

$$\text{Degree of acidification } (\%) = (S_p/S_i) \times 100 \qquad (4.1)$$

The COD equivalents of each volatile acids for the conversion were taken as follows: acetic acid, 1.066; propionic acid, 1.512; butyric acid, 1.816; valeric, 2.036; caproic acid, 2.204 (Kasapgil, 1994).

4.3.2. Improvement in Acidification of Cattle Manure due to Enzyme Addition

4 parallel batch-wise digesters with 500 ml volumes were incubated for 5 days at 37±1^{o}C with a mixing rate of 100 rpm. The active volumes were 300 ml and pH was adjusted to 5.5±0.1. The enzyme concentrations were 1, 2, 3 and 4 grams, respectively. The enzyme tablets used in this study, SEPT, were commercially supplied from ALFA Kimya, Istanbul. SEPT is a dry preparation in tablet form of non-pathogenic bacterial cultures, enzymes, inorganic salts and fragrance, used in the maintenance of septic systems, drains and lines. The tablets were first pounded and then added to the digesters.

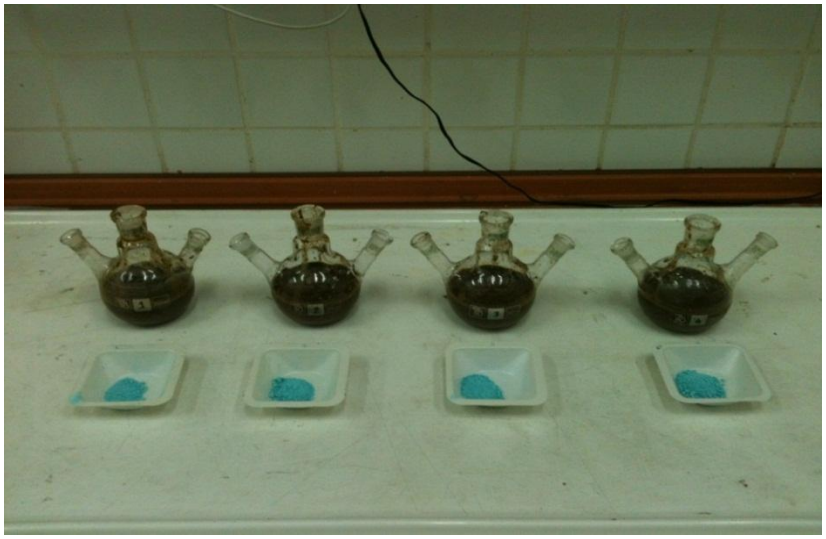

Figure 4.1. Set-up for the determination of optimum enzyme concentration.

4.3.3. Comparison of Single and Two-phase Anaerobic Digestion of Cattle Manure in Presence of OTC

The experimental set-up used in this study is depicted in Fig. 4.2. Six completely mixed digesters were used in 3 pairs, including single-phase digesters (Single 1 & Single 2), two-phase digesters operated with non-medicated manure (Acid 1 & Methane 1) and two phase digesters operated with OTC-medicated manure (Acid 2 & Methane 2). The single-phase conventional digesters were run as the control for the two-phase digesters. The volume of the single phase digesterrs were 1.0 L with a working volume of 800 mL while the methanogenic phase digesters were 1.0 L with a working volume of 600 mL and

the volume of the acidogenic digesters were 500 mL with a working volume of 350 mL. The digesters were maintained at 37±1^{o}C and continuously stirred at 100 rpm in an incubator shaker. This digestion system was performed in semi-continuous mode and all the digesters were fed with a withdraw/feed method once in every five days. The effluent of the first-phase digesters were fed into the second-phase as influent. Samples were taken from the digesters during the feeding days for chemical and molecular analysis. Organic loading rates of two-phase and single phase digesters were 6.25 ±0.15 g TVS/L-day and 1.50±0.02 g TVS/L-day, respectively.

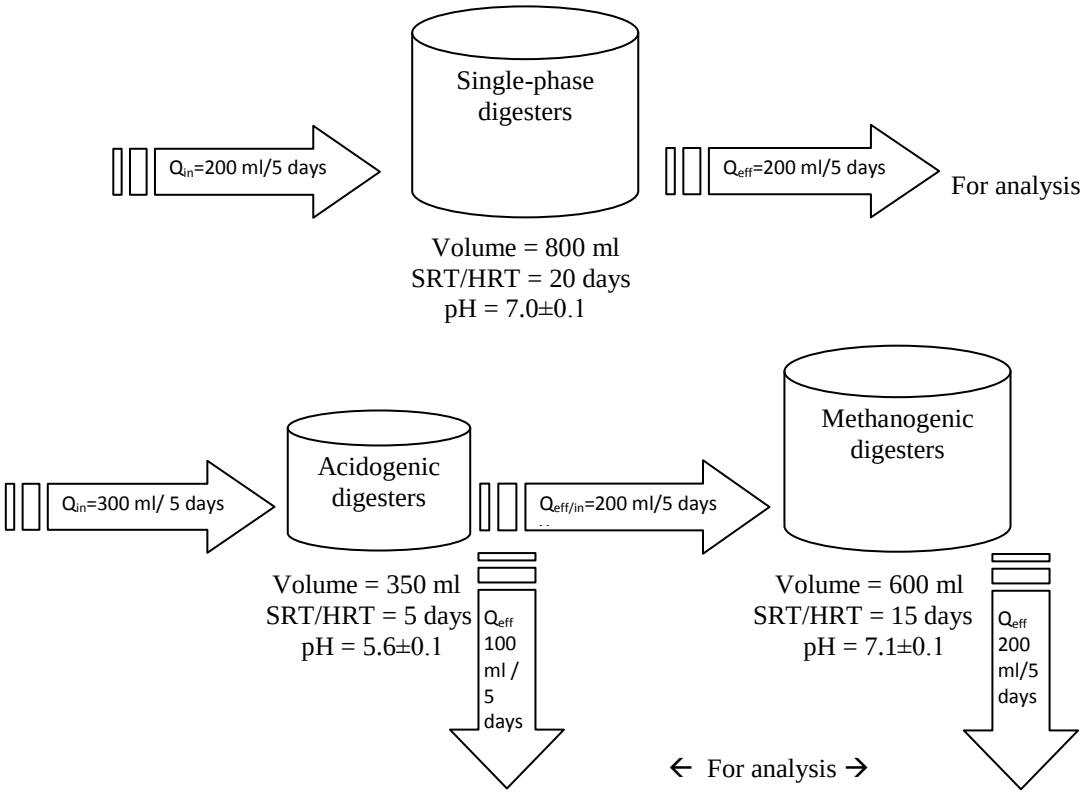

Figure 4.2. Experimental set-up used in semi-continuous single and two-phase anaerobic digesters.

Figure 4.3. The digesters and milligas counters used in the study.

4.4. Analytical Techniques

Every 5 days, samples were taken from the digesters for analytical and molecular analyses. Total solids, total volatile solids, alkalinity, COD and ammonia-N tests were done according to Standard Methods for the Examination of Water and Wastewaters (APHA, AWWA-WEF, 1998). pH was measured and also adjusted using HANNA HI 221 Microprocessor pH meter. The volatile fatty acids (VFA) concentrations were measured with Perkin Elmer Clarus 600 Gas Chromotograph equipped with a flame ionization detector (FID). The column used was Elite FFAP (30 m x 0.32 mm). The set point of the oven and maximum temperature of inlet are 100^{o}C and 240^{o}C, respectively. Helium gas was used as a carrier gas at a rate of 0.8 ml/min.

The gas produced from the digesters were measured by milligas counters and noted every day cumulatively. Gas compositions were measured once on every 5 days using HP Agilent 6850 Gas Chromatograph (GC) with a thermal conductivity detector (HP Plot Q column 30 m x 0.53 mm). As a carrier gas, helium was used at a range of 2 mL/min. The oven temperature was 70^{o}C during the measurements. Air tight syringe (2.5 mL) was used to collect the sample accumulated in the headspace of the digesters. 2 ml of gas was taken from the digesters and 0.5 mL of it was injected to GC for the analysis.

Shimadzu High Performance Liquid Chromatography (HPLC) instrument (Schimadzu LC-10 AD) was used for OTC measurement, equipped with an UV detector (UV VIS Detector, SPD 10-A), operating at 357 nm. During this study, Intersil ODS-3 HPLC column was used as the analytical column, 25 cm x 4.6 mm ID, 5 μm, used at ambient temperature. For the injection, an autosampler, SIL-10 AD, was used and the injection volume was 20 μL.

The chemicals, oxalic acid dehydrate (Merck), methanol and acetonitrile (LiChrosolv), were commercially supplied. Methanol and acetonitrile were all HPLC-grade. The other chemicals were analytical grade. Oxytetracycline was purchased from Agros Chemicals. Double distilled water was used during the analysis.

The mobile phase consisted of 75% 0.1 M oxalic acid buffer, 15% acetonitrile and 10% methanol, which was degassed prior to use via sonication in a Transonic ultrasonic bath, ELMA D-78224 Singen/Htw. Before every analysis, analytical column was conditioned with the mobile phase until a proper baseline was observed. After an acceptable baseline was obtained, standards and then the samples were analyzed. The mobile phase was delivered isocratically at a flow rate of 1 mL/min and the total run time was 20 min.

Stock standard OTC solution was prepared by dissolving 100 mg of OTC in HPLC-grade methanol and stored at -20^{o}C. In order to plot a calibration curve, serial dilutions of OTC standard solution were prepared and then analyzed via HPLC. All solutions were protected from direct sunlight and/or artificial light in order to prevent the photodegredation of OTC.

Prior to the extraction of samples, extraction efficiency was determined. 5 g of non-medicated wet manure was spiked with OTC solution in methanol, incubated for 4 hours in dark and extracted as mentioned earlier. Spiking levels were; 2 mg/kg, 20 mg/kg, 200 mg/kg and 1000 mg/kg. Recovery rate was calculated by equation 4.2.

$$\text{Recovery rate} = \frac{\text{Amount of OTC detected by HPLC}}{\text{Amount of OTC added to manure}} \text{x}100 \quad (4.2)$$

The analytical conditions maintained were mentioned earlier. Retention time of OTC was found to be 7.3±0.1 min. In order to confirm the correctness of the method, duplicate analysis of five working standard solutions covering the range from 1 to 100 mg/L were made. 20 μL of these standards were injected into the HPLC system and its concentrations were calculated by the software. A calibration curve was plotted with concentration against area (Ertekin, 2010).

The accuracy of extraction was verified by extracting a known amount of OTC spiked into non-medicated manure, and analyzing with HPLC. The spiking concentrations were 2, 20, 200 and 1000 mg/kg manure. After each extraction OTC was collected in 50 mL of extract. The extract was injected into HPLC. All of the analyses were conducted triplicate. The extraction efficiencies are given in Table 4.2. After plotting the calibration curve and calculating the extraction efficiency, samples were extracted and analyzed.

Table 4.2. Extraction efficiencies.

Amount Collected in 50 mL Extract (mg/L)	**Concentration in Manure (mg/kg)**	**Recovery Rate (%)**
100	1000	99
20	200	92
2	20	85
0.2	2	80

Extraction was done according to a method modified from Yuan et al. (2010). Extraction solution includes 1.5 g oxalic acid, 3 g citric acid and 22.5 ml HPLC-grade methanol for each sample to be extracted. 5 g manure and 7.5 ml extraction solution were put into 50 ml polycarbonate centrifuge tubes and placed on a shaker for homogenization

at 100 rpm for 10 minutes. Then the tubes were sonicated for 15 minutes and later on centrifuged at 11000xg for 10 minutes. This procedure was repeated for 3 times and the supernatants were collected in 50 mL falcon tubes and kept in the dark until the end of the extraction. After the final step, falcon tubes were diluted to 50 mL with double distilled water and then filtrated through 0.2 μm filters into 2 mL amber vials and stored at -20^{o}C (Ertekin, 2010).

4.5. Molecular Techniques

4.5.1. DNA Extraction

Approximately 500 μL sample was added up to lysing matrix tubes along with 978 μL sodium phosphate and 122 μL MT buffer solution. The tubes contain mixture of ceramic and silica particles to lyse all microorganisms in sample. The lysing matrix tubes were spinned in Ribolyser (Fast Prep TM FP120 Bio 101 Thermo Electron Corporation) for 45 seconds at speed of 6.5 m/s. The tubes were then centrifuged at 14000xg at 4^{o}C for 5 minutes. After centrifugation, supernatants were transferred to clean 1.5 ml micrufuge tubes and added 250 μL PPS reagent. To mix the composition the tubes were shaked for 30 seconds. After mixing, the tubes were centrifuged again at 14000xg for 5 minutes to pellet the precipitate. Supernatants were transferred to 15 mL conical tubes and 1 ml of binding matrix suspension was added to supernatant. The tubes were inverted for 3 minutes to allow binding of DNA to matrix. To settle the silica, matrix tubes were incubated at room temperature for 3 minutes. 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 by centrifugation at 14000xg for 1 minute in filter spin tubes 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 then centrifuged at 14000xg for 2 minutes. Application-ready DNA was obtained in the tube. 1/10 and 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.

4.5.2. 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 4.3 and their sequences were given in Table 4.4.

Table 4.3. Bacterial and *Archaeal* oligonucleotide primers used for PCR amplification.

Primer	**Experimental Stage**	**Annealing Temperature, (°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	55	8-27	Edwards et al., 1988
Bact1541r	First round of nested PCR	55	1541-1522	Edwards et al., 1988
Arc07f	First round of Nested PCR	52	07-24	Lueders et al., 2004
Arc1384r	First round of nested PCR	52	1384-1368	Lueders et al., 2004
Arc344f-GC	DGGE	53	344-358	Raskin et al., 1994
Univ522r	DGGE	53	522-504	Amann et al., 1995

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 512). Conditions are given in Table 4.5. 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 4.4. 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
Arc07f	TTCYGGTTGATCCYGCC	Lueders et al., 2004
Arc1384r	CGGTGTGTGCAAGGAGCA	Lueders et al., 2004
Arc344f-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 4.5. 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 45 sec.	40 °C 30 sec.	72 °C 60 sec.	35
Arc344f-Univ522r	94 °C 45 sec.	53 °C 30 sec.	72 °C 60 sec.	35

4.5.3. Denaturating Gradient Gel Electrophoresis (DGGE)

Acrylamide solution (30%), deionised formamide, urea and molecular biology grade ammonium persulfate 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 1 mm spacers 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 persulfate (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 which took approximately 3 hours, a stacking solution (6-10 ml) excluding denaturants was mixed with 60 μL APS and 6 μL TEMED and added over the polymerized gel.

Electrophoresis tank was filled with 1xTAE and temperature was set to 60°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. 30 μl of 1:100000 diluted SYBR Gold DNA staining dye was added to 500 ml 1xTAE washing buffer and gels were incubated for 20 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 6.0 Software (Applied Maths, Belgium). Similarities between tracks were calculated by using the Dice coefficient (*SD*) (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.

4.5.4. Fluorescence in situ Hybridization (FISH)

For FISH analysis, 5 ml samples from each digester were transferred to Falcon tubes and diluted 1:1 with absolute ethanol and stored at -20^{o}C and fixed with paraformaldehyte (PFA) within 3 days.

For the standard PFA preparation, 5-10 mL 1x PBS was added into 0.8g PFA in a beaker, covered and mixed at 70-80^{o}C until it was homogenized. Then, the beaker was placed on ice to cool down. pH of the solution were adjusted to 7.2 with HCl or NaOH and then the the final volume was fulfilled to 20 mL with 1x PBS. At the end, the solution was strelized by filtrating through 0.2 μm filters. For the fixation, 1 mL ethanol-sample mixture was transferred to 2.0 ml microcentrifuge tubes and centrifujed for 3 minutes. The supernatant was removed and the pellet was washed with 0.5 mL 3X phosphate buffer baline (PBS) for two times and resuspended in 0.25 mL 3xPBS and 0.75 mL freshly prepared 4% PFA and incubated for minimum 3 hours at +4^{o}C. After incubation, cells were washed once with 1x PBS and resuspended in 0.5 mL ethanol + 1x PBS mixture and stored at -20^{o}C until hybridization.

For the hybridization, oligonucleotid probes targeting 16S ribosomal RNAs (rRNAs) listed in Table 4.6 were used. The methanogen targeted probe sequences and classification of the methanogens are given in Figure 4.4 (Raskin et al., 1994).

For each hybridization, two negative controls were prepared; one for assessing non-specific bindings (with NonEub probe), and the other (lacking a probe) monitoring autofluorescence.

Table 4.6. 16S rRNA-targeted oligonucleotide probes used in this study.

Probe	**Target Group**	**Probe sequence (5'-3')**	**Labelling (5')**	**Reference**
LGC534Mix	*Firmicutes*	TGGAAGATTCCCTACTGC	CY3	Meier et al., 1999
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	CY3	Manz et al., 1992
MB310	*Methanobacteriales*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin et al., 1994
MSMX860	*Methanosarcinales (all Methanosarcina and Methanosaeta)*	GGCTCGCTTCACGGCTTCCCT	CY3	Raskin et al., 1994
NONEUB	*Non sense probe*	ACTCCTACGGGAGGCAGC	TAMRA	Wallner et al., 1993

10 µL of the fixed samples were transferred to new microfuge tubes and centrifuged for 3 minutes. This amount was determined by the microorganism density in the sample. The pellets were than washed 3 times with 1 mL 3xPBS and once with 1 mL ddH_2O. After washing, the pellet was resuspended in 2 mL ddH_2O so that the 1:200 dilution was obtained. Meanwhile, hybridization buffer containing 0.5 M EDTA, 200µM Tris HCl (pH 7.2), 250 µM NaH_2PO_4, 4.5 M NaCl (pH 7.0), 10 % SDS, 10x Denhards, was prepated and kept at 46 ^oC. 20 µl of the samples were taken into the wells of the slides and the slides were dried at 46 ^oC. Afterwards, the slides were dehydrated through ethanol series (50%, 80%, 96%) for 3 minutes and then again dried at 46^oC. 17 µL hybridization buffer was added into the each well and the slides were kept at 46^oC in a humid container for 10 minutes. Then, 3µl targeted probes were added and incubated at the optimal hybridization temperature for the given probe for 4 hours. Following hybridization, the cells were washed twice in a wash buffer containing 200 µM Tris-HCl (pH 7.2), 10% SDS, 4.5 M NaCl, 250 µM NaH_2PO_4, for 7 minutes at 48^oC before a final wash in MilliQ water for 10 seconds. After the washing step, 10 µL DAPI were added to each sample and kept at dark in the room temperature for 10 minutes. Then, the slides were washed agan with MilliQ water and dried at 46^oC. Finally, 10 µL of DABCO (1,4-diazabicyclo[2.2.2]octane) [Sigma D-2522]: 0.233g DABCO 800 µL ddH_2O 200 µL Tris-HCl (pH=7.2) was added to

the cells, and a coverslip was applied and sealed with nail polish before epifluorescence microscopy**.**

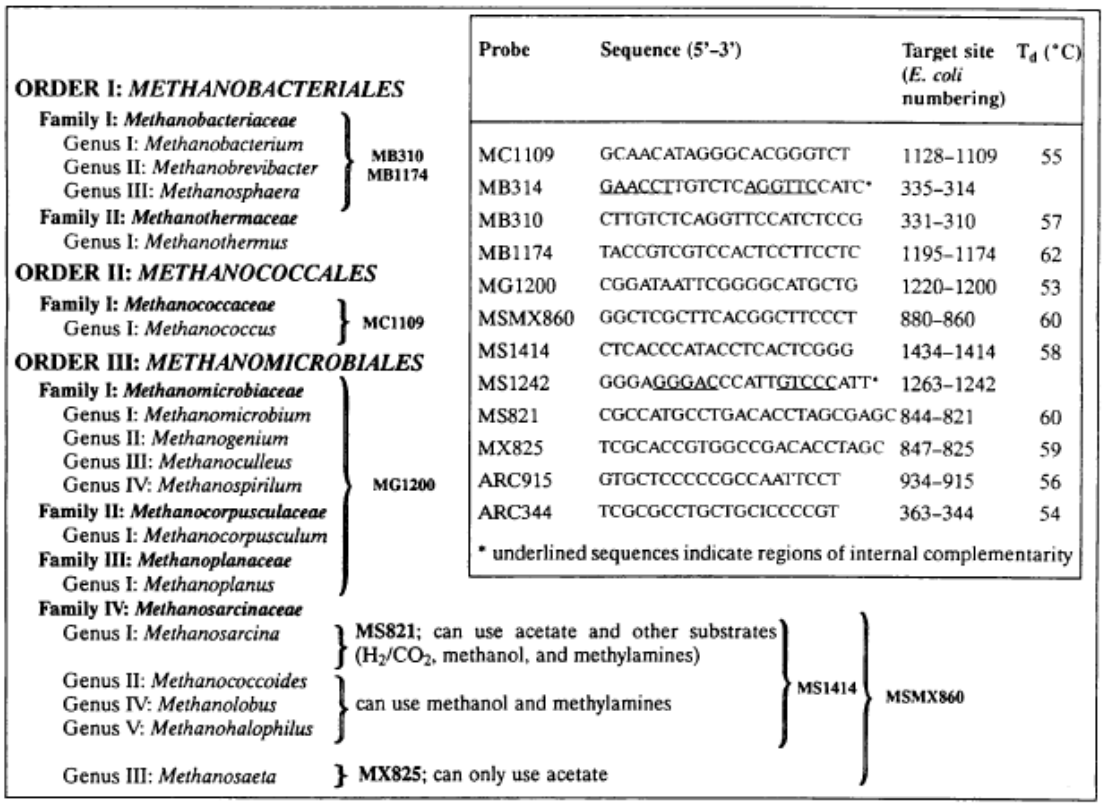

Figure 4.4. Classification of methanogens in relationship to the oligonucleotide probes characterized.

Slides were examined under Olympus BX 50 epifluorescence microscope equipped with a 100 W high-pressure mercury lamp, U-MWIB and U-MWG filter cubes. Images were captured using a Spot RT charged coupled device (CCD) camera having special software supplied by the camera manufacturer (Diagnostic Instruments Ltd., UK) The dilution percent needed is determined by counting DAPI added cells. For all times, counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated. Average of the counts gave the representative number of total microorganisms in each sample. Images were processed and analyzed using Image-Pro Plus version 6.3 image analysis software (Media Cybernetics, USA).

5. RESULTS AND DISCUSSION

In the context of this study, OTC-medicated cattle manure was used. Previous studies of the TUBITAK project showed that, highest OTC concentrations in the excreted manure were observed within the first 5 days after medication (Türker, 2013). Therefore, manure was collected for 5 days, equally mixed and used for digestion experiments. The excretion pattern of OTC in manure is shown in Figure 5.1.

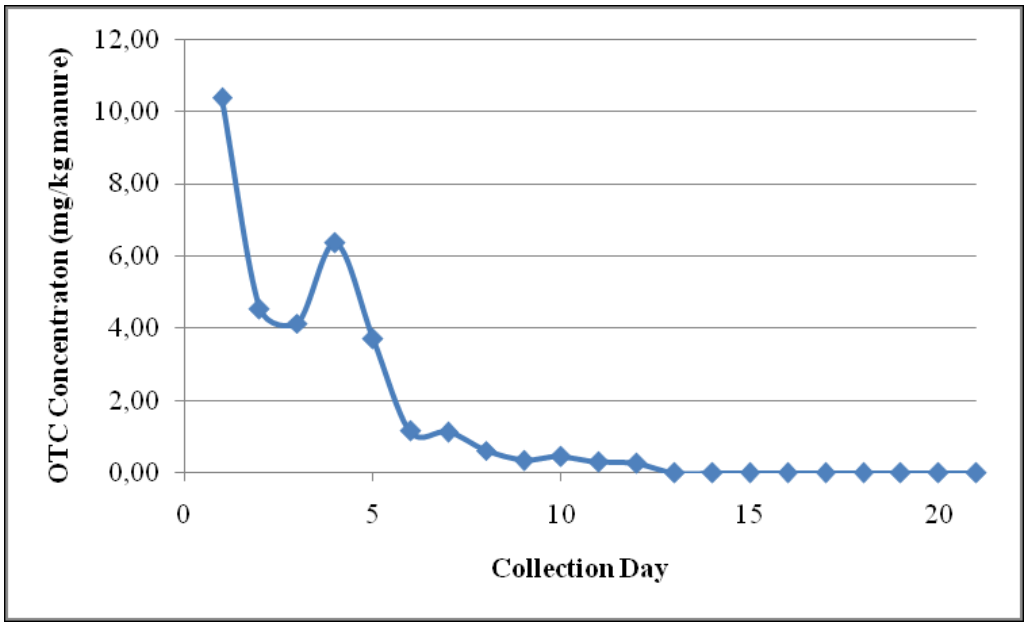

Figure 5.1. Excretion pattern of OTC in manure (Türker, 2013).

Excreted OTC concentration was found to be highest on the first day of medication as 10.38 mg/kg manure. The concentration decreased below the detection limit after 13th day of medication. Cumulative OTC concentraiton of 13 days was calculated as 33.34 mg/kg manure. Assuming that a dairy cattle produces approximately 20- 30 kg/day of solid manure, 6-10% of injected OTC was excreted (Türker, 2013).

In the studies on the effect of tetracyclines on anaerobic digestion, generally oral administration of the drug was 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 a wide application. 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 (Arikan 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 of 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 mostly due to administration of the drug and also sampling and storage conditions, the diet, general health of the animal and type of the animal.

5.1. Optimization of Operational Conditions for Acidification of Cattle Manure

5.1.1. The Effect of Digestion Period and pH

In order to examine the effect of digestion period and pH on the performance of acidogenesis phase of the anaerobic digestion, 3 sets, and each containing 2 batch anaerobic digesters were incubated at different pH values, at 37±1^{o}C in an incubator shaker for approximately 7 days. One digester was operated with non-medicated cattle manure and the other digester was operated with medicated manure. TS contents of the digesters were maintained at 5-6% (80-85% of TVS). The active volume of the digesters were 800 mL and in the sets, set 1 was operated at pH of 5.2±0.1, set 2 was operated at pH of 5.5±0.1 and set 3 was operated at pH of 5.8±0.1. Once or twice a day, minimum amount of samples were taken from the digesters to monitor the volatile fatty acids concentrations. The volatile acids concentrations (mg/L) are given in Appendix A.

Regarding the VFA concentrations, pH of 5.5 was observed as the most favorable condition for acidification compared to that of pH of 5.2 and 5.8. Considering maximum total VFA concentration which was measured on Day 3 was 960 mg COD/L and soluble COD was approximately 8000 mg/L, degree of acidification can be calculated as follows:

Degree of acidification = (960 mg COD/L) / (8000 mg COD/L) x 100 = 12% (5.1)

In the study of Yılmaz and Demirer (2008b), effects of (SRT/HRT), OLR and pH control on anaerobic acidification of cattle manure in terms of VFA formation and VS reduction was investigated. They selected SRT of 2 days and OLR of 15 g VS/L.day as the optimum operational conditions. They maintained pH value around 5.0-5.5 in the controlled reactor but found out that pH control did not improve the extent of acidification, on the contrary to this study. However, peak VFA concentration reached up to 2300 mg/L and degree of acidification was much higher. The difference may come from the fact that their study was operated in continuous-mode and this study was operated in batch-wise for optimization purposes. In another study of Yılmaz and Demirer (2008a), their acidification rate for anaerobic cattle digestion was around 30%. Li et al. (2010) investigated the co-digestion of dairy manure and food waste in a two-phase anaerobic digestion system. They studied different mixing ratios of substrates at different HRT for both acidification and methanogenesis. They concluded that HRT of 1 day for acidification was the optimal ones with the mixing ratio of 6:1 (food waste to dairy manure). This short time of HRT might be the result of higher acidification properties of food waste.

5.1.2. The Effect of Different TVS Concentrations

During the optimization step of pH and digestion period, TVS values were maintained between 4-5%. In order to determine the organic loadings during the digestion operation, a set of 3 batch digesters with different TVS concentrations were run. In this set, TVS content of the digesters R1, R2 and R3 were 4%, 6% and 8%, respectively. The digesters were incubated at pH of 5.5 at 37±1^{o}C in an incubator shaker at rpm of 100 for 5 days. Volatile fatty acids concentrations of this set are given in Appendix A.

VFA concentrations of R1 (TVS = 4%) were similar to the previous step's digesters' concentrations, which were operated with TVS of 4-5%. The maximum VFA concentration for R2 was 1464 mg/L as acetic acid equivalence. According to the VFA results of R1, R2 and R3, maximum VFA concentration was achieved by R3 (TVS = 8%) on day 3, with 2547 mg/L as acetic acid equivalence and 2715 mg COD/L as COD

equivalence. While the soluble COD concentration was approximately 9200 mg/L due to higher organic content, this time the acidification degree was:

Degree of acidification = (2547 mg COD/L) / (9200 mg COD/L) x 100 = 29% (5.2)

Although operating the digesters with TVS = 8% resulted in a higher acidification efficiency, increasing the degree of acidification from 12% to 29%, working with higher TS content, approximately 10%, showed up some difficulties such as loading/feeding problems and/or mixing problems in the digesters. Therefore, for the continuance of the study, TVS content of 6% was chosen.

The aim of two-phase anaerobic digestion systems is to improve the process stability and efficiency. Comparing the results of this pre-study to the literature, acidification of cattle manure during the acidogenesis phase was not sufficient to perform such a system due to the effect of hardly biodegradable particulate organic matter such as straw and other effects including mixing.

In two-phase anaerobic digestion systems, acidification is an important parameter to evaluate the system performance (Yılmaz and Demirer, 2008). In literature, there are many studies to improve the hydrolysis and acidification step of anaerobic digestion processes, such as operating in thermophilic conditions (El-Mashad et al., 2004; Boe and Angelidaki, 2009), sonication (Chu et al., 2002; Aldin et al., 2010), co-digestion (Li et al., 2010; Panichnumsin et al., 2010 and enzyme addition (Sonakya et al., 2001; Romano et al., 2009; Luo et al., 2011). Although these studies resulted in better acidification efficiencies, practical and cost-effective methods are the most significant considerations in full-scale applications. For example; thermophilic anaerobic digestion must be very well-operated to keep the system stable during the process and in full-scale applications; its costs are high due to heating of the system. Co-digestion is a very common practice in anaerobic digestion, especially in manure digestion, but it’s a study by oneself instead of an improvement method. Sonication might be a good solution in lab-scale studies but its full-scale applications are not feasible. On these grounds, enzyme addition seemed to be the best solution for our goal and its full-scale applications are also practical.

5.2. Improvement in Acidification of Cattle Manure due to Enzyme Addition

Although recommended dosage was given in the recipe of the enzyme tablets supplied, it was mostly utilized for domestic sewage which is more biodegradable compared to manure. Therefore, digesters with different enzyme concentrations were operated in acidific conditions, at pH = 5.5±0.1. The enzyme concentrations in the digesters E1, E2, E3 and E4 were 1, 2, 3 and 4 grams, respectively. E0 represents the initial VFA concentrations of the manure fed into the digesters, with no enzyme addition. The digesters were incubated at 37±1^{o}C for 5 days in an incubator shaker and at the end of the digestion, volatile fatty acid concentrations (Table 5.1) and gas productions (Figure 5.2) of each digester were taken into account for decision. 5 days were chosen as the digestion time because in the previous steps it was observed that after the 5th day, VFA concentrations started to decrease due to the consumption by existing methanogens.

Table 5.1. Volatile fatty acids concentration in enzyme-added digesters (mg/L).

Name	**Acetic Acid**	**Propionic Acid**	**Isobutyric Acid**	**Butyric Acid**	**Isovaleric Acid**	**Valeric Acid**	**Isocaproic Acid**	**Caproic Acid**	**Heptanoic Acid**
E0- No enzyme	1121	210	31	111	70	15	4	1	0
E1- Day 5	2378	979	58	387	97	113	4	7	0
E2- Day 5	1023	2330	112	501	120	87	3	10	0
E3- Day 5	2514	2275	110	566	110	85	2	11	0
E4- Day 5	1862	2116	92	619	111	97	6	13	4

Table 5.2. Total volatile fatty acids concentration in enzyme-added digesters as acetic acid equivalence (mg/L).

Name	**Total VFA Concentration as Acetic Acid Equivalence (mg/L)**
E0 - No enzyme	1827
E1 - Day 5	4461
E2 - Day 5	4927
E3 - Day 5	5746
E4 - Day 5	5591

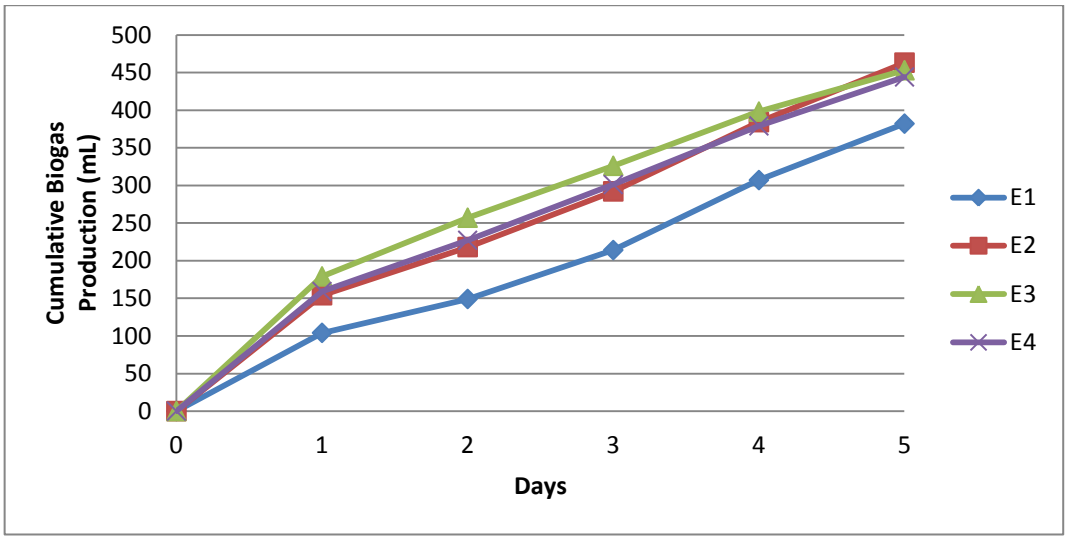

Figure 5.2. Cumulative biogas production of enzyme-added digesters.

According to final VFA concentrations, digester E3 had the best performance with 5746 mg/L VFA as acetic acid. The cumulative gas productions in acidogenic phase were approximately same in all enzyme concentrations. However, due to high amount of enzyme addition in digesters E3 and E4, scum problem occurred during the digestion, which is not desired in the treatment systems. Besides the scum problem, these high amounts of enzyme additions would increase the costs in full-scale plants treating tons of wastes. Therefore, the reactors were fed with an enzyme concentration of 4 mg/mL (1.5 grams).

5.3. Comparison of Single and Two-phase Anaerobic Digestion of Cattle Manure in Presence of OTC

After the completion of determination of optimum operational conditions for two-phase anaerobic digestion of cattle manure, in this part of the study, 2 different two-phase digesters (Acid 1 - Methane 1, operated with non-medicated manure and Acid 2 - Methane 2, operated with medicated manure) and 2 different single-phase digesters (Single 1, operated with non-medicated manure and Single 2, operated with medicated manure) were set-up. Single phase digesters were run as control for two-phase digesters.

For the start-up period, all 6 digesters were operated in batch-wise mode for 15 days. At the end of 15 days, their operation type was changed into semi-continuous mode and all digesters were fed with a withdraw/feed method once on every five days and the digesters were operated for extended period of 30 days. During this period, there was no enzyme addition into the system and all optimum operational conditions investigated earlier were maintained such as pH and TVS content. The only difference was that SRT/HRT for acidogenic phase was increased from 3 to 5 days because it was observed in the previous study that within enzyme addition, total VFA production made a peak at 3rd day and remained almost at the same concentration until 5th day. All throughout the start-up prediod for 45 days, samples were taken from the acidogenic digesters to monitor the VFA concentrations as given in Appendix A. The results confirmed that there was a necessity for enzyme addition to increase acidification. The importance of start-up periods during the anaerobic digestion systems have been highlighted in many studies, especially with respect to microbial populations (Morgan et al., 1991; Anderson et al., 1994). During start-up, the VFA concentration should be monitored on a daily basis. If the VFA concentrations decrease after approximately 3 days of feeding or remain at a stable low level, the hydraulic retention time can be lowered. By repeating this pattern and, at the same time, monitoring the concentration of VFA carefully, it is possible to reach the desired final retention time in approximately 1 month (Ahring, 2003).

After the start-up period, the digesters were operated for 60 days in semi-continuous mode within enzyme addition. According to results in the optimization step and typical parameters in the literature, HRT of 5 days for acidogenic phase, 15 days for

methanogenic phase and 20 days for single-phase digesters were applied; which makes organic loading rates of 6.25 ±015 g TVS/L day for two-phase digesters and 1.50±0.02 g TVS/L day for single-phase digesters.

The enzyme was added into the acidogenic digesters and single-phase digesters in predetermined amounts. In two-phase digesters, the effluent of the acidogenic phase digester was fed into the methanogenic phase as influent. Samples were taken from the digesters during the feeding days for chemical, analytical and molecular analysis.

In the study of Demirer and Chen (2005), one-phase and two-phase configurations were run for the anaerobic digestion of unscreened dairy manure. They investigated the optimum OLR and SRT/HRT values for the two-phase configuration and compared it to single-phase configuration. Their results indicated that two-phase reactor at a SRT/HRT of 10 days (2 days acidogenic and 8 days methanogenic) resulted the most efficient performance. Within two-phase configuration, working with OLR of 12.6 g TVS/L day was possible which was not achievable for conventional single-phase configuration. Yılmaz and Demirer (2008b) selected SRT/HRT of 2 days and OLR of 15 g TVS/L day as the optimum operational conditions for the anaerobic acidification phase. Dinsdale et al. (2000) investigated two-phase anaerobic digesion of fruit/vegetable and waste activated sludge and achieved stable performance at an overall OLR of 5.7 g TVS/L day. The performance of a laboratory-scale mesophilic (35^{o}C) two-phase anaerobic digestion system was evaluated by Hutnan et al. (2000) using sugar beet pulp as the substrate. The acidification reactor was operated in a pH range of between 4.0 and 4.5 and a HRT of 4 days, while the methanogenic reactor was operated in a pH range of 6.7 to 7.2 and a HRT of 8.9 to 13.3 days. Li et al. (2010) operated a two-phase digestion system of dairy manure and food waste up to 6.1 g TVS/L day and HRT of 3 days for acidification with a stable system performance.

5.3.1. Total Solids and Total Volatile Solids

During the study, TS and TVS contents of the digesters were maintained mostly at 38.5 g TS/L (7%) and 33 g TVS/L (6%), respectively. TS/TVS ratio was approximately 80%. TS contents in the digesters are given in Tables 5.3-5.4.

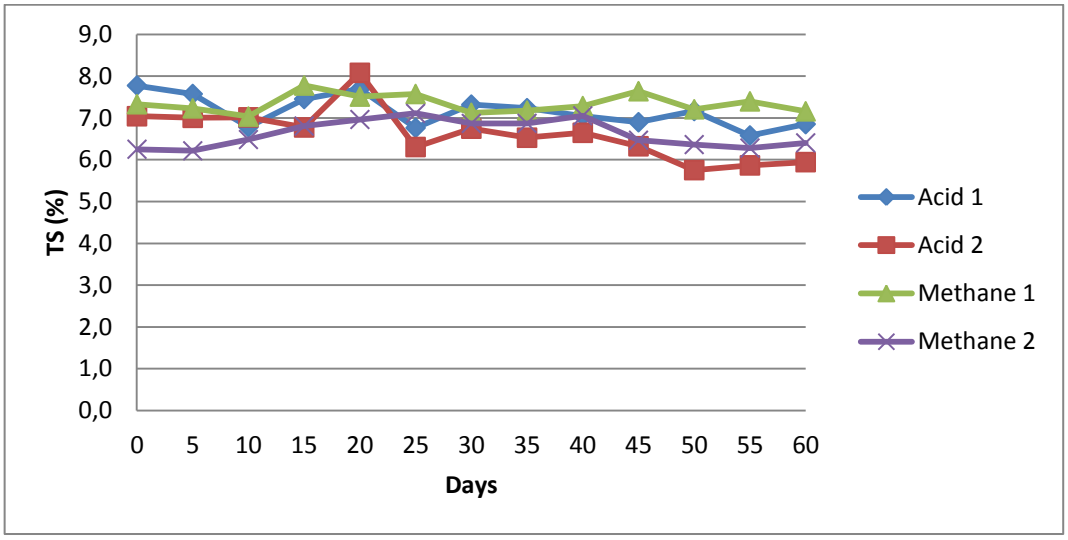

Figure 5.3. TS content of two-phase digesters.

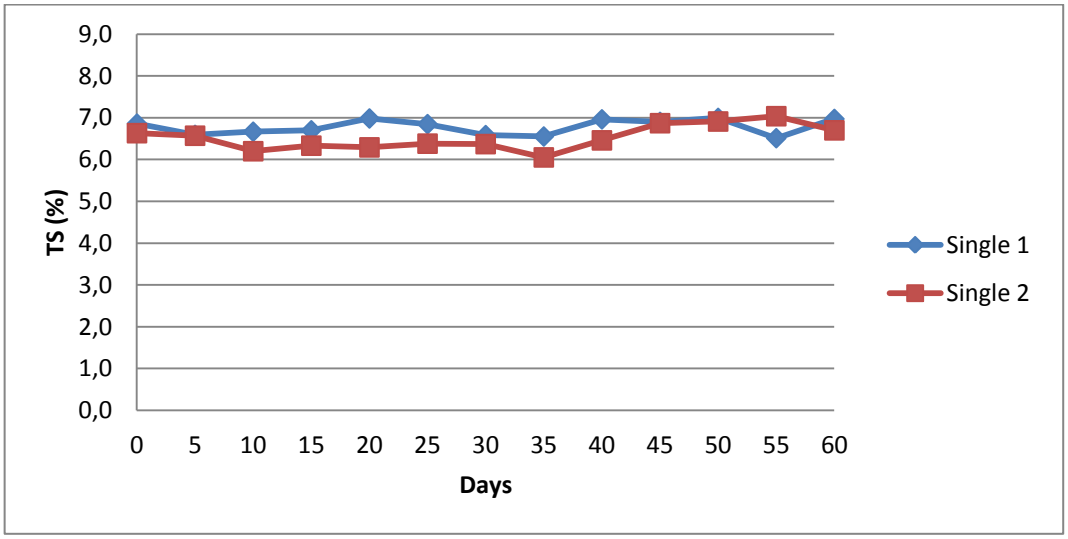

Figure 5.4. TS content of single-phase digesters.

5.3.2. Alkalinity

During the study, due to the high buffer capacity of cattle manure, alkalinity concentrations were sufficient enough as shown in Figures 5.5-5.6 and there was no need to add any extra buffering materials.

In two-phase digesters, there had been a significant decrease on the first days because of the production of volatile fatty acids in higher concentrations due to the enzyme

addition. But still, minimum 1500 mg $CaCO_3$/L alkalinity existed in the system which was enough for the stability of the system. A typical anaerobic reactor should have an alkalinity of 2000 to 3000 mg/L as $CaCO_3$. This amount is considered as a safety factor for the pH changes rendered from loading. Studies also state that in a balanced anaerobic digestion system alkalinity should not be less than 1500 mg/L as $CaCO_3$ (Gunaseelan, 1997).

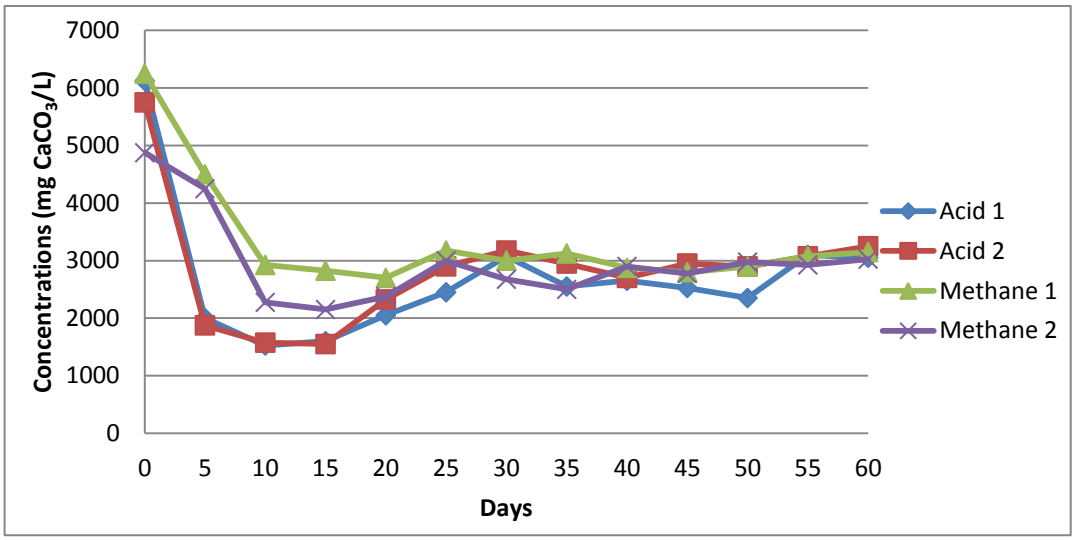

Figure 5.5. Alkalinty trend in two-phase digesters.

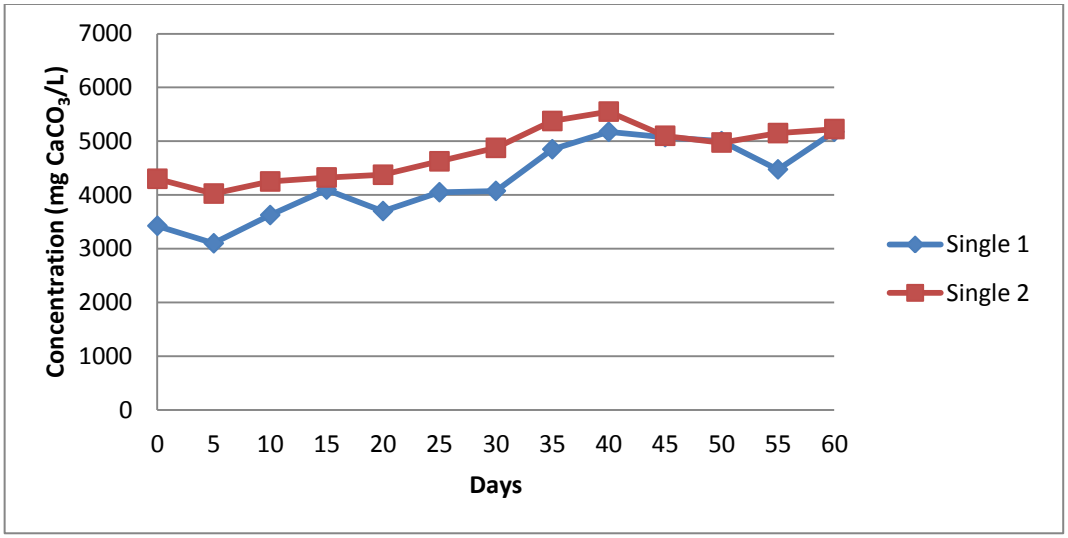

Figure 5.6. Alkalinity trend in single-phase digesters.

5.3.3. NH_3-N

During this study, C:N ratios were 27.3:1 for non-medicared manure and 24.3:1 for medicated manure. Soluble NH_3-N concentrations ranged between 50-350 mg/L in two-phase digesters (Figure 5.7) and 30-145 mg/L in single-phase digesters (Figure 5.8). The studies have shown that maximum safety limit for the ammonia concentration is approximately 3000 mg/L in anaerobic digestion systems, while 50-200 mg/L of ammonia concentration can have a beneficial effect and 200-1000 mg/L of ammonia concentration has no adverse effect on the system (McCarty, 1964). Besides, the highest methane contents are obtained with C:N ratios between 13:1 and 28:1 (Yılmaz, 2007). According to the concentrations in the literature, these concentrations can be accepted as beneficial for the system performance.

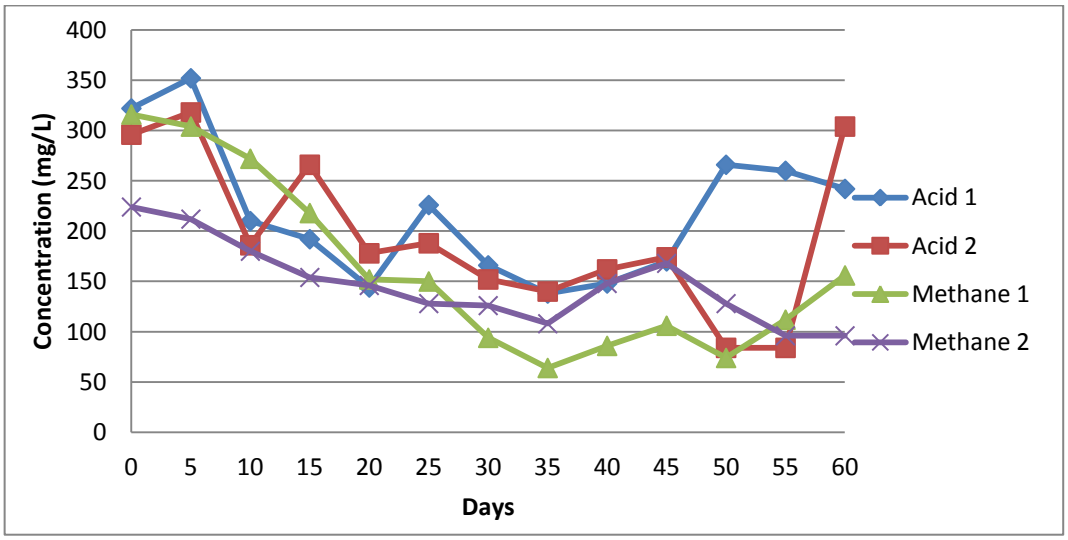

Figure 5.7. NH_3-N concentration in two-phase digesters.

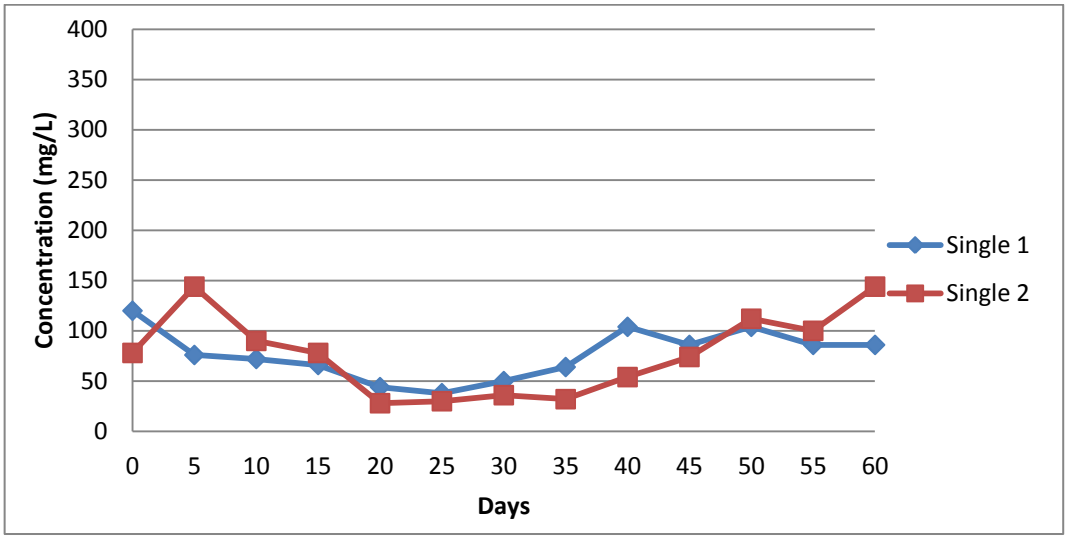

Figure 5.8. NH_3-N concentration in single-phase digesters.

5.3.4. OTC Analysis

The behaviour of OTC was monitored throughout the digestion period as following: The samples were taken on each feeding day from the two-phase and single phase medicated manure digesters and also from the manure fed as substrate on that day. The results are shown in Tables 5.3-5.4 and Figures 5.9-5.10. The influent of the acidogenic digester (Acid 2) left the acidogenic phase as effluent after 5 days, which was also the influent of the methanogenic digester (Methane 2). The effluent of the methanogenic digester left the system then after 5 days. So that there were 10 days between the influent and effluent of the overall two-phase digesters, and 5 days in single-phase digesters.

Table 5.3. OTC amounts in two-phase digestion of medicated cattle manure.

	Days/Amounts of OTC (mg/kg dry manure)												
	0	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**
Acid Inf.*	84.3	82.9	82.9	81.4	77.1	78.6	81.4	82.9	84.3	82.9	90.0	84.3	84.3
Acid Eff.$^{+}$/ Methane Inf.*	74.3	72.9	67.1	62.9	52.9	51.4	54.3	60.0	65.7	65.7	67.1	74.3	70.0
Methane Eff.$^{+}$	38.6	38.6	34.3	32.9	31.4	34.3	41.4	37.1	38.6	44.3	48.6	51.4	62.9

*Inf: Influent, $^{+}$: Effluent

Table 5.4. OTC amounts in single-phase digestion of medicated cattle manure.

	Days/Amounts of OTC (mg/kg dry manure)												
	0	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**
Inf.*	77.1	78.6	81.4	82.9	84.3	82.9	90.0	84.3	75.7	75.7	78.6	81.4	80.0
Eff.$^{+}$	61.4	41.4	41.4	41.4	48.6	42.9	52.9	44.3	51.4	57.1	57.1	55.7	65.7

*Inf: Influent, $^{+}$: Effluent

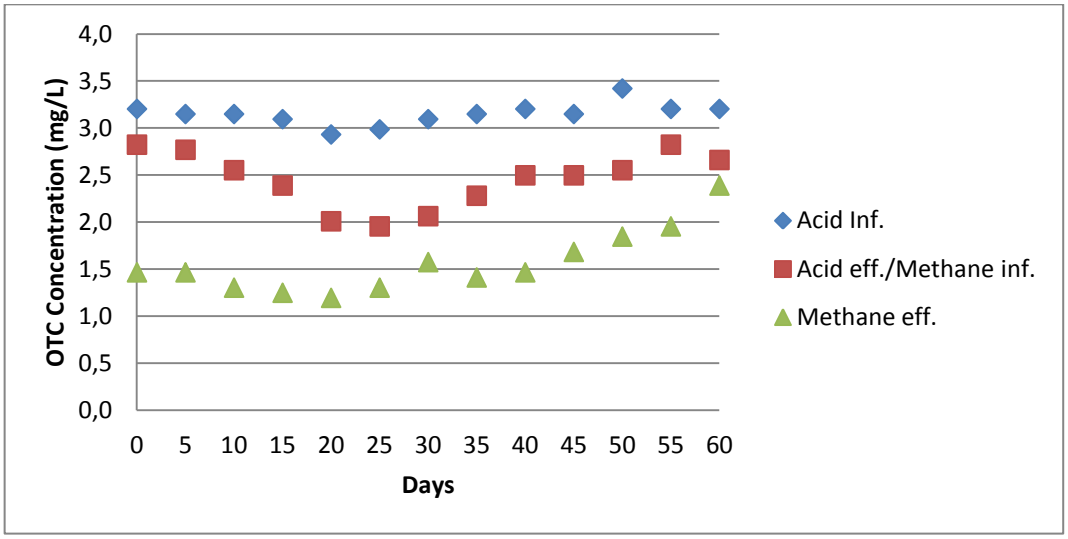

Figure 5.9. OTC concentration in two-phase digestion of medicated cattle manure.

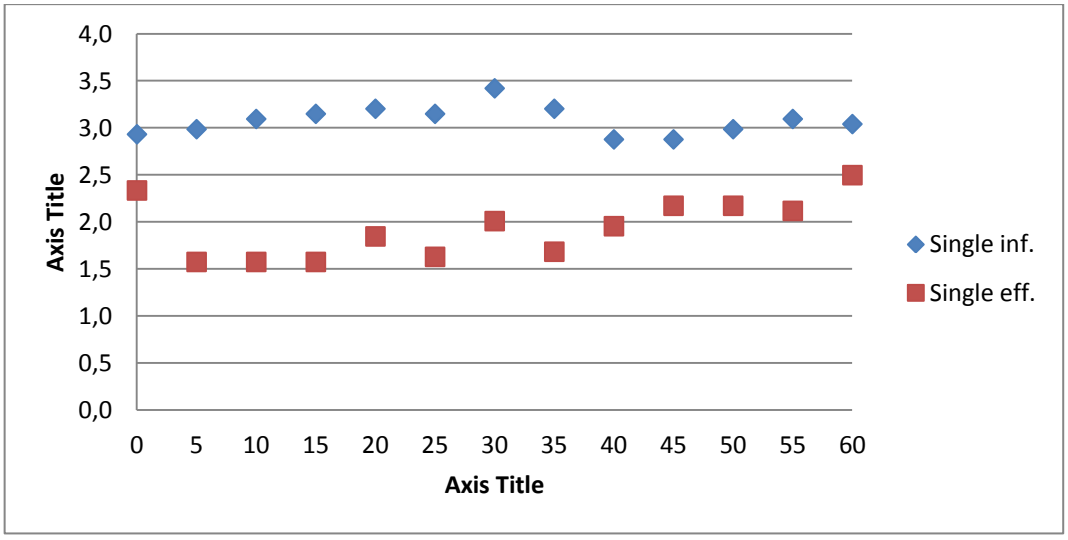

Figure 5.10. OTC concentration in single-phase digestion of medicated cattle manure.

According to results OTC analysis, the average OTC amounts in the digestion system was 82.5±3.1 mg OTC/kg dry-manure (3.11±0.12 mg OTC/L) in two-phase digesters and 81±4 mg OTC/kg dry-manure (3.07±0.14 mg OTC/L in single-phase digester. The highest decrease in OTC concentration was observed at the beginning of the methanogenic phase between days 0 and 15; meanwhile in the acidogenic phase, between days 20 and 35. In both two-phase and single-phase digesters, OTC reduction in the system decreased on the last days of the digestion and there had been OTC accumulation in the system.

5.3.5. Soluble COD

In anaerobic digestion, COD analysis is mostly suitable for wastewater treatment systems since homogeneous liquids in influent and effluent can easily be diluted and analyzed. In manure digestion systems viscosity is low and slurry is not homogeneous (Türker, 2012). Therefore, changes in the organic strength in digesters were monitored by soluble COD (sCOD) analysis. On the other hand, low solubility of organic part of manure may not reflect total COD of samples. Soluble COD concentrations in the digesters are given in Figures 5.11-5.12.

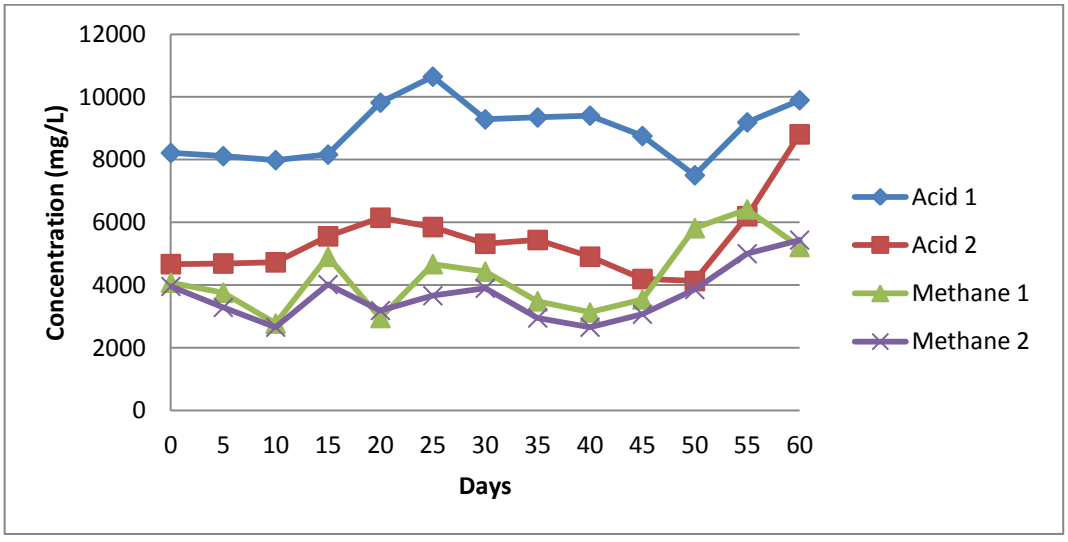

Figure 5.11. Soluble COD concentration in two-phase digesters.

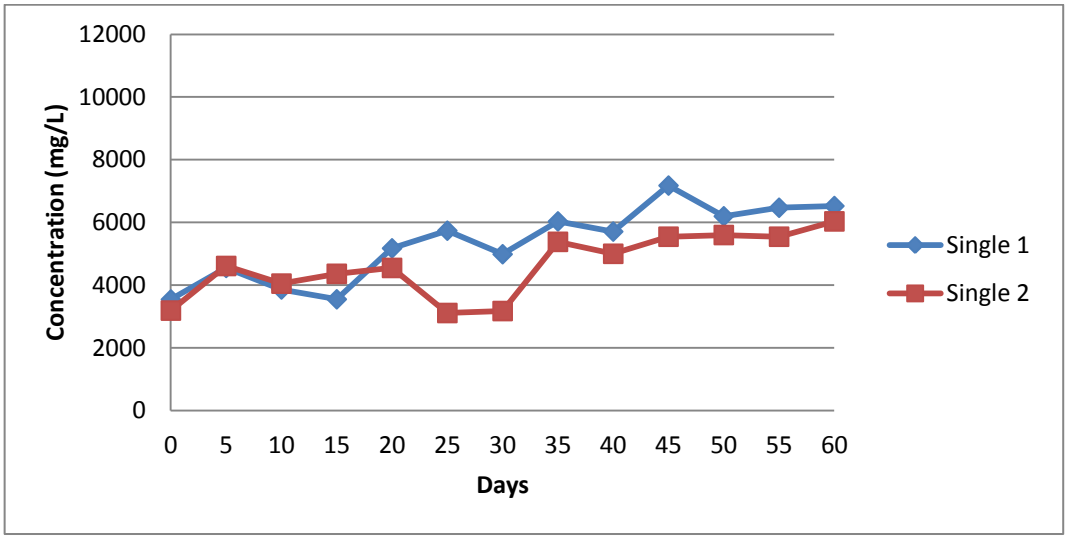

Figure 5.12. Soluble COD concentration in single-phase digesters.

Table 5.5. Soluble COD removal efficiency in "Methane 1" digester.

Days	**Influent concentration (mg/L)**	**Effluent concentration (mg/L)**	**Soluble COD removal efficiency (%)**
0-5	8114	3762	54
5-10	7985	2775	65
10-15	8162	4906	40
15-20	9820	2953	70
20-25	10649	4670	56
25-30	9287	4433	52
30-35	9346	3486	63
35-40	9406	3130	67
40-45	8754	3545	60
45-50	7504	5817	22
50-55	9190	6416	30
55-60	9897	5219	47

Table 5.6. Soluble COD removal efficiency in "Methane 2" digester.

Days	Influent concentration (mg/L)	Effluent concentration (mg/L)	Soluble COD removal efficiency (%)
0-5	4690	3288	30
5-10	4729	2657	44
10-15	5558	4018	28
15-20	6150	3190	48
20-25	5854	3663	37
25-30	5321	3900	27
30-35	5439	2953	46
35-40	4906	2657	46
40-45	4196	3071	27
45-50	4131	3859	7
50-55	6198	5001	19
55-60	8809	5436	38

Considering the TVS concentrations of acidogenic digesters' effluents, which were the influents of methanogenic digesters; organic loading of methanogenic digesters can be calculated 2.05±0.5 g TVS/L day at HRT of 15 days. According to the sCOD removal efficiencies, at OLR of 2.05 g TVS/L day, there was 52±15% sCOD removal in the digester Methane 1 and 33±12% in the digester Methane 2. Demirer and Chen (2005) achieved 59% COD removal in two-phase anaerobic digestion of unscreened and non-medicated dairy manure at OLR of 2.39 g COD/L day and HRT of 10 days, which is similar to the results of this study.

5.3.6. Volatile Fatty Acids

During the evaluation of the performance of the acidogenic digesters, soluble COD and VFA concentrations and as well as acidification rates were undertaken together. Acidification efficiencies of the acidogenic digesters are given in Tables 5.7-5.8. Within the first 10 days of the digestion, it was observed that VFA concentrations were lower than to be expected with respect to the earlier optimization studies, 1626 and 1858 mg/L as acetic acid in Acid 1 digester and 1019 and 1249 mg/L as acetic acid in Acid 2 digester. Therefore, the enzyme amount added, which was 1.5 grams, increased by 50% to 2.25 grams. The increase in the enzyme amount also increased the VFA production and

eventually acidification rates after 15th day. Acidification rates were calculated according to the Formula 4.1. VFA concenctrations were recorded during the study on each feeding/wasting day and the results are given in Appendix A.

Table 5.7. Acidification efficiency in "Acid 1" digester.

Days	**Total VFA concentration as soluble COD equivalent (mg/L)**	**Soluble COD concentration (mg/L)**	**Acidification rate (%)**
0	450	8217	5
5	1733	8114	21
10	1981	7985	25
15	3648	8162	45
20	2625	9820	27
25	3426	10649	32
30	3294	9287	35
35	2219	9346	24
40	2136	9406	23
45	1935	8754	22
50	2872	7504	38
55	3321	6198	36
60	2272	9897	23

Acidogenic non-medicated manure digester, Acid 1, had an average acidification rate of 30±7%. The highest acidification rate was achieved on the 15th day, when enzyme concentration was increased. There was also the highest sCOD removal of the system in methanogenic digesters, Methane 1 and Methane 2, removal of 70% and 48 %, respectively. There is a slight decrease during days between 35 and 45. It might have been because manure samples might have lost their biodegradability in time. The decrease in sCOD removal can also be observed during these days in methanogenic digesters. Since new manure samples were introduced to the system, solubilization and acidification efficiencies increased eventually after 50th day.

Table 5.8. Acidification efficiency in “Acid 2” digester.

Days	Total VFA concentration as soluble COD equivalent (mg/L)	Soluble COD concentration (mg/L)	Acidification rate (%)
0	135	4670	3
5	1087	4690	23
10	1331	4729	28
15	1329	5558	24
20	1414	6150	23
25	1362	5854	23
30	1280	5321	24
35	961	5439	18
40	1047	4906	21
45	1030	4196	25
50	1208	4131	29
55	1418	6198	23
60	3008	8809	34

In digester Acid 2, digesting medicated-manure, there was an average acidification rate of 24±4%. Compared to digester Acid 1, VFA concentrations and sCOD concentrations were much lower as summarized in Tables 5.5-5-8 and Appendix A. However, there was not much difference between the acidification rates.

In spite of high VFA concentrations, acidogenesis phases maintained stable conditions. This could be related to the strong buffer capacity due to high alkalinity of above 2000 mg $CaCO_3$/L in the digesters. Additionaly, there was no decrease in pH of acidogenic digesters where the peak pH value was measured as 6.5. pH in methanogenic and single digesters were more stable between of 7.1 and 7.3.

Compared to acidogenic digesters, the VFA concentrations in methanogenic and single phase digesters were significantly lower. This is a major indicator of stability in methanogenic phase where there was no VFA accumulation. The peak at the acidification rate on Day 15 was because of the increase in the enzyme addition and on Day 50, manure samples fed into the digesters were renewed, therefore; the acidification rates increased eventually.

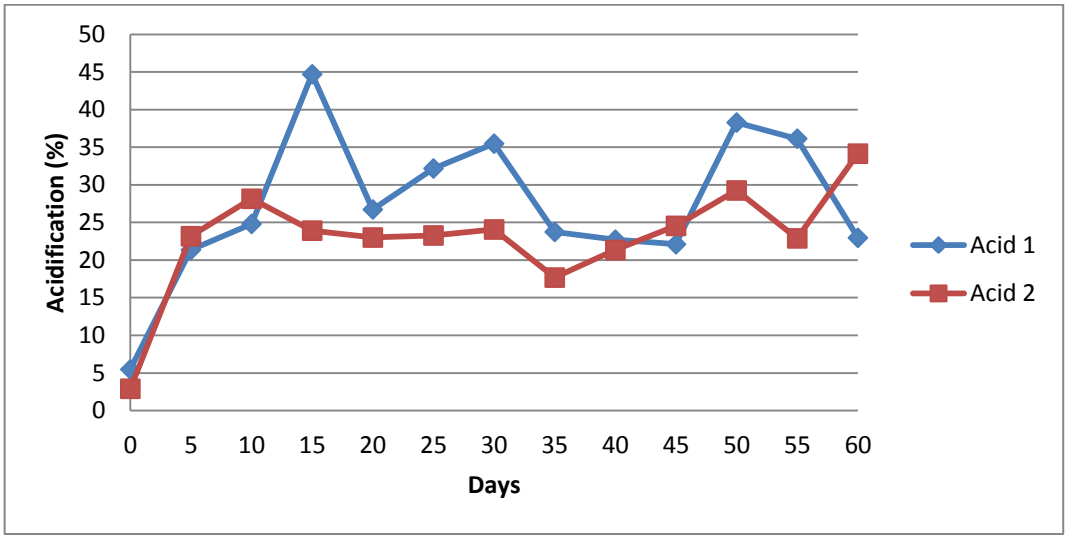

Figure 5.13. Acidification trend in acidogenic digesters.

A well operated acid digester should contain few methanogens, since optimum operational and environmental conditions for acidification seriously retard methanogenic activity but do not eliminate all methanogens (Ince and Ince, 2000). The fluctuations in VFA concentrations during the digestion might be the result of VFA consumption by those few methanogens.

In the study of Yılmaz (2007), the degree of acidification of non-medicated cattle manure varied between 10 and 25% in two-phase anaerobic digestion. The difference was probably due to enzyme addition or different operation conditions. Yılmaz and Demirer (2008a) reported that total VFA concentration was 806 mg/L by HRT of 4 days and OLR of 5 g VS/L day during the improvement of anaerobic acidification of dairy manure. They received the highest VFA concentration 2236 mg/L by HRT of 4 days and OLR of 15 g VS/L day. These results are in agreement with this study.

5.3.7. Biogas Production

Biogas productions of the digesters were recorded with milligas counters and biogas yields were calculated with respect to the TVS concentrations fed to the digesters. Methane yields were calculated according to the methane content of the biogas produced. Biogas productions in the digesters are given in Figures 5.14-5.15.

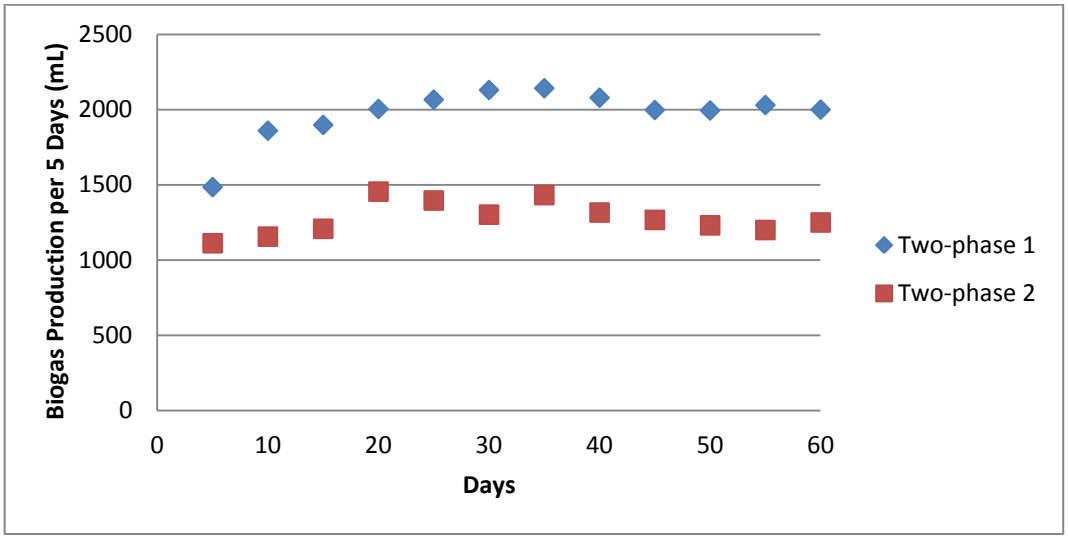

Figure 5.14. Biogas production in two-phase digesters.

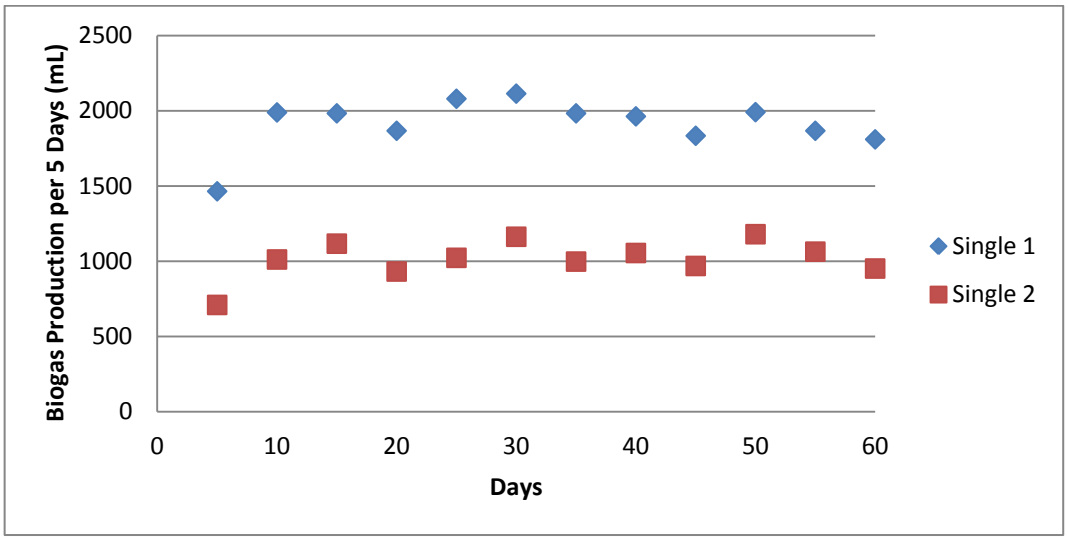

Figure 5.15. Biogas production in single-phase digesters.

At the end of 60 days of digestion biogas yields of two-phase digesters, Two-phase 1 and Two-phase 2, were 299±26 L/kg TVS-added and 193±16 L/kg-TVS-added, respectively. In single- phase digesters, Single 1 and Single 2, biogas yields were 289±25 L/kg TVS-added and 154±19 L/kg-TVS-added, respectively.

Every 10 day, biogas compositions were monitored by GC analysis. Average biogas contents of the digesters are given in Table 5.9.

Table 5.9. Biogas composition in the digesters.

Digester	**CH_4 (%)**	**CO_2 (%)**
Acid 1	39±5	61±7
Acid 2	35±6	65±11
Methane 1	58±5	42±6
Methane 2	51±5	49±7
Single 1	52±11	48±11
Single 2	47±10	53±9

In non-medicated manure digesters, two-phase and single-phase digesters performed similar in the name of biogas yields. Differently, two-phase digesters achieved a higher methane yield than single-phase (Table 5.10). Many studies have shown that two-phase system can be more effective than single-phase system in terms of increasing the stability of the process, higher organic loading rates, shorter HRT and increasing the biogas production (Zhang and Noike, 1991; Ince, 1998; Azbar and Speece, 2001; Demirer and Chen, 2005; Panichnumsin, 2010) But in some studies this acceptance was not found as satisfactory (Liao and Lo, 1985; Lo et al., 1986). Lo and his colleagues (1986) studied both completely-mixed and fixed-film reactors using screened dairy manure. In terms of overall systems performance the two-phase systems did not perform better than the one-phase systems. Mtz.-Viturtia et al. (1995) stated that although the two-phase system has been suggested for enhancing the performance, on the other hand, it is also sensitive to the substrate with high easily degradable organic load, and in that case, a single CSTR can achieve almost the same yield as the two-phase system.

Acidogenic phase digestion products may be affected by the specific characteristics of wastes, operational parameters such as HRT, SRT, and environmental factors such as pH, temperature, reactor configuration, oxidation-reaction potential (ORP), and available trace minerals. Compared to methanogenic and single phase digesters, there was little gas production in acidogenic digesters. Generally in acidogenic digesters, methane content of biogas ranges between 10-20%. In this study, methane contents of the acidogenic digesters were higher than usual. Therefore, acidogenic digesters played a role as pre-acidification step in two-phase digestion process. Biogas and methane yields of both two-phase and single-phase digesters are given in Table 5.10.

Table 5.10. The relation between the OTC presence and total biogas and methane yields.

Digester	**OTC Amount (mg/kg dry manure)**	**OTC Concentration (mg/L)**	**Biogas Yield (L/kg TVS added)**	**Methane Yield (L/kg TVS added)**
Two-phase 1*	0	0	299±26	173±15
Two-phase 2^{+}	82.5±3.1	3.11±0.12	193±16	99±8
Single 1	0	0	289±25	150±13
Single 2	81±4	3.07±0.14	154±19	72±9

* Acid1 + Methane 1, $^{+}$ Acid 2 + Methane 2

OTC concentrations here were the mean of initial influent concentrations fed into the digesters. Since two-phase digesters are considered as a whole in the process, the OTC concentration in the influent of acidogenic phase were taken into account in the calculation.

In this study, biogas yields of non-medicated two-phase and single phase digesters were 299±26 and 289±25 L biogas/kg TVS added, respectively. Typical biogas yield for the anaerobic digestion of non-medicated cattle manure is 150-350 L biogas/kg TVS (Abdal-Hadi et al., 2002; Martinez and Burton, 2003), which is in agreement with the results of this study. In medicated two-phase digesters (Two-phase 2), OTC concentration of 3.11±0.12 mg/L caused 35% inhibition on biogas yield and 43% inhibition on methane yield during the 60 days of digestion. In medicated single-phase digester (Single 2), OTC concentration of 3.07±0.14 mg/L caused 47% and 52% inhibitions on biogas and methane yields, respectively. The results indicated that two-phase anaerobic digestion performed more efficient in the presence of OTC with respect to the biogas and methane yields.

The inhibitory effects of tetracyclines in anaerobic manure digestion systems have been studied earlier. In most of these studies different from this study, the drug was administered orally to the animal. Summary of some of these studies are given in Table 5.11.

Table 5.11. Summary of methane reduction in manure digestion systems in the presence of OTC and CTC (Alvarez et al., 2010).

Compound	**Concentration (mg/L)**	**CH_4 Production Decrease (%)**	**Reference**
OTC	125	No inhibition	Lallai et al., 2002
	250		
OTC	3.1	27	Arikan et al., 2006
CTC	5	20	Sanz et al., 1996
	40	50	
	152	80	
OTC	1	2	Loftin et al., 2005
	5	5	
	25	7	
CTC	1	32	Loftin et al., 2005
	5	33	
	25	44	
OTC and CTC	10	45.2	Alvarez et al., 2010
	50	56.5	
	100	64.1	

In the study of Arikan et al. (2006), 3.1 mg/L OTC caused 27% inhibition of methane production and 60% of OTC reduction was reported in 64 days of anaerobic calf manure digestion in batch-wise mode. They reported the methane yields for medicated and non-medicated manure as 184±1 and 256±91 L/kg VS, respectively. Although the OTC concentration in the digestion system is the same, the inhibition was different from this study. This might be because of different operation modes. Since this study was operated in semi-continuous mode, there had been OTC addition continuously in every feeding. In another study, Türker (2013) reported that 1-3 mg/L OTC caused 15-35% inhibition on biogas production with a yield of 121 L/kg TVS in the anaerobic digestion of cattle manure in batch assays at mesophilic temperature. Sankvist et al. (1984) investigated the effect of OTC on the anaerobic pig manure digestion systems for batch and semi-continuous operations at mesophilic (37^oC) and thermophilic (55^oC) temperatures. They reported a reduction of 50% on methane production in the presence of 100 mg/L OTC in semi-continuous flow thermophilic fermenters with a HRT of 5-7 days. There are some differences and disagreements about the inhibitory effect of OTC on the anaerobic manure

digestion systems. These inconsistencies might be the result of different operational and environmental conditions used in the studies such as inoculum and manure sources, inoculum/manure ratio, antibiotic concentrations, reactor size, SRT/HRT, OLR, batch or continuous operation, etc. (Alvarez et al., 2010).

In this study, approximately 3.1 mg/L OTC concentration caused higher inhibitons in both two-phase and singe-phase digesters. Meanwhile, similar biogas and methane yields were obtained compared to the literature. In the presence of OTC, two-phase digestion were superior to single-phase digestion, resulted in a higher reduction in OTC concentrations and less inhibition on biogas and methane productions. The reduction in OTC concentrations can be attributed to abiotic and biotic conditions such as photodegradation, temperature, pH, humidity, binding to the organics, mineralization, dilutions during the feeding of digesters and microbial degradation.

5.3.8. DGGE Results

Community fingerprinting of each digester was performed with DGGE. Dice correlation which is based on the presence-absence of bands was used to observe the changes in community diversity. Every ten-day of the digesters were analyzed and phylogenetic trees were constructed. Changes in diversity with time and phylogenetic trees are given in Figures 5.16-5.21.

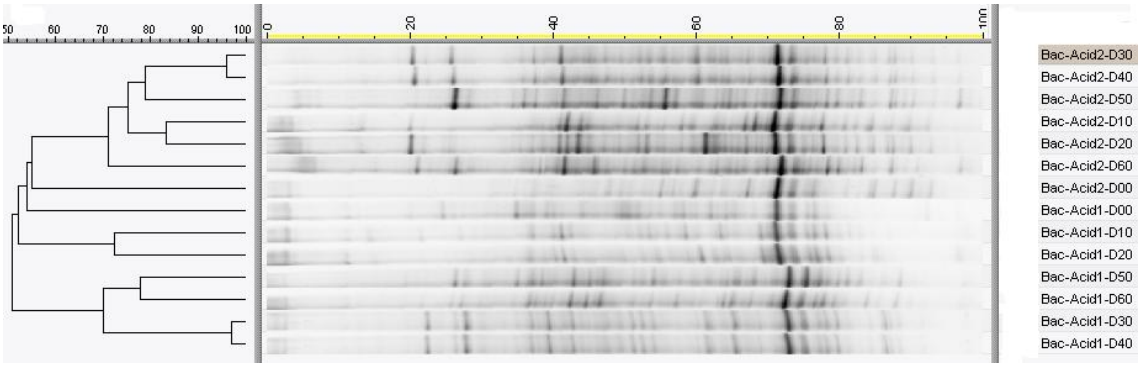

Figure 5.16. Phylogenetic tree constructed (based on Dice correlation) from the bacterial DGGE profiles in the acidogenic digesters.

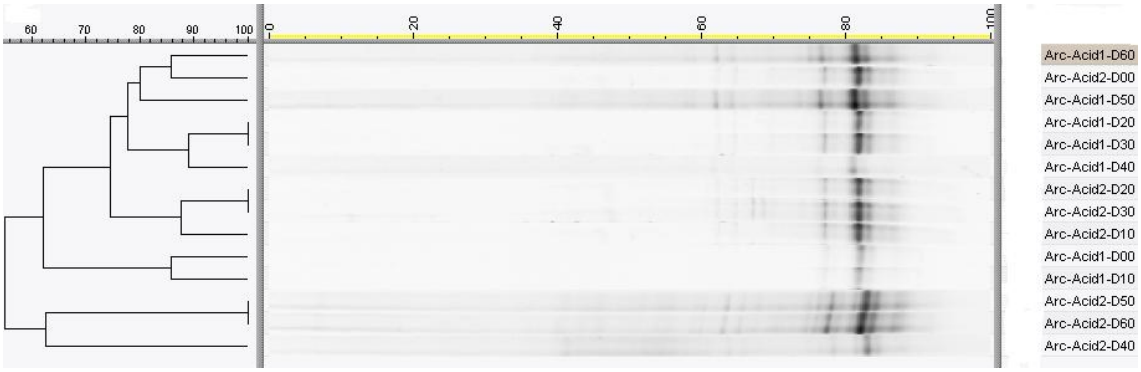

Figure 5.17. Phylogenetic tree constructed (based on Dice correlation) from the *Archaeal* DGGE profiles in the acidogenic digesters.

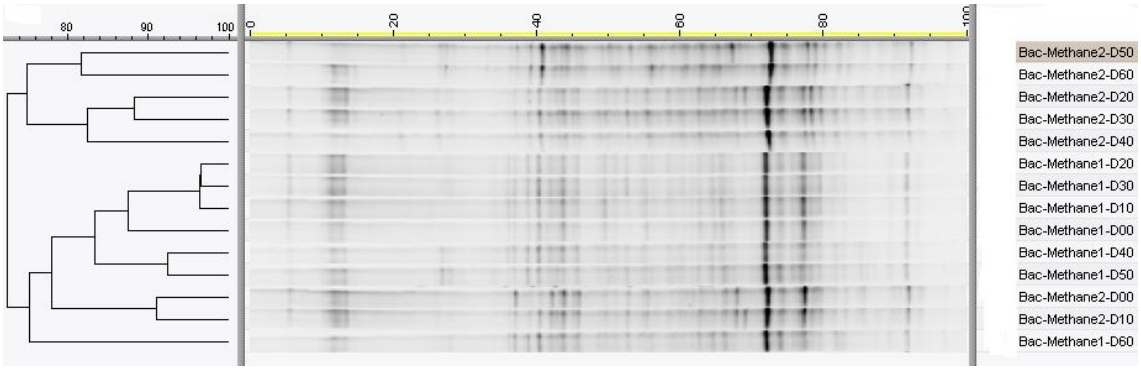

Figure 5.18. Phylogenetic tree constructed (based on Dice coefficient) from the bacterial DGGE profiles in the methanogenic digesters.

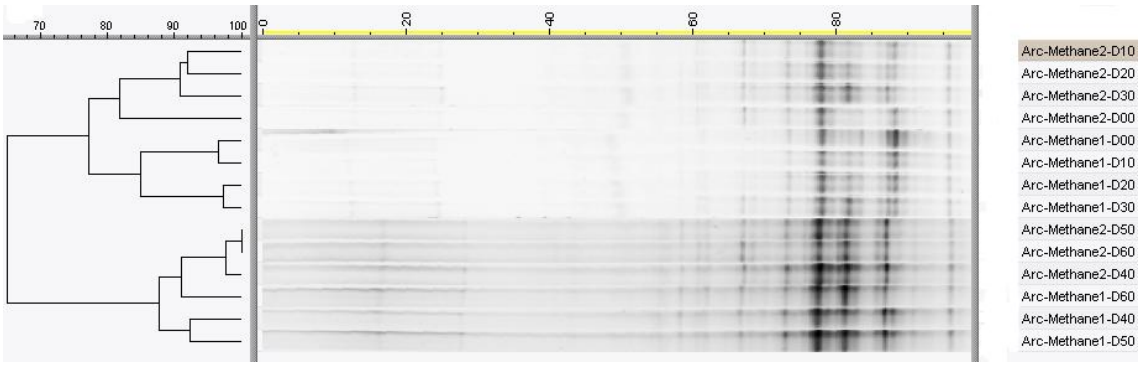

Figure 5.19. Phylogenetic tree constructed (based on Dice correlation) from the *Archaeal* DGGE profiles in the methanogenic digesters.

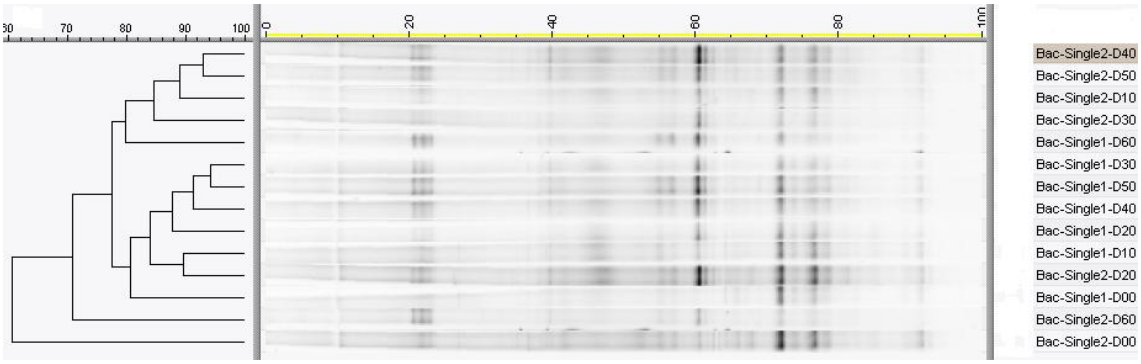

Figure 5.20. Phylogenetic tree constructed (based on Dice correlation) from the bacterial DGGE profiles in the single-phase digesters.

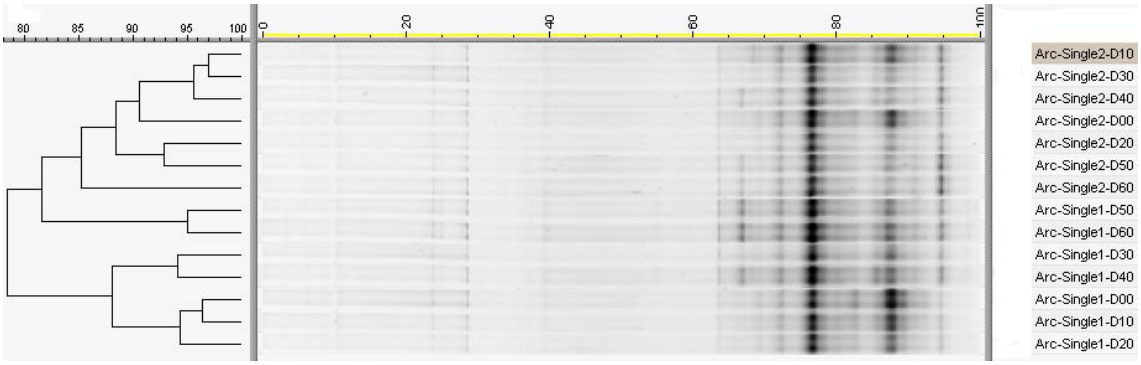

Figure 5.21. Phylogenetic tree constructed (based on Dice correlation) from the *Archaeal* DGGE profiles in the single-phase digesters.

The analysis of community fingerprinting indicated that most observable difference was according to the presence of OTC in the digesters, both in bacterial and *Archaeal* communities. Phylogenetic analysis showed that 0-30 days and 40-60 days samples were clustered into two major groups. The similarity of the community profile involved in digestion for the 0-30 days was in the range of 95-97 % whereas that of 40-60 days was 90-92%. Similarity between 0-30 day and 40-60 day communities was observed as 60%. Considering the digester performance efficiencies with respect to COD removal, OTC removal and biogas and methane production, such a difference might be as a result of acculumation and eventually inhibitory effects of OTC and by-products.

According to Bionumerics data, a total of 25 bacterial and 9 *Archaeal* species in acidogenic digesters and 17 bacterial and 17 *Archaeal* species in methanogenic digesters

were matched within the 16S rDNA clone library that was prepared by Çoban (2010). Meanwhile, a total of 13 bacterial and 14 *Archaeal* species were matched in single-phase digesters. Comparing the two-phase and single-phase digesters, bacterial diversity in acidogenic digesters and *Archaeal* diversity in methanogenic digesters were higher than single-phase digesters. This result may be one of the supporting evidences that the performance efficiencies of the two-phase digesters, with respect to VFA production and biogas and methane yields, were higher than single-phase digesters.

According to comparison analysis, many species in bacterial community matched with species in the clone library such as *Clostridium glycolicum, Clostridium lituseburense, Acinetobacter spp., Bacteriodetes, Bacillales, Solibacillus silvestris, Bacillus odyssey spp., Bacillus psychrodurans, Bacillus odysseyi,* and *Clostridium disporicum.* Among these species, the most abundant groups of bacteria were observed as *Acinetobacter* (belonging to the class *Gammaproteobacteria*), *Clostridium* and *Bacillus* (both belonging to the phylum *Firmicutes*). It is known that species of *Clostridium* is resistant to environmental conditions; therefore they might be possibly resistant to OTC as well (Kuesel et al., 2001). In the study of Demirel (2007), the bacterial 16S rDNA patterns showed at least 5 different major species including *Acinetobacteria*, *Firmicutes*, *Gammaproteobacteria*, *Bacteroidetes* and *Alphaproteobacteria* which represented the most abundant bacterial sequences of the digester community at the different times of sampling in two-phase anaerobic digestion of cattle manure. In the study of Leung and Topp (2001), dominant members of microbial community in swine manure were monitored by DGGE and found that *Clostridium spp.* was related with dominant bands. Another study dentified microbial community structure of thermophilic cattle manure digester by SSCP showed that 80% of community was made of *Bacillus* and *Clostridium spp.* Remaining percentage was made up by *Bacteriodetes* and *Preoteobacteria spp.* (Chachkhiani et al., 2004). The dominant bacteria species found in this study are in good agreement with literature data.

In *Archaeal* diversity, DGGE bands were related to *Methanobacteriales spp., Methansarcina mazei, Methanosaeta spp., Methanosarcina spp.,* and other uncultered *Archaea* species. In the digesters, dominance of hydrogenotrophic methanogens represented by *Methanobacteriales and* acetoclastic methanogens by *Methanosaeta spp.*

and *Methanosarcina spp.*, belonging to the order *Methanosarcinales. Methanosarcina spp*. are very versatile and can able to use all methane pathways.

These results showed that even in *Archaeal* domain if the conditions were met, archaeal species may increase in number and dominate the system. In the study of Hachkhiani and his co-workers (2004), *Archaeal* diversity of thermophilic cattle manure digesters was dominated by *Methanobacteriales and Methanosarcina spp.* This hydrogenotrophic pathway to methane is mainly supported by activity of syntrophic bacterial species like *Clostridium spp.* and hydrogenotrophic methanogens like *Methanobacteriales* and *Methanomicrobiales* spp. which also explains the microbial interactions and species observed in this study (Schnurrer et al., 1997; Hattori et al., 2000).

5.3.9. FISH Results

FISH analysis was conducted for the determination of active species available in the digesters. FISH probes were selected according to clone library analysis. The activity of the most abundant groups of bacteria and *Archaea* as mentioned above were investigated on the first day of the digestion as Day 0, in the middle of the digestion period as day 30 and on the last day of the digestion as Day 60. The activity of *Gammaproteobacteria* and *Firmicutes* were investigated in the acidogenic digesters and compared to single-phase digesters. Similarly, the activity of *Methanobacteriales* and *Methanosarcinales* were investigated in the methanogenic digesters and compared to the single-phase digesters. The results are expressed as Total Cell/DAPI (%). This expression gives the ratio of the related active cells with respect to DAPI count.

The ratios of active *Firmicutes* and *Gammaproteobacteria* cells in acidogenic digesters are given in Table 5.12 and Figures 5.22-23. The acitivity of *Firmicutes and Gammaproteobacteria* cells in the non-medicated manure digester, Acid 1, increased on the 30^{th} day of the digestion and then decreased on the 60^{th} day of the digestion. In the medicated manure digester, Acid 2, the trend was different. The activity of *Firmicutes* cells decreased continuously throughout the digestion and *Gammaproteobacteria cells* decreased sharply on the 30^{th} day of the digestion and increased slightly on the 60^{th} day.

Table 5.12. The ratios of active *Firmicutes* and *Gammaproteobacteria* cells with respect to DAPI count in acidogenic digesters (Total cell/DAPI, %).

Digesters	**Days**	***Firmicutes***	***Gammaproteobacteria***
Acid 1	0	21.6	17.1
	30	25.8	21.1
	60	11.1	9.5
Acid 2	0	17.3	17.2
	30	11.9	3.8
	60	3.2	6.0

Compared to Acid 1, the performance efficiency of the digester Acid 2 were lower with respect to the parameters such as acidification rate and VFA production. Within FISH results, this fact has been supported that the activity of *Firmicutes* and *Gammaproteobacteria* in Acid 2 digester were much lower. Especially, the difference between the active cells of the digesters showed itself clearly on Day 30. The activity of *Firmicutes* and *Gammaproteobacteria* cells were 54% and 82% higher in Acid 1, respectively.

Among all sampling days in the digesters, last day of Acid 2 digester had the lowest activity. This result is likely to be expected when considering the OTC results in Table 5.3 that there had been an OTC accumulation on the last days of the digestion, which also caused the highest inhibition on the active *Firmicutes* and *Gammaproteobacteria* cells.

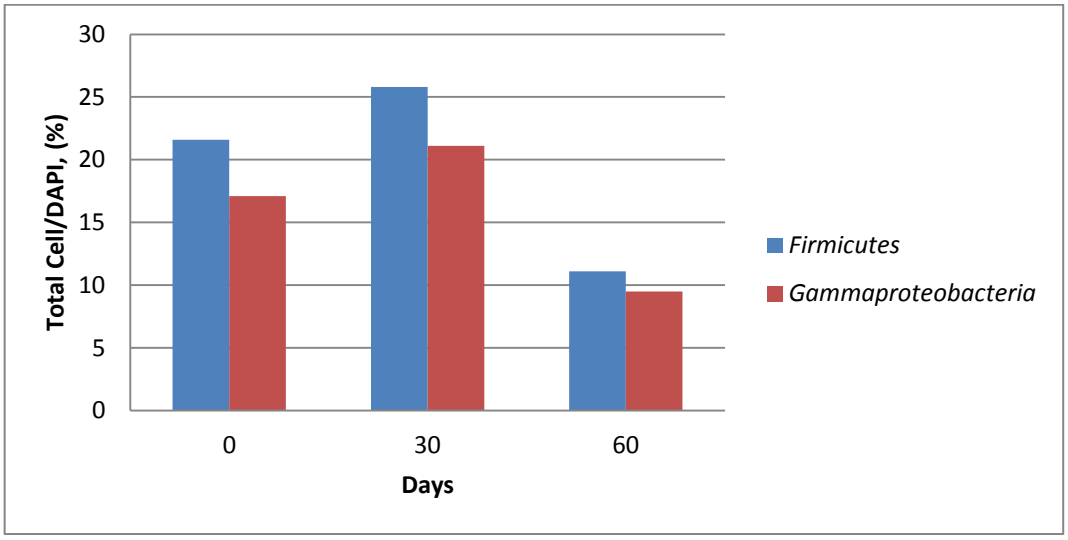

Figure 5.22. Changes in active *Firmicutes* and *Gammaproteobacteria* cells in "Acid 1" digester (Total cell/DAPI, %).

In Acid 2 digester, although the ratio between the active cells of *Firmicutes* and *Gammaproteobacteria* was the same on Day 0, probably *Gammaproteobacteria* have been affected more than *Firmicutes* by the inhibitory effects of OTC during the digestion.

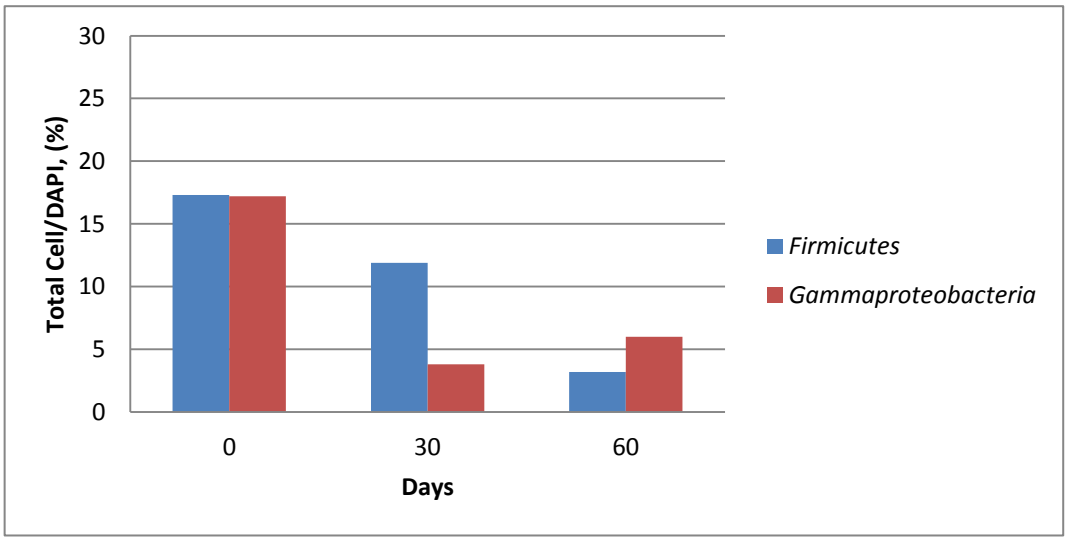

Figure 5.23. Changes in active *Firmicutes* and *Gammaproteobacteria* cells in "Acid 2" digester (Total cell/DAPI, %).

The ratios of active *Methanobacteriales* and *Methanosarcinales* cells in methanogenic digesters are given in Table 5.13 and Figures 5.24-25. The trend in the

activity of these methanogens in methanogenic digesters was different than the bacteria in acidogenic digesters. In acidogenic digesters, the activity of investigated bacteria groups had changed significantly in time during the digestion. But in methanogens, it is hard to establish a relationship between the digestion time and the active methanogen cells.

Table 5.13. The ratios of active *Methanobacteriales* and *Methanosarcinales cells* with respect to DAPI count in methanogenic digesters (Total cell/DAPI, %).

Digesters	**Days**	***Methanobacteriales***	***Methanosarcinales***
Methane 1	0	15.4	6.2
	30	15.7	12.7
	60	16.6	11.1
Methane 2	0	13.5	2.7
	30	16.8	1.9
	60	12.6	2.6

The activity in both digesters was quite stable during the digestion, except *Methanosarcinales* in Methane 1 digester, increased on Day 30. When looked at the percentages of *Methanobacteriales* and *Methanosarcinales*, it can be seen that the effect of OTC in medicated manure was most on the order Methanosarcinales. Although the activity of *Methanobacteriales* was close to each other in non-medicaated and medicated manure digesters, the low activity of *Methanosarcinales* all through the digestion was encountered in Methane 2 digester showing the inhibition on biogas and methane yields (Table 5.10). There was 76% and 85% difference between the ratio of active *Methanosarcinales* cells in the digesters Methane 1 and 2 on day 60 and on day 30, respectively. This significant difference might have been one of the reasons for lower performance efficiencies of Methane 2 digester compared to Methane 1. Since *Methanosarcinales* are acetoclastic methanogens, comparatively low acetic acid production in medicated acidogenic digester, Acid 2, might have affected the activity of *Methanosarcinales* in Methane 2 digester.

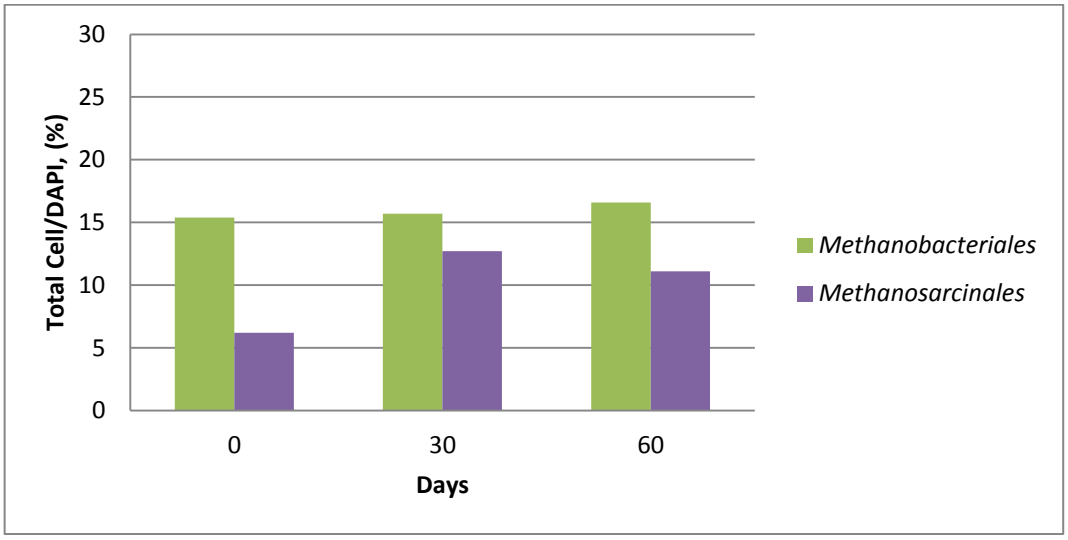

Figure 5.24. Changes in active *Methanobacteriales* and *Methanocarcinales* cells in "Methane 1" digester (Total cell/DAPI, %).

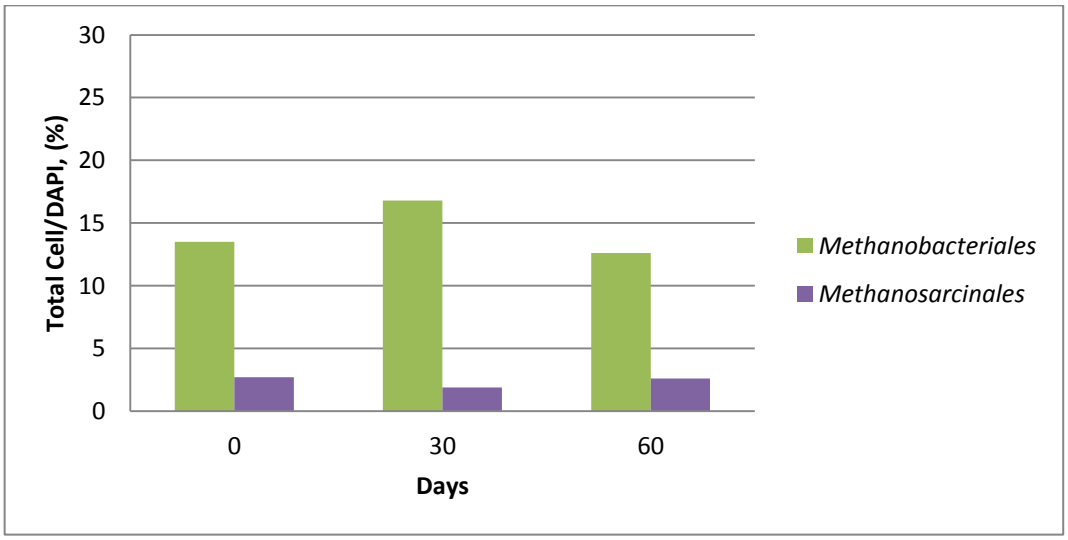

Figure 5.25. Changes in active *Methanobacteriales* and *Methanocarcinales* cells in "Methane 2" digester (Total cell/DAPI, %).

The ratios of active *Firmicutes*, *Gammaprotepbacteria*, *Methanobacteriales* and *Methanosarcinales* cells in single-phase digesters are given in Table 5.14 and Figures 5.26-27.

Table 5.14. The ratios of active *Firmicutes*, *Gammaprotecobacteria*, *Methanobacteriales* and *Methanosarcinales cells* with respect to DAPI count in single-phase digesters (Total cell/DAPI, %).

Digesters	Days	Firmicutes	Gammaproteobacteria	Methanobacteriales	Methanosarcinales
Single 1	0	15.8	9.4	16.5	1.6
	30	15.8	10.6	7.2	2.1
	60	8.0	3.2	3.3	3.1
Single 2	0	11.6	5.8	7.9	0.7
	30	14.3	11.2	10.1	4.7
	60	7.0	9.7	4.3	2.7

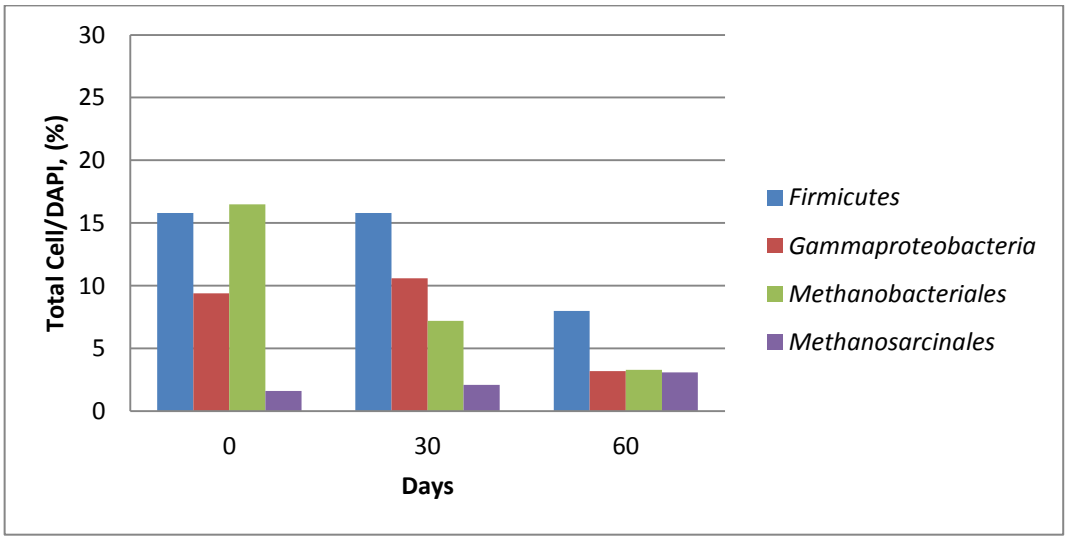

Figure 5.26. Changes in active *Firmicutes*, *Gammaproteobacteria*, *Methanobacteriales* and *Methanocarcinales* cells in "Single 1" digester (Total cell/DAPI, %).

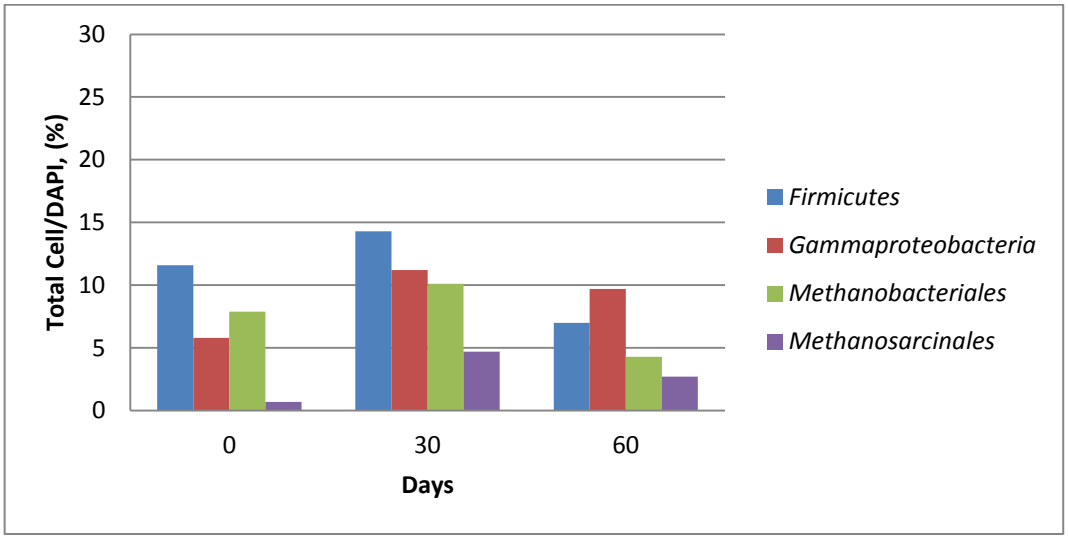

Figure 5.27. Changes in active *Firmicutes*, *Gammaproteobacteria*, *Methanobacteriales* and *Methanocarcinales* cells in "Single 2" digester (Total cell/DAPI, %).

Comparing the single-phase digesters among themselves, as a common incident, it can be said that OTC did not play an important role on the acitivity of the related microorganisms; unlike it did in two-phase digesters among each other. The most observable difference is that in non-medicated single phase digester, Single 1, the activity of *Firmicutes* was higher than Single 2. On the other hand, the active cells of *Gammaproteobacteria*, *Methanobacteriales* and *Methanosarcinales* did not differ much among two single-phase digesters.

When single-phase and two-phase digesters are compared, their difference is obvious. The ratio of both active *Firmicutes* and *Gammaproteobacteria* cells in Acid 1 digester were much higher than Single 1 digester, which were operated with non-medicated manure. In both digesters, the activity of Firmicutes and *Gammaproteobacteria* decreased between days 30 and 60. According to the final comparison, the least affected group from the difference between two-phase and single-phase operations in the presence of OTC was *Firmicutes*. Moreover, single-phase operation in the presence of OTC seemed to be more favourable for both of them. The ratio of active cells in Single 2 digester was higher than Acid 2, even on 60th day of digestion.

In non-medicated methanogenic digester, Methane 1, the activity of *Methanobacteriales* was stable around 15% during digestion. Meanwhile, in non-medicated single-phase digester, Single 1, the activity of *Methanobacteriales* decreased from 16% on Day 0 to 3% on Day 60. Same trend was also observed in the activity of *Methanosarcinales* between Methane 1 and Single 1. The activity of *Methanosarcinales* increased from 6% to 12% during the digestion in Methane 1 but the ratio of activity ranged between 1-3% in Single 1. Although biogas yields were almost the same between these two digesters, the difference in the ratio of active cells might be the reason for Methane 1 to have a higher methane yield than Single 1. In the presence of OTC, *Methanobacteriales* were more favoured in methanogenic digester, Methane 2. Both on days 0, 30 and 60, the activity ratio of *Methanobacteriales* were 13, 17 and 12% in Methane 2 and 8, 10, 4% in Single 2, respectively. Comparatively much lower activity of *Methanosarcinales* did not differ much between the digesters Methane 2 and Single 2.

In this assay, the dominant active bacteria group was *Firmicutes* both in the absence and presence of OTC and both in acidogenic and single-phase digesters which are fermentative hyrdolitic bacteria along with *Gammaproteobacteria*. The common indicent in the comparison of methanogens is that *Methanobacteriales* was dominant in all digesters, which is condidered as the most abundant hydrogenotrophic methanogen in manure digesters (Sharp et al., 1998; Dworkin, 2006). According to most studies, methanogenesis uses hydrogenotrophic pathway in manure digesters (Angelidaki and Ahring, 1993; Karakashev et al., 2006). The active *Methanosarcinales* cells were only abundant in methanogenic non-medicated manure digester, Methane 1, which had the highest methane yield among the other digesters. Comparatively abundance of active *Methanobacteriales* cells in Methane 2 digester might have caused the higher biogas and methane yields than Single 2 since the activity of *Methanosarcinales* did not change differently.

In the study of Karakashev and co-workers (2005), *Archaeal* structure of 6 sludge digesters and 9 manure digesters were monitored by FISH method. The study showed that two distinct *Archaeal* structures for two digester types. Manure digesters were dominated by *Methanobacteriales* and *Methanosarcinacaea* while sludge digesters were dominated by *Methanosaeta spp.* In the study of Schmidt and his colleagues (2000), abundance of

Methanosarcina spp. instead of *Methanosaeta spp.* was reported in manure digesters. Most of the studies investigating manure digesters by FISH technique did not focus on the inhibitory effects of toxic compounds such as oxytetracycline. However, the studies of Ertekin (2010) and Türker (2013) investigated the inhibitory effects of OTC on microbial communities in conventional single-phase cattle manure digestion operated in batch-wise mode. Ertekin (2010) stated that number of bacteria and *Archaea* decreased against the increasing degree of inhitibition and the order *Methanomicrobiales* was abundant in all serum bottles. Both *Methanosarcinacea* and *Methanobacteriales* decreased in control and medicated serum bottles showing high and moderate inhibition. Türker (2013) stated that the activity of related microorganisms increased in time and then decreased on the last days of digestion. It was found out that there was a negative relation with the activity of *Methanomicrobiales* and OTC; meanwhile, no negative relation was found with *Methanosarcinales*. In this study, *Methanosarcinales* did not show any significant changes in the absence and presence of OTC in single-phase digesters, as well.

6. CONCLUSIONS

The major objective of this study was to determine the behaviour and inhibitory effecst of oxytetracycline on biogas production, system efficiency and microbial communities in the two-phase digesters using cattle manure as substrate.

First, a preliminary study was conducted to determine the optimum environmental and operational conditions during digestion such as pH, TVS content and digestion period. Under the optimized conditions, acidification efficiency was enhanced through enzyme addition. Two-phase and single-phase digesters were operated for 60 days for comparative determination of behaviour and effects of OTC parameters such as biogas and VFA production, and digestion efficiency. Organic loading rates of two-phase and single phase digesters were 6.25±0.15 g TVS/L-day and 1.5±0.02 g TVS/L-day, respectively. SRT/HRT for acidogenic, methanogenic and single-phase digesters were 5 days, 15 days and 20 days, respectively. Biogas yields of non-medicated single and two-phase digesters were almost the same as 289±25 and 299±26 L/kg-TVS added, respectively. On the other hand, highest methane yield was achieved by non-mediacted two-phase digester as 173±15 L/kg-TVS added. In the presence of OTC, biogas yields were 193±16 and 154±19 L/kg TVS in two-phase and single-phase digesters, respectively. In medicated digesters, approximately 3.1 mg/L OTC concentration caused 35% inhitibition on biogas yield and 43% on methane yield in two-phase digester. The inhibitory effects of OTC on biogas and methane yields were higher in single-phase digester as 47% and 52%, respectively.

In bacteria *Firmicutes and Gammaprotecobacteria*, in *Archaea Methanobacteriales* and *Methanosarcinales* were dominated. Almost all bacterial and *Archaeal* species were inhibited by OTC. Comparing whole communities, the presence of OTC resulted in much significant differences in bacterial community structure than *Archaeal* community structure. Phylogenetic analysis showed that 0-30 days and 40-60 days samples were clustered into two major groups. Considering the digester performance efficiencies with respect to COD removal, OTC removal and biogas and methane production, such a difference might be as a result of accumulation and eventually inhibitory effects of OTC and by-products.

The presence of OTC in the digesters was mostly effective on activity of *Gammaproteobacteria* and *Methanosarcinales*. Digestion time was inversely proportional with the activity of *Firmicutes* and *Gammaproteobacteria*. Generally, there was an increase in the activity of the related microorganisms on Day 30 and then a decrease on Day 60. The activity of *Methanobacteriales* was most stable and highest both in the absence and presence of OTC in methanogenic digesters; however, showed a decreasing trend in single-phase digesters.

In this study, inhibitory effects of OTC on performances of single and two-phase anaerobic digestion of cattle manure and microbial communities were studied. However, the results of OTC analysis do not specify whether the reduction in OTC concentration due to degradation, mineralization or binding of OTC to the organic matrix (Arikan et al., 2006) Most antibiotics are adsorbed quickly and their antibiotic potency decreases by sorption and fixation which does not mean a complete elimination of the antimicrobial activity (Sengeløv et al., 2003). In literature, it has been stated that OTC had negative effect on biogas and methane yields in digestion systems, but did not cause any complete system failures (Sanz et al., 1996; Lallai et al., 2002, Liguoro et al., 2003; Arikan et al., 2006, Alvarez et al., 2010), which is in agreement with this study.

7. FURTHER RECOMMENDATIONS

In this study, behaviour and inhibitory effects of OTC on two-phase anaerobic digestion of cattle manure was studied. The main focus was oriented to biogas and methane productions, system efficiencies, determination of the diversities among the digesters and most abundant active bacterial (*Firmicutes* and *Gammaproteobacteria*) and methanogenic (*Methanobacteriales* and *Methanosarcinales*) populations. For further investigations, FISH studies can be extended to target other available species and taxa. Also, a future study is can be conducted 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 may result in better understanding of inhibition on microbial communities.

Although OTC removal in anaerobic digestion of manure was extensively studied in the context of this thesis and other publications (Lallai et al., 2002; Loftin et al., 2005; Arikan et al., 2006; Alvarez et al., 2010), there is still a gap in the literature whether this removal occurs due to biotic or abiotic conditions during manure digestion. Therefore, further experiments can be designed and conducted under different operational conditions in biotic and abiotic conditions. Additionally, determination of OTC and its metabolites during the digestion should be investigated, as well.

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APPENDIX A: VOLATILE FATTY ACIDS (VFA) CONCENTRATIONS

Table A.1. VFA concentrations in acidogenic digesters at pH = 5.2±0.1 (mg/L).

Digester	Hours	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Isocaproic Acid	Caproic Acid	Heptanoic Acid
Acid 1	0	129	8	4	7	9	0	0	0	0
	19	128	9	4	9	9	0	0	0	0
	25	135	9	5	13	11	0	0	0	0
	40	138	10	4	43	7	0	0	0	0
	51	181	13	7	50	14	0	0	0	0
	66	176	17	10	48	17	0	0	0	0
	74	210	23	10	52	18	0	0	0	0
	91	194	25	11	56	20	3	0	0	0
	98	213	20	8	46	13	3	0	0	0
	120	211	22	10	63	17	4	0	0	0
	144	352	44	18	179	32	8	0	0	0
Acid 2	0	63	11	0	10	0	0	0	0	0
	19	114	11	0	18	0	0	0	0	0
	25	132	10	0	28	0	0	0	0	0
	40	102	6	0	13	10	0	0	0	0
	51	111	6	5	14	11	0	0	0	0
	66	100	0	5	12	10	0	0	0	0
	74	100	5	6	13	11	0	0	0	0
	91	80	5	4	10	9	0	0	0	0
	98	80	0	0	7	5	0	0	0	0
	120	99	9	6	23	10	0	0	0	0
	144	68	66	0	5	4	0	0	0	0

Table A.2. VFA concentrations in acidogenic digesters at pH = 5.5±0.1 (mg/L).

Digester	Hours	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Isocaproic Acid	Caproic Acid	Heptanoic Acid
Acid 1	18	301	73	16	79	29	6	0	0	0
	25	318	84	18	86	32	8	0	3	0
	41	303	85	18	83	31	8	0	0	0
	49	305	87	18	85	31	8	0	3	0
	65	207	70	14	64	25	7	0	0	0
	73	245	67	13	63	20	7	0	0	0
	90	268	121	27	118	46	14	0	5	0
	98	259	129	30	126	50	15	0	5	0
	114	137	93	21	88	34	11	0	4	0
Acid 2	18	366	105	20	98	32	10	0	0	0
	25	377	113	22	104	35	12	0	3	0
	41	345	112	22	99	34	11	0	3	0
	49	356	115	22	101	34	11	0	3	0
	65	195	68	12	60	19	7	0	0	0
	73	354	158	33	75	52	17	0	5	0
	90	294	164	35	151	56	19	0	5	0
	98	261	135	28	118	42	15	0	4	0
	114	111	107	23	94	34	12	0	3	0

Table A.3. VFA concentrations in acidogenic digesters at pH = 5.8±0.1 (mg/L).

Digester	Hours	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Isocaproic Acid	Caproic Acid	Heptanoic Acid
Acid 1	24	199	77	13	45	19	6	0	0	0
	48	235	92	14	47	20	6	0	0	0
	72	288	122	16	54	22	7	0	0	0
	96	211	147	18	59	22	8	0	0	0
	120	70	157	19	59	22	9	0	0	0
Acid 2	24	196	71	10	48	17	5	0	0	0
	48	235	85	11	45	16	6	0	0	0
	72	250	100	12	47	17	6	0	0	0
	96	243	123	14	53	18	7	0	0	0
	120	165	135	15	54	18	7	0	0	0

Table A.4. VFA concentrations in acidogenic digesters at different % TVS (mg/L).

Digester	Hours	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Isocaproic Acid	Caproic Acid	Heptanoic Acid
Acid 1*	24	197	55	12	47	21	0	0	0	0
	48	298	73	17	77	29	5	0	0	0
	72	324	102	23	102	39	13	0	4	0
	96	338	123	26	110	41	14	0	5	0
Acid 2^	24	431	145	24	118	31	11	0	0	0
	48	538	180	30	158	41	15	0	0	0
	72	582	237	39	195	51	27	0	0	0
	96	467	249	41	191	52	27	0	4	29
Acid 3^{+}	24	808	248	35	212	42	24	0	0	0
	48	1074	322	53	334	66	39	0	4	0
	72	1065	372	59	369	75	42	0	5	0
	96	937	341	60	352	73	44	0	6	0

*4% TVS, ^6% TVS, $^{+}$8% TVS

Table A.5. VFA concentration in the acidogenic digesters during the start-up period (mg/L).

Digester	Days	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Isocaproic Acid	Caproic Acid	Heptanoic Acid
Acid 1	3*	587	213	41	108	68	9	93	0	0
	11*	61	352	62	185	100	23	0	4	0
	15*	153	264	42	139	79	119	1	4	1
	3	564	298	56	161	102	25	2	7	0
	7	364	293	46	128	81	38	0	7	0
	13	212	517	51	2	80	39	1	6	0
	16	175	722	13	1	86	6	2	1	3
	19	117	920	12	3	130	13	4	2	4
	23	175	826	28	11	131	19	3	3	3
	25	178	823	28	8	147	17	1	3	0
	28	189	914	82	113	138	46	0	9	0
	30	172	815	92	157	146	37	6	6	0
Acid 2	3*	552	232	30	129	47	12	0	0	0
	11*	62	416	50	144	73	21	0	6	0
	15*	281	394	43	139	71	22	3	5	0
	3	470	242	34	110	57	20	2	6	0
	7	122	324	39	115	63	32	0	7	0
	13	159	511	39	1	63	4	2	1	0
	16	123	96	0	1	2	0	1	0	5
	19	182	131	1	1	7	2	1	1	5
	23	164	191	10	2	24	8	1	2	3
	25	171	240	11	3	33	11	0	1	0
	28	378	281	27	73	38	20	1	8	0
	30	522	311	38	109	55	23	1	5	0

*During the batch-wise operation

Table A.6. VFA concentration in two-phase digesters (mg/L).

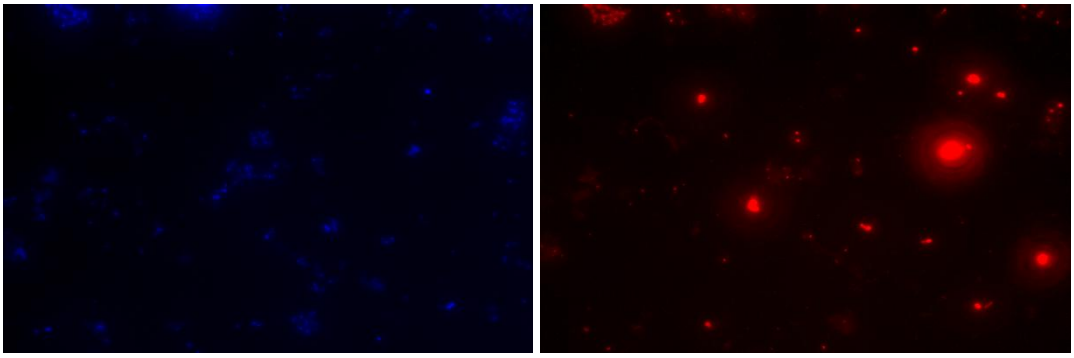
Digester	Days	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Isocaproic Acid	Caproic Acid	Heptanoic Acid
Acid 1	0	303	33	7	19	8	4	2	0	0
	5	165	538	49	177	69	82	5	13	0
	10	141	505	40	164	52	80	1	15	0
	15	1163	791	47	338	58	144	13	40	3
	20	187	798	42	281	54	102	12	21	2
	25	312	1172	62	415	73	131	2	29	1
	30	189	1269	60	361	74	99	2	15	0
	35	217	732	32	306	47	82	1	8	0
	40	154	814	36	251	44	61	4	7	0
	45	158	736	30	229	38	49	6	5	0
	50	169	1219	56	248	78	66	8	7	0
	55	286	1579	69	104	87	68	6	7	0
	60	173	1279	55	0	33	2	2	2	0
Acid 2	0	124	2	0	0	0	0	0	0	0
	5	147	370	31	81	41	37	1	7	0
	10	224	450	31	83	44	45	5	10	0
	15	269	450	27	69	39	40	6	12	1
	20	304	565	30	3	39	37	2	13	2
	25	238	576	29	2	40	36	8	9	1
	30	259	594	16	0	21	3	6	3	2
	35	138	470	23	0	31	2	2	0	0
	40	129	546	20	0	20	5	2	1	2
	45	146	515	20	0	27	2	5	0	0
	50	204	623	8	0	10	2	6	3	0
	55	226	759	14	0	4	2	2	1	0
	60	249	1666	75	0	43	2	10	3	0
Methane1	0	62	1	0	0	0	0	0	0	0
	5	101	3	0	0	22	0	1	0	0
	10	74	3	0	0	0	0	2	0	0
	15	101	2	0	0	0	0	2	0	0
	20	109	2	0	0	0	0	2	0	0
	25	148	5	0	0	0	1	6	1	0
	30	130	2	0	1	3	0	0	0	0
	35	89	2	0	0	0	0	0	0	0
	40	104	2	2	0	0	0	0	0	0
	45	79	0	0	0	1	0	3	0	0
	50	110	2	0	0	0	0	3	0	0
	55	112	2	0	0	0	0	1	0	0
	60	110	3	0	0	0	0	1	0	0
Methane2	0	62	0	0	0	0	0	0	0	0
	5	68	0	0	0	0	0	0	0	0
	10	87	1	1	1	0	0	0	0	0
	15	107	3	0	0	0	0	3	1	0
	20	124	2	0	0	0	1	3	1	0
	25	118	4	0	0	0	0	3	2	0
	30	98	2	0	0	0	1	0	1	1
	35	66	0	0	0	0	0	2	0	0
	40	70	0	0	0	0	0	0	0	0
	45	91	1	0	0	0	0	3	0	0
	50	105	1	0	0	0	0	3	0	0
	55	114	2	0	0	0	0	0	0	0
	60	114	2	0	0	0	0	1	0	0

Table A.7. VFA concentration in single-phase digesters (mg/L).

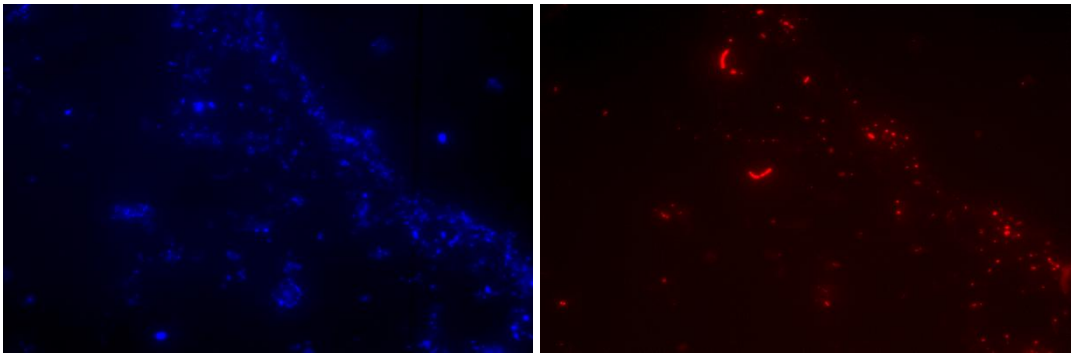
Digester	Days	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Isocaproic Acid	Caproic Acid	Heptanoic Acid
Single 1	0	89	0	0	0	1	0	1	0	0
	5	103	3	0	0	0	0	0	1	0
	10	66	0	0	0	0	0	0	0	0
	15	82	2	0	0	0	0	0	0	0
	20	111	1	0	0	0	0	0	0	0
	25	82	0	0	0	0	0	3	0	0
	30	129	1	0	0	0	0	1	0	0
	35	142	3	0	0	0	0	2	0	0
	40	131	1	0	0	0	0	2	1	0
	45	113	2	0	0	0	0	2	1	0
	50	73	2	0	0	0	0	0	0	0
	55	126	2	0	0	0	0	1	1	0
	60	99	2	0	0	0	0	1	1	0
Single 2	0	86	0	0	0	0	0	0	0	0
	5	122	3	0	0	0	0	0	1	0
	10	90	0	0	0	0	0	0	0	0
	15	103	0	0	0	1	1	0	0	0
	20	102	0	0	0	0	0	0	0	0
	25	83	1	0	0	0	0	0	0	0
	30	93	1	0	0	0	0	0	0	0
	35	103	2	0	0	0	0	1	0	0
	40	119	2	0	0	0	0	1	0	0
	45	124	2	0	0	0	0	1	0	0
	50	60	0	0	0	0	0	0	0	0
	55	110	2	0	0	0	0	1	1	0
	60	103	2	0	0	0	0	0	1	0

APPENDIX B: EPIFLUORESCENCE MICROGRAPHS

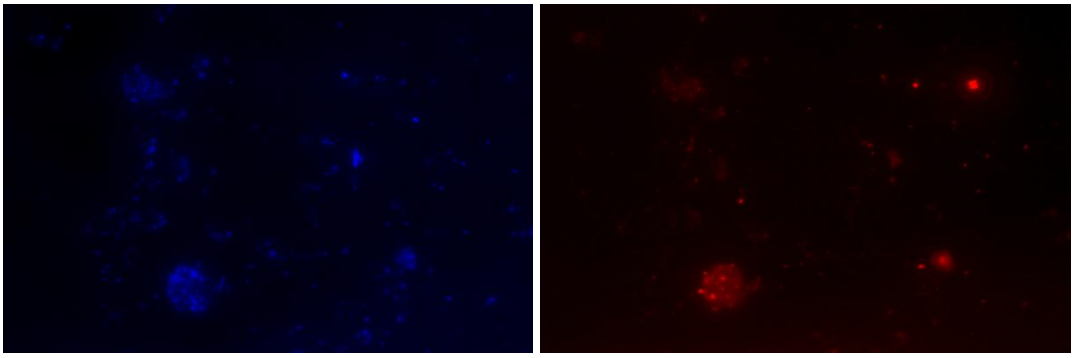
Dapi Probe



Acid 1 digester - Gam42a probe

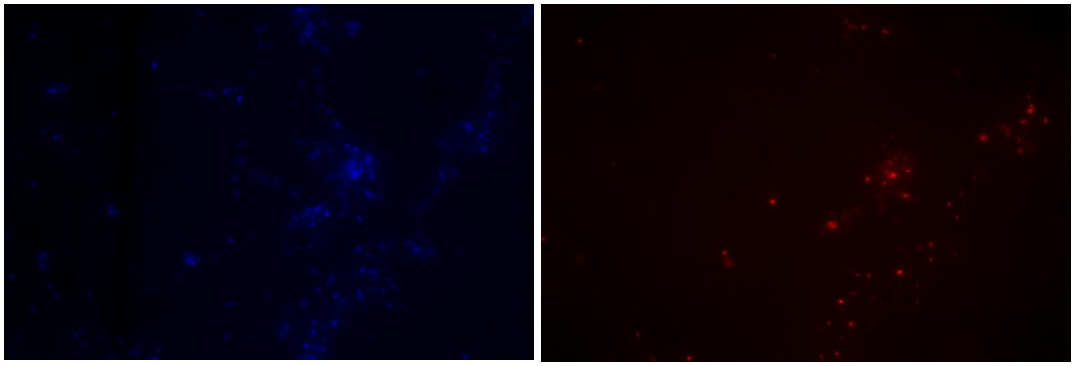


Acid 1 digester – LGC354Mix probe



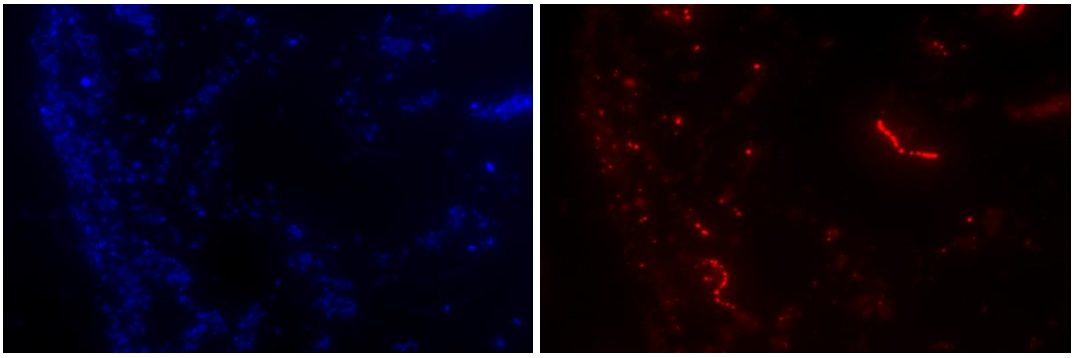
Acid 2 digester - Gam42a probe

Figure B.1. Epifluorescence micrographs of acidogenic digesters on Day 30.

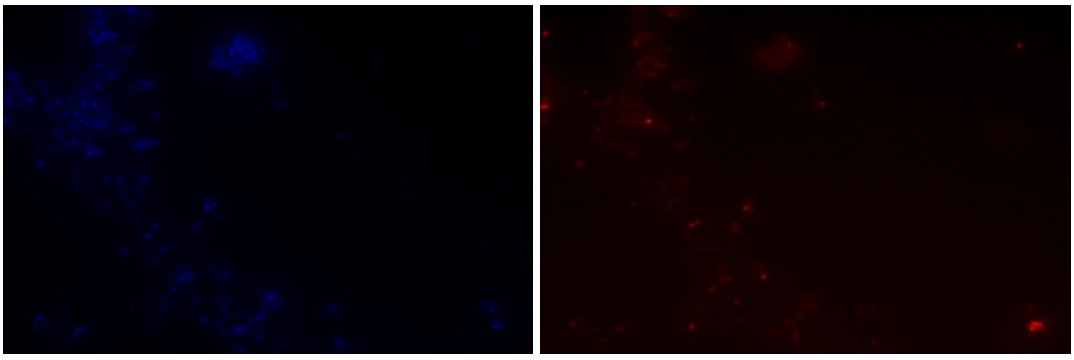


Acid 2 digester – LGC354Mix probe

Figure B.1. (continued) Epifluorescence micrographs of acidogenic digesters on Day 30.

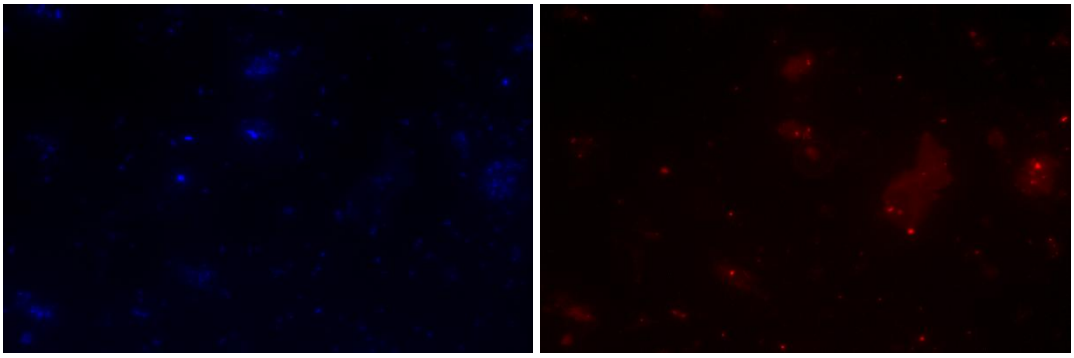


Methane 1 digester – MB310 probe

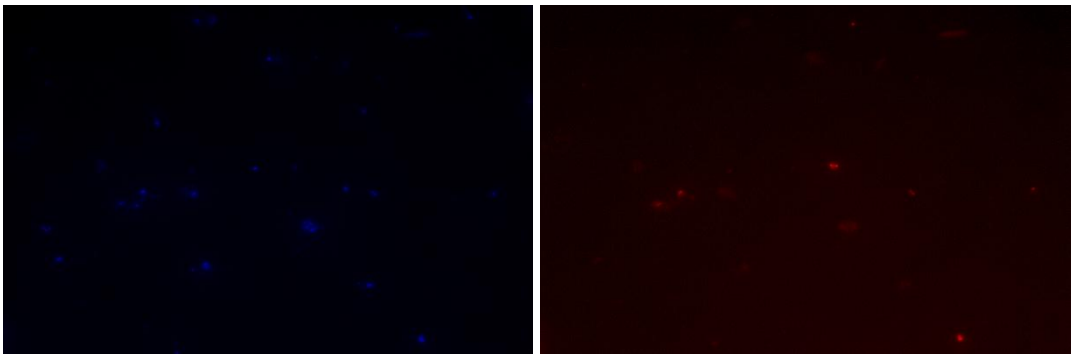


Methane 1 digester – MSMX860 probe

Figure B.2. Epifluorescence micrographs of methanogenic digesters on Day 30.

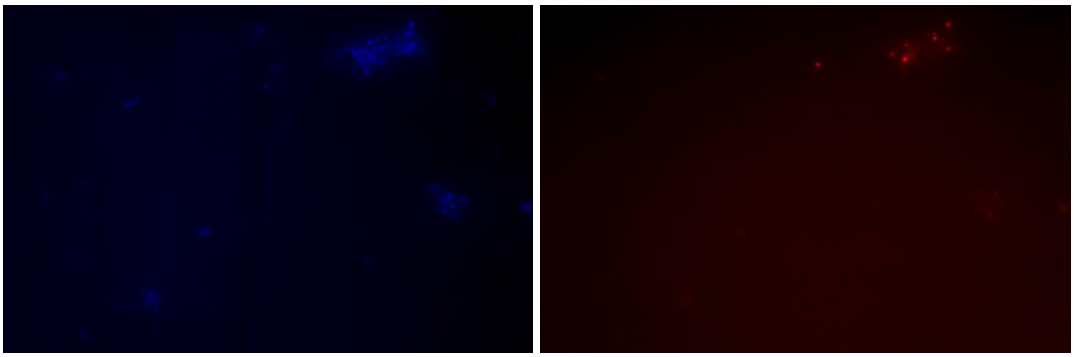


Methane 2 digester – MB310 probe



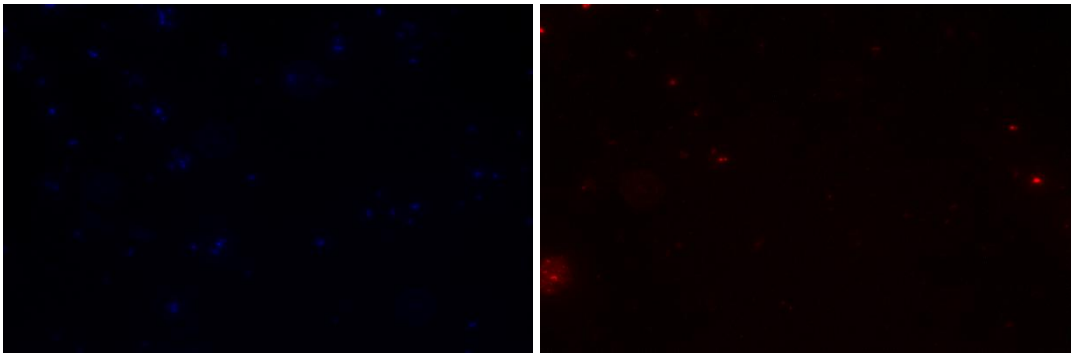
Methane 2 digester – MSMX860 probe

Figure B.2. (continued) Epifluorescence micrographs of methanogenic digesters on Day 30.

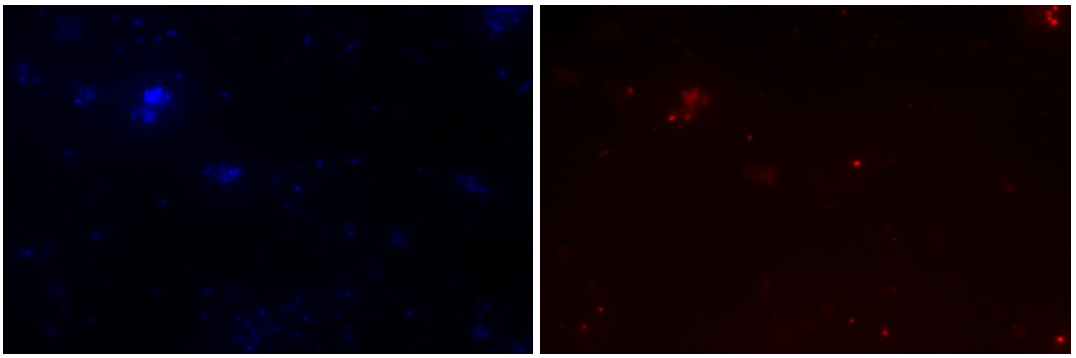


Single 1 digester – LGC354Mix probe

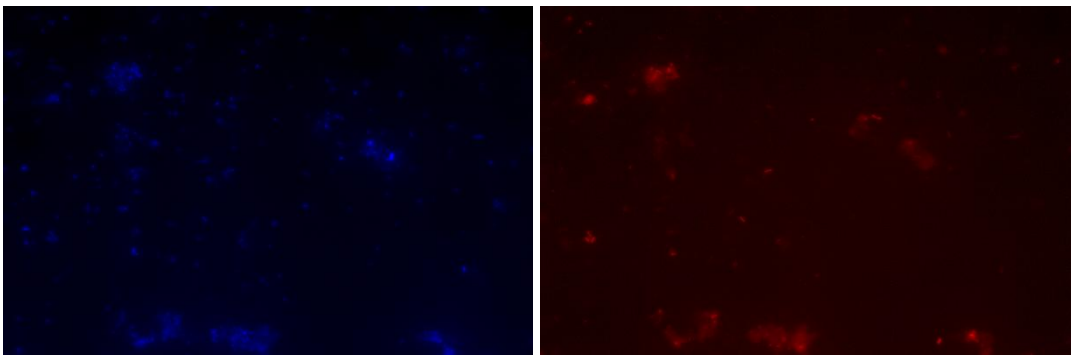
Figure B.3. Epifluorescence micrographs of single-phase digesters on Day 30.



Single 1 digester – MB310 probe



Single 2 digester – GAM42a probe



Single 2 digester – MSMX860 probe

Figure B.3. (continued) Epifluorescence micrographs of single-phase digesters on Day 30.