DETERMINATION OF EFFECTS OF SELECTED VETERINARY ANTIBIOTICS ON BIOGAS PRODUCTION IN ANAEROBIC DIGESTION SYSTEMS AND ANALYSIS OF RESISTANCE GENE PROMOTION

by
GÖKHAN TÜRKER
B.Sc. in Molecular Biology and Genetics, Boğaziçi University, 2002
M.Sc. in Environmental Sciences, Boğaziçi University, 2007

Submitted to the Institute of Environmental Sciences in partial fulfillment of the requirements for the degree of
Doctorate
of
Philosophy
in
Environmental Sciences

Boğaziçi University

2013

…Biraz da serüvendi yaşamak
Belki yatkındı büyük yolculuklara
Ki serüvenler daima büyük aşklar
Ve büyük yolculuklarla başlar…

To my family, Nevin and Dağhan Ayhan

ACKNOWLEDGEMENTS

I would like to thank my thesis supervisor Prof. Dr. Bahar K. İnce for her support, her guidance and “timely calibrations” during all years under her supervision. Without her this study cannot be done. I also want to express my gratidutes for letting me join her lab group where I conduct my researches and grew as an academic being. Her advices both in academic and personal terms help me to overcome my problems. Since we met in 2000, she has been more than a thesis supervisor and I consider myself as privileged student for being chosen to be supervised by her.

I would also like to thank Prof. Dr. Orhan İnce, who has opened the doors of his laboratories in Istanbul Technical University, where I find the chance to work with brilliant fellows. He was there when I need advice; sharing his experiences and expanding my vision.

I want to express my deepest gratidutes and thanks to my lab mates Emine Ertekin, Çağrı Akyol, Halil Çoban and Fulya Süzen. This thesis contains also your effort; without you it cannot be done.

I would also like to thank to all members of the Microbial Ecology Group (MEG) in Istanbul Technical University. Your support was very helpful.

I would also like to thank my jury members for spending their valuable time to evaluate this thesis.

I express my heartiest thanks to my roommates Evrim Kalkan, Murat Örok and Derya Aydın Sarıkurt for enduring my nuisance without complaining during my study. I also want to thank Assist. Prof. Dr. Ulaş Tezel for his availability whenever I knock on his door to get an advice. My thanks to Filiz Ayılmaz for her help during my analyses. I owe very special thanks to Gülhan Özkösemen and Ayşe Tomruk for answering my continuous question. I also want to send my special thanks to Department of Toxicology and

Pharmacology in Faculty of Veterinary at Istanbul University. Your friendship and contribution help me greatly in succeeding this study.

This study was mainly supported by TUBİTAK Project No: 109Y275, “Effects of a commonly used veterinary antibiotic on biogas production in anaerobic digestion systems and its fate in receiving environments”. The financial support partly provided also by the Boğaziçi University Research Fund projects, 10Y00M12, 10Y00D7 and 11Y00P4, was greatly acknowledged. I also kindly acknowledge Bogazici University (BU) administrative personnel especially working in Research Fund Projects dealing with funds, administration and bureaucratic matters.

The most special thanks go to my dear wife Nevin Türker for her love, support, patience and understanding. My son, my love, Dağhan Ayhan Türker, your mere presence is enough to me. Everything was hastened since you come into our lifes. Days became short; nights became shorter. In every obstacle your mommy put on my path, I build up my determination stronger. I thank you both for giving me a purpose to work on.

Finally, I would like to thank everybody who was important to the realization of thesis, as well as expressing my apology that I could not mention personally one by one.

ABSTRACT

Animal husbandry developed into a gigantic business sector to meet demands of modern society. Manure produced in animal husbandry is a suitable substrate for anaerobic digestion since it brings solution to the problem of manure accumulation. Although anaerobic digestion is an old and well known process; it is still far from understood completely. Microbial communities and its importance have been revealed recently. A better understanding of operational and microbiological parameters should be reached to modify and reshape anaerobic digestion systems according to demands.

In recent years, antibiotic build up has been detected in receiving environments due to common use in treating both men and animals. Due to their persistence, they begin to accumulate in environment. Antibiotics may create a selective pressure on microbial communities. Microorganisms can become resistant to antibiotics when antibiotics are in non-therapeutic concentrations.

This study presents results of investigation of many laboratory scale anaerobic digesters operated with cattle manure of OTC vaccinated animal. Excretion pattern of oxytetracycline after treatment was observed and excreted amount of antibiotic was calculated. Anaerobic digestion of cattle manure was monitored and performances were recorded in presence of antibiotic and changing operational parameters. Manure amendment to soil was performed to monitor fate and effect of oxytetracycline and digester microbial communities on soil microbial communities

Within the scope of the dissertation, it was found that OTC inhibits digester performance significantly. Effect of OTC was higher on bacterial population rather than archaea. OTC excreted from animal body as at least 18% of initial amount within solid manure. This amount will further increase because of repetitive injections and liquid manure addition. These manures were used as feedstock in anaerobic digesters operated under common operational parameters for farm type anaerobic digesters. 50% inhibition in biogas production was observed. Temperature was most influential parameter of digester performance followed by solid content and mixing rate. Temperature has been also most effective parameter on degradation of OTC. Mixing rate also helped degradation of OTC slightly. Also solid content was in negative relation with OTC inhibition. It has been found that half-life of OTC was ranging from 22-28 days in mesophilic and 16-18 days in thermophilic digesters, respectively.

Molecular analyses showed that bacterial community of digesters was dominated by fermentative and hydrolytic bacteria belonging groups of *Firmicutes* and acidogenic *Proteobacteria*. *Methanobacteriales, Methanomicrobiales* and *Methanosarcina* spp. were found as methanogenic community. FISH analyses indicated an increase in microbial activity after 20 days; after that activity dropped significantly.

Manure and digestate were amended to soil to monitor the elimination of antibiotic and its effects on microbial structures. It has been found that the half-life of OTC is far longer (135 days) in soil than in digesters (18-24 days) and microbial structures were changed after amendment. Tetracycline resistance gene analyses showed presence of 10^4-10^6 resistance gene copies in digester studies and 10^3-10^5 resistance gene copies in soil studies Analyses also showed that ratio of antibiotic resistance genes to bacterial 16S rRNA genes were increased 50 fold in mesophilic digesters and 100 fold in thermophilic digesters. In soil studies ratio don’t showed a significant change.

ÖZET

Hayvancılık modern hayatın isteklerini karşılamak için devasa bir işe dönüşmüştür. Hayvancılık çalışmaları sırasında üretilen dışkı havasız çürütme sistemleri için uygun bir besin olduğundan dışkı birikmesi sorununa da çözüm getirmektedir. Havasız çürütme eski ve iyi bilinen bir süreç olsa da, tamamıyla anlaşılmaktan çok uzaktır. Yakın zamanda mikrobiyal komüniteler ve önemleri ortaya çıkmıştır. Havasız çürütme sistemlerinin iyileştirilmesi ve yeniden şekillendirilmesine için hem işletim hem de mikrobiyolojik parametrelerin daha iyi anlaşılması gereklidir

Geçen yıllarda, alıcı ortamlarda insan ve hayvanların iyileştirilmesi için kullanılan antibiyotiklerin birikimi tespit edilmiştir. Antibiyotiklerin çoğu bozunmaya dirençli olduklarından, gün be gün doğada birikmektedirler. Antibiyotikler bulundukları ortamda mikrobiyal komüniteler üzerinde seçici bir baskı oluşturmaktadırlar. Antibiyotiklerin terapik dozun altındaki konsantrasyonlarda olması mikrokorganizmalara direnç kazandırmaktadır.

Bu çalışma OTC ile aşılanmış büyükbaş hayvan dışkısıyla çalıştırılmış birçok lab ölçekli çürütücü çalışmasının sonuçlarını sunmaktadır. OTC tedavisi sonrasında atılım düzeni gözlemlenmiş ve atılan miktar hesaplanmıştır. Büyükbaş gübresinin havasız çürütme süreci gözlemlenmiş ve sistem performansları antibiyotik ve değişen işletme parametreleri altında incelenmiştir. Toprak gübre ile karıştırılmış ve çürütücü mikrobiyal komünitelerin ve oksitetrasiklinin toprak mikrobiyal yapıları üzerindeki etkisi ve akıbeti gözlemlenmiştir.

Bu doktora tezi kapsamında, OTC'nin çürütücü performansını ciddi bir şekilde düşürdüğü tespit edilmiştir. OTC'nin etkisi bakteriler üzerinde arkelerdeki etksine kıyasla daha fazladır. OTC hayvan vücudundan aşılanan miktarın en az %18'i oranında katı dışkı ile atılmaktadır. Bu miktar ardı ardına enjeksiyon ve sıvı dışkı eklenmesiyle daha da artacaktır. OTC içeren dışkılar çiftlik tipi çürütücüler için kullanılan genel işletme şartlarında işletilmiş ve sistem performansı üzerindeki inhibisyonu incelendiğinde biyogaz üretimini %50 azalttığı görülmüştür. Sıcaklık en etkin parametre olarak bulunmuş katı oranı ve karışım hızı onu takip etmiştir. Sıcaklık OTC bozunması üzerinde en etkili parametre olmuştur. Karışma hızı az da olsa OTC'nin bozunmasına yardım etmektedir. Katı oranı da OTC inhibisyonu ile negatif etkileşim halindedir. OTC'nin yarı ömrü mezofilik çürütücülerde 22-28 gün arasında, termofilik çürütücülerde 16-18 gün arasında bulunmuştur.

Çürütücülerdeki bakteriyal komünite *Firmicutes* grubuna bağlı fermantasyon ve hidroliz yapan bakterilerden ve *Proteobacteria* grubuna bağlı asidojen bakterilerden oluşmaktadır. *Methanobacteriales, Methanomicrobiales* ve *Methanosarcina* türleri metanojen populasyonunu oluşturmaktadır.ur. FISH analizleri mikrobiyal aktivitenin 20 gün boyunca arttığını sonrasında ciddi bir biçimde düştüğünü göstermiştir.

Dışkı ve çürütücü çıkışı toprağa karıştırılarak antibiyotiğin topraktaki akıbeti ve mikrobiyal yapılar üzerindeki etkisi gözlemlenmiştir. OTC'nin topraktaki yarı ömrü (135 gün) çürütücülerdekinden (18-24 gün) çok daha fazla olarak bulunmuş ve dışkı eklenmesi mikrobiyal yapıları değiştirmiştir. Tetrasiklin direnç geni analizleri çürütücü çalışmalarında 10^4-10^6 direnç geni kopyası, toprak çalışmalarında ise 10^3-10^5 direnç geni kopyası tespit etmiştir. Analizler direnç genlerinin bakteriyal 16S rRNA genlerine oranının mezofilik çürütücülerde 50 kat, termofilik çürütücülerde ise 100 kat arttığını göstermiştir. Toprak çalışmalarında bu oran belirgin bir değişiklik göstermemiştir.

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Symbol	**Explanation**	**Units**
AD	Anaerobic Digestion	
ARG	Antibiotic Resistance Gene	
COD	Chemical Oxygen Demand	mg/l
cDNA	Complementary DNA	
CTC	Chlorotetracycline	
DABCO	1,4-diazabicyclo[2.2.2]octane	
DGGE	Denaturing Gradient Gel Electrophoresis	
DNTP	deoxynucleoside triphosphate	
EDTA	Ethylenediamine Tetraacetic Acid	
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	
LC-MS/MS	Liquid Chromatography- Mass Spectrophotometry/ Mass Spectrophotometry	
OHPA	Obligate Hydrogen Producing Acetogenic Bacteria	
OMG	OTC medicated Microcosms	
OTC	Oxytetracycline	
OTU	Operational Taxonomic Unit	
PBS	Phosphate Buffered Saline	
PCR	Polymerase Chain Reaction	
PFA	Paraformaldehyde	
rDNA	Ribosomal DNA	
rRNA	Ribosomal RNA	

RT	Room temperature	Celcius
RT-PCR	Reverse Transcriptase PCR	
Q-PCR	Real Time PCR (Quantitative PCR)	
SDS	Sodiumdodecylsulfate	
SRB	Sulphate Reducing Bacteria	
SRT	Sludge Retention Time	day
SS	Suspended Solids	mg/l
SSU	Small Subunit	
TAE	Tris-Acetic Acid-EDTA	
TC	Tetracycline	
TS	Total Solid	mg/l
TVS	Total Volatile Solid	mg/l
VFA	Volatile Fatty Acids	mg/l

1. INTRODUCTION

Use of fossil fuels as primary energy source caused release of greenhouse gases and climate change due to those gases. In last fifty years, greenhouse gases increased approximately 25% (IPCC, 2001). Renewable energy sources become popular in recent century due to problem generated by greenhouse gases and depletion of fossil fuel reservoirs. Energy production from biomass is a versatile renewable energy source which can be present nearly in all places and easy to storage. It can also be used as in form of electricity and heat. Due to flexibility of biomass energy, it can be used in range from family farms to centralized electricity production facilities. Various waste substances can be used as substrate of biomass energy. In urban areas, domestic and solid wastes can be used where in rural areas waste of agriculture and animal husbandry can be used. Animal waste receives attention in that part since natural biodegradation of animal manure produce greenhouse gases.

Animal manure has a great potential in respect to organic matter (COD: 90000 mg O_2/l). Anaerobic biodegradation uses this potential to produce methane. Anaerobic degradation is a biochemical process where many different species of bacteria and archaea involved. Therefore, the system itself is delicate and susceptible to inhibitor which may hinder performance of the process. One of main inhibitors encountered in animal manure digesters was antibiotics used in veterinary treatments. The importance of antibiotics cannot be neglected in respect of treatment and preventing diseases. Although use of antibiotics as growth promoter has been banned in 2006 across the European Union countries, USA still uses 10 different antibiotics for that manner (Thiele-Bruhn, 2003;Thiele-Bruhn and Beck, 2005; Arikan, 2008).

Tetracyclines, sulfonamids, beta lactams, aminoglicosides and macrolides are commonly used antibiotic families (Kumar et al., 2004). The main problem with antibiotics is their poor transformation in animal body which results in excretion of antibiotic in manure without or slightly changing (Loscher et al., 2002; Kumar et al., 2005a). Antibiotics were detected from minute amounts to 216 mg/l concentrations in animal manure (Kumar et al., 2005a). Different antibiotics detected in different concentrations like 46 mg/kg, 91 mg/kg and 8.3 mg/kg for tetracylines, sulfonamids and flouroquinolons respectively (Martinez-Carballo, 2007). Antibiotics present in manure cause inhibitions in manure digester systems. Antibiotics interact with microbial consortium in digesters and disturb balance of the system. Affected microorganisms cause disharmony in the biodegradation pathway of organic material. 10 mg/l OTC in excreted manure caused 27% inhibition in biogas production (Arikan et al., 2006) where another study show inhibitions of 32%, 40% and 49% for concentrations of 12.5 mg/l, 37.5 mg/l and 75 mg/l (Gamel-El-Din, 1986).

Antibiotics in manure not only cause problem in digesters but also in soil where they are applied as fertilizers. Antibotics are recalcitrant compounds and highly susceptible for accumulation. In recent years, there are many studies have been published in subject of detection of various antibiotics in soil medium (Baguer et al.., 2000; Kuepper, 2003; Kümmerer, 2004; Martinez, 2008; Karci and Balcioglu, 2009). Presence of antibiotics in manure also causes another problem which may also become a public health issue. Low concentrations of antibiotics present in manure and fertilized soil may also trigger resistance gene promotion. Bacteria may gain resistance over time and this gene also can be transferred in the ecosystem where it may end up as a major outbreak of resistance pathogens in a town or hospital. Understanding the fate of antibiotics after excretion is important to prevent such scenarios.

There are numerous studies about inhibition in manure digesters and effect of operational parameters on digester performance. But nearly all of these studies investigate the topic from engineering point of view (Masse et al., 2000, Arikan et al., 2006, Arikan, 2008, Lallai et al., 2002). Also there are not enough study regarding each antibiotic since their number is quite high (Lallai et al., 2002; Arikan et al., 2006; Arikan, 2008). With the increase of using molecular analysis on environmental biotechnology area, many biochemical processes can be analyzed at microbial level and help to increase efficiency of biological treatment systems. The composition of the microbial community in the bioreactors is determined by physical, chemical and biological selective pressures. This selection plays an important role in degradation process and resisting to changes in environmental conditions and disturbances. The ecology of microbial populations remains largely unexplored in engineered systems. In order to be able to operate anaerobic systems effectively, it is becoming much more important to understand the factors affecting both microbial diversity and activity of the biomass. The studies in the literature focusing on problems in manure digesters are rather scarce (Chachkhiani et al., 2004; Kaparaju and Angelidaki, 2008; Montero et al., 2008). In order to better understand manure digesters, access higher efficiencies and cope with the stress conditions, these biological processes should be identified and studied. Results of these investigations can be then used to enhance performance of digesters and design of better systems for antibiotic containing manures. Evaluation of microbial communities together with other operational parameters is crucial. Therefore, understanding microbiology of manure digesters, determine relation between antibiotic and microorganisms and identification of microorganisms involving in the process are important.

In the light of the discussion above, anaerobic manure digesters can be modified for a better performance if the microbial communities defined better in respect of activity, diversity and function in presence of antibiotics. Therefore, in the context of this study, effects of oxytetracycline, a tetracycline group antibiotic, on biogas production in anaerobic manure digestion systems are analyzed in terms of microbial communities, diversities and dynamics under different operating conditions. The elimination of antibiotics in the system is also monitored. The presence and concentration of antibiotics are determined by High Pressure Liquid Chromatography (HPLC) method in fresh manure and during anaerobic digestion. Presence of antibiotics is monitored in soil where digestate and manure applied as fertilizer. Effects of antibiotics on microbial structures, microbial diversity and resistance genes which are promoted in soil were determined. Composition and diversity of microbial communities was analyzed by culture independent molecular techniques like Denaturant Gradient Gel Electrophoresis (DGGE), Fluorescence in situ hybridization (FISH), Real Time Polymerase Chain Reaction (Q-PCR) and 16S rRNA-rDNA clone libraries. The abundance of resistance genes was then determined by using specific primers coding resistance genes with Q-PCR. The findings help us to understand the effect of antibiotics on biogas production in anaerobic digestion systems in respect of microbial structures and dynamics. With this understanding, the process may be operated with a greater efficiency. The monitoring of antibiotics after anaerobic digestion is important in order to understand the elimination process of antibiotics in natural environments, change in microbial structures caused by antibiotics during that process and antibiotic resistance gene promotion.

2. THEORETICAL BACKGROUND

2.1. Anaerobic Digestion Systems

2.1.1. Fundamentals of Anaerobic Digestion

Anaerobic digestion is the biochemical process conducted by a microbial consortium which degrades the organic matter into a basic structure, methane and carbon dioxide (biogas) in the absence of oxygen. A basic pathway can be described as follows; hydrolysis of organic matter into simple forms, volatile fatty acid production from them by fermentation, production of acetate from VFAs, formation of methane via acetate or H_2-CO_2 pathways (Zinder et al., 1984). These four stages can be expanded also into subgroups:

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

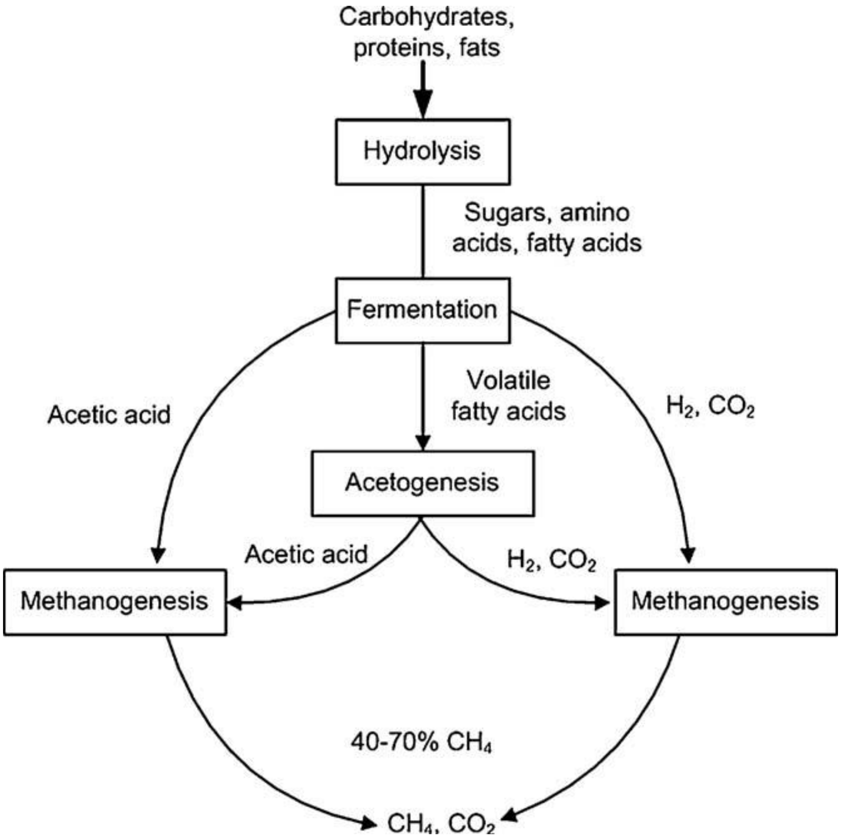


Figure 2.1. Process flow of the degradation of organic material through anaerobic digestion (Li et al., 2010).

<u>2.1.1.1. Hydrolysis.</u> First step in anaerobic degradation pathway is the hydrolysis. Complex organic matters are break down into simpler units such as lipids into fatty acids, proteins into aminoacids and carbohydrates into simple sugars by specific extra cellular enzymes (Hassan, 2003; Khanal, 2010). Reaction of hydrolysis is affected by pH, cell residence time and organic matter content. Mainly members of *Firmicutes* group especially *Clostridium spp.* and *Bacillus* spp. are highly active in this step (Noike et al., 1985; Lema et al., 1991). Specific extracellular enzymes such as amylases, proteinases, lipases and nucleases are responsible in breaking down of organic matters.

Since hydrolysis is the initial step in anaerobic degradation, it can be a rate-limiting step (Pavlostathis and Giraldo-Gomez, 1991). It becomes especially a critical step in digesters operated with particulate matter like manure (Li et al., 2010; Coats et al., 2011). Studies are in agreement with each other; hydrolysis is main rate-limiting step in high particulate substrates like manure where for highly soluble substrates like wastewaters methanogenesis is the rate-limiting step (Boe, 2006).

2.1.1.2. Acidogenesis. In the acidogenesis phase, monomers are degraded further by facultative and obligatory anaerobic bacteria into short chain organic acids as acetic, butyric, propionic, valeric acid and alcohols, hydrogen and carbon dioxide (Hassan, 2003; Gerardi, 2003; Li et al., 2010). Type of the products heavily depends on the type of bacteria and environmental parameters like temperature and pH. Group *Firmicutes and Proteobacteria* are main contributors to this step.

2.1.1.3. Acetogenesis. Among the fatty acids, acetate is the most important one since methanogenic archaea mainly use acetate to produce methane (Gerardi, 2003), and about two thirds of biologically generated CH_4 is coming from acetate (Schmidt et al., 2000). Production of acetate was performed by obligate hydrogen producing acetogenic bacteria (OHPA) who convert acids to acetic acid, CO_2 and H_2. But for some acids conversion is an endothermic reaction. Therefore, this step can be a rate-limiting step. Acetic acid is produced from propionic acid by *Methanobacterium bryantii*, *Desulfibrio,* and *Synthrophobacter wolinii* (Stronach et al., 1987; Malina et al., 1992). The common organisms which convert butyric, caproic and valeric acids to acetic acid are *Synthrophomonas wolfei* and *Syntrophus buswellii* (Pandey, 2011).

2.1.1.4. Methanogenesis. Methanogenesis is the final and may be the most important step of the anaerobic digestion process which is carried out by a group of strictly anaerobic microorganism called *Archaea* (Hassan, 2003). Two conversion mechanisms are performed by methanogens; decarboxylation of acetic acid and reduction of carbon dioxide in the absence of other electron acceptors such as oxygen, nitrate, and sulfate except bicarbonate and protons as terminal electron acceptors (Garcia et al., 2000; De Bok et al., 2004; Stams et al., 2006). In anaerobic pathway, methanogenesis can be most rate limiting step due to slow growth rate of the methanogens (Malina et al., 1992). Methanogens are highly susceptible to inhibition from accumulation of H_2 and short chain fatty acids.

Methanogenesis pathway is not only from acetate but also from CO_2 and H_2/formic acid and methylated products (Table 2.1. and Table 2.2.). All methanogenic reactions are thermodynamically exergonic at standard conditions; therefore, they can be performed if substrate level is adequate.

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

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

Table 2.2. Characteristics of methanogenic Archaea (Madigan et al., 2002).

Genus	**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, pyruvate+CO_2, formate
Methanomicrobiales		
Methanomicrobium	Short rods	H_2+CO_2, formate
Methanogenium	Irregular cocci	H_2+CO_2, formate
Methanospirillum	Spirilla	H_2+CO_2, formate
Methanoplanus	Plate-shaped cells	H_2+CO_2, formate
Methanocorpusculum	Irregular cocci	H_2+CO_2, formate, alcohols
Methanoculleus		H_2+CO_2, alcohols, formate
Methanosarcinales		
Methanosarcina	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; halophile
Methanothrix	Long rods to filaments	Acetate
Methanopyrales		CO_2, hyperthermophile, growth at 110 °C
Methanopyrus	Rods in chains	

2.1.2. Microbiology of Anaerobic Degradation

Anaerobic degradation of organic matter and liberation of biogas is a complex process which is performed by many species of Bacteria and Archaea. Main groups that perform this degradation are hydrolytic and fermentative bacteria, hydrogen-producing acetogenic bacteria, homoacetogens, hydrogenotrophic methanogens and acetoclastic methanogens (Khanal, 2008).

2.1.2.1. Hydrolytic and Fermentative Bacteria. At the first st ep of anaerobic biodegradation, organic compounds like proteins, fats and cellulose are degraded down into their monomers by action of hydrolytic and fermentative bacteria (Ivanov, 2011). A number of Firmicutes groups' bacteria are responsible in degradation of complex organic material. It was proposed that *Clostridium spp.* are responsible in degradation of cellulose, *Peptococcus spp.*, *Bacteroides spp*., *Peptostreptococcus spp.* in degradation of proteins, genera of *clostridia* and *micrococci* in degradation of lipids and *Clostridium butyricum, Bacillus subtilis* in degradation of amino acids. Also hydrolytic bacteria (*Lactobacillus spp*., *Bifidobacterium spp*., *Butyrivibrio spp)* break down some intermediate compounds to Volatile Fatty Acid (VFA), CO_2, H_2 and ethanol (Khanal, 2008; Ivanov, 2011).

2.1.2.2. Hydrogen-Producing Acetogenic Bacteria. This group mainly consists of *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., *Syntrobacter* spp. and is responsible of forming acetate, CO_2 and H_2 from propionic acid, butyric acid and some alcohols. Mostly this group is in symbiotic relationship with hydrogen consuming methanogens. As hydrogen is consumed by actions of methanogens, partial pressure drops and favors formation of hydrogen (Khanal, 2008; Deublein and Steinhauser, 2008).

2.1.2.3. Homoacetogens (hydrogen consuming acetogens). This group receives an increasing attention since they produce acetate from an alternative route which is CO_2 and H_2. *Clostridium spp*. and *Acetobacterium spp*. are responsible of production of acetate from this reaction. Also some homoacetogens use another route which utilizes CO and organic compounds like formate and methanol as substrates (Khanal, 2008).

2.1.2.4. Hydrogenotrophic and Acetoclastic Methanogens. Most common pathway of methanogenesis use production of methane from acetate, followed by from CO_2 and H_2. Use of methylated substrates is rather uncommon (Ivanov, 2011). Studies indicate 70% of methane produced was due to action of acetoclastic methanogens where remaining 30% is from hydrogenotrophic methanogens (Deublein and Steinhauser, 2008). Most common acetoclastic methanogens include *Methanosarcina* spp., *Methanosaeta* spp., and *Methanolobus* spp. where common hydrogentorphic methanogens include

Methanomicrobium spp., *Methanobacterium* spp., *Methanobrevibacter* spp., *Methanococcus* spp., *Methanogenium* spp., and *Methanospirillum* spp.

In most anaerobic reactors, methanogenesis is the main rate-limiting step. As methanogens are at the end of food chain, their doubling time is quite high. Minimum 3-4 days are required to double the methanogenic population where acidogenic and hydrolytic bacteria can double their population in mere of hours. Methanogens are strictly anaerobic and can be inhibited by free oxygen as low as 0.01 mg/l. Also their pH range of living is around 7.

There are three types of methanogenesis as described in Table 2.2. Their reaction efficiencies can be seen in equations 2.1.-2.4. (Deublein and Steinhauser, 2008; Madigan, 2009).

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

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

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

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G° = -31 kJ/mol \tag{2.4}$$

Formation of methane from CO_2 and H_2 is a highly hydrogen dependent reaction and needs a constant feed of hydrogen which is sustained by action of hydrogen-generating acidogens. Formation of methane from methylated compounds can be done by an external electron donor such as H_2 and also without H_2. Formation of methane from acetate is most common version of methane synthesis which is simple and straightforward.

2.2. Environmental and Operational Factors Affecting Anaerobic Treatment Processes

Although it is assumed that the rate-limiting step in anaerobic degradation is methanogenesis, all steps can be actually rate-limiting. But the low growth rates of methanogens make them sensitive to environmental changes more than other microorganisms performing other steps (Xing et al., 1997). Also their diversity is much narrower than bacteria; so a disturbance in methanogenic community may affect other trophic levels (Raskin et al., 1996). The most important factors which may affect the performance of anaerobic systems are listed below.

2.2.1. Temperature

Temperature is one of the most important environmental parameter by affecting directly efficiency of reactions, specific growth rate of microorganisms and half saturation constants. Anaerobic degradation can occur in a wide range of temperature ranging 10^0C to 80^0C. Mesophilic temperatures are most common temperatures encountered in process. Temperature is important for methanogens since their low growth rate is highly susceptible to change in environmental parameters. Temperature changes coupled with other environmental parameters like pH and organic loading rate increases its effect significantly. Table 2.3. shows a brief summary of advantages and disadvantages of temperature.

Table 2.3. Advantages and disadvantages of the mesophilic and thermophilic anaerobic digestion process.

Mesophilic Conditions	**Thermophilic Conditions**
Advantages	**Advantages**
*Mesophilic microflora are able to tolerate temperature fluctuations within ±3°C *Need less energy input for heating *Less influenced by inhibitory factors of ammonia released during the mineralization of proteins	*Higher metabolic rates *Higher specific growth rates *Process is faster and more efficient *High total biogas production *Low content of volatile solids in the stabilized digestion residues *Ability to fed with higher organic loading rates at lower hydraulic retention times *More efficient killing (%90) of pathogens present in the waste
Disadvantages	**Disadvantages**
*Efficient killing of pathogens cannot be said for mesophilic digestion when used alone. This is a significant criterion for the animal waste treatment since the effluent can be used as a soil fertilizer	*Frequently higher death rates *More vulnerable to temperature fluctuations *Lower microbial diversity *More sensitive to toxicants or inhibitors and temperature fluctuation *Leads to the system imbalanced and susceptible to failure *Additional energy requirements (reduction in the net energy production) *The pH increases through a reduced solubility of carbon dioxide, this presents to a higher proportion of free ammonia

2.2.2. Effect of pH

Most methanogens prevail in a pH range between 6.7 and 7.4 (Table 2.4), but optimally at pH between 7.0-7.2. pH below 6.0 is moderately inhibitory to methanogens where it is preferable for acidogens. As the pH gets below 6.7 methanogenic activity decreases as acidogenic activity increases. This event ends up accumulation of fatty acids produced by acidogens where low activity of methanogens is not sufficient to consume produced acids (Pohland, 1987; Malina et al., 1992). Normally changes in acid concentration buffered by bicarbonate produced by methanogens. As the acid concentration increases, bicarbonate buffer destroyed which ends up in termination of methanogenesis. Therefore, in anaerobic systems increases in fatty acid concentration is an early warning for failure in methanogenesis.

Table 2.4. Optimum pH for some methanogenic *Archaea* (Gerardi, 2003)

Genus	**Optimum pH Range**
Methanothermus	6.5
Methanogenium	7.0
Methanolacinia	6.6-7.2
Methanomicrobium	6.1-6.9
Methanospirillium	7.0-7.5
Methanococcoides	7.0-7.5
Methanohalobium	6.5-7.5
Methanolobus	6.5-6.8
Methanosaeta	7.1-7.2

2.2.3. Nutrients and the C/N Ratio

C/N ratio is the relationship of amount of carbon and nitrogen in an organic compound (Monnet, 2003). Studies show that carbon is utilized approximately 25-30 times faster than nitrogen so at least 20-30:1 C/N ratio is needed to meet the requirement (Hassan, 2003; Yadvika et al., 2004). There are risks present when the necessary ratio cannot be established. A high ratio leads to over consumption of nitrogen by methanogens which result in low methane production where a low ratio leads to ammonia accumulation which is toxic to methanogens at high pH values.

Methanogens not only need balanced nitrogen and carbon but also need trace amounts of elements called as micronutrients (Speece et al., 1983). The most significant micronutrients are iron, nickel, magnesium, calcium, sodium, barium, tungstate, molybdate, selenium and cobalt (Henze et al., 1983). Some of the elements such as selenium, tungsten and nickel are important in the enzyme systems of acetogenic and methanogenic bacteria (Stronach et al., 1987).

2.2.4. Mixing

Mixing is an important operational parameter since it does not only increase degradation but also prevents inhibitory conditions. An adequate mixing is necessary to maintain contact of substrates and microorganisms (Gerardi, 2003). By mixing it can also prevented local accumulation of inhibitors like ammonia or fatty acids (Hassan, 2003; Karim et al. 2005; Hoffmann et al., 2008; Kaparaju et al., 2008; Pandey 2011). Also mixing is needed to distribute pH and temperature evenly (El-Mashad and Zhang, 2010).

Type of mixing varies in anaerobic digestion systems mostly depended also reactor configuration. It can be either done by mechanical mixers, liquid or gas recirculation (Hassan, 2003). Mixing strategy is a parameter of mixing; strength of mixing, duration etc. But there is no accepted strategy (Kaparaju et al., 2008).

2.2.5. Retention Time

The hydraulic retention time (HRT) and sludge retention time (SRT) is an important parameter. The substrates must be maintained in reactor short enough for a sufficient elimination of organic content where microorganisms need to reside long enough to meet doubling time and population increase.

2.2.6. Organic Loading Rate (OLR) and Solid Content

Organic loading rate can be defined as daily amount of substance fed into the volume of digester. Low OLR leads to lower metabolic activity due to starvation also low biogas yield. High OLR leads up accumulation of fatty acids which also decreases produced biogas (Khanal, 2010).

Solid content can be defined as amount of organic material of feed in a unit volume of slurry. Solid content and OLR is a critical operational parameter since they are directly affecting system performance, stability and amount of biogas. For digesters whose substrate is particulate matter like manure 8-10% solid content is preferable (Yadvika et al., 2004). It is proposed an OLR of 2.5-3.5 kg VS/m^3.day is optimal for manure digesters.

2.2.7. Toxicants

Inhibitory materials are rather common in anaerobic digestion. Some of these inhibitors come from with substrate some of them produced during process. Methanogens are sensitive to inhibitors due to slow metabolic rates. But opposite to bacteria, methanogens show acclimatization to some inhibitors with time which enables biogas production in moderate levels of inhibitors (Speece and Parkin, 1983).

2.2.7.1. Sulfide Inhibition. Sulfide is produced mostly from sulfate by action of sulfate reducing bacteria (SRB). SRBs also outcompete with methanogens for hydrogen and acetate (Anderson et al., 1994). A sulfide concentration 50-100 mg/l can be tolerated in non acclimated in anaerobic treatment, sulfide concentration 200 mg/l or higher needs an acclimated community (Stronach et al., 1987).

2.2.7.2. Ammonia-Nitrogen Inhibition. Ammonia is an important buffer in anaerobic reactors and can be founding form of ammonium ion (NH_4^+) or dissolved ammonium gas (NH_3). pH is main player in this equilibrium where at high pH levels equilibrium shifts toward ammonium gas. Although ammonia level up to 1000 mg/l has no adverse effect on methanogens whereas 1500 - 3000 mg/l may have inhibitory effects at higher pH values.

2.2.7.3. Volatile Fatty Acids (VFA) Inhibition. VFA inhibition is one of the most common inhibitions encountered in anaerobic treatments. Main players of the inhibition are acetic, propionic and butyric acids. VFA accumulation mainly occurs after shock loadings, nutrient depletions and introduction of some inhibitors. VFA accumulation is a self feeding process since increasing concentration of acids drops pH which leads to a decrease in the activity of methanogens which ends up decreased VFA elimination. Propionic acid as high as 3000 mg/l and butyric acid as high as 1000 mg/l has toxic effects on the system (Madigan et al., 2009).

2.2.7.4. Heavy Metal Inhibition. Although presence of some heavy metals are essential at trace amounts in anaerobic processes, they are highly toxic if accumulate. Heavy metals may affect certain enzymes thereby killing organism. They also have toxic effect on process depending on redox potential, pH and ionic strength.

2.2.7.5. Organic Chemicals. Organic chemicals present in low concentrations can be metabolized while high concentrations have inhibitory effect. Those compounds are mostly poorly soluble in water and tend to accumulate. Toxicity of an organic compound is mostly affected by toxicant concentration, biomass concentration, exposure time, acclimation and temperature (Yang and Speece, 1986).

2.3. Anaerobic Digesters as a Renewable Energy Source

As the civilization progress, energy demand increased significantly. Modern civilization of earth reached six billion people who consume nearly 500 Quadrillion Btu (QBtu) of energy, of which over 90% come from non-renewable sources such as petroleum, coal, natural gas and nuclear (Khanal, 2010). This energy sources will soon be consumed. Therefore, renewable energy sources are needed. Another problem of current energy sources is the emission of greenhouse gases. All fossil fuels emit greenhouse gases upon consumption and accelerating global climate change. In last fifty years CO_2 level in the air increased from 313 ppm to 375 ppm where methane level increased from 1200 ppb to 1600 ppb. (http://pratclif.com/climatechange/Greenhouse%20Gases.htm; http://www.grida.no/publications/other/ipcc_tar/?src=/climate/ipcc_tar/wg1/107.htm#331).

It has been accepted that biomass is carbon neutral. Biomass is more versatile than most renewable energy sources. It is widely available and can be used in almost all places. Biogas produced by anaerobic degradation can be stored or can be used for heat and electricity. Therefore, anaerobic digestion systems can be used in from family farms to industrial scale electricity generators.

Biogas is the main output of anaerobic digestion of biomass. Biomass used in digesters is mostly under waste classification like animal manure, agricultural waste and domestic sludge. Biogas production from animal manure and agricultural wastes is a fast growing market in the world. Energy produced from biogas in Europe reached to 6 million tons of oil equivalent in 2007. Germany is the leading country for biogas where more than 4000 biogas plants can be found (Weiland, 2010).

2.3.1. Substrates for Anaerobic Digesters

One of main advantage of biogas is the origin of gas. Any type of organic material can be converted into methane with time (Alvarez et al., 2010). As energetic balance fats give a higher biogas yield than proteins and carbohydrates but opposite to those substances need a longer processing (Steffen et al., 1998; Weiland, 2010). Biogas yields of these substances can be seen in Table 2.5.

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

Organic matter	**Biogas (Nm^3/t TS)**	**CH_4 (%)**	**CO_2 (%)**	**Heat efficiency (KJ/Nm^2)**
Carbohydrates	800	50	50	17782
Raw Proteins	700	70	30	24894
Raw Fats	1200	67	30	23639

Feeds used in anaerobic digestion vary in biogas yield mostly depending on their content of organic matter and substrate composition. Common feeds and their biogas equivalency can be seen in Figure 2.2.

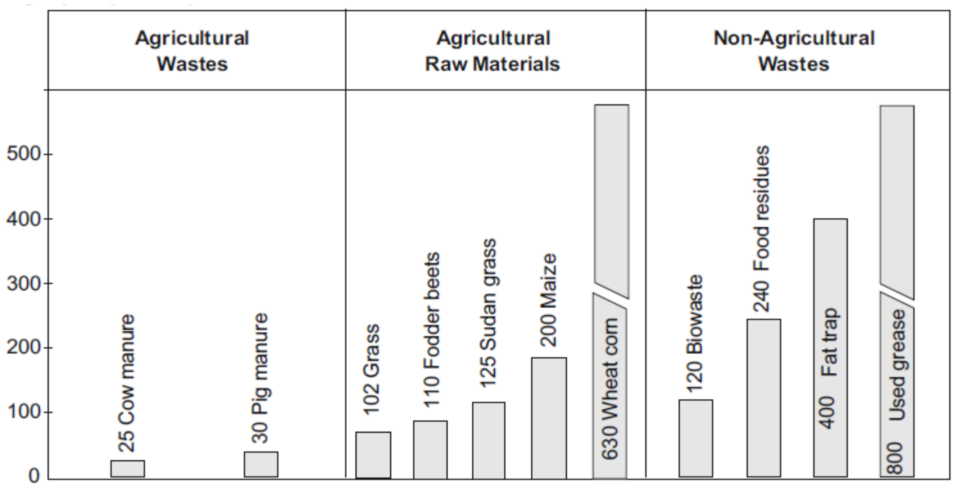


Figure 2.2. Mean Biogas yields of different substrates (m^3/t Fresh Material) (Weiland, 2010).

Animal manure is the most common feed for anaerobic digesters throughout the history. Cattle manure is a perfect substrate for a digester since it contains also methane producing Archaea. Nowadays, animal manure mostly co-digested with other suitable feeds like agricultural wastes, harvest residues, energy crops and food waste from households to increase biogas production (Weiland, 2010). High percentage of nitrogen and high lignin content in manure is preventing quick fermentation which can be overcome by carbon and microorganism addition by co-digestion (Steffen et al., 1998; Li et al., 2010; Monteiro et al., 2011).

Animal manure has a high organic matter content of 50-85%. It also has a very high COD equivalent (>50 000 mg O_2/l). This potential will be lost to entropy if not controlled. Also natural degradation of manure will release greenhouse gases. Therefore, anaerobic digestion of animal manure has advantages in many angles. Another advantage of using animal manure for digester feed is presence of naturally occurring microorganisms in manure. It has been known that cattle manure contains hydrolytic, acidogenic and methanogenic microorganisms along with fungi and protozoa. Presence of pathanogenic bacteria in manure is very common and harmful for human health. Anaerobic digestion of manure reduces the number of these pathogens significantly. The digestate is far more safe and healthier for land application than raw manure itself (Lague et al., 2005).

2.3.2. Biogas Production in Anaerobic Digesters

Methane is the main gas produced during degradation of organic materials in the absence of oxygen. It is highly flammable. Carbon dioxide is another gas produced in anaerobic degradation. The composition of biogas can be varying according to waste type and operational parameters (Ilkilic and Deviren, 2011). Digestion time, temperature, pH and diversity of microorganisms can be considered as main parameters for biogas production. There are also other parameters like solid content, organic content, mixing rate and nutrient balance affecting the process (Demirbaş, 2006). In general, biogas contains 40-70% of methane (CH_4), 30-60% of carbon dioxide (CO_2), moisture and small amounts of hydrogen sulfide (H_2S) (Ilkilic and Deviren, 2011).

Mostly, biogas produced from digesters need to be treated to produce electricity in Combined Heat and Power (CHP) generators (Monnet, 2003; Ahring, 2003). Biogas need to be stripped from its CO_2, H_2O and H_2S in order to reach to a higher calorific value (17000 to 25000 kJ/Nm$^{3)}$ and prevent system malfunction due to corrosion (Ilkilic and Deviren, 2011). In these systems more than 40% of the energy can be converted into electricity (Weiland, 2010). This value tends to increase with developing technologies for energy production instruments.

Use of biogas will help us to fight with climate change since methane is 25 times effective than CO_2 in respect to warming the atmosphere. Due to low half life of methane (12 years) it has been calculated 5-10% of global greenhouse gas (GHG) effect was contributed by methane (Attwood et al., 2011).

2.3.3. Technology of Biogas Plants

Anaerobic digestion can be performed at high Total Solid (TS) values (dry fermentation) or at low TS values (wet fermentation) (Ward et al., 2008). Dry fermentation can be run at TS concentration higher than 15% as high as 35%. The process can be operated both batch and continuously. It is rather an uncommon process since wet fermentation can be achieved easily by co-digestion (Weiland, 2010). Wet fermentation is more common method and can be done in continuously stirred tanks. Manure slurry can be obtained by diluting raw manure with water if water is plenty or by co-digesting low TS residues.

Vertical continuously stirred tank fermenter is the most common digester type used in biogas production from agricultural feed. Gastight mobile double membrane dome is the most common application in fermenter technology where gas is stored and pressured simultaneously. Mixing is essential in fermenters and can be achieved by means of mechanical, hydraulic or pneumatic equipments. Mixing not only increases substrate-microorganisms contact but also help forming a uniform temperature in digesters. Although vertical design is the most common design in anaerobic digestion; horizontal plug-flow system can be seen also especially in dry fermentation systems.

Various process designs also can be seen in anaerobic digestion. Most common process designs are thermophilic and two-phase systems. Thermophilic systems need a higher energy input and are sensitive to changes but they produce more biogas with higher methane content. Thermophilic systems also further improve pathogen elimination up to 99%. Two phase systems are anaerobic digesters where hydrolysis and acidogenesis steps are separated to another digester from methanogenesis step. Phase separation improves stability of the system by separating two delicate processes from each other (Ward et al., 2008). Both digesters can be optimized differently to maximize end products (Hassan, 2003). Two phase systems have a higher biogas yield than single phase systems. Furthermore, these process designs can be mixed together ending up thermophilic two stage digesters or thermophilic acid phase digesters to meet needs of operator. Studies show that the two phase thermophilic digesters have 6-8% higher methane yield and 9% more VS removal than conventional single phase digester (Nielsen et al., 2004; Ward et al., 2008).

2.3.4. Digestate Production and Application

The residues coming from the anaerobic digestion process are called digestate. Digestate is more balanced in C/N ratio than raw manure and can be used as organic fertilizer on farmlands (Holm-Nielsen et al., 2009; Rico et al., 2011). After digestion effective ratio of slurry increases significantly which increases short-term N fertilization (Monnet, 2003; Weiland, 2010; Masse et al., 2011). Use of digestate as fertilizer decreases use of mineral fertilizer (de Vries et al., 2010). Digestate is also less odorous and hygenic than raw manure. Soil penetration capacity of digestate is high, so loss of nitrogen to ammonia is low. These attributes make digestate highly suitable for usage as organic fertilizer (Weiland, 2010).

2.4. Antibiotics in the Environment

Veterinary antibiotics (VAs) are commonly used to treat diseases and protect the health of animals. Also in some countries they are added to feed of the animal to improve growth rate and feed efficiency (Thiele-Bruhn and Beck, 2005; Arikan et al., 2006; Alvarez et al., 2010). According the data obtained in 2000, more than 10 million kg of antimicrobial drugs were in non-therapeutic purposes (Sarmah et al., 2006). Although using antimicrobial drugs as feed additives improves health and growth of animals, this kind of use is banned in many countries including European Union (Kümmerer, 2008).

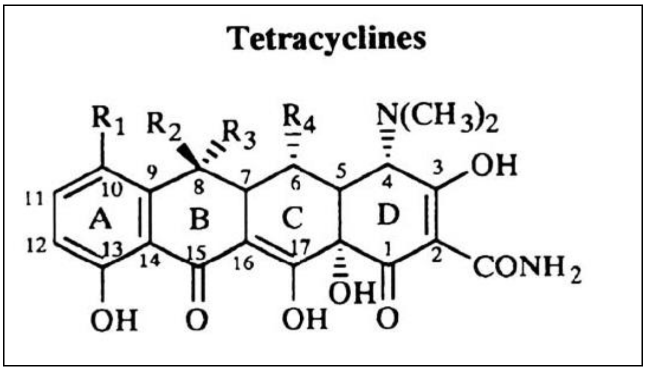
There are many types of antibiotics, working in their own way on microorganisms. Main families of antibiotics are sulfonamides, macrolides, fluroquinolones; tetracyclines. These drugs are known for their partially conjugated four-ring structure with a carboxyamide functional group.

Antibiotics are complex in structure and highly xenobiotic. They are poorly metabolized in animal body and excreted in great amount without or slightly changing in feces and urine (Kemper, 2008) (Table 2.6.). Antibiotics and their metabolites are detected in feces and urine in percentages of 17% and 76% of administered concentration (Alvarez et al., 2010).

Table 2.6. Metabolism rate of the main antibiotic groups in animal body (Kümmerer, 2008).

Antibiotics group	Metabolism
Tetracyclines	Minimal (<20%)
Sulfonamides	High (>80%)
Macrolides	Minimal (<20%)
Fluoroquinolones	Moderate high (20-80 %)
Beta-lactams	High (>80%)

Tetracycline family antibiotics are protein synthesis inhibitors and distrupting cell growth by binding ribosome (Bowman, 2009) (Figure 2.3.). Their main target is gram-negative bacteria but some gram-positive bacteria are also affected (Madigan, 2009). Because of their hydrophilic structure due to presence of hydroxyl groups; they can cross outer membrane of gram negative bacteria with ease. Tetracycline family antibiotics show strong tendency of adsorption to solid matter like soil, clay and sediments (Halling-Sorensen, et al., 2002).



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

Figure 2.3. Molecular Structure of Tetracyclines (Sarmah et al., 2006).

Oxytetracycline (OTC) is a tetracycline family antibiotic isolated from *Streptomyces rimosus* in 1940s (Madigan, 2009). OTC is favored among veterinary antibiotics because of its broad range of activity, low side effects and low cost. It can be administered to a wide range of animals including cattle, swine, poultry and fish (Alvarez et al., 2010). In animal body, OTC is degraded into its metabolites 4-epi-Oxytetracycline (EOTC), α-apo-oxytetracycline (α-Apo-OTC), and β-apo-oxytetracycline (β-Apo-OTC) (Arikan et al.,

2006) (Figure 2.4.). OTC and its metabolites are strongly adsorbed in manure due to their tendency to form complexes with metal ions, humic acids, proteins and organic matter (Alvarez et al., 2010).

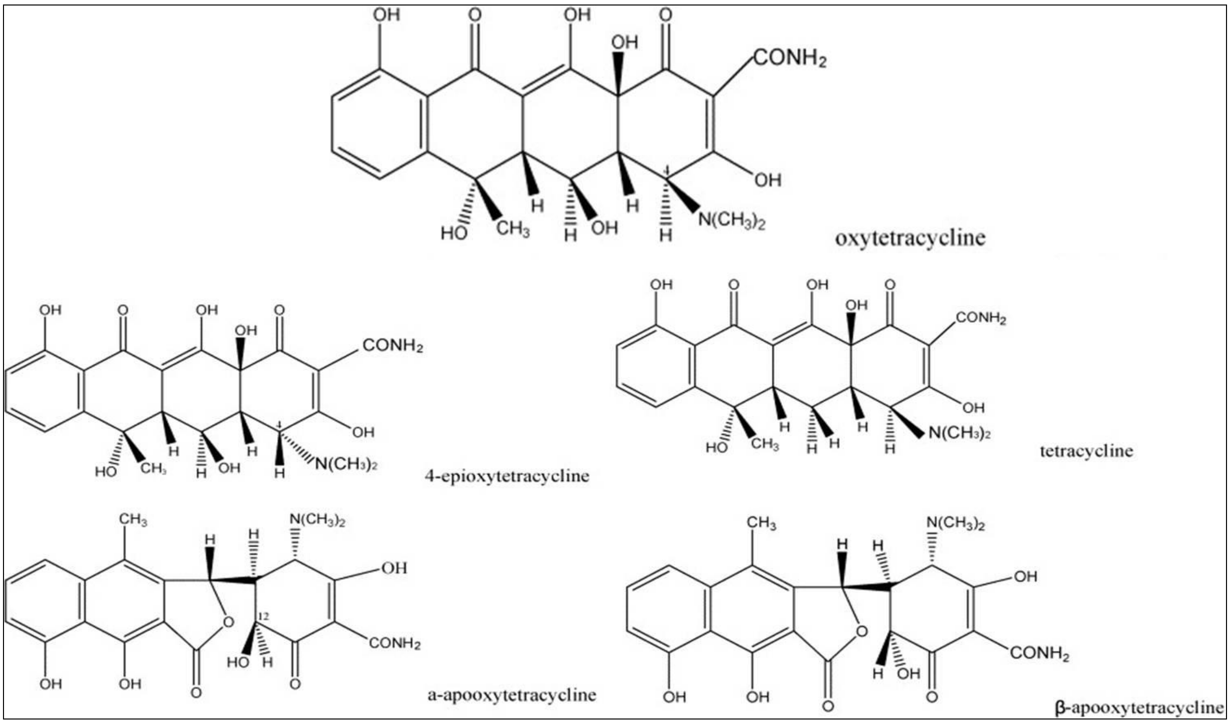


Figure 2.4. Molecular structures of OTC and its metabolites

2.4.1. Occurrence of Antibiotics in Environment

Main pathway of antibiotic release is excretion. Both animals and humans excrete their antibiotic intake and drugs were present rather in manure or Sewage Treatment Plant (STP). From that point antibiotics find their way to water bodies as described briefly in Figure 2.5.

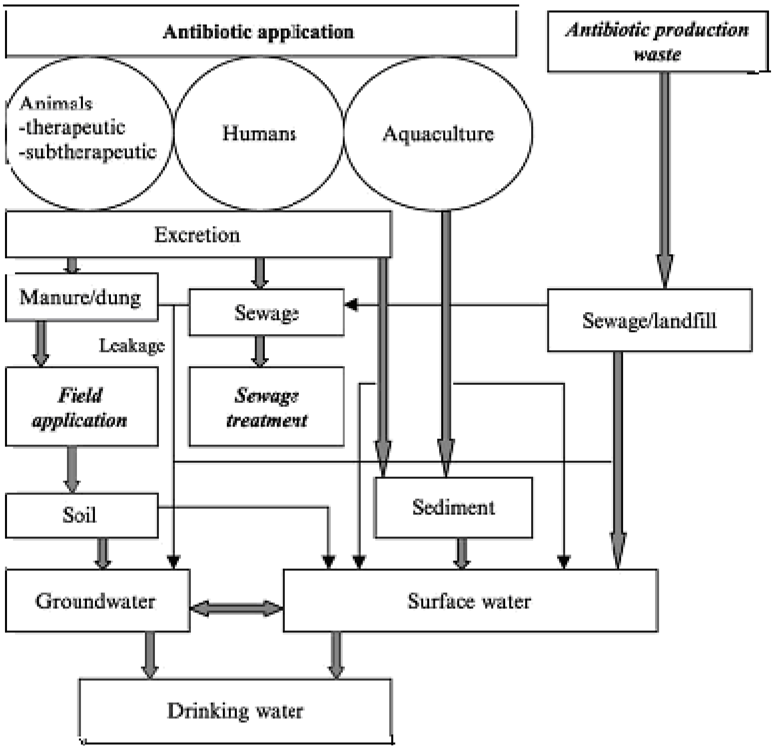


Figure 2.5. Anticipated pathways of human and veterinary antibiotics in the environment (Kumar et al., 2005a).

2.4.1.1. Antibiotics in Soil. Antibiotics are introduced to soil environments by field application of manure and sewage sludge. Antibiotics have strong binding abilities which increase their concentration in sewage sludge or manure (Jorgensen and Sørensen, 2000). Antibiotics with weak binding abilities like sulfonamids are also detected in sludge, mostly because of their common and extensive use in human medicine. Other than these sources wastewaters from municipal STP or animal feeding operations may also contain antibiotic compounds where they introduced to soil by irrigation of fields (Bradford et al., 2008; Kinney et al., 2006).

2.4.1.2. Antibiotics in Water. Antibiotics presence is confirmed in sewage treatment plants due to human excreta. Also wastewaters from hospitals and antibiotic manufacturing companies contain considerable amount of antibiotics. At the influent of sewage treatment plant, antibiotics can be detected at µg/l level; where the effluent of hospitals and antibiotic manufacturing plants contains much higher concentration of antibiotics. STP are very weak in removing antibiotics therefore, effluent also contains antibiotic compounds. Surface and groundwater contamination can be traced to natural causes like rainfall and runoffs but main points of origins are anthropogenic. Aquaculture practices, leaching from agricultural fields, improper discharge of STP effluents and irrigation with wastewaters are main anthropogenic sources for antibiotic input.

Studies show that sulfonamides are detected in high amounts in surface and ground water due to extensive use and their mobile characteristics. Antibiotics with strong binding affinity like fluoroquinolones and tetracyclines are also detected in groundwater samples.

2.4.2. Fate of Antibiotics in Environment

2.4.2.1. Sorption of Antibiotics. Octanol water partition coefficient (log K_{OW}) is a common parameter for organic carbon normalized sorption coefficients (log K_{OC}) of hydrophobic compounds. But this prediction cannot be used in ionizable compounds like antibiotics where many sorption processes occur simultaneously (Tolls, 2001). Sorption of antibiotics is mainly depending to pH, molecular structure of antibiotic, cation exchange capacity and texture of solid matrices. Antibiotics like tetracyclines and fluoroquinolones can be considered as strongly adsorbed antibiotics. They tend to make complex with divalent and trivalent cations; thus have a lower mobility. A soil leaching experiments showed OTC prefers to stay in soil along with fluoroquinolone group antibiotics (enrofloxacin, ciprofloxacin or norfoloxacin) as imidazoel and sulfonamide group antibiotics leached through soil matrix (Rabølle and Spliid, 2000; Golet et al., 2003; Blackwell et al., 2007; Uslu et al., 2008).

2.4.2.2. Biotic Degradation of Antibiotics. Antibiotics are highly resistant to biodegradation. Some aerobic and anaerobic water simulation experiments have been carried out to investigate biodegradation of tylosin, metranidazole and oxytetracycline by Ingerslev and his coworkers (2001). Half lives were found 10-40 days for tylosin, 14-104 days for metranidazole and 42-46 days for OTC. Closed bottle biodegradation tests have been performed for 28 days for various antibiotics and none has been found biodegradable after the incubations (Kümmerer et al., 2000). Another study classified some antibiotics according to their biodegradability like non biodegradable (Erythromycin) to 50% degradable (Ampicilin) (Richardson and Brown, 1985).

Sulfonamides are commonly used and widely detected in soil environments. Their low sorption characteristics decrease their lifespan in soil. 50% and 90% of removal has been observed in manure amended sandy loam soil for sulfachloropyridazine in 3.5 and 19 days, respectively (Blackwell et al., 2007). But after 127 days, antibiotic still can be detected in top soil. Another study concluded that 15% of initial concentration can be detected in manure amended soil after 90 days of fertilization (Stoob et al., 2007). Macrolide antibiotics are also less persistent as sulfonamides in soil. Half lives of Tylosin and erythromycin has been found 4-20 days in soil (Gavalchin and Katz, 1994; Ingerslev and Halling-Sørensen, 2001; Schlüsener and Bester, 2005). In study of Blackwell et al., 2007, no observable Tylosin has been detected after 127 days in manure amended soil. A longer half life (up to 67 days) has been reported for Tylosin A in sandy soil (Halling Sørensen et al., 2005). A half life of eight days has been reported for Tylosin A in both blank and sterile soil in another study (Hu and Coats, 2007). Results indicated that elimination of Tylosin A was rather abiotic or due to sorption.

Strong sorption characteristics of some antibiotics make them highly resistant for biodegradation. The half-life of enrofloxacin has been found as 90 days in cattle manure (Wetzstein et al., 2002). Similar results have been reported for tetracycline and oxytetracycline where half lives have been found as 55-105 days and 30 days respectively (Winckler and Grafe, 2001, De Liguoro et al., 2003). Half-life of OTC has been calculated as 64 days in anaerobic digestion of calf manure in another study (Arikan et al., 2006). A recent study showed that degradation of OTC (8.1 days) was achieved in high moisture content aerobic treatment (Wang and Yates, 2008). But as the available concentration of antibiotic decrease due to degradation, remaining compounds become more stable to degradation. Slower degradation of OTC in anaerobic treatment compared to aerobic has been confirmed in a study (Kühne et al., 2000). Some composting experiments show high percentage of elimination of antibiotic like 99.8% for OTC in 37 days and 99% for CTC in 22-35 days (Arıkan et al., 2007, Dolliver et al., 2008). But in both experiments, no clear conclusion for elimination has been reached whether removal was due to biodegradation or sorption or abiotic process. Antibiotics with low tendency of sorption are more degradable as seen in a study (Wang, et al, 2006). A sulfonamide group antibiotic has half-life of 2.56 days in fresh manure and 10.2 days in sterilized manure under aerobic conditions. A macrolide group antibiotic Tylosin A has been studied and found as easily degradable. Half life has been calculated as less than two days (Loke et al., 2000). But as mentioned before authors do not clarify of loss of antibiotics whether from biodegradation or sorption. Another study supported previous results where a rapid degradation of Tylosin A has been monitored in 10 days of microcosm experiment conducted with liquid manure (Oliveira et al. 2002). They found that halflife of Tylosin was 2.4 days. A similar half life, 3.6 days, was also calculated in the study of De Liguoro et al. (2003). Study of Kolz et al., (2005) showed that concentration of Tylosin was reduced by 90% in 5 days of anaerobic degradation where same concentration was achieved by aerobic degradation in 26 hours. Although half life of easily biodegradable antibiotics is short, their residuals can be detected for long time in manure. Sulfadimethoxine has a short half life as reported in Wang et al. (2006), but study of De Liguoro et al. (2007) detected low amounts of antibiotic after 90 days. Dolliver et al., (2008) detected residues of sulfamethazine after eight months of manure incubation. As most of antibiotics are excreted in manure, agricultural application of manure transfers these compounds into soil matrix. Persistence of antibiotics in soil matrix is much higher than manure, possible due to humidity of the environment. In a study no degradation was observed for 180 days in manure amended soil (van Gool, 1993). A similar result has been found in another study where detectable tetracycline compound declined slowly over five months after amendment in soil (Aga et al., 2005). Results were confirmed by other studies where OTC removal of 50% and 90% was established in 22 and 98 days, respectively (Blackwell et al., 2007). Half lives of CTC was found as 25 and 34 day in two different soil types, sandy loam and sandy soil, respectively (Halling-Sørensen et al., 2005). Like tetracyclines, fluoroquinolones are also persistent in soil. Ciprofloxacin and norfloxacin persisted in sludge amended soil for 21 months (Golet et al., 2003). Another study showed nearly no degradation of sarafloxacin in different soil types, loam, silt loam and sandy loam, within 80 days (Marengo et al., 1997).

2.4.2.3. Abiotic Degradation of Antibiotics. Abiotic processes like photodegradation can also play an important role in the elimination of antibiotic compounds. Antibiotics of β-lactam group showed different tendencies to photodegradation. Amoxicillin has half life of 2-9 days in pure water where rate of photodegradation increases with increasing pH (Andreozzi et al., 2004). But ceftiofur sodium was more resistant to photodegradation with half life of 30 days (Gilbertson et al., 1990). Sulfonamides were more resistant to photodegradation as revealed in the study of Lunestad et al., (1995). After 21 days of light exposure no degradation has been monitored in sea waters. Tylosin has also shown slow photodegradation where half life in pond water has been calculated as 200 days.

Although tetracyclines and fluoroquinolones are highly persistent in soil matrix, they are subject to photodegradation in water. A study showed half life of enrofloxacin, danofloxacin, ciprofloxacin, and norfloxacin, fluoroquinolones group antibiotics, in range of 21-106 minutes in water (Burhenne et al. 1997). In the study of Lunestad et al., (1995), loss of antibacterial activity of flumequine and oxolinic acid was established in 21 days at sea water. An experiment was designed to monitor effect of water types on photodegradation efficiency. Oxolinic acid and ciprofloxacin was tested in pure and river waters. The results showed rapid degradation of ciprofloxacin compared to oxolinic acid due to different molecular structure. It was also found that photodegradation efficiency was higher in pure water than river water probably because of humic substances present in river water (Turiel et al., 2005).

Beside photodegradation, hydrolysis is considered another abiotic process for degradation of antibiotics. β-lactam group antibiotics can be hydrolyzed easily in both basic and acidic conditions (Hou and Poole, 1969). Like photodegradation, hydrolysis is also very effective in degradation of tetracycline in water. In a study, it has been found that 20% of OTC degradation was achieved in 14 days in deionized, fresh and sea waters, respectively. It was also reported that degradation increased with increased pH. But flumequine and oxolinic acid showed no tendency to hydrolysis in these 14 days (Pouliquen et al. 2007). It has been also reported in another study that pH played a significant role in hydrolysis of tetracyclines (Loftin et al., 2008). Hydrolysis of tetracyclines was also affected by temperature but not by ionic strength. Although Tylosin was degraded by hydrolysis in low and high pH values, several sulfonamides showed no degradation in different pH, temperature and ionic strength values.

2.4.3. Effects of Antibiotic Residues in Environments

2.4.3.1. Toxic Effects to Aquatic and Soil Organisms. Antibiotics are compounds that are detrimental to some bacteria as well as some other organism, micro or macro. As antibiotics begin to be detected in terrestrial and aquatic environments, studies have been conducted to determine their toxic effects on organisms. Water species like *Daphnia magna* and *Artemia salina* are commonly used in toxicity studies in water samples. A study showed that furazolidone had an acute toxicity on *Daphnia manga* (Macri et al., 1988). Studies showed different toxicities of different antibiotics. As oxolinic acid and tiamulin had an acute toxicity, oxytetracycline, sulphadiazine, and tetracycline had a reproductive toxicity in mg/l range (Wollenberger et al., 2000). Another study showed toxic effects of aminosidine, bacitracin, erythromycin, flumequine and lincomycin on *Artemia salina* (Migliore et al., 1997).

Algae are another organisms studied for toxicity of antibiotics. Toxic effect of metranidazole has been studied on *Chlorella sp.* and *Selenastrum capricornutum*. Drug showed toxic effect within 72 hours EC_{10} values of 2.03 and 19.9 mg/l (Lansky and Halling-Sørensen, 1997). Toxic effect of some selected antibiotics on algal species has been analyzed in the study of Halling-Sørensen, 2000. Antibioitics, tetracycline, chlorotetracycline, benzylpenicillin, streptomycin, tiamulin, olaquindox, tylosin and spiramycin, are found toxic with EC_{50} 0.005-5.1 mg/l for algae cyanobacteria, 0.133-40 mg/l for green algae. Another type of algae (*Tetraselmis chuii)* has been subjected to antibiotic exposure, oxytetracycline and florfenicol. IC_{50} values for 96 hours were 11 and 6 mg/l for oxytetracycline and florfenicol, respectively (Halling-Sørensen, 2000).

Toxic effects of antibiotics have been seen also in soil organisms. But in soil matrix sorption of antibiotics limits bioavailability of the compound (Jjemba, 2002). A study demonstrated that oxytetracycline and tylosin were adsorbed more in clay loam than sandy loam decreasing inhibitory effects of antibiotics. Different studies have been conducted to monitor effects of antibiotics on soil fauna. Oxytetracycline had inhibitory effects on enzymatic activities of soil microorganisms (phosphates, dehydrogenase) at concentrations 1-10 mg/kg (Boleas et al., 2005). A similar study showed effective dose of sulfapyridine and oxytetracycline in Fe (III) reduction test was in range of 0.003-1.14 mg/kg and 5.5-7.35 mg/kg, respectively (Thiele-Bruhn and Beck, 2005).

It has been long speculated that metabolites of antibiotics are more detrimental for microbial life than the parent compounds. A study showed that metabolites of tetracycline showed significant toxicity in sewage sludge and soil (Halling-Sørensen et al., 2002). But lower toxicity of metabolites has been reported from same author in a later study where selected antibiotics were tylosin, sulfadiazine, ciprofloxacin, olaquindox, and streptomycin (Halling-Sørensen et al., 2003).

2.4.3.2. Plant Uptake. Antibiotics presence in plants has been recently encountered due to presence of antibiotics in soil and in water where plants grow. Presence of antibiotics in plants is due to uptake of antibiotics from soil matrix and depended to both structure and species of the plant. An earlier study showed effect of some antibiotics on different plants. In this study chlortetracycline and oxytetracycline had negative effect on pinto beans but not on corn. Radish and wheat had positively affected by antibiotics by increasing nutrient uptake (Batchelder et al. 1982). Although negative effects had been observed for sulfonamides on rice growth, no effect had been monitored for tetracyclines (Liu et al., 2009). This result was explained by higher bioavailability of sulfonamides than tetracyclines due to low sorption characteristics of the sulfonamide antibiotics.

A study showed plant uptake of CTC was higher in corn than cabbage and green onions (Kumar et al., 2005b). In the same study, Tylosin antibiotic was also used but opposite to predictions, compound was not detected in plant. It was thought low sorption of Tylosin will increase bioavailability of compound and it will be more readily present for plant uptake. Molecular structure of compound was believed to hinder uptake.

2.4.4. Problem of Antibiotic Resistance

Presence of antibiotics in environmental bodies triggers another problem of great concern which is antibiotic resistance gained over time by pathogenic bacteria. In order to reduce risk and decrease amount of antibiotics in the environment, use of antibiotics as growth promoter in livestock animals were banned in EU countries in 2006. Therefore, antibiotics used in EU countries are only for therapeutic uses. In USA still some antibiotics are used as feed additive and make a considerable percentage of used antibiotics in total.

Although effluents of sewage treatment plants, hospitals and antibiotic manufacturing plants contain antibiotic residues, main route of antibiotics into receiving environments is through animal manure. Raw manure or slurry is used often for soil conditioning. Many antibiotics are highly persistent in soil matrix and can accumulate in soil over time. This problem not only cause resistance gene proliferation in soil microorganisms but also introduce resistance genes to humans by consumption of antibiotic contaminated food like plant, livestock and water (Batt et al., 2006). Mostly concentration of antibiotics in soil is at sub therapeutic level where exposing long periods may cause resistance gene development (Hirsch et al., 1999, Boxall et al., 2002, Kümmerer, 2004). Obtained resistanct gene can be transferred to other microorganism or may create selective environment for resistant bacteria.

Antibiotic production and resistance gene is a naturally occurring process. Presence of antibiotics in a system may promote proliferation of resistance genes. This process is then a forced selection and may change community dynamics (Kümmerer, 2004). Resistance genes can be obtained by two ways, natural or acquired. There are three main mechanism used in gaining resistance over an antibiotic (Aminov et al., 2001). They are:

1. Inactivation of antibiotic compound
2. Transferring antibiotic out of cell
3. Protection of antibiotics target

Resistance genes are present mainly in microorganisms that produce antibiotic compounds. Vancomycin resistance in *Enterobacteriaceae* and polymixin resistance in gram positive bacteria are good examples of this type of resistance. On the other hand, resistance can be acquired later rather due to chromosomal mutation or transfer of mobile genetic elements between organisms. Presence of antibiotics in the environments comes into the scene at this point. Acquired resistance has been promoted by presence of antibiotics at sub therapeutic level. Increase in resistant microorganisms also increase probability of resistance gene transfer. Acquired resistance by chromosomal mutation also cause change in cell of the organism. Resistance genes on mobile elements can be multiplied independently from cell multiplication and transferred by different pathway. Those resistance genes on mobile elements can easily move vertically and horizontally move in the environment with help of bacteriophages, plasmids and transposons (Witte, 1998; Smalla et al., 2000; Kümmerer, 2004; Aarestrup, 2005). This movement of resistance gene increased with increasing bacterial diversity and density (Muray, 1997). Activated sludge sytems, waste and sewage treatment sludges and drinking water treatment biofilms can be prospective systems for gene transfer (Hirsch et al., 1999, Esiobu et al, 2002, Giger et al., 2003, Schwartz et al., 2003, Kümmerer, 2004)

In general resistance gene acquiring described as mentioned above. But, this process itself is very complicated and cannot be understand fully (Kümmerer, 2004, Martinez 2008). Table 2.7. shows inhibitory mechanism of main antibiotic families and their resistance gene characteristics.

Table 2.7. Main antibiotic families, their inhibitory and resistance mechanism

Antibiotic	**Inhibition**	**Resistance**
Tetracyclines	Protein synthesis inhibition	*Inactivation of antibiotic compound *Transferring antibiotic out of cell *Protection of antibiotics target
Beta-lactams	Cell wall synthesis prevention	*beta lactamase production *Change of target molecule *Decreased permeability
Aminoglycosides	Protein synthesis inhibition	*Methylation of ribososmal target *Change in ezymes
Macrolides	Protein synthesis inhibition	*Methylation of 23S RNA
Sulfonamides	Inhibition of PABA	*Increase in PABA production *Change in metabolic enzymes

Tetracycline group antibiotics are protein synthesis inhibitors. Upon entering bacterial cell they bind on ribosome and prevent binding of aminoacyl tRNA (Schnappinger and Hillen, 1996). Tetracycline resistant bacteria prevent this binding by three different mechanism, modifying ribosome to prevent binding of antibiotic, pumping antibiotic out of cell by efflux proteins and producing enzymes that disrupt structure of tetracycline (Chopra and Roberts, 2001). Studies showed that by adding TC and CTC containing manure into soil, resistance gene can be promoted. Tetracycline resistance gene increased in number in first week but declined down to control level after 45 days. During this study, it was concluded that resistance gene number increased only with manure amendment and bacteria carried *tet*M resistance gene do not increased in number after manure addition. After five months, these bacteria can be detected in soil, suggesting they were natural dwellers of soil microbiota (Agerso et al., 2006). Another study also showed that animal manure increases resistance gene diversity if the concentration of antibiotic is high where low concentrations promote resistance gene proliferation by pathogens (Schmitt et al., 2006). In another study, tetracycline resistance was monitored in *Basillus subtilis* (Tn916 transposon carrier) contaminated soil. Samples collected within a week of incubation showed high resistance to tetracycline, but no *tet* M gene was detected (Natarajan and Oriel, 1992). Another study confirmed other results by finding increase in number of resistant bacteria after prolonged exposure of tetracycline where total heterotrophic bacteria decreased in number (Rysz and Alvarez, 2004).

Recent studies showed that resistant pathogens are beginning to be monitored more often. As the contact with resistant pathogens and by consumption of resistance gene containing food, these genes can be transferred to human and animals (Teuber, 2001). As the resistance gene and resistant pathogens are more common than before, it pose a great threat for public health due to difficulties in treating infections (Kanay, 1983).

2.5. Characterization of Microbial Communities using Molecular Tools

Microbial communities in environmental systems are only predicted somehow in the past due to the limitations of available methods for characterisation of microorganisms by pure culture (Amann, 1995b). Until introduction of molecular tools into environmental studies, most common method for identification of microorganisms cultivation or microscopic tools. Main limitation of these techniques is identification of small percentage of community. Many microorganisms live in syntrophic relation and cannot be cultivated independently. Also cultivation of anaerobic organisms is especially hard. Cultivation may give false information since in culture community shifts may occur due to favoring of a normally not favorable microorganism. Due to these limitations of cultivation, cultured microorganisms cannot reflect whole community. Studies showed that culturable microorganisms make only small fraction of all microorganisms of earth (Muyzer et al., 1993; Amann et al., 1995a; Hugenholtz et al., 1998; Muyzer, 1999).

In the last 25 years, many studies used molecular tools to identify microbial communities, monitor temporal and spatial changes in communities and explain microbial population and community dynamics in defined environments and the impact of specific factors, such as pollution by xenobiotics on microbial diversity (Ranjard et al., 2000; Morris et al., 2002).

In molecular microbiology, some target molecules help scientist to identify and monitor microorganisms. RNA, DNA, proteins and lipids can be targeted. Nucleic acids, both RNA and DNA, are biomarkers and hereditary molecules due to their role in protein synthesis (Woese, 1987). Microorganisms can be detected, identified and enumerated by the analysis of genes. Mostly ribosomal RNA molecules used for phylogenetic marker since ribosomes are abundant and obligatory component of cell. Ribosomes are directly responsible for protein production. Therefore, they can be used as growth and activity indicators, too (Amann, 1995b). 16S rRNA studies created a phylogenetic network of organisms which created then tree of life what contains 3 main branches including *Archaea*, *Bacteria* and *Eukarya* (Figure 2.6.).

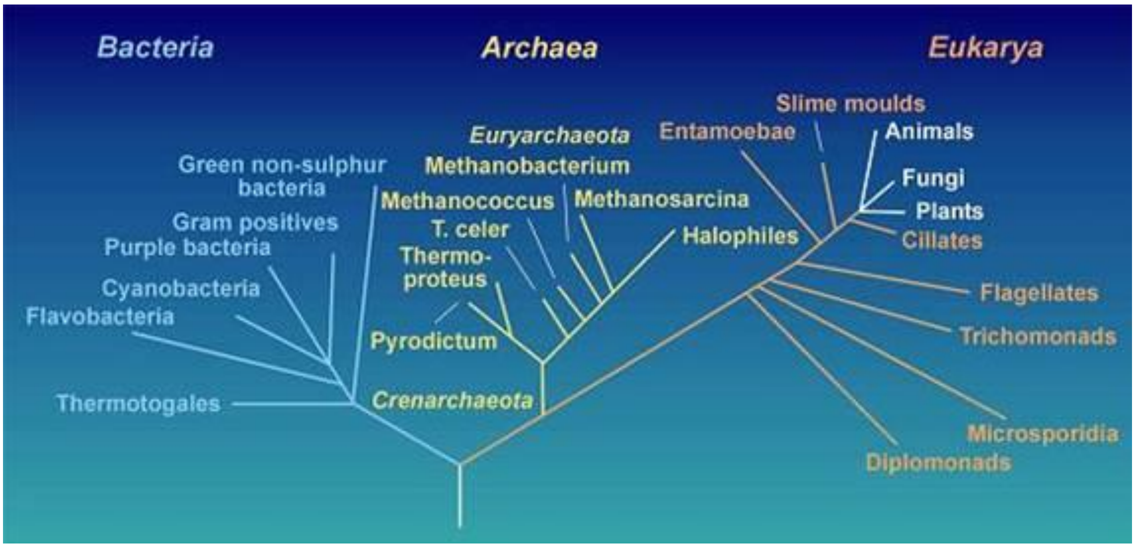


Figure 2.6. The rRNA tree of life (Madigan et al., 2002).

16S rDNA contains more than 1500 bp and is sufficiently large for any phylogenetic identification (Saiki et al., 1988) (Figure 2.7.). Sequence analysis of 16S rDNA molecules are collected in a database which helps phylogenetic classification. 16S rDNA contains both conserved and variable regions (Lane et al., 1985). It is highly conserved since its secondary structure and function was the same through years of evolution. It also has variable regions where changes occur time to time. Those changes reflect evolutionary relationship of organisms (Olsen et al., 1986). Among the variable regions, V3 region is mostly used in molecular analysis (Neefs et al., 1990; Øvreas et al., 1997). All sequenced 16S rDNA fragments are collected in databases creating an enormous source of information. But as databases offer a large amount of data, they also bring some problems with it. 16S sequences of same species may contain heterogeneity and may confuse interpretation of diversity obtained during experiments (Dahllöf, 2002).

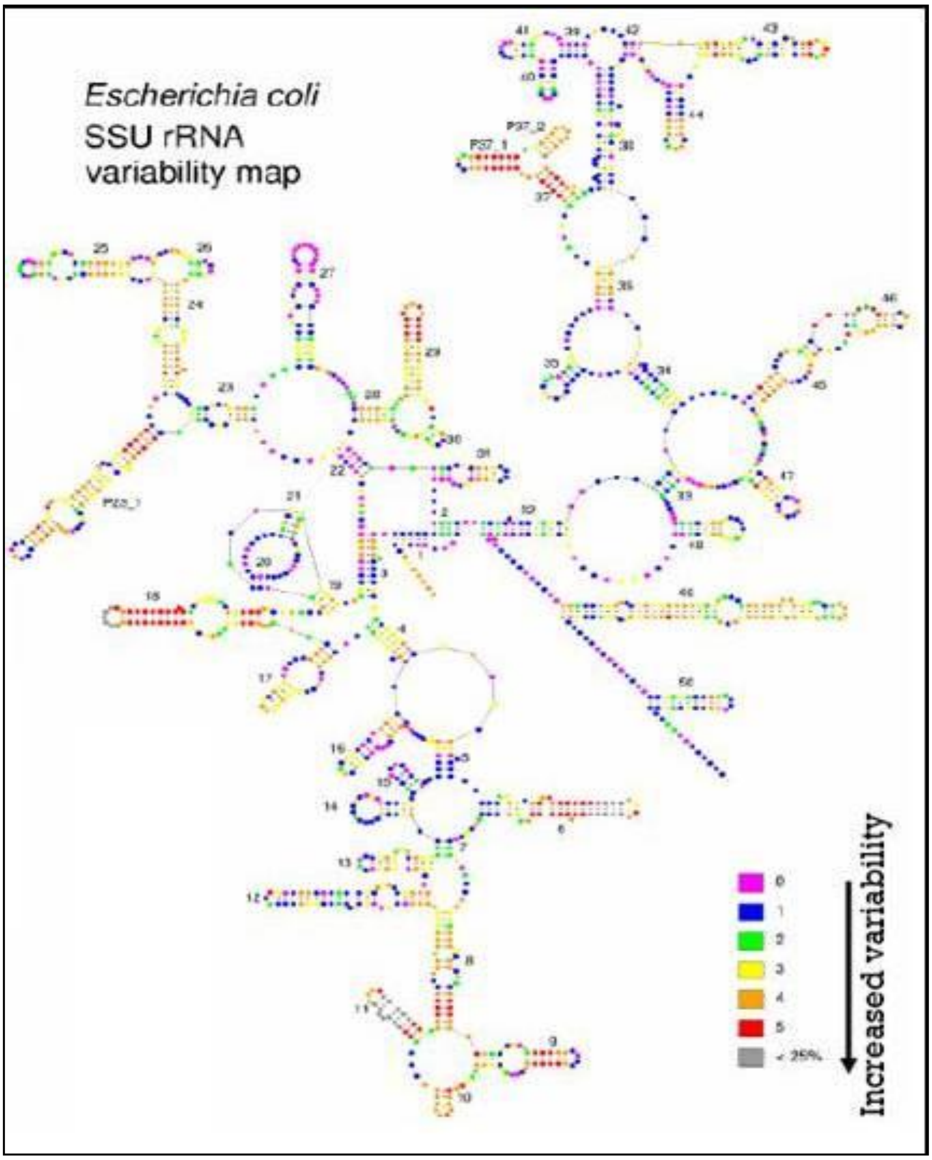


Figure 2.7. Secondary structure of the 16S rRNA of *E. coli*, showing conserved and variable regions (Van de Peer et al., 1996).

As considered by most, molecular microbial ecology started with the study of Pace et al, (1986). In their study, they use 16S rRNA genes for identification, enumeration and classification of previously uncultured microorganisms. Later molecular techniques become more available and used in many other studies (Muyzer et al., 1993; Amann et al., 1995a; Muyzer and Ramsing, 1995). Common pathways and common techniques used in environmental microbiology were summarized in Figure 2.8. and Table 2.8. More detailed information will be given in following chapters.

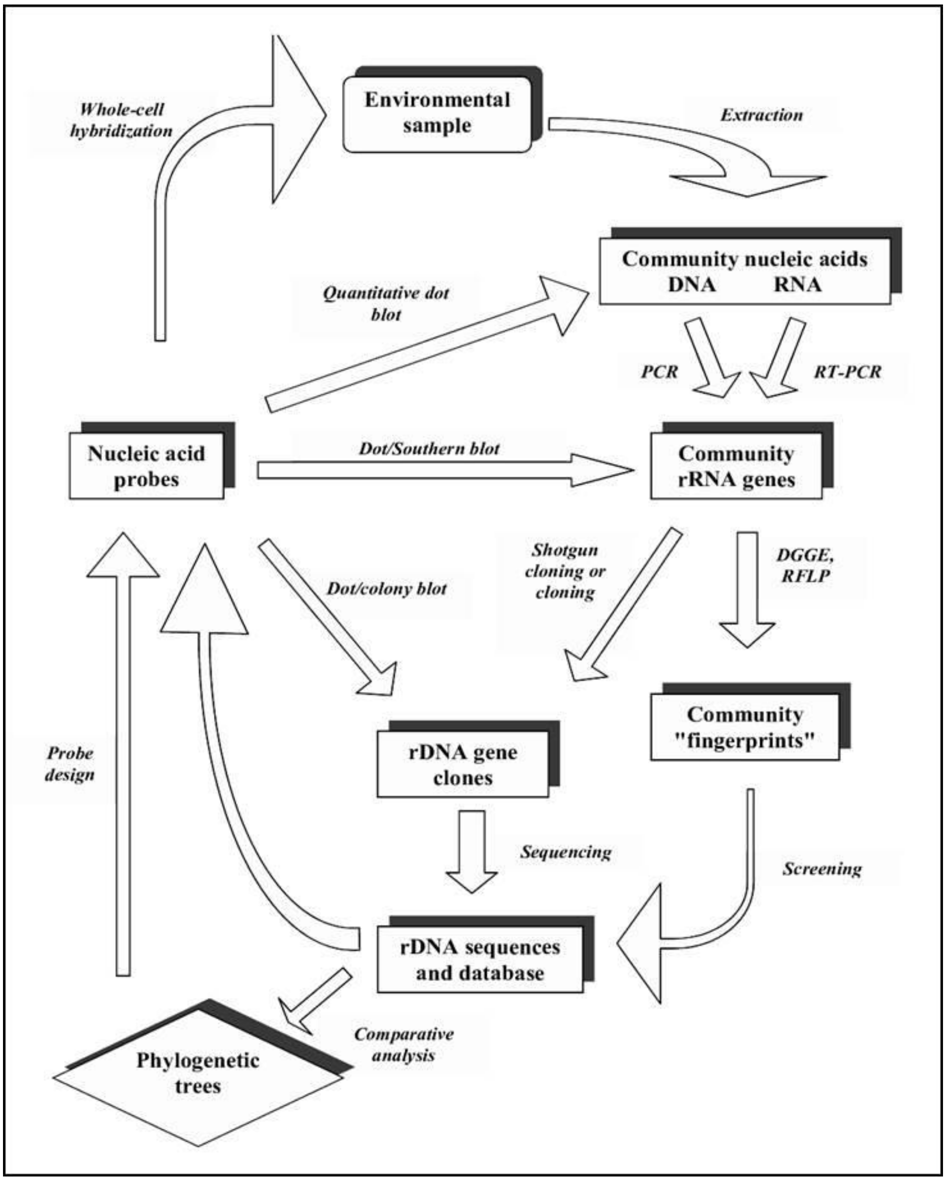


Figure 2.8. Summary of overall methodologies used in the phylogenetic studies (Muyzer et al., 1998).

Table 2.8. Summary of common molecular methods used in microbial ecology

Approach	Description	Remarks
Cultivation	Study micro-organisms in defined circumstances.	*Only a minor fraction of the microorganisms can be cultivated.
PCR	Specific and sensitive amplification of genetic material (DNA/RNA).	*Primers developed from known sequences and can cause bias.
Real-time PCR	Sensitive quantitative amplification suitable for high-throughput over a wide dynamic range.	*Sensitive quantitative amplification suitable for high-throughput over a wide dynamic range.
Fingerprinting (DGGE/SSCP/TRFLP)	Rapid overview of diversity. Ideal for comparisons of ecosystems in time or between different samples.	*Bias in nucleic acids extraction and PCR. * Only dominant populations can be visualised.
Sequencing	Gold standard for sequence retrieval.	*Nucleic acids extraction, PCR and cloning can be biased.
FISH *In situ* isotope tracking	Enumeration of micro-organisms *in situ*. Allows localization and quantification.	*Laborious without automatisation *Requires sequence information for probe development. *Cell permeabilization and fixation can cause bias.

2.5.1. Polymerase Chain Reaction (PCR)

Amplification of DNA segments using thermostable DNA polymerase was a total breakthrough in molecular biology and opens wide range of alternatives of usage DNA in many fields including environmental microbiology (Saiki et al., 1985).

PCR is used amplify a specific region of a double stranded DNA. This is actually replication of natural occurring process. DNA replication needs certain ingredients to perform. A reaction need to contain a template DNA, a heat-stable DNA polymerase enzyme, dNTPs, buffer containing magnesium and two oligonucleotide fragments called primers. At the end of reaction a single copy of DNA template amplified a billion times so fragments are available enough for experimenting on. PCR based on repetition of three steps called Denaturation, Annealing and Extension (Elongation). In denaturation, high temperature is applied to melt double stranded DNA. In annealing, temperature decreases to a point where primers can bind single stranded DNA template. In extension, temperature raised again for thermo stable polymerase to work. Enzyme adds then free dNTPs to end of primer, elongating second strand. These three phases repeated 30-40 times where original template amplified as 2^n where n is the repeat number. Then end product monitored for its correct size and amount on an agarose gel electrophoresis. PCR should be done with great care and cleanliness since as it can be seen a foreign DNA fragment can be amplified to a number which may affect downstream analyses. Therefore, a negative control without a DNA template was also included to the reaction. In some reactions, a positive control was also added to monitor reactions performance. Although the general steps and ingredients are well defined, steps and ingredients can be manipulated to meet demands.

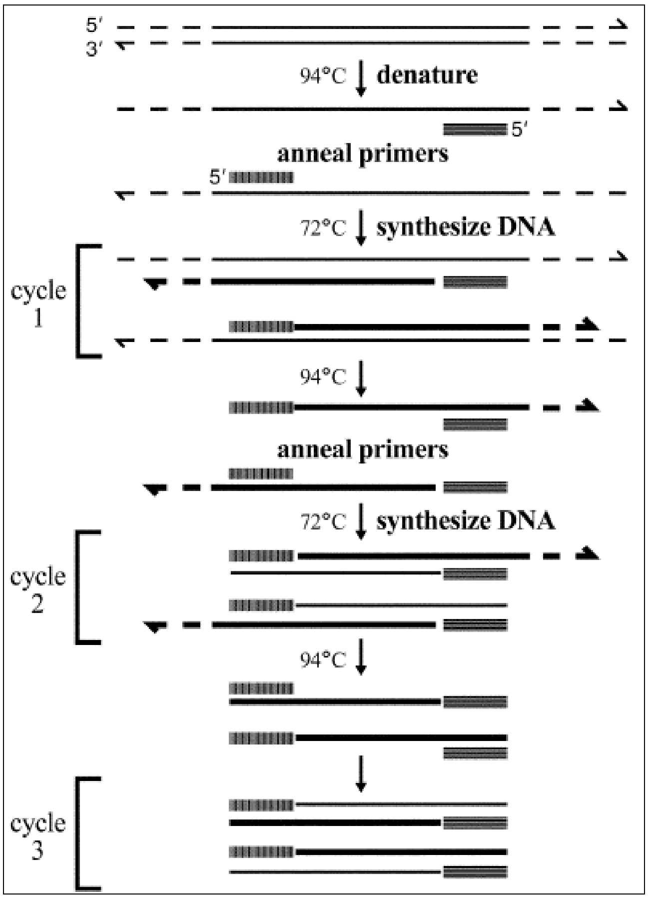


Figure 2.9. Mechanism of PCR

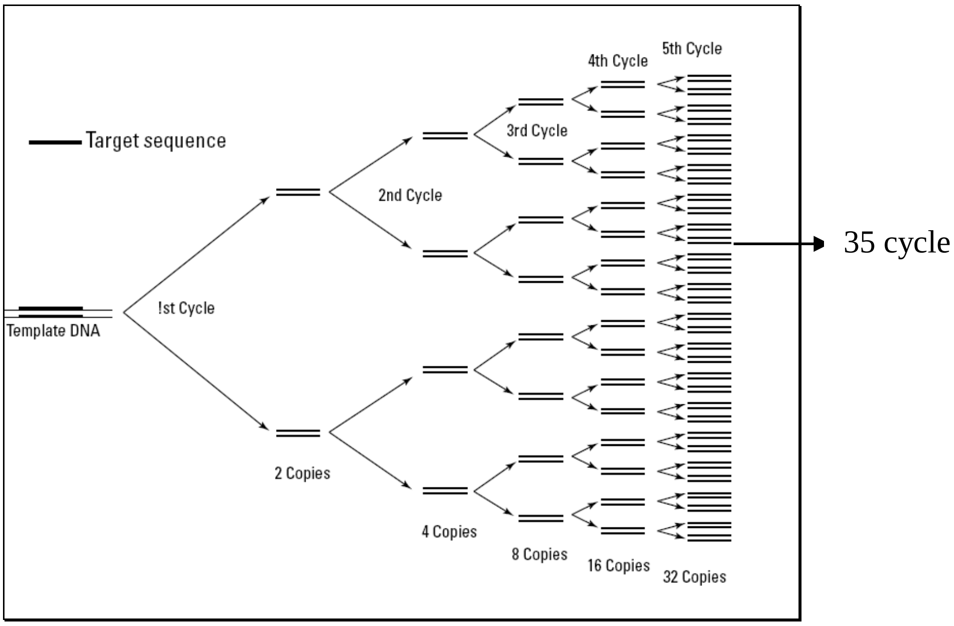


Figure 2.10. Exponential growth of selected fragment by PCR

PCR also itself a technique to analyze microbial communities but also is used as beginning of many other techniques. Most of fingerprinting techniques like DGGE, SSCP and T-RFLP can only be performed after PCR. PCR also used in cloning which allows identification of community members in an environment (Hofman-Bang et al., 2003).

2.5.2. Denaturant Gradient Gel Electrophoresis (DGGE)

Since introduction into environmental microbiology, DGGE become one of the most used fingerprinting techniques. Although there are similar techniques like SSCP and TGGE, advantages of DGGE make it popular among its similars. DGGE is an electrophoretic technique based on the same principle with polyacrylamide gel. Opposite of agarose gel electrophoresis, DGGE separates PCR amplified DNA fragments of same length. The idea behind DGGE is to melt dsDNA fragments of the same length. dsDNA melt at discrete segments called melting point where each melting point is sequence specific (Myers et al., 1987). Therefore, each specific sequence creates a different melting point where each separation occurs at a specific melting temperature (Tm). In acrylamide gel, partially melted dsDNA decreases its mobility so each sequence stops at specific place according to melting points of DNA fragments. When run on polyacrylamide gel, the mobility of the molecule is retarded when the first melting domain is reached resulting in partial dissociation of the fragment. Complete strand separation is prevented by the presence of a high melting domain, known as GC clamp, which is added to one primer (Dorigo et al., 2005) (Figure 2.10.). Community richness was estimated through number of bands appear on the gel lane. Also intensity of bands can be taken into account but since DGGE is a PCR dependent technique results are not fully reliable (Nübel et al., 1999). When DNA from a microbial community runs in a DGGE gel, a fingerprint specific to the community has been established (Figure 2.11).

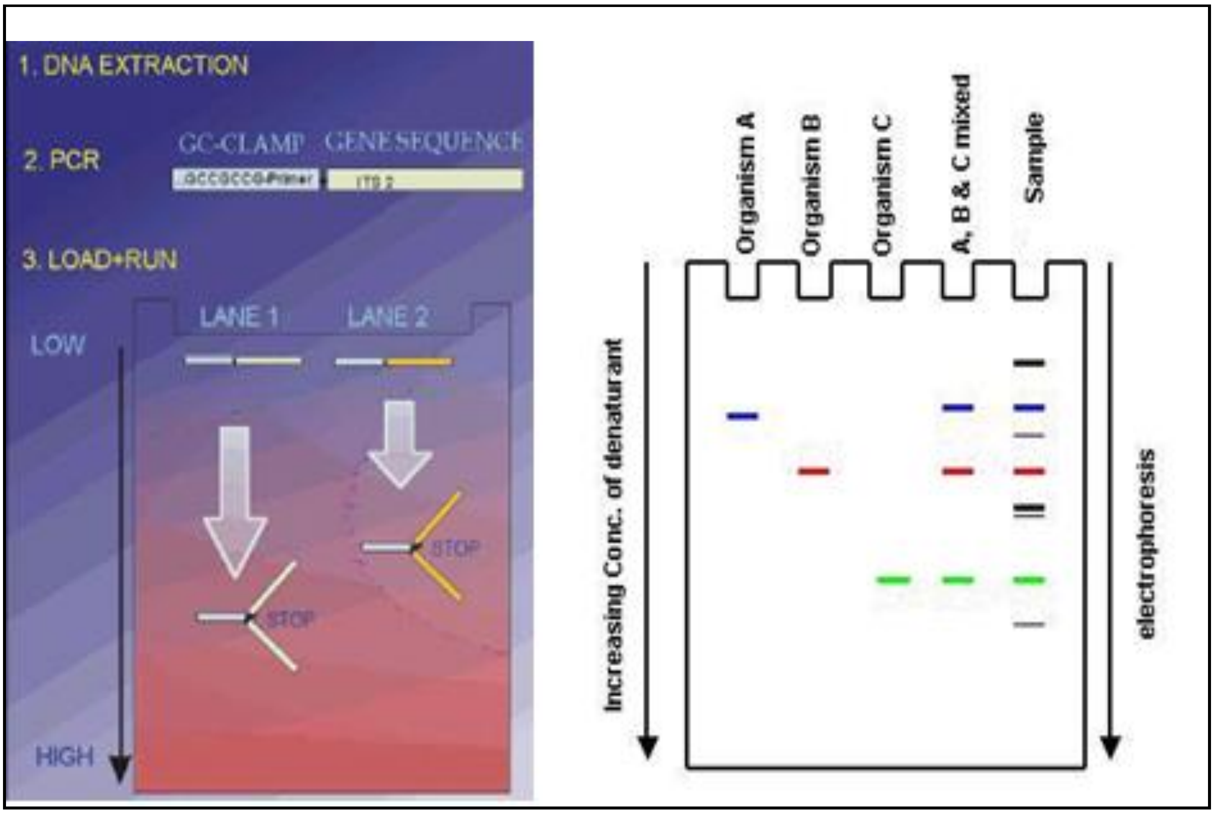


Figure 2.11. Principle of DGGE

DGGE was introduced to environmental microbiology in study of Muyzer et al., (1993). Then, it is widely used in monitoring microbial diversity and dynamics in soil, (Heuer and Smalla, 1997), activated sludge (Curtis and Crane, 1998), sediments (Muyzer et al, 1993), lake water (Øvreas et al., 1997), hot springs (Santegoeds et al., 1996), biofilm (Santegoeds et al., 1997).

DGGE can be used to analyze diversities of communities like two sludge plants (Curtis and Crane, 1996), soil samples (Heuer et al., 1997), bacterial and archaeal communities (Øvreås et al., 1997). Community changes over time can also be monitored through DGGE (Donner et al., 1996; Santegoeds et al., 1997). Monitoring of enrichment cultures can be done by DGGE (Santegoeds et al., 1996; Ward et al., 1996; Muyzer, 1997). Band excision, amplification and sequencing make DGGE an alternative method for cloning. Among other uses, DGGE can be used to screen clone libraries into groups of same Operational Taxonomic Units (OTU) (Kowalchuk et al., 1997).

DGGE has also some biases which can be circumvented with delicate handling. DGGE is a PCR based technique therefore, affected by biases of PCR. Another bias is co-migration of the bands. It may occur during DGGE run which result in giving false or non conclusive results if bands were excised and sequenced. Some bands separate very close to each other preventing successful band excision.

2.5.3. Molecular Cloning, Sequencing, Phylogenetic Analysis

Molecular cloning simply means to copy a selected DNA fragment into new cell where it can be expressed abundantly (Madigan et al., 2002). DNA fragments for cloning can be obtained from digestion of restriction enzymes (shotgun cloning) or by PCR (Hofman-Bang et al., 2003). In environmental microbiology, cloning of 16S rRNA gene can be used to identify members of a community (Giovannoni et al., 1990). 16S rRNA clone libraries are constructed with the 16s rDNA from mixed sample of a community. Each clone has one rDNA fragment which belongs to one member of the original community. As more clones are sequenced, community structure takes shape.

In general molecular cloning can be divided into three main steps (Madigan et al., 2002) Figure 2.12.;

Amplification. Isolation and fragmentation of the source DNA rather with restriction enzymes of PCR

Ligation. DNA fragment of interest put into a cloning vector with DNA ligase. Cloning vectors are self replicating genetic elements mostly obtained from plasmids or viruses. There are many vectors modified for cloning purposes.

Transformation. Cloning vector was placed in a host organism. Host is then enriched in culture to create a field of cells where each colony carries a single type of DNA fragment of interest. These colonies need to be harvested and monitored for insert of correct size. Sequencing of these colonies reveals community structure. In shotgun cloning, gene library was made from DNA fragment digested with restriction enzymes where then library contains random fragments of a genome. After sequencing, the data need to be arranged to construct the genome.

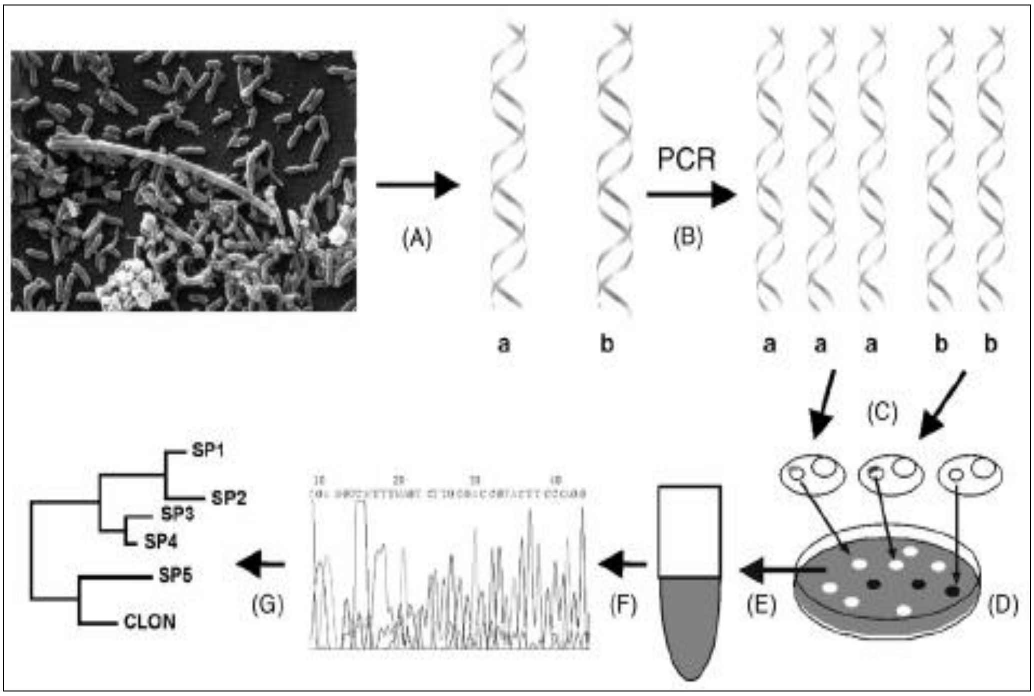


Figure 2.12. Outline of the cloning procedure for studying a microbial community (Sanz and Köchling, 2007). The work cycle is as follows: (A) direct nucleic acid extraction, (B) amplification of 16S rRNA gene by PCR (C) cloning of the PCR products into vector and transformation of competent E. coli cells with this vector; (D) selection of transformed clones with an indicator contained in the plasmid, (E) extraction of plasmid DNA; (F) sequencing of the cloned gene, creating a clone library; (G) determination of the phylogenetic affiliation of the cloned sequence with the help of dedicated computer programs (ARB, Seqlab, PAUP, PHYLIP).

Cloning is a PCR based technique which suffers from biases from PCR (Ward et al., 1992; Pace, 1996). Shotgun cloning has bias and produces clones of multiple genes at the same time (Pace, 1996). The problem is then different rRNA gene fragments may be cloned with different efficiencies. Constructing a shotgun library is time consuming and labor-intensive, thus not appropriate for large samples.

2.5.4. Quantitative Real Time PCR

Normal PCR amplifies a template but only DNA concentration at the end of the reaction can be monitored. A real life PCR do not amplify template as theory indicates. At the end of reaction, acquired DNA concentration cannot be related to the concentration of the template. Real time PCR was developed to monitor whole PCR process with fluorescence detectors so template concentration can be calculated according to amplified concentrations of control templates. Main idea behind Q-PCR is to record fluorescence spikes on each amplification phase with detectors and by confronting data of unknown DNA concentration with standard DNA template with known concentration (Malinen et al., 2003). It is sensitive, easy and straightforward technique which can process a large amount samples in relatively short time (Harms et al., 2003; Yu et al., 2005). Q-PCR can be modified by different add-ons to be more sensitive than standard Q-PCR. For example, TaqMan system use three probes, two primers and one fluorescent probe complementary to target DNA (Harms et al., 2003). By using different primer for the need, Q-PCR can be used to monitor activity of the selected communities and environments (Yu et al., 2005). Q-PCR can be performed on RNA templates so active microbial populations and metabolic reactions can be monitored.

2.5.5. Fluorescent in situ Hybridization (FISH)

Fluorescent labeled probes can be used to investigate cells *in situ* (i.e., in a culture or in an environmental sample) in a technique called fluorescent *in situ* hybridization (FISH) (Giovannoni et al., 1988; Amann et al., 1990). The technique first proposed in the study of Olsen et al., (1986) and rRNA gene fragments was used firstly in study of De Long et al., (1989). FISH is now an accepted technique in environmental microbiology (De

Long, 1992; Raskin et al., 1994; Wagner et al., 2003). It has been shown that the signal intensity of hybridized cells is directly related to the cellular rRNA content. This allows a quantification of rRNA concentrations both in single cells and in the environment (Poulsen et al., 1993, De Long et al., 1989). This made FISH technique also available for quantitative analysis (Amann et al., 1990). Probes for FISH studies increased with each study and considerable sized database (over 700 probes) has been created for further studies (Loy et al., 2003). Methanogenic groups of anaerobic digesters were monitored by FISH in the study of Raskin et al., (1994). Same probes were used in different studies during years with different experimental conditions. Studies showed that probes were still fairly accurate to target most of defined phylogenetic groups of methanogens (Merkel et al., 1999; Imachi et al., 2000; Tagawa et al., 2000; Upton et al., 2000; Yu and Fang, 2001). Modification of the technique allow scientist to label seven different microbial groups simultaneously. FISH targeting DNA and mRNA was also studied (De Long et al., 1989).

The practical application of the technique contains four main steps. A short explanation of the technique was also presented in Figure 2.13.

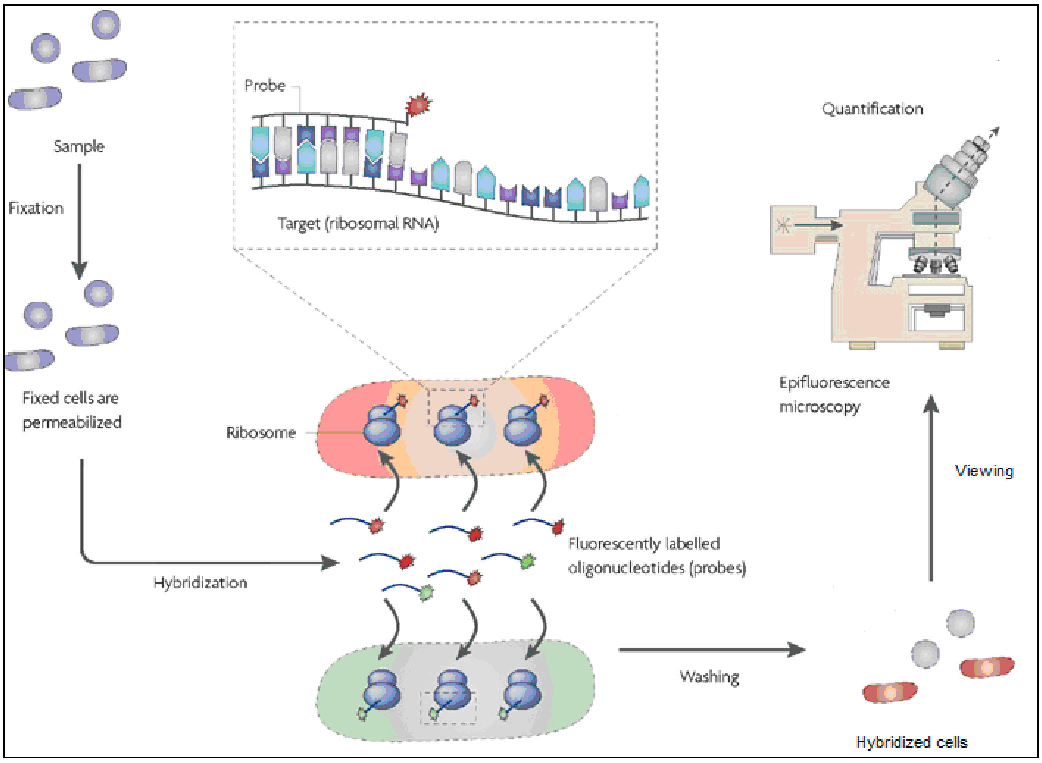


Figure 2.13. Schematic diagram of the steps involved in FISH.

Fixation. FISH starts with fixation step where cell wall and membrane made permeable to probe enter the cell. In fixation step also morphology of the cell was maintained. Mainly, alcohols and aldehydes are used as fixative agents. Paraformaldehyde is the most common fixative among many. Although fixation step is successful, probes may have problem penetrating Gram-positive cells (Muyzer and Ramsing, 1996). Gram-negative cells are easier to penetrate with probes than Gram-positive bacteria due to different cell wall structure.

Hybridization. In this step, fluorescent labeled probes bind to rRNA in fixed cells. In active cells, rRNA present in abundance and easy to target. Probes are 15-25 nucleotides in length and can be specific from kingdom to species depending of its sequence (De Long et al., 1989; Amann et al., 1995a). Hybridization step was done with help of hybridization buffers. Two commonly used buffers are buffer with Denhardt's solution and buffer with formamide. First buffer is a cocktail of substances which binds possible steric binding locations lowering non-specific binding of probe. This buffer is more suitable in analysis of complex microbial communities with high background noise. In this buffer, hybridization temperature is crucial and need to be calculated with care. As for another option hybridization buffers with formamide can be used. In this buffer, hybridization temperature was kept stable where concentration of formamide was changed according to probe (Manz et al., 1992). The hybridization step is a delicate step in FISH technique where complementarity and stringency becomes highly important parameters. Complementarity is sequence homology between probe and its target. Stringency is specific binding of the probe to its target. Stringency is affected by temperature, salt concentration and denaturant concentration during FISH. Low temperatures promote non specific binding where high temperatures favor specific bindings. Conditions for probes were obtained from studies of pure cultures with different parameters have been used to pinpoint optimal conditions. At least 700 probes are now present in databases and more can be developed from known sequences of 16S rRNA of uncultured organisms (Pernthaler et al., 1998; Ravenschlag et al., 2001).

Washing. In this step, unbound probes are removed to decrease background noise and false true signals. Stringency was also affected by washing so washing buffer was also prepared for this purpose.

Visualization. After washing step, the cells can be viewed by means of epifluorescence and confocal laser scanning microscopy or by flow cytometry with fluorescent microscopy.

In FISH technique DNA or RNA extraction is not needed. It can be used directly on environmental sample. Therefore, it is easy and fast. It also allows visualization of the cell morphology. It identify active cells by targeting rRNA which is abundant especially in active cells; therefore, it can be used for quantitative analysis (Sanz and Köchling, 2006; Wilderer et al., 2002). Signal intensity is related to probe therefore, related to cellular rRNA content. Many probes can be used on same sample to characterize a complex environmental community.

Although having many advantages FISH technique has also its own limitations. Main problem of FISH technique is limited hybridization of some cell types. Many microorganisms cannot be fixed by standard protocols or hybridized due to strong interactions of ribosome and other cell components (Amann et al., 1995a, Head et al., 1998). Microorganisms detected by FISH technique need to be known for its rRNA sequence for probe production and binding site location. Therefore, unknown groups of microorganisms cannot be detected. Designing new probe is an option but analysis with new probes it is rather laborious and complex.

2.5.6. Other techniques

2.5.6.1. Fluorescence in situ Hybridization-Microautoradiography (MAR-FISH). MAR-FISH combines use of radioactive probing with fluorescent probing on the same time. In environmental sample incubated with radioactive substrates, microorganisms incorporate these radioactive elements into their body and are detected by microautoradiography. Image of it was then superimposed with image of FISH probe revealing groups using these substrates (Lee et al., 1999). This technique has been used in different studies to reveal microbial communities. In study of Ariesyady et al., (2007), glucose, propionate, butyrate, and acetate degraders were studied in anaerobic sludge digesters with this technique.

2.5.6.2. Amplified Ribosomal DNA Restriction Analysis (ARDRA). In ARDRA, rRNA was amplified by PC and digested by restriction enzymes producing unique fragments due to sequence of template (Moyer et al., 1994). With this technique, a rapid comparison of rRNA genes can be performed and community specific patterns can be obtained (Martinez-Murcia et al., 1995)

2.5.6.3. Terminal Restriction Fragment Length Polymorphism (T-RFLP). T-RFLP is also a fingerprinting technique and uses idea of length of terminal restriction fragment to categorize samples. The gene of interest is labeled with a fluorescent dye at the end and digested with a four-cutter restriction enzyme. The length of labeled fragment is then sequence specific gives both quantitative and qualitative information about the sample (Liu et al., 1997) (Figure 2.14). T-RFLP differs from ARDRA or RFLP since only terminal fragment is analyzed rather than all fragments. T-RFLP gives reproducible data in a short time but because it is a PCR based technique sometimes underestimation or over estimation of communities can occur (Liu et al., 1997).

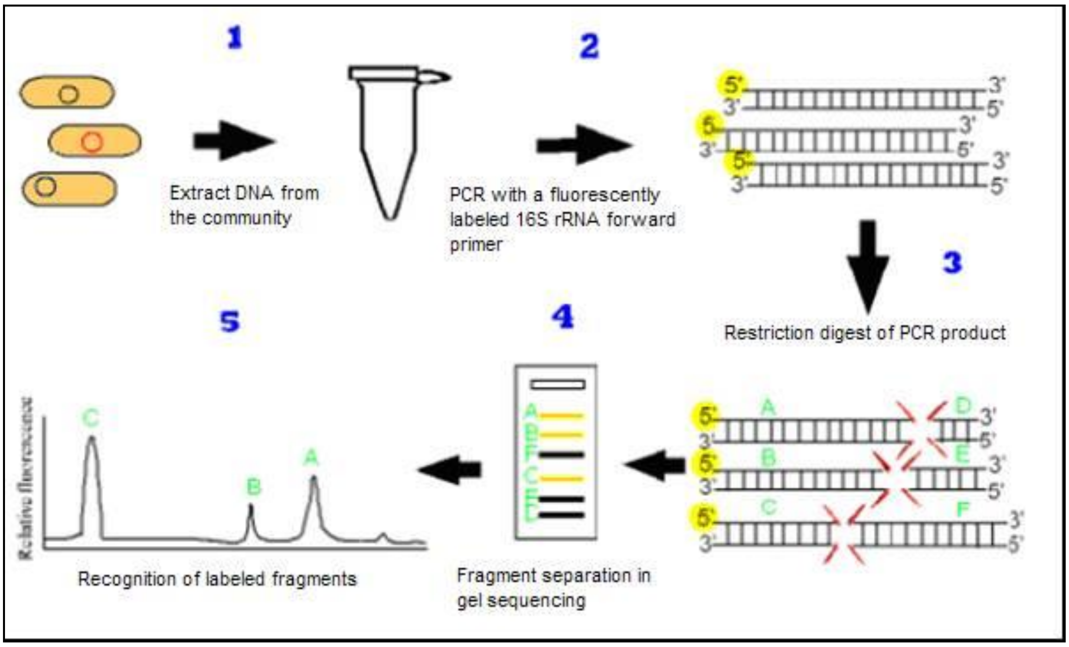


Figure 2.43. Principle of T-RFLP

2.5.6.4. Single Strand Conformation Polymorphism (SSCP). SSCP is an electrophoretic fingerprinting technique very similar to DGGE in principle. In SSCP, different than DGGE single stranded gene fragments are separated by their secondary structures; each SSCP peak representing a different structure meaning a different gene sequence (Leclerc et al., 2001; Lee et al., 1996). SSCP has same limitations like DGGE, where a single band may contain multiple bands due to co-migration (Tiedje et al., 1999). Lack of GC clamps and denaturing gradient makes SSCP easier and straightforward.

2.5.6.5. Dot-blot Hybridization-based Approaches. Dot-blot Hybridization has been developed to circumvent amplification biases of PCR. The idea is hybridization of nucleic acid mixture by specific probes of interest. Total RNA was extracted and immobilized on a membrane and hybridized with oligonucleotide probes. Relative abundance of a probe can be derived from abundance of universal probe hybridization (Raskin et al., 1994; Amann et al., 1995a). The sensivity of the technique can be improved to 0.1% by using radioactive labeled probes. Although it is an accurate technique in quantification, data is hard to relate cell number since different species and microorganisms may have different number of ribosome in their cell. Technique is highly favored for its accuracy therefore, probes are designed and a database has been established (Loy et al., 2003). It can be used in all kind of samples therefore; it is an expensive alternative for FISH (Amann and Ludwig, 2000).

2.5.6.6. Stable Isotope Probing (SIP). SIP uses stable isotopes of elements to label cell. Like in MAR, some substrates are labeled with heavy isotopes (e.g. 13C) and incubated in sample. During incubation substrates are used for cell compartments like DNA and RNA making nucleic acids “heavy”. After incubation, heavy isotope containing cells are separated from other cells and can be used for downstream analyses. SIP enables to follow a specific substrate through metabolic pathways. One of main advantage of SIP is the heavy and normal cells can be used in all downstream analyses like Phylogenetic sequencing or fingerprinting (Radajewski et al., 2000) (Figure 2.15.). Factors affecting the efficiency of the technique are operative anabolic pathway, the substrate for assimilation, the duration of label addition, the relative abundance of naturally occurring unlabeled substrate, and the rate of DNA synthesize of the microbial populations involved. SIP is much cheaper than MAR-FISH and can be used identify substrate degrader in a community (Collins et al., 2006). Several studies used SIP technique in identification of selected members of metabolic pathways like syntrophic propionate oxidation in flooded rice field soil and phenol-consuming populations in an aerobic bioreactor (Manefield et al., 2002; Lueders et al., 2004).

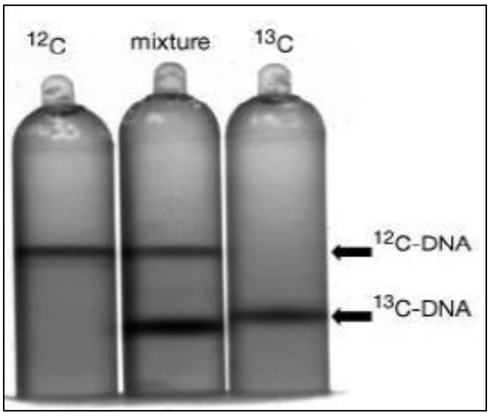


Figure 2.15. Principle of SIP (Radajewski et al., 2000)

2.5.6.7. Phylogenetic Micro-arrays. In micro-arrays, DNA fragments are immobilized on a coated surface and hybridized with different probes. Relative abundance of specific DNA is used to analyze similarities or differences of various microorganisms (Call et al., 2003). Although micro arrays are very suitable for using in microbial ecology; complex systems like environmental samples are hard to process.

3. PROBLEM DEFINITION AND AIM

The veterinary antibiotics used in animal husbandry cause several problems after excretion from animal body. Anaerobic manure digester performances are decreasing due to inhibition of various microbial communities by antibiotics present in animal manure. In order to operate these system efficiently, the microbial dynamics in digesters should be identified. There is a lack of research in the area of characterization of microbial communities and diversities during the anaerobic digestion of manure. Also veterinary antibiotics pass to soil by manure amendment which introduces antibiotics to soil ecosystem. Antibiotics tend to bioaccumulate in soil due to chemical structure and characteristics of the compound. The level of antibiotics in soil ecosystem then rise with every mixing of manure with soil. The presence of antibiotics may also trigger antibiotic resistance gene formation which helps opportunist pathogens to gain resistance which may pose a serious threat to public health.

Therefore, this study aims to analyze microbial ecology of anaerobic digestion of manure under different operating conditions in the presence of selected antibiotic. In the light of this information, effect of antibiotics on microbial communities and their dynamics within the system can be understood. Change in soil microbial communities will be also investigated due to manure amendment and introduction of antibiotic. In addition to this, the antibiotic resistance gene promotion in systems due to presence of antibiotics will be analyzed to determine the size of the threat. Based on this, the objectives of the research are outlined below as to investigate:

- the antibiotic level in fresh manure, excretion pattern and recovered concentrations of antibiotic
- the inhibitory effect of antibiotic on biogas production
- identification of microbial communities involving biogas production process
- effect of selected operational parameters on biogas production and antibiotic eliminations well as on microbial structures and dynamics
- the change in microbial communities in soil due to manure application

- the resistance gene promotion due to presence of antibiotic in manure digestion and soil ecosystems

4. MATERIALS AND METHODS

4.1. Animal Medication and Manure Sampling

Female, Holstein race, 2.5-3.5 years old, 400-500 kg body mass dairy cows were kept in a pen at the Istanbul University Veterinary Faculty Barn. Blank manures were collected from unmedicated animals. Medicated animals were injected with 50 ml Oxytetracyline injection solution (20 mg/kg) under commercial name Teknomycin LA 200 (TEKNOVET, Turkey) once. This is a standard dosage in veterinary practice. Equal doses were injected to right and left body between *musculus semitendinosus* and *musculus semimembranosus* muscles. Medicated manures were collected from rectum every 24 hours for 20 days and used in microcosm tests. Later manure was collected only for the first five days and mixed together until homogenous state has been established. This mixed manure was then lableled as “medicated manure” and used throughout the digester experiments. All collected manure samples were stored in volume of 1 L containers and brought to laboratory in cold chain. Until the experiments, samples were kept in +4^{0}C.

4.2. Digester Operations

4.2.1. Microcosm Digesters

Two sets of microcosm digesters have been prepared in the study. OTC in different concentrations was added to first set (name of the first set was OTC) in order to monitor inhibitory effect of pure parental compound. The second set (name of the first set was OMG) was prepared with manure samples of OTC medicated animal in order to see effect of metabolized compound. Both sets were prepared same wise little exceptions.

Manure samples were diluted with tap water and mixed with the seed sludge (1:5 (v/v)) to a final TS concentration of 5%. Prior to microcosm set-up, pH was set to 6.8. 40 ml slurry was added into 120 ml serum bottles (Figure 4.1.). For the first set OTC concentrations of 50, 100 and 200 mg/l were added into non medicated slurries. In the second set, medicated manure were collected on 1st, 2nd, 3rd, 5th, 10th, 15th and 20th day of animal excretion and were used in microcosm test. All samples and negative control microcosms (for each set) containing no oxytetracycline were set-up in triplicate. Microcosms were flushed with nitrogen gas for 3 minutes to obtain anaerobic conditions. After that, microcosms were incubated on a shaker in a warm room (37.1 ± 1.0 °C) at 100 rpm. Digestion time of the first set was 60 days where 30 days of digestion was applied to second set.

Figure 4.1. 120 ml serum bottle with 40 ml active volume.

4.2.2. Batch Digesters

Digesters were prepared in 1 L glass reactors in which active volume was 600 ml. For each reactor, 1 liter slurry was prepared which contains 1/10 seed sludge. While 600 ml slurry was put in the reactor, 100 ml slurry was analyzed as the sample of the time zero. The remaining 300 ml slurry was stored at 4°C and used to replace the lost volume which was taken from the digester for sampling. The pH of each digester was set to be pH 7.0. Digesters were flushed with nitrogen gas for 5 minutes to remove oxygen. After the controlling of the air-tightness, digesters were placed on the temperature controlled incubator shaker as seen Figure 4.2.

Figure 4.2. Digesters placed on the temperature controlled incubator shaker.

The amount of biogas produced was measured with the miligas counters. Gas compositions were determined using Gas Chromatograph HP Agilent 6850 with a thermal conductivity detector and HP Plot Q Column (30 m, 530 μm). At the each sampling time approximately 100 ml slurry was taken for physicochemical, analytical and molecular analysis.

In the Set1, temperature was 37 ± 1.0 °C and mixing rate was 90 rpm. In this set, digesters which contain low solid content of 5-6% and high solid content of 8-9% TVS were designed for the investigation of the effect of different solid contents. For this set, hydraulic retention time was set to 30 days and samples were collected in every 10 days.

In the Set2, temperature was 37 ± 1.0 °C and mixing rate was 120 rpm. In this set, digesters which contain low solid content of 5-6% and high solid content of 8-9% TVS were designed for the investigation of the effect of different solid contents. For this set, hydraulic retention time was set to 30 days and samples were collected in every 10 days.

In the Set3, temperature was 55 ± 1.0 °C and mixing rate was 90 rpm. In this set, digesters which contain low solid content of 4% and high solid content of 5.0-6% TVS were designed for the investigation of the effect of different solid contents. For this set, hydraulic retention time was set to 20 days and samples were collected in every 5 days. Temperature was as follows: 37 °C for 1.day, 40 °C for 2.day, 45 °C for 3. day, 50 °C for 4. day and 55 °C for 5. day for acclimatization of microorganisms to increasing temperature.

In the Set4, temperature is 55 ± 1.0 °C and mixing speed is 120 rpm. In this set, digesters which contain low solid content of 4 % and high solid content of 5-6% TVS were designed for the investigation of the effect of different solid contents. Set4 was clone of Set3 except mixing rate. Initial conditions of all digesters for both two sets are given in Table 4.1.

Table 4.1. Initial conditions in the digesters.

Sets	Digesters	Temperature (^{0}C)	Mixing Rate (rpm)	TVS (%)
Set1	S1	37	90	Low TVS content (5-6% TVS)
	S3			
	S4			High TVS content (8-9% TVS)
	S6			
Set2	S1	37	120	Low TVS content (5-6% TVS)
	S3			
	S4			High TVS content (8-9% TVS)
	S6			
Set3	S1	55	90	Low TVS content (4% TVS)
	S3			
	S4			High TVS content (5-6% TVS)
	S6			
Set4	S1	55	120	Low TVS content (4% TVS)
	S3			
	S4			High TVS content (5-6% TVS)
	S6			

4.3. Soil Experiments

Soil experiments were done by incubating selected soil sample at RT for 150 days after amendment with manure and digestate. Suitable soil samples (loam, sandy loam or silt loam) were collected with the help of Department of Forestry in Faculty of Forestry at Istanbul University. Soil samples from several sampling sites were analyzed for their texture and suitable samples were selected for soil microcosm experiments.

For soil microcosm experiments, 2 sets (Sandy loam and loam) microcosms were prepared. Each set contains one control and three different amendments (Spiked manure, medicated manure and digestate) (Table 4.2.). All microcosms were incubated in the dark at RT. Humidity was maintained by adding 10 ml of water each week. Each microcosm contains 400 g of soil-manure amendment with amendment ratio 1:10 (w/w). Soil-manure was mixed well until homogenous state was reached. Sampling was done each 15 days for the first two months and 30 days for the rest of the incubation period.

Other than these samples, previously a sample was obtained from an orchard at Geyve County in city of Adapazarı. After texture analysis, this sample found out not meeting criteria. Therefore, it was used to conduct prestudy experiments. Microcosms were set as described above. Several results were obtained from this microcosm experiment and these were also added to this study.

Table 4.2. Details of soil microcosm sets

Soil Type	Control	Spiked Manure	Medicated Manure	Digestate
Silt Loam (Belgrad)	BelB	BelS	BelF	BelD
Sandy Loam (Arboretum)	ArbB	ArbS	ArbF	ArbD
Clay soil (Geyve)	GeyveB	GeyveS	GeyveF	GeyveD

Figure 4.3. 400g soil-manure containing 1 L soil microcosms

4.4. Physical-Chemical Analyses

Table 4.3. Analysis carried out during the operation of anaerobic reactors.

PARAMETER	METHOD	INSTRUMENT
pH	4500-H B Method Electrometric (APHA, AWWA-WPCF-1995)	ORION SA 520 pH meter
COD	5220 D Method Closed Reflux, Colorimetric (APHA, AWWA-WPCF-1995)	HACH COD digester ; HACH DR/3 Spectrophotometer.
VFA	Gas Chromatograph	VFA measurements were carried out in a Perkin Elmer Gas Chromatograph (Clarus 600) with an FID detector and Elite-FFAP column (30 m, 0.32 mm)
Alkalinity	2320 B Method Titration (APHA, AWWA-WPCF-1995)	
TKN	4500 E Method Titration (APHA, AWWA-WPCF-1995)	Gerhardt Vapodest Digester Apparatus
Ammonia-N	4500 E Method Titration (APHA, AWWA-WPCF-1995)	Gerhardt Vapodest Disstillation Apparatus
Orthophosphate	4500-P E Method Ascorbic Acid (APHA, AWWA-WPCF-1995)	HACH DR/3 Spectrophotometer
SS/VSS	2540 Method (APHA, AWWA-WPCF-1995)	-
TS/TVS	2540 Method (APHA, AWWA-WPCF-1995)	-
Biogas	Milligas counter	Ritter (digital counter)
CH_4, CO_2	Gas Chromatograph	HP Agilent 6850 with a thermal conductivity detector and HP Plot Q Column (30 m, 530 µm)

4.5. High Performance Liquid Chromatography (HPLC) analyses

Oxytetracycline concentrations in medicated animal manure, digester sludge and soil-manure amendment were measured by routinely by HPLC analysis.

4.5.1. Chemicals Used

Acetic acid glacial (BDH-GPR), oxalic acid dihydrate (Merck), methanol and acetonitrile (LiChrosolv) were commercially supplied. Oxytetracycline, 4-epi-oxytetracycline, α-Apo-oxytetracycline and β-Apo-oxytetracycline were purchased from Agros Chemicals. Methanol and acetonitrile were HPLC grade. The other chemicals were of analytical grade. Double distilled water was used throughout the analysis.

4.5.2. Instruments and Conditions

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

The Inertsil ODS-3 analytical HPLC column was used at ambient temperature. The mobile phase consisted of 75% 0.1M oxalic acid buffer and 25% Methanol: Acetonitrile (1:1.5) solution which was delivered isocratically at a flow rate of 1 ml/min. The mobile phase was degassed prior to use. The total run time was 30 min. Wavelength for the detection of oxytetracycline was 357 nm. Before every analysis, analytical column was conditioned with the mobile phase, until a clean baseline was observed.

4.5.3. Standard Solutions and Calibration Curves

Stock standard solution of OTC was prepared by dissolving 100 mg of OTC in HPLC grade methanol and stored at -20 C^o. A total of five working standard solutions were prepared in methanol at concentrations 1 mg/l, 10 mg/l, 20 mg/l, 50 mg/l and 100 mg/l. All solutions were protected from direct sun and artificial light in order to prevent photodegredation of OTC. In order to plot a calibration curve, serial dilutions of OTC standard solution (1 mg/l, 10 mg/l, 20 mg/l, 50 mg/l and 100 mg/l) prepared in 100%

methanol, were analyzed by HPLC. Measurements of standard solutions were made as duplicate and OTC peak at 7.3 min confirmed. All results of measurements were analyzed by Class VP (Shimadzu Scientific Instruments Inc.) software (Figure 4.4.).

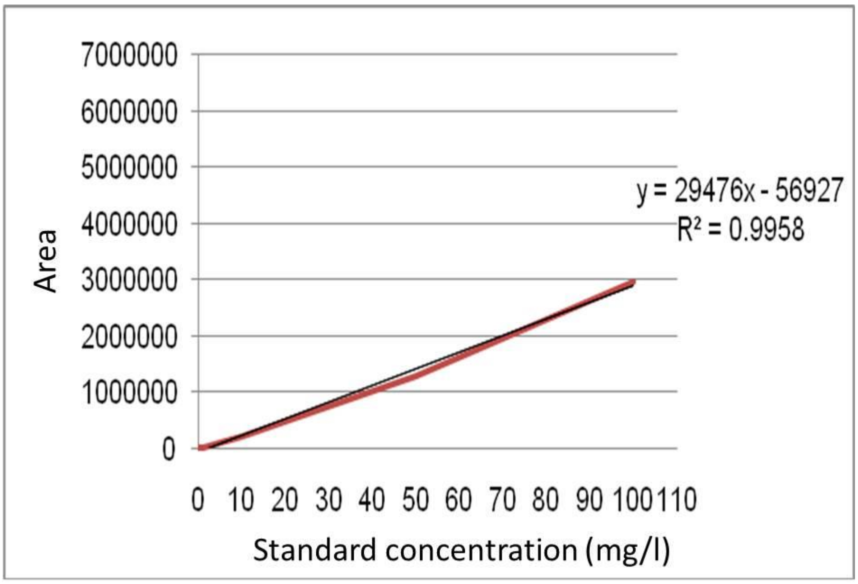


Figure 4.4. Calibration curve of HPLC analysis

4.5.4. Extraction and Recovery Rates

Prior to the extraction of samples, extraction efficiency was determined. 5 g nonmedicated wet manure was spiked with OTC solution in methanol, incubated for 4 hours in dark and extracted as mentioned down below. Spiking levels were 1g/kg, 200 mg/kg, 20 mg/kg and 5 mg/kg OTC in manure. For the soil samples 5gr of soil-manure amendment was spiked with OTC solution in methanol, incubated for 4 hours in dark and extracted. Spiking levels were 25 mg/kg, 12.5 mg/kg, 5 mg/kg and 2.5 mg/kg OTC in soil (Table 4.4.).

Table 4.4. Recovery rates of OTC in manure and soil

Spiked Manure (mg/kg)	**Recovery rate (%)**	**Spiked Soil (mg/kg)**	**Recovery rate (%)**
1000	99±0.02	25	99±0.1
200	92±0.1	12.5	99±0.1
20	85±0.1	5	95±0.6
5	80±0.1	2.5	80±0.5

Extraction was done according to a method modified from Yuan et al. (2010). 5 g wet manure was put into 50 ml polycarbonate centrifuge tubes with 0.5 g oxalic acid ($C_2O_4H_2{\cdot}2H_2O$), 4 ml acetic acid and 7.5 ml of 96% methanol and shaked at 100 rpms for 30 minutes. The tubes were further centrifuged at 11000 rpm for 10 minutes. This procedure was repeated for 3 times and the supernatants were collected in 50 ml volumetric flasks. Flasks were diluted to 50 ml with double distilled water and centrifuged again at 14000 rpm for 3 minutes and filtrated through 0.2 μm Millipore filters. The extracts were kept in 2 ml amber vials at -20 ºC until the day of HPLC analysis.

Recovery rate was calculated according to Equation 4.1.

$$\text{Recovery rate (\%)} = \frac{\text{Amount measured in HPLC analysis}}{\text{Amount spiked to the sample}} \times 100 \qquad (4.1)$$

4.6. Liquid Chromatography-Mass Spectrometry (LC-MS/MS) Analyses

Metabolite analyses of OTC containing samples were done by LC-MS/MS at TÜBİTAK MAM Food Institute. Standard solution were prepared and measured; calibration curve was drawn and recovery rate was calculated. Measurements of samples were done after getting satisfactory results from recovery rate. All analysis was carried out as described elsewhere (Arikan et al., 2006).

4.6.1. Instruments and Conditions

Metabolite measurements were carried out in LS-MS/MS instrument Applied Biosystems 4000 QTRAP (A.B.D.) having Waters XTerra MS C18 column. First mobile phase of the system was 5:95 (methanol:water) containing 0.308μl formic acid where second mobile phase was 5:95 (water:methanol) containing 0.308μl formic acid. Oven temperature was 50^0 C, flow rate was 0.25 ml/min and injection volume was 10 μl. For MS measurements weight table and other parameters were given in Table 4.5.

Table 4.5. Calculated mass and retention time of metabolites in LC-MS/MS

Compound	**Parent compound**	**Daughter Compound**	**Peak time (min)**	**DP**	**EP**	**CE**	**CXP**
OTC	461,121	426,1	8.77	61	10	27	12
4-epi-OTC	461,158	426,2	5.15	76	10	25	12
α-Apo-OTC	443,158	426,2	12.1	76	10	27	12
β-Apo-OTC	443,136	426,2	18	86	10	23	12

4.7. Molecular Tools for Identification of Microbial Community Structures

Identification of molecular community structures was done with the help of DNA and RNA based molecular analyses. On DNA level, PCR, DGGE and 16S rDNA clone library formation has been performed. On RNA level, FISH and Q-PCR has been performed.

4.7.1. Sample Collection and Preparation

Samples for molecular analysis were collected from anaerobic microcosms, lab scale anaerobic digesters and soil microcosms. For DNA-based molecular methods, the GDNA of microbial communities were extracted from collected samples, diluted for downstream molecular analyses and stored at -20ºC. For RNA-based molecular methods total RNA of microbial communities were extracted immediately from collected samples and stored at -80ºC until cDNA synthesis reaction. After synthesis, cDNAs were stored at -20ºC. For FISH studies, sludge and soil samples were transferred into 15 ml sterile falcon tubes with the addition of absolute ethanol (1:1 (v/v)) and fixed in a week, stored at -20ºC.

4.7.2. DNA-based Molecular Analysis

4.7.2.1. Genomic DNA Extraction. DNA was extracted from 0.5 g or 500µl sample by using Fast DNA Spin Kit for Soil (Q-Biogene, Bio 101 Thermo Electron Corporation, Belgium) and a Ribolyser (Fast PrepTM FP120 Bio 101 Thermo Electron Corporation, Belgium) according to the manufacturers' instructions. The methodology of Genomic DNA extraction of by Fast DNA Spin Kit for Soil was as follows: Approximately 0.5 g sludge was added up to lysing matrix tubes provided by the kit. The tube contains mixture of ceramic and silica particles to lyse all microorganisms in sample. Then lysing matrix tubes were spinned in Ribolyser (Fast PrepTM FP120 Bio 101 Thermo Electron Corporation) for 45 seconds at speed of 6.5 m/s. The tubes were then centrifuged at 14000 rpm for 5 minutes. After centrifugation supernatants were transferred to clean 1.5 ml eppendorf tubes and added 250 µl PPS reagent. To mix the composition tubes were shaked by hands for 30 seconds. After mixing the tubes centrifuged again at 14000 rpm for 5 minutes to pellet the precipitate. Supernatants were transferred to 2 ml eppendorf tubes and 1 ml of Binding Matrix Suspension was added to supernatant. Tubes were inverted by hand for 2 minutes to allow binding of DNA to matrix. To settle the silica matrix tubes were incubated 3 minutes at room temperature. 500 µl of supernatant was removed carefully without disturbing settled silica matrix. Then the binding matrix was resuspended in the remaining supernatant. All mixture was filtered and filter was placed to a new tube. Filter was washed by 500 µl SEWS-M wash solution. After washing, filter was dried by centrifugation at 14000 rpm for 2 minutes. Filter was removed to a new tube and 50 µl DES (DNase/Pyrogen free water) was added. The filter with DES was vortexed and then centrifuged at 14000 rpm for 1 minute. Application-ready DNA was obtained in the tube. 1/100 diluted genomic DNA was run on the %1 (w/v) agarose gel, prestained with Ethidium Bromide (EtBr) in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA, pH 8) at 7 V/cm. Gel was visualized by using a gel documentation system, Chemi-Smart 3000 (Vilber Lourmat, France).

4.7.2.2. Polymerase Chain Reaction (PCR). Amplification of 16S rDNA gene sequences was performed by PCR using archaeal and bacterial specific primers. Primers used in this study are given in Table 4.6. and Table 4.7. Bact8f-Bact1541r 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. Bact8f-Bact1541r and Arch07f-Arch1384r amplification products were also used for cloning and sequencing analysis. Bact341fGC-Bact534r and Arch344f-Univ522r primers were used to amplify approximately 200 bp region of 16S rDNA of bacteria and archaea. These PCR products were used in DGGE analysis. PCR reactions were performed in a 50 µl (total volume) mixture containing 0.2 µM forward primer, 0.2 µM reverse primer, each DNTP (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 1 µl of template. Amplification was performed with a thermal cycler (TECHNE-TC 512 and TECHNE-TC 5000). PCR programs in thermal cycler were given in Table 4.7. and 4.8. 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, Chemi-Smart 3000 (Vilber Lourmat, France).

Table 4.6. Tetracycline resistance gene coding primers used in this study

Primer	**Sequence of primer**	**Reference**
tet(M) f	ACA GAA AGC TTA TTA TAT AAC	Aminov et al., 2001
tet(O) f	ACG GAR AGT TTA TTG TAT ACC	Aminov et al., 2001
tet(B) f	TAC GTG AAT TTA TTG CTT CGG	Aminov et al., 2002
tet(K) f	GAT CAA TTG TAG CTT TAG GTG AAG G	Kumar at al., 2005b
tet(M) r	TGG CGT GTC TAT GAT GTT CAC	Aminov et al., 2001
tet(O) r	TGG CGT ATC TAT AAT GTT GAC	Aminov et al., 2001
tet(B) r	ATA CAG CAT CCA AAG CGC AC	Aminov et al., 2002
tet(K) r	TTT TGT TGA TTT ACC AGG TAC CAT T	Kumar et al., 2005b

Table 4.7. Bacterial and archaeal oligonucleotide primers used for PCR amplification

Primer	**Target**	**Experimental Stage**	**Annealing (°C)**	**Position[1]**	**Reference**
Bact341f_GC[2]	Bacterial 16S rDNA	DGGE	55	341-357	Muyzer et al., 1993
Bact534r				534-518	
Bact8f		Cloning		8-27	Edwards et al., 1988
Bact1541r				1541-1522	
Bact342f		Sequencing		342-361	
Arch07f	Archaeal 16S rDNA	First round of nested PCR - Cloning	52	07-24	Lueders et al., 2004
Arch1384r				1384-1368	
Arch344f_GC[2]		DGGE	53	344-358	Raskin et al., 1994
Arch855r				855-836	Shinzato et al.,1999
Univ522r				522-504	Amann et al., 1995
M13f	β-galactosidase	Clone screening	54	–	Schrenk et al., 2003
M13r					

[1] *Escherichia coli* numbering.

[2] 5′-GC clamp on Arch344f and Bact341f (GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGG).

Table 4.8. PCR conditions used in the study

Primers	**Denaturation (°C)**	**Time (s)**	**Annealing (°C)**	**Time (s)**	**Elongation (°C)**	**Time (s)**	**#of cycles**
Bact8f-Bact1541r Bact341fGC-Bact534r Bact342f	94	45	55	45	72	60	30
M13f-M13r	94	45	55	45	72	60	30
Arch07f-Arch1384r	94	45	52	45	72	60	35
Arch344-Arch855 Arch344-Univ 522	94	30	53	30	72	60	35

4.7.2.3. Denaturant Gradient Gel Electrophoresis (DGGE). Acrylamide solution (30%), deionised formamide, urea and molecular biology grade ammoniumpersulfate were commercially supplied (Applichem, Germany). Both samples and the positive clones were run on an Ingeny phorU DGGE system (the Netherlands) (Figure 4.5.). The first step was the assembly of the perpendicular gradient gel sandwich. The thickness of the sandwich was established by using between two glass plates. Before assembly, glass plates were cleaned carefully with 70% EtOH to avoid any particle matter which may affect the gel. The position of spacers were checked to avoid any leakage and glass plate sandwich then placed on the casting stand. The next step was preparation of the denaturing gradient gel. 10% (w/v) acrylamide 40% denaturant solution was prepared by mixing 83 ml of 30% acrylamide with 5 ml 50xTAE (2.0 M Tris, 50 mM EDTA, and 1.0 M acetic acid) and 40 ml formamide and 42 g urea. 70% of denaturant concentration was reached by adding 70 ml formamide and 73.5 g urea to 83 ml of 30% acrylamide and 5 ml 50xTAE (2.0 M Tris, 50 mM EDTA, and 1.0 M acetic acid). Into both solutions, distilled water was added up to 250 ml. After solutions were prepared, they were filtered through 0.45 μm filter and sonicated for 15 minutes. The bottles were wrapped with foil paper to avoid sunlight and stored at 4 °C for further uses. Into two beakers, 25 ml of 10% (w/v) acrylamide solutions containing 40% and 70% denaturants were poured. To both solutions, 75 μl freshly prepared 20% ammonium per sulfate (APS) and 7.5 μl TEMED was added and immediately transferred to gradient forming system. With the gradient forming system and a pump, solutions were transferred to the form gel sandwich. After polymerization, a stacking solution (6-10 ml) excluding denaturants was mixed with APS and TEMED and added over the polymerized gel.

Figure 4.5. DGGE equipment used in the study (Ingeny phorU)

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

The last step was staining and visualizing gels. The core was taken from the tank and gel sandwiches were separated from it. Glass plates were disassembled and the direction of gel was marked with a cut on the upper left corner. 50 µl of 1:100000 diluted SYBR Gold DNA staining dye was added to 500 ml 1xTAE washing buffer and gels were incubated for 15 minutes. Gels were washed 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 6.1 Software (Applied Maths, Belgium). Similarities between tracks were calculated by using the Dice coefficient (S_D) (unweighted data based on band presence or absence) and UPGMA clustering. For analysis using Dice coefficient 0.7% optimization and 0.5% band position tolerance was applied. This was the minimum tolerance at which all marker lanes clustered at 100%. For intensity analysis, samples were clustered depending on band weights by using Pearson coefficient and UPGMA clustering.

4.7.2.4. Analysis of DGGE Gels using Bionumerics. DGGE gels were analyzed by using Bionumerics 6.1 software (Applied Maths, Kortrijk, Belgium) to determine the phylogenetic relationship between the samples. Presence-absence data of DGGE fingerprint were created based on similarity matrix obtained using Bionumerics software. Band intensity analysis was done automatically by the software if selected. Bionumerics software also used to create relation analysis tree from DGGE gels photos. Different correlation analysis can be performed with the software. Similarities between tracks were calculated by using the Dice coefficient (S_D), unweighted data based on band presence or absence, whole-densitometric-curve-based Pearson product-moment correlation coefficients (r) and Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) clustering (Figure 4.6-4.8.).

Dice Coefficient. For analysis using Dice coefficient a band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at 100%. The Bionumerics package used the Dice algorithm with the numbers of common and total bands to generate similarity coefficients between banding patterns. For two banding patterns A and B, the Dice similarity coefficient is calculated as,

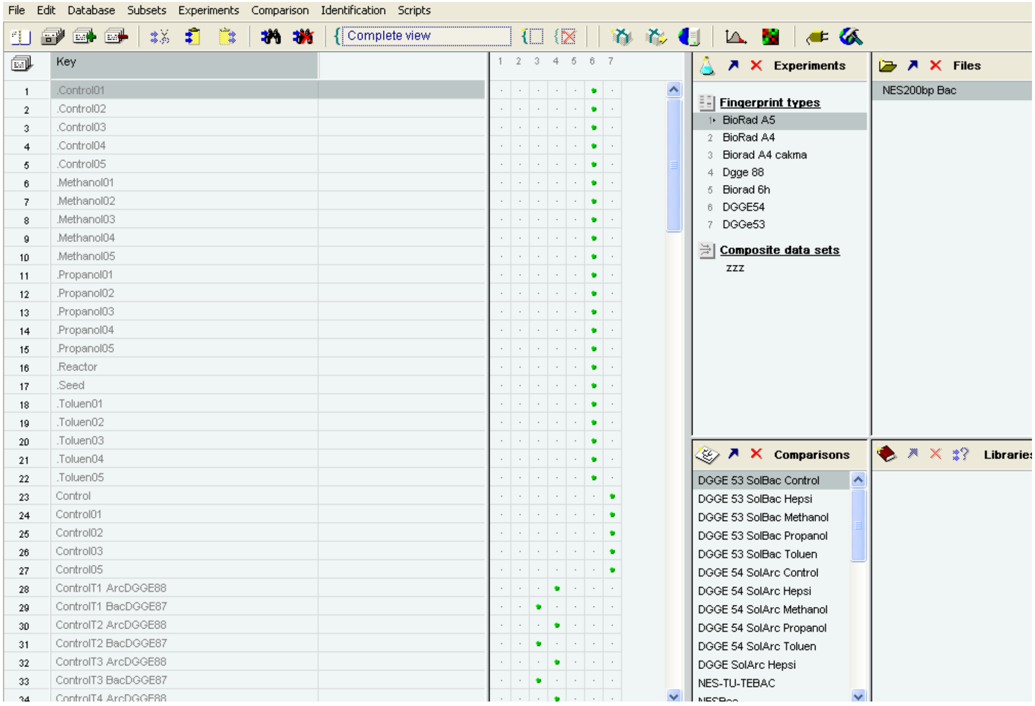
$$\text{Similarity}_{\text{Dice}} = 2x\sum AB \, / \, [(2x\sum AB)+(\sum Ab)+(\sum aB)] \tag{4.2}$$

where AB is the number of bands common to both banding patterns, Ab is the number of bands found in banding pattern A but not banding pattern B, and aB is the number of bands found in banding pattern B but not banding pattern A. The Bionumerics program was used to calculate the best optimization and tolerance values for the comparison. This index ranges from 0 (no common bands) to 1 (identical band patterns). Within Bionumerics, optimization refers an adjustment of bands beyond normalization and was necessary when imperfect normalization resulted in residual shifts. Likewise, tolerance refers to the total distance that bands in different lanes differed by before they were determined to be distinct. The default values were used for optimization and tolerance and were 0.5 and 0.7%, respectively. Use of fuzzy logic in Bionumerics results in bands being scored as identical only when there was zero distance between them, meaning that with additional distance (in pixels) in band position, the matching score was decreased until the maximal position tolerance was reached, after which the similarity score was zero. This allowed larger tolerances to be chosen while still obtaining meaningful clustering.

Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation coefficient.

Unweighted Pair-Group Method with Arithmetic Mean (UPGMA). UPGAMA is an example of clustering method and has built on the assumption that tree is additive. Thus, all taxa are equally distant from a root in UPGMA tree which is an unlikely assumption.

Neighbour Joining Method (NJ). NJ is similar to UPGMA in manipulating a distance matrix as reducing it in size at each step, and then reconstructs the tree from that series of matrices. It differs from UPGMA in that it does not construct clusters but directly calculates distances to internal nodes. NJ does not assume that all taxa are equally distant from a root. NJ is like parsimony, a minimum-change method, but it does not guarantee tree with the smallest overall distance.



	Key
1	Control01
2	Control02
3	Control03
4	Control04
5	Control05
6	Methanol01
7	Methanol02
8	Methanol03
9	Methanol04
10	Methanol05
11	Propanol01
12	Propanol02
13	Propanol03
14	Propanol04
15	Propanol05
16	Reactor
17	Seed
18	Toluen01
19	Toluen02
20	Toluen03
21	Toluen04
22	Toluen05
23	Control
24	Control01
25	Control02
26	Control03
27	Control05
28	ControlT1 ArcDGGE88
29	ControlT1 BacDGGE87
30	ControlT2 ArcDGGE88
31	ControlT2 BacDGGE87
32	ControlT3 ArcDGGE88
33	ControlT3 BacDGGE87
34	ControlT4 ArcDGGE88

Fingerprint types

1 BioRad A5
2 BioRad A4
3 Biorad A4 cakma
4 Dgge 88
5 Biorad 6h
6 DGGE54
7 DGGe53

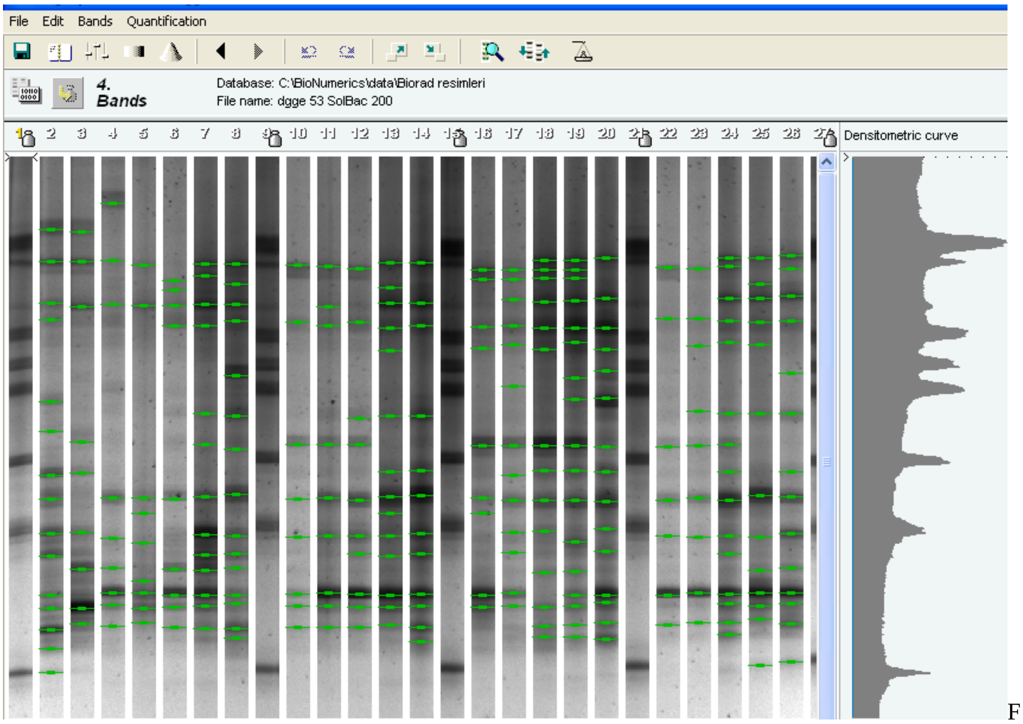
Composite data sets

zzz

NES200bp Bac

DGGE 53 SolBac Control
DGGE 53 SolBac Hepsi
DGGE 53 SolBac Methanol
DGGE 53 SolBac Propanol
DGGE 53 SolBac Toluen
DGGE 54 SolArc Control
DGGE 54 SolArc Hepsi
DGGE 54 SolArc Methanol
DGGE 54 SolArc Propanol
DGGE 54 SolArc Toluen
DGGE SolArc Hepsi
NES-TU-TEBAC

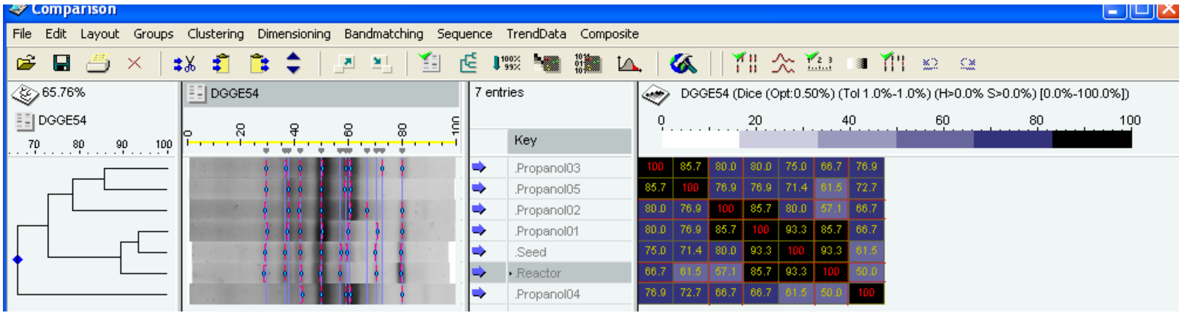
Figure 4.6. Main operations window of Bionumerics software.



Database: C:\BioNumerics\data\Biorad resimleri
File name: dgge 53 SolBac 200

F

Figure 4.7. Selection of bands in fingerprinting of photos window.



Key							
.Propanol03	100	85.7	80.0	80.0	75.0	66.7	76.9
.Propanol05	85.7	100	76.9	76.9	71.4	61.5	72.7
.Propanol02	80.0	76.9	100	85.7	80.0	57.1	66.7
.Propanol01	80.0	76.9	85.7	100	93.3	85.7	66.7
.Seed	75.0	71.4	80.0	93.3	100	93.3	61.5
.Reactor	66.7	61.5	57.1	85.7	93.3	100	50.0
.Propanol04	76.9	72.7	66.7	66.7	61.5	50.0	100

Figure 4.8. Relation analysis window of Bionumerics software.

4.7.2.5. Generation of 16S rDNA Cloning Library. Two clone libraries, a bacterial and an archaeal, were generated to analyze microbial communities. Whole bacterial 16S rDNA (ca.1.5 kb) was amplified by using primers Bact8f-Bact1541r, as described above. PCR products were cloned with a TOPO TA cloning kit (Invitrogen Ltd.) and gene libraries were screened by DGGE. Nearly whole archaeal 16S rDNA (ca.1.3 kb) was amplified by using primers Arch07f-Arch1384r, as described above. PCR products were cloned with a TOPO TA Cloning Kit for Sequencing (Invitrogen Ltd.) and gene libraries were screened by DGGE. The procedure of generating 16S rDNA Clone Library was as follows:

The process was started with preparing 6 µl reaction mix by adding 3 µl PCR product, 1 µl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), 1 µl TOPO vector and 1 µl Sterile Water. The solution was mixed gently and incubated at room temperature (R.T.) for 20 minutes. After incubation reaction mix was placed on ice before its usage in One Shot TOPO transformation step. One shot TOPO transformation was started by thawing one vial of One Shot TOPO reaction mix on ice. After thawing, 2 µl of reaction mix was added to One Shot vial. The solution was mixed gently without pipetting or shaking. The solution was incubated on ice for 30 minutes. After incubation, tube was subjected to a heat shock at 42°C for 30 seconds and transferred immediately to ice and 300 µl of S.O.C. medium at R.T. (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The solution was shaked horizontally for 60 minutes. Three LB plates containing 50 µg/ml Kanamycin were warmed to R.T. 100 µl of solution was spread on plates using glass spreader. The plates were incubated overnight and white colonies were observed after incubation. Colonies were picked from plate and transferred into 200 µl PCR tubes containing 50 µl TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). Colonies were boiled at 95°C for 5 minutes then frozen at -20°C overnight. Thawed solution was used as templates for PCR. The 16S rDNA fragments were isolated from vector by PCR with primers M13f-M13r (M13 Forward 5′-GTA AAA CGA CGG CCA G-3′/ M13 Reverse 5′-CAG GAA ACA GCT ATG AC-3′). From PCR products of reaction with M13f-M13r primers nested PCRs were done to screen the clones in DGGE. Nested PCRs were performed for bacterial and archaeal separately as described above. The screening of gene library was done with DGGE. The gels were stained with 25 µl EtBr (10 mg/ml) instead of SYBR Gold.

4.7.2.6. Phylogenetic Analysis of Microbial Community. Phylogenetic analysis of microbial community was done by sequencing clones. Sequencing of the clones was done commercially by Macrogen (Holland).

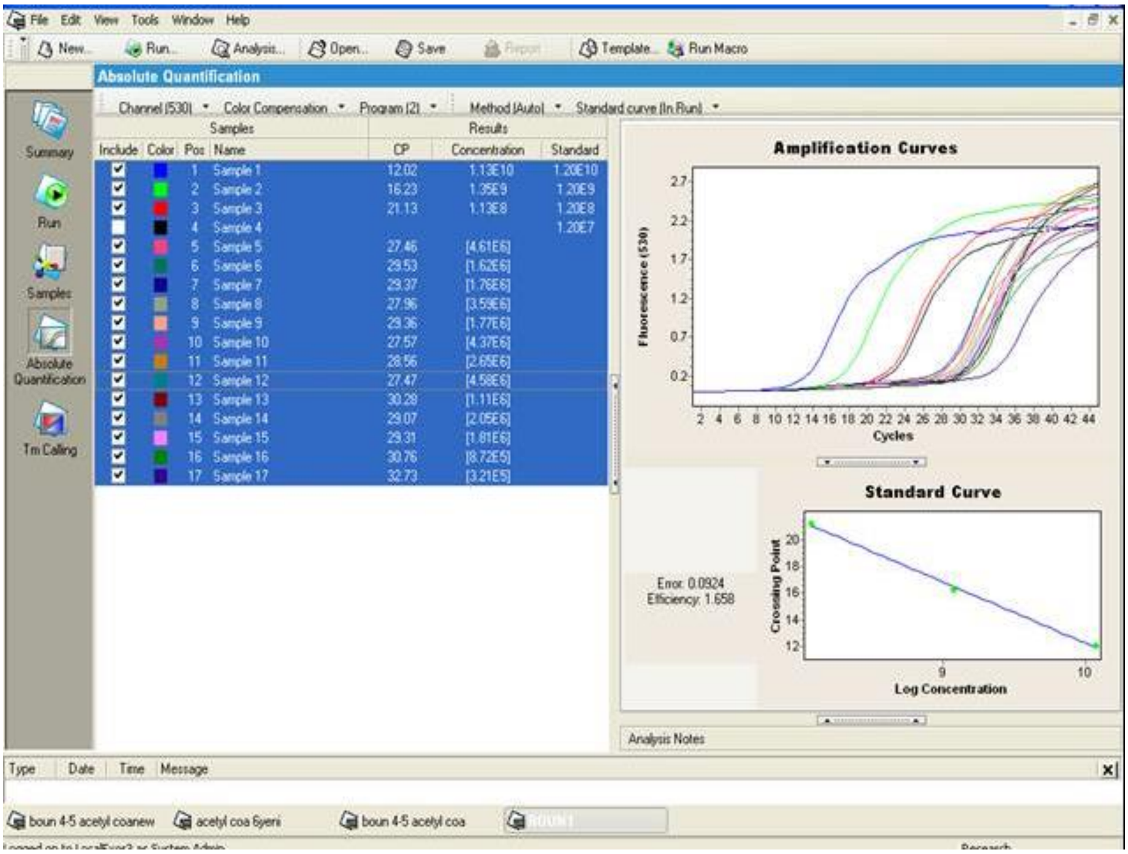
Partial 16S rRNA gene sequences were analyzed and manually edited in Chromas software package version 1.45 (http://www.technelysium.com/au/chromas.html). The 16S rDNA sequences were checked for chimerical constructs by using the CHECK-CHIMERA program of the Ribosomal Database Project (Cole et al., 2007). Homology searches of the EMBL and GenBank DNA databases for the 16S rRNA gene sequences were performed with FASTA (Pearson, 1988) provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/ nucleotide.html) to identify putative close phylogenetic relatives. 16S rRNA genes sequences showing 95% similarity or higher were considered to belong to the same phylotype. Related 16S rRNA gene sequences were placed within tentative taxa (between Phylum and Order) by determining the taxonomic class (using the NCBI taxonomy database) of the closest relative in GenBank of sequences that formed a phylogenetic clade. Sequences that showed no or low (below 70%) relation with known bacterial or archaeal phylogenetic groups were listed as unclassified. The distribution of clone types present in the clone libraries was determined and used to calculate the Shannon-Weaver index {$H = -\Sigma[n_i \cdot \log(n_i)]$, where n_i is the relative contribution of clone type i to the whole library}. Coverage was calculated as $1 - (n_1/N)$, where n_1 is the number of clone types that was encountered only once in the library and N is the total number of clones analyzed. The Chao1 estimator of species (here, clone type) richness (S_{chao1}) was calculated as; $S_{obs} + n_1^2/2n_2$, where S_{obs} is the number of observed different clone types, n_2 is the number of clone types encountered twice in the library and m is n_1/n_2.

4.7.3. RNA-Based Molecular Analysis

4.7.3.1. Total RNA Extraction. Total RNA was extracted according to the manual instructions. 0.5 g soil or 500 µl sludge was added to Lysing Matrix tubes. 1 ml of RNApro™ Soil Lysis Solution was added to the tube and vortexed to mix. Tubes were spinned in FastPrep® instrument for 40 sec at 6 m/s. Tubes were then centrifuged at 14000 rpm for 5 minutes. Supernatant was transferred to new tube. 750 µl phenol: chloroform (1:1) was added to the tube and vortexed to mix. Tubes were incubated for 5 min at R.T. and then centrifuged at 14000 rpm for 5 min. at 4 ^{0}C. Upper aqueous layer was taken to a new tube and 200 µl Inhibitor Removal Solution was added. Tubes were mixed by 5 min incubation on shaker table at 140 rpm. After incubation tubes were centrifuged for 5 min at 14000 rpm. Supernatant was taken to new centrifuge tube. 660 µl of cold absolute isopropanol was added to sample and mixed by vortex. Tubes were incubated at -20 ^{0}C for at least 30 min (can be extended to overnight). After incubation tubes were centrifuged at 14000 rpm for 15 min at 4 ^{0}C. Supernatant was discarded and pellet was washed with 500µl cold 70% ethanol (made with DEPC-H_2O). Ethanol was removed and pellet was air dried for 5 min. Pellet was then resuspended in 200µl of DEPC-H_2O. 600µl of RNAMATRIX®Binding Solution and 10 µl of RNAMATRIX®Slurry was added to the tube and incubated on shaker table for 5 min at 140 rpm. Tubes were pulse spinned for 10 sec to pellet RNA and supernatant was discarded. Pellet was resuspended with the addition of 500µl of RNAMATRIX® Wash Solution. Tubes were pulse spinned for 10 sec to pellet RNA and supernatant was discarded. Pellet was air dried for 5 min at RT. 50 µl of DEPC-H_2O was added to pellet and incubated for 5 min. Tubes were pulse spinned for 10 sec and RNA containing supernatant was transferred to a new tube. 50 µl of DEPC-H_2O was added to pellet and incubated for 5 min. Tubes were pulse spinned for 10 sec and RNA containing supernatant was transferred to a new tube. Total RNA in the tube was evaluated by visualization and stored at -80^{0}C until cDNA synthesis.

4.7.3.2. cDNA Synthesis. cDNA was synthesized using SuperScript® VILO™ cDNA Synthesis Kit according to procedure. The following components were taken in a tube on ice: 5X VILO™ Reaction Mix (4 µl), 10X SuperScriptR Enzyme Mix (2 µl), RNA (up to 2.5 µg) x µl, DEPC-treated water (20 µl). Tube contents was mixed gently and incubated as following at 25°C for 10 minutes, at 42°C for 60 minutes and the reaction was terminated at 85°C at 5 minutes. The cDNA generated from total RNA quantities above 100 ng will need to dilute. In this study 20 times diluted cDNA was used for Q-PCR experiment.

<u>4.7.3.3. Q-PCR Analyses.</u> Roche LightCycler DNA Master SYBR Green I kit and Roche Light Cycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) were used for the Q-PCR assays. Light Cycler Software 4.05 was used to analyze output of the Q-PCR assays (Figure 4.9.). The primers used in this study are in Table 4.9.



Channel (530) Color Compensation Program (2) Method (Auto) Standard curve (In Run)

Samples				Results		
Include	Color	Pos	Name	CP	Concentration	Standard
☑	■	1	Sample 1	12.02	1.13E10	1.20E10
☑	■	2	Sample 2	16.23	1.35E9	1.20E9
☑	■	3	Sample 3	21.13	1.13E8	1.20E8
☐	■	4	Sample 4			1.20E7
☑	■	5	Sample 5	27.46	[4.61E6]	
☑	■	6	Sample 6	29.53	[1.62E6]	
☑	■	7	Sample 7	29.37	[1.76E6]	
☑	■	8	Sample 8	27.96	[3.59E6]	
☑	■	9	Sample 9	29.36	[1.77E6]	
☑	■	10	Sample 10	27.57	[4.37E6]	
☑	■	11	Sample 11	28.56	[2.65E6]	
☑	■	12	Sample 12	27.47	[4.58E6]	
☑	■	13	Sample 13	30.28	[1.11E6]	
☑	■	14	Sample 14	29.07	[2.05E6]	
☑	■	15	Sample 15	29.31	[1.81E6]	
☑	■	16	Sample 16	30.76	[8.72E5]	
☑	■	17	Sample 17	32.73	[3.21E5]	

Error: 0.0924
Efficiency: 1.658

Figure 4.9. Analysis of output by Light Cycler Software 4.05

Table 4.9. Information regarding the 16S rDNA spesific primers used

Primer	**Target**	**Annealing Temperature**	**Reference**
Bac519f	Bacteria	53C°	Lane,1991
Bac907r			
Arc349f	*Archaea*	55C°	Takai and Horikoshi, 2000
Arc806r			
Met348f	Methanogens	55C°	Sawayama et al., 2006
Met786r			
MSaeta170f	*M.Saeta* spp.	59C°	Yu et al., 2006
MSaeta390r			
MBac857f	*M.Bacteriales*	60C°	Yu et al., 2006
MBac1996r			
MMic282f	*M.Microbiales*	59C°	Yu et al., 2006
MMic832r			
MSarles812f	*M. Sarcinales*	55C°	Yu et al., 2006
MSarles1159r			
Tet(M)f	*Tet (M)*	55C°	Aminov et al., 2001
Tet(M)r			
Tet(O)f	Tet (O)	60C°	Aminov et al., 2001
Tet(O)r			

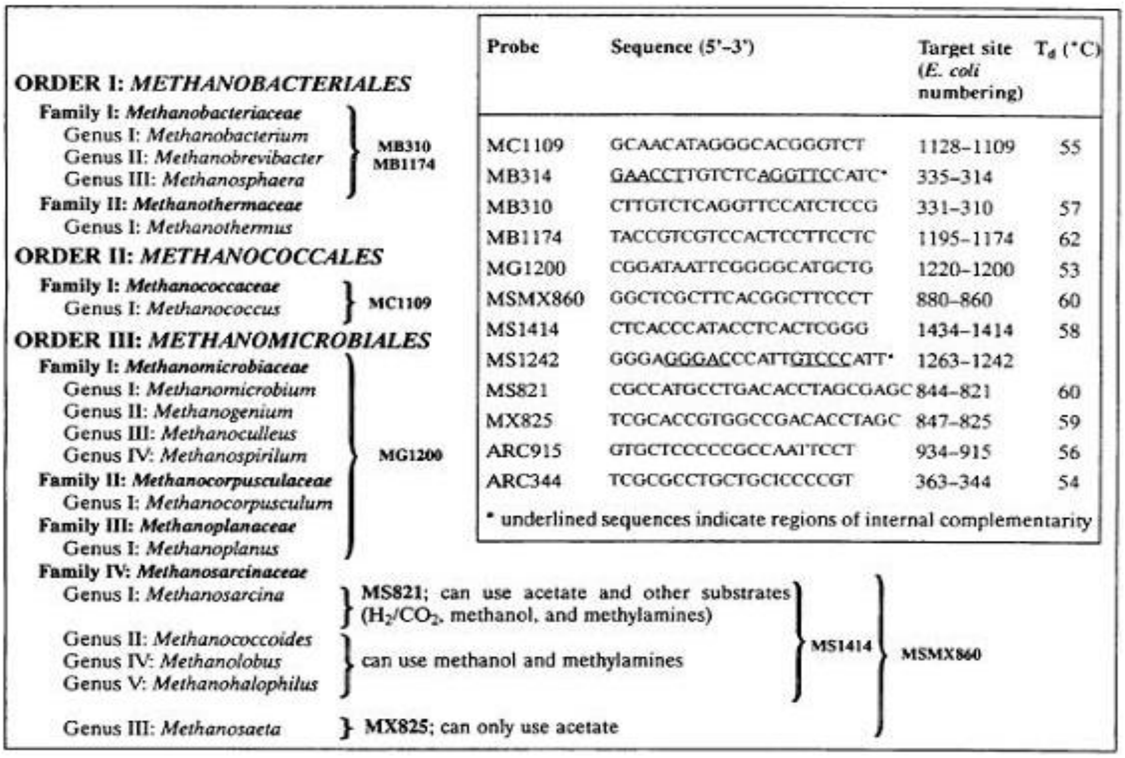
4.7.3.4. Fluorescent in situ Hybridization (FISH). For the FISH analysis, standard steps of FISH technique have been followed.

Short term fixation. Samples were transferred into 15 ml sterile falcon tubes and fixed with absolute ethanol (1:1 (v/v)). Samples were stored at -20°C and fixed with PFA within a week.

PFA fixation. 1 ml ethanol-sample mixture was transferred to 1.5 ml Eppendorf tubes and washed with 0.5 ml 3X Phosphate Buffer Saline (PBS) [130 mM NaCl, 10 mM sodium phosphate, pH 7.2] for two times and resuspended in 0.25 ml 3x PBS. 0.75 ml freshly prepared 4% PFA was added and incubated for at least 3 hours (or overnight) at +4 ^{0}C. After incubation, cells were washed once with 3XPBS and resuspended in 1 ml PBS-absolute ethanol (1:1, v/v) mixture and stored at -20 ^{0}C until hybridization.

DAPI staining. The total cell concentrations present in the samples were previously determined by counting 4,6-diamine phenylindol (DAPI) stained cells. 200 µl fixed samples were put into the eppendorf tubes and centrifuged at 13000 rpm for 3 minutes. After the centrifugation, 500 µl 1XPBS was added to tubes and resuspended by syringe. Then, the mixture was centrifuged at 13000 rpm for 3 minutes again. Following centrifugation, supernatant was put out without destroying the pellet. 500 µl 1XPBS was added to the tube and resuspended secondly. The supernatant was put out and 500 µl MQ water was added to tubes for the dilution. After the suspension, 20-30 µl samples were taken on each well and dried in the incubator. The slides were dehydrated in the ethanol series (50%, 80%, and 100%) for 3 minute at each concentration. After the dehydration, 49 µl 1XPBS, then 1 µl DAPI stain was added on each well. The slides were incubated at 46°C for 20 minutes. After that, slides were washing into two washing buffer (40 ml 1XPBS) for 7 minutes in each of them. Finally, slides were put in two 40 ml MQ water for 1 minute in each of them. Slides were dried in incubator and covered with lamel by enamel.

Hybridization. 20-25 µl of the fixed samples were transferred to new microfuge tubes. The amount was determined by the microorganism density in the sample (DAPI staining). The samples were than washed 2 times with 3X PBS and once with ddH_2O. After washing, the pellet was resuspended in 0.5 ml ddH_2O. The slides were dehydrated through ethanol series (50%, 80%, 96%) for 3 minutes. 17 µl hybridization buffer (2 mg/ml Ficoll, 2 mg/ml Bovine serum albumin, 2 mg/ml polyvinyl pyrolidone, 5 mM EDTA, Tris HCl, pH 7.2, 25 mM NaH_2PO_4, NaCl, pH 7.0, 0.1% SDS) and 3µl targeted probes were added and incubated at the optimal hybridization temperature for the given probe for 4 hours (Figure 4.10.). Following hybridization, the cells were washed twice in a wash buffer containing 20 mM Tris-HCl (pH 7.2), 0.01% SDS, 4.5 M NaCl before a final wash in MilliQ water. The cells were resuspended in 200 µl of MilliQ water, and then dried. 10 µl of DABCO [(1,4-diazabicyclo[2.2.2]octane) [Sigma D-2522]: 0.233g DABCO 800 µl ddH_2O 200 µl TRIS-HCl (pH=7.2)] was added to the cells, and a coverslip was applied and sealed with nail polish before epifluorescence microscopy (Table 4.10. and 4.11.).



Probe	Sequence (5'–3')	Target site (*E. coli* numbering)	T_d (°C)
MC1109	GCAACATAGGGCACGGGTCT	1128–1109	55
MB314	GAACCTTGTCTCAGGTTCCATC*	335–314	
MB310	CTTGTCTCAGGTTCCATCTCCG	331–310	57
MB1174	TACCGTCGTCCACTCCTTCCTC	1195–1174	62
MG1200	CGGATAATTCGGGGCATGCTG	1220–1200	53
MSMX860	GGCTCGCTTCACGGCTTCCCT	880–860	60
MS1414	CTCACCCATACCTCACTCGGG	1434–1414	58
MS1242	GGGAGGGACCCATTGTCCCATT*	1263–1242	
MS821	CGCCATGCCTGACACCTAGCGAGC	844–821	60
MX825	TCGCACCGTGGCCGACACCTAGC	847–825	59
ARC915	GTGCTCCCCCGCCAATTCCT	934–915	56
ARC344	TCGCGCCTGCTGCTCCCCGT	363–344	54

* underlined sequences indicate regions of internal complementarity

Figure 4.10. Classification of methanogens in relationship to the oligonucleotide probes used in this study.

Table 4.10. Hybridization buffer solution

Chemical	Concentration	Volume
NaCl	4,5 M	200 µl
Tris HCl pH 7.2	200 mM	100 µl
Denhardt's reagent	10x	200 µl
EDTA	0.5 mM	10 µl
NaH_2PO_4; pH 7.0	250 mM	50 µl
SDS	%10	10 µl
2x dH_20		Up to 1 ml
Total Volume		1 ml

Table 4.11. Washing buffer solution

Chemical	Concentration	Volume
NaCl	0,5 M	10 ml
Tris HCl pH 7.2	200 mM	5 ml
NaH_2PO_4; pH 7.0	250 mM	2.5 ml
EDTA	0.5 M	> 20% FA; 200 µl
SDS	10%	200 µl
Total Volume		50 ml

16S rRNA-targeted oligonucleotide probes used in this study and their target microbial groups nucleotide sequences are listed in Table 4.12. For each hybridization, two negative controls were prepared; one of these controls was used to assess non-specific binding (containing the non-sense probe Non338. This probe does not hybridize with any known bacteria and the other (lacking a probe) was used to monitor autofluorescence. In addition to negative controls, one positive control was prepared to assess success of cell permeabilization and rRNA content of the cells (with universal probe UNIV1392). Whole microbial community in the sludge samples was also stained using DAPI to visualize intact cells in the samples.

Table 4.12. 16S rRNA-targeted oligonucleotide probes used

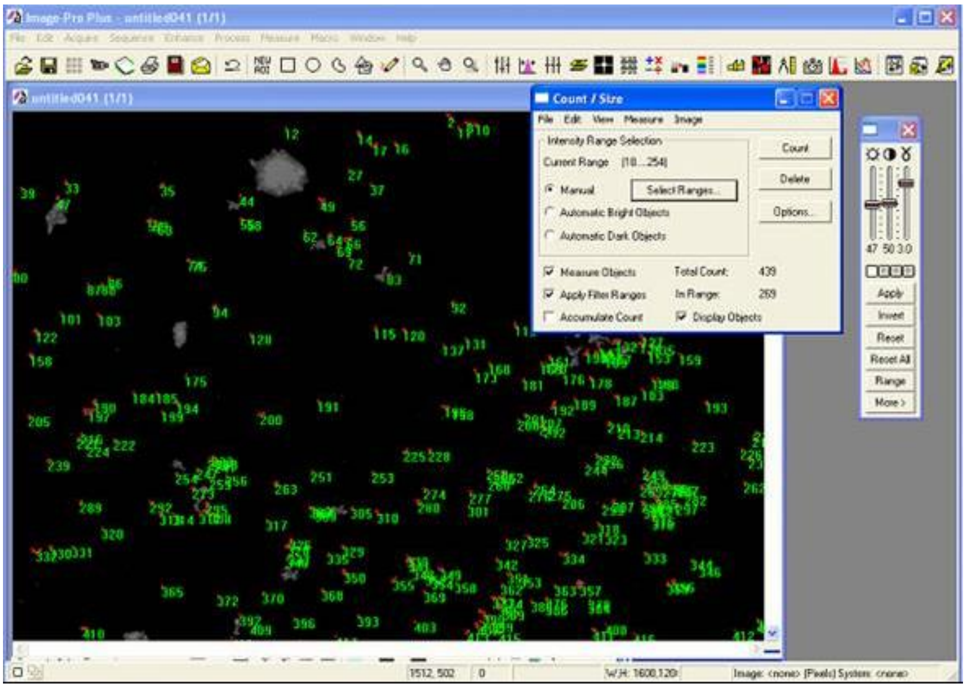
Probe	**Target**	**Sequence (5’-3’)**	**Labeling (5’)**	**Reference**
UNIV1392	All known organisms	ACGGGCGGTGTGTAC	CY3	Pace et al., 1986
ARC915	Archaea	GTGCTCCCCCGCCAATTCCT	CY3	Stahl et al., 1988
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	CY3	Amman et al., 1990
MB310	*Methanobacteriales*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin et al., 1994
MG1200	*Methanomicrobiales*	CGGATAATTCGGGGCATGCTG	CY3	Raskin et al., 1994
MS1414	*Methanosarcina* + relatives	CTCACCCATACCTCACTCGGG	CY3	Raskin et al., 1994
MSMX	*Acetoclastic methanogens*	GGCTCGCTTCACGGCTTCCCT	CY3	Raskin et al., 1994
NON338	Nonsense probe	ACTCCTACGGCAGGCAGC	CY3	Raskin et al., 1994
LGC354A	*Firmicutes*	TGGAAGATTCCCTACTGC	CY3	Meier et al., 1999
LGC354B	*Firmicutes*	CGGAAGATTCCCTACTGC	CY3	Meier et al., 1999
LGC354C	*Firmicutes*	CCGAAGATTCCCTACTGC	CY3	Meier et al., 1999
SPIRO1400	Subgroups of *Spirochaetacea*	CTCGGATGGTGTGACGGGCG	CY3	Daly and Shirazy-Beechey, 2003
GNSB-941	*phylum Chloroflexi*	AAACCACACGCTCCGCT	CY3	Gich et al., 2001
PLA46	*Planctomycetales*	GACTTGCATGCCTAATCC	CY3	Neef et al., 1998
HGC69A	*Actinobacteria*	TATAGTTACCACCGCCGT	CY3	Roller et al., 1994
CFX1223	*phylum Chloroflexi*	CCATTGTAGCGTGTGTGTMG	CY3	Björnsson et al., 2002
CFB1082	*Most member of Bacteroidetes*	TGGCACTTAAGCCGACAC	CY3	Weller et al., 2000
ALF968	*Alphaproteobacteria, except Rickettsiales*	GGTAAGGTTCTGCGCGTT	CY3	Neef, 1997
BET42a	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	CY3	Manz et al., 1992
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	CY3	Manz et al., 1992

Visualisation. 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). For all times, counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated. Average of the counts gave the representative number of total microorganisms in each sample. Images were processed and analyzed using Image-Pro Plus version 6.3 image analysis software (Media Cybernetics, USA). Different fluorochromes are excited and emitted at different wavelengths. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used in this study are given in Table 4.13.

Table 4.13. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used in this study

Fluorochrome	**Color of Fluorescence**	**Maximum excitation wavelength (nm)**	**Maximum emission wavelength (nm)**	**Filter cube used**
CY3	Red	550	565	U-MWG
DAPI	Blue	365	397	U-MWIB

Quantification. Quantification of microoorganisms in the sludge samples collected during three different periods was conducted by using version 5.1 of Image-Pro Plus image analysis software. Quantification involves counts of total microorganisms with DAPI staining and counts of specific methanogenic groups with other oligonucleotide probes using FISH (Figure 4.11.).



Intensity Range Selection
Current Range [10...254]
● Manual
○ Automatic Bright Objects
○ Automatic Dark Objects
☑ Measure Objects
☑ Apply Filter Ranges
☐ Accumulate Count
Total Count: 439
In Range: 269
☑ Display Objects

1512, 502 0 W,H: 1600,1200 Image: <none> [Pixels] System: <none>

Figure 4.11. Capture image of Image Pro Plus 6.3.

5. RESULTS AND DISCUSSION

The results and discussion part of this dissertation is divided into subchapters ranging from 5.1 to 5.5.

Part 5.1. focuses on OTC medication and its metabolites formed in the animal body. A common knowledge is that inhibitory effect of an antibiotic is much lower than the inhibitory effect of an antibiotic after animal medication. It is hypothetized that metabolites formed in animal body have much greater impact on microbial communities than parent compound itself. It is also important to have the knowledge of excretion pattern of antibiotic in animal manure if that manure is considered to be used as substrate for anaerobic digestion or organic fertilizer. In this part, excretion pattern of OTC was monitored after OTC injection to a dairy cow. Metabolites of OTC in manure was determined

Part 5.2. shows inhibitory effects of OTC and its metabolites on anaerobic digestion process. It has been hypothetized that metabolites of an antibiotic have more inhibition potential on the system than the parent compound itself. Effects of OTC on microbial communities present in anaerobic digesters were also investigated.

Part 5.3. shows effects of selected operational parameters (temperature, solid content and mixing rate), on biogas production efficiency in presence and absence of OTC. Presence of OTC and its metabolites on biogas yields production under various operational parameters were determined. Changes in molecular community structures were determined due to OTC and changing operational parameters and statistical analyses were also supplied

Part 5.4. contains outcomes of soil experiments where OTC contained manure used as fertilizer. In fate of OTC in soil environments was investigated by amending soil with different manures and digestate. Microbial diversities in soil microbiota and active microbial communities were also determined. Finally, 5.4.5 Quantitative analysis of soil microbial communities was also discussed.

Part 5.5. focuses on tetracycline resistance gene proliferation due to the presence of OTC in environment. Resistance gene presence in microcosms and resistance gene proliferation in anaerobic digesters operated in different operational parameters were separately investigated. Finally, 5.5.2 resistance gene proliferation in soil microbial communities was determined.

5.1. Excretion Pattern of OTC in Animal Body and Concentrations

Dairy cow was injected with Oxytetracycline solution once with dosage of 20 mg/kg bodyweight. Manure samples were collected for 20 days each 24 hours following injection. OTC was extracted from manure and analyzed with HPLC (Figure 5.1.).

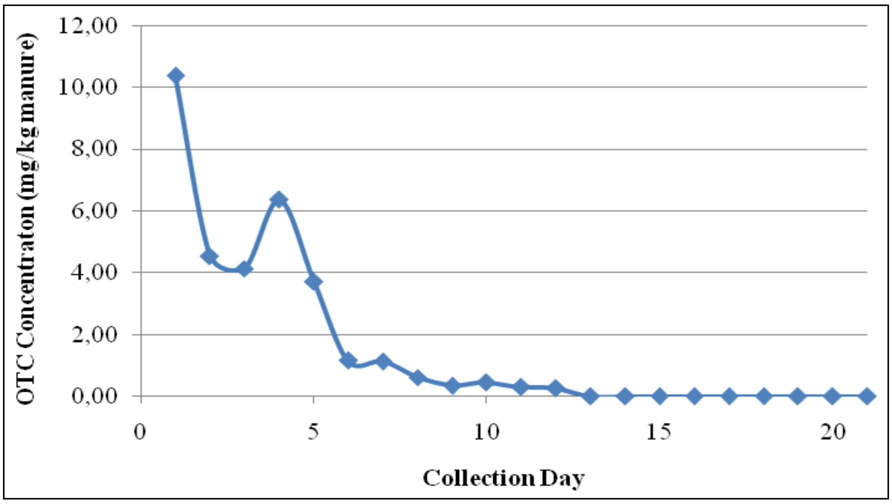


Figure 5.1. Excretion pattern of OTC in manure

Highest concentration of OTC was detected at first day of sampling. OTC concentration started with 10.38 mg/kg and began to decrease in following days. There was a sudden increase in day 4. After that sampling no increase has been observed and OTC concentraions drop down to detection limit after 13 days. On the 13th day the concentration of excreted OTC was calculted as 33.34 mg/kg. As dairy cattle produce 20-30 kg of solid manure in a day approximately 6-10% of injected OTC was excreted. It will also be expected to see OTC presence, maybe even higher, in liquid manure since it will be excreted in liquid manure due to intramuscular injection. This situation was also supported by reports and prospectus of the vaccine.

Studies on the effect of tetracyclines on anaerobic digestion in literature consist of manure containing antibiotic after oral administration. Especially in USA, tetracyclines are one of 10 antibiotics permitted as growth promoter applications. In EU countries, the usage of antibiotics as growth promoters is banned and all antibiotic used in EU countries are for theraupetic usages. In a study about, 10 mg/kg OTC was detected in 5 fold diluted manure slurry of oral medicated calves (Arıkan et al., 2006). A much higher concentration was detected in swine manure. In another study, 5.88 mg/kg CTC was detected in manure samples collected from different farming. The excreted concentrations and amounts were in defined limits of literature since it has been recorded that tetracyclines can be detected in manure in concentrations of 0.1 mg/kg to 173 mg/kg (Hamsher et al., 2002). The excretion concentration of tetracyclines was mainly depended on concentration of administration, method of administration, type of the animal, diet and general health of the animal; therefore, range of detected tetracyclines in manure is quite wide.

After revealing excretion pattern of OTC, only first five days manure was collected to use in downstream experiments. HPLC analysis was continued for these samples to monitor concentrations of excretion of OTC (Table 5.1).

Table 5.1. Average concentrations of OTC excretion

	OTC conc.(mg/kg)	**Average OTC conc.(mg/kg)***
1. day manure	10.4	10.5±0.1
2. day manure	4.5	5.8±1.0
3. day manure	4.1	4.8±2.0
4. day manure	6.4	7.4±2.0
5. day manure	3.7	4.0±0.5

* n: 8 cattle

Addition to HPLC analysis, LC-MS/MS analyses have been carried out for extracted samples to monitor transformation of OTC in animal body (Table 5.2.). Accordingly, OTC excretion was recalculated. Antibiotic concentration excreted for the selected days was

86.4 mg/kg. Cattle produce 20-30 kg of manure each day, so for those 6 days approximately 17-26% of injected antibiotic was excreted in solid manure. This ratio will further increase due to untested days and liquid manure.

Table 5.2. OTC and its metabolites detected in manure

	OTC	**4-epi**	**α-apo**	**β-apo**	**Total**
	mg/kg				
1. day manure	13.5	0.7	6.6	14.5	35.2
2. day manure	4.4	0.3	1.7	7.1	13.5
3. day manure	4.5	0.4	1.6	7.2	13.6
4. day manure	6.1	0.3	9.6	6.9	22.9
10. day manure	0.4	0.1	0.1	0.2	0.8
20. day manure	0.1	0.0	0.0	0.1	0.3
Total Excretion	**29.0**	**1.8**	**19.6**	**36.0**	**86.4**

LC-MS/MS analyses showed that OTC has been transformed into its metabolites inside of the cattle. Two main metabolites of OTC, α-apo-OTC and β-apo-OTC, present in high concentration in manure. These two metabolites are isomers but concentration of β-apo-OTC was higher in manure than both OTC and α-apo-OTC. LC-MS/MS analyses showed the presence of high concentration of metabolites in manure. It presents on arguments for why inhibitions in anaerobic digestion with spiked manures have been seen only in high antibiotic concentrations.

5.2. Inhibitory Effects of OTC and its Metabolites in Anaerobic Digestion

After monitoring of excretion pattern of OTC, anaerobic digestion experiments have been performed with parameters of farm type digesters. In digester microcosms, inhibitions caused by OTC and its metabolites, half-lives and effects on microbial communities were investigated.

5.2.1. Concentrations of OTC and its Metabolites in Microcosms Experiments

In microcosm experiments, OTC and its metabolites concentrations has been monitored. HPLC analysis has been performed in each sampling time and LC-MS/MS analyis has been performed at the end of digestion.

HPLC analysis of microcosm showed OTC has half-life of 20-27 days in anerobic microcosms (Table5.3.).

Table 5.3. Change of OTC concentration in anaerobic microcosm with time

Microcosm	**OTC concentration (mg/l)**			
	Day 0	**Day 10**	**Day 20**	**Day 30**
Slurry OTC 50	50	41.9	31.5	22.9
Slurry OTC 100	100	66.6	58.0	42.4
Slurry OTC 200	200	153.2	88.2	34.6
Slurry Day 1	3.34	1.36	1.56	1.45
Slurry Day 2	1.4	1.07	0.85	0.16
Slurry Day 3	1.39	0.85	0.4	0.6
Slurry Day 5	1.01	0.29	0.3	N.D.*
Slurry Day 10	0.15	N.D.*	N.D.*	N.D.*
Slurry Day 15	N.D.*	N.D.*	N.D.*	N.D.*
Slurry Day 20	N.D.*	N.D.*	N.D.*	N.D.*

*N.D.: not detected

30th day samples of microcosms were also analyzed with LC-MS/MS to determine concentrations of OTC metabolites (Table 5.4.). In OTC added microcosms, main metabolite of OTC was 4-epi OTC which was produced at a ratio of 5-10% of initial parent compound. The production of α-apo-OTC and β-apo-OTC was in negligible levels. In medicated manure microcosms, like solid manure analysis β-apo-OTC was present in concentrations equal to OTC followed by α-apo-OTC and 4-epi-OTC. Ratio of 4-epi-OTC to OTC was higher in microcosms analysis indicating production of 4-epi-OTC as a result of anaerobic digestion.

Table 5.4. OTC metabolites concentrations at the end of microcosm experiments

Microcosm	OTC	4-epi	α-apo	β-apo	Total
	mg/l				
Slurry OTC 50	24.27	6.66	1.335	1.44	**33.7**
Slurry OTC 100	41.22	7.11	1.56	1.83	**51.7**
Slurry OTC 200	130.13	36.36	1.56	10.47	**178.5**
Slurry Day 1	1.39	0.276	0.57	0.849	**3.1**
Slurry Day 2	1.58	0.337	0.639	1.38	**3.9**
Slurry Day 3	0.614	0.147	0.208	0.534	**1.5**
Slurry Day 5	0.245	0.07	0.089	0.213	**0.6**
Slurry Day 10	0.03	0.016	0.021	0.037	**0.1**
Slurry Day 15	0.01	0.012	0.016	0.027	**0.1**

5.2.2. Biogas Yield in Microcosm under Presence of OTC and its Metabolites

Presence of OTC and its metabolites was detrimental to microorganisms. This effect showed itself on biogas production efficiency of the system. Therefore, biogas yield was inhibited significantly in OTC containing microcosms. For microcosm containing 100 mg/l OTC, the biogas yield was equal to microcosm containing manure of day 1 excretion. Also microcosm containing 200 mg/l OTC had same biogas yield as microcosm fed with manures excreted in day 2 and 3.

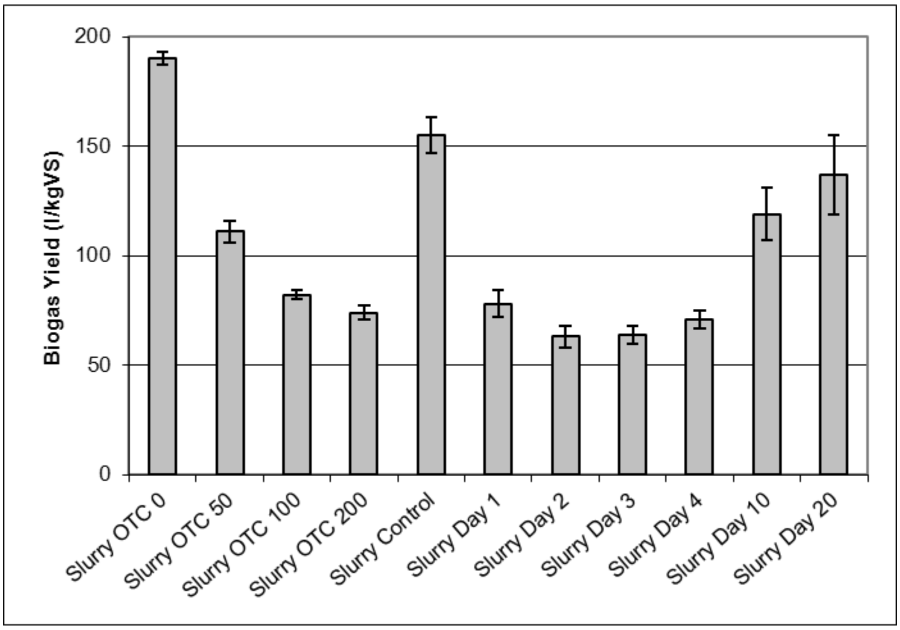


Figure 5.2. Biogas yields of serum bottle microcosms after 30 days.

Values for biogas and methane yields for day 20 and day 30 of operation can be seen in Table 5.5.

Table 5.5. Relation of OTC concentrations (mg/l) with biogas and methane yields (l/kgTVS)

		20 days of operation		30 days of operation	
Microcosm	**OTC conc.**	**Biogas yield**	**Methane yield**	**Biogas yield**	**Methane yield**
Control	0.0	146.02±3.04	85.05±2.44	190.11±3.13	90.10±1.55
50 mg/l OTC	50	76.57±6.36	44.04±2.23	111.49±5.11	63.59±5.19
100 mg/l OTC	100	61.07±3.27	38.14±1.80	82.30±2.20	47.95±1.08
200 mg/l OTC	200	53.04±1.72	29.83±3.55	74.12±2.97	40.11±2.40
0 (Control)	0.0	132.43±5.72	80.07±0.92	155.22±7.91	86.96±6.41
1	3.3	70.46±6.46	40.83±0.56	78.04±6.29	45.82±2.13
2	1.4	51.73±0.68	32.85±1.26	62.57±4.75	38.62±3.24
3	1.4	48.32±1.54	29.45±1.12	64.30±3.88	37.35±2.16
5	1.0	54.10±2.17	33.96±2.16	71.00±4.09	43.93±3.53
10	0.2	95.67±12.12	51.52±17.68	119.08±11.97	67.53±10.02
15	0.0	103.08±20.61	60.38±15.27	139.17±23.01	83.93±15.67
20	0.0	94.66±13.65	54.19±8.58	136.85±18.56	74.77±1.23

In this study, inhibitory effects of OTC and its metabolites were monitored. For OTC as sole antibiotic, biogas production was decreased by 41-61% for concentrations 50-200 mg/l OTC. In study of Gamel-El-Din et al., (1986), biogas production was inhibited by 32%, 40% and 49% at OTC concentrations of 12.5 mg/l, 37.5 mg/l and 75 mg/l, respectively. Another similar study showed that biogas production inhibition was increased from 20% to 80% where 5 mg/l CTC concentration was increased to 150 mg/l (Sanz et al., 1996). Study of Alvarez and his co workers (2010) monitored 45%, 57% and 64% inhibition in anaerobic digesters efficiency treating swine manure where OTC+CTC concentrations were 10 mg/l, 50 mg/l and 100 mg/l, respectively. Similar study showed 28 mg/l CTC caused 28% inhibition in methane production in swine manure digesters (Stone et al., 2009).

5.2.3. Microbial Community Structures in Microcosms

Microbial community structures were identified by RNA and DNA targeting molecular techniques.

5.2.3.1. Community fingerprinting with DGGE. Both bacterial and archaeal diversity was determined by DGGE (Figures 5.3.-5.6). Nomenclature was as follows: microbial domain, microcosm code, microcosm indicator and sampling time (for example analysis of 10th day sample of microcosm fed with manure collected 3rd day of excretion will be named as omg 3 10 where “omg” is microcosm code and “3” is microcosm indicator).

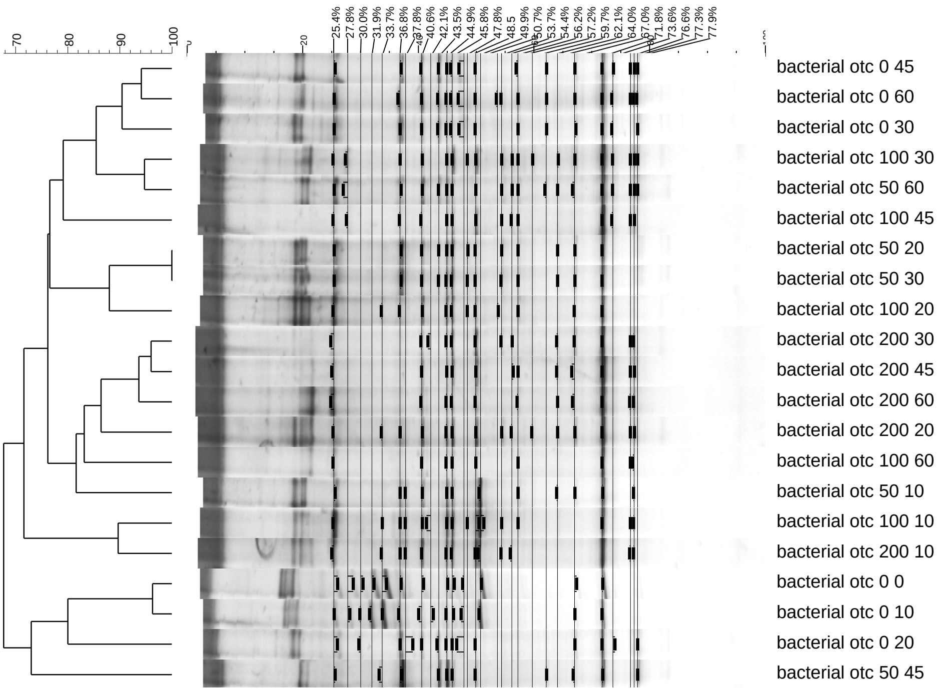


Figure 5.3. Bacterial community dendogram of OTC spiked Microcosms (OTC).

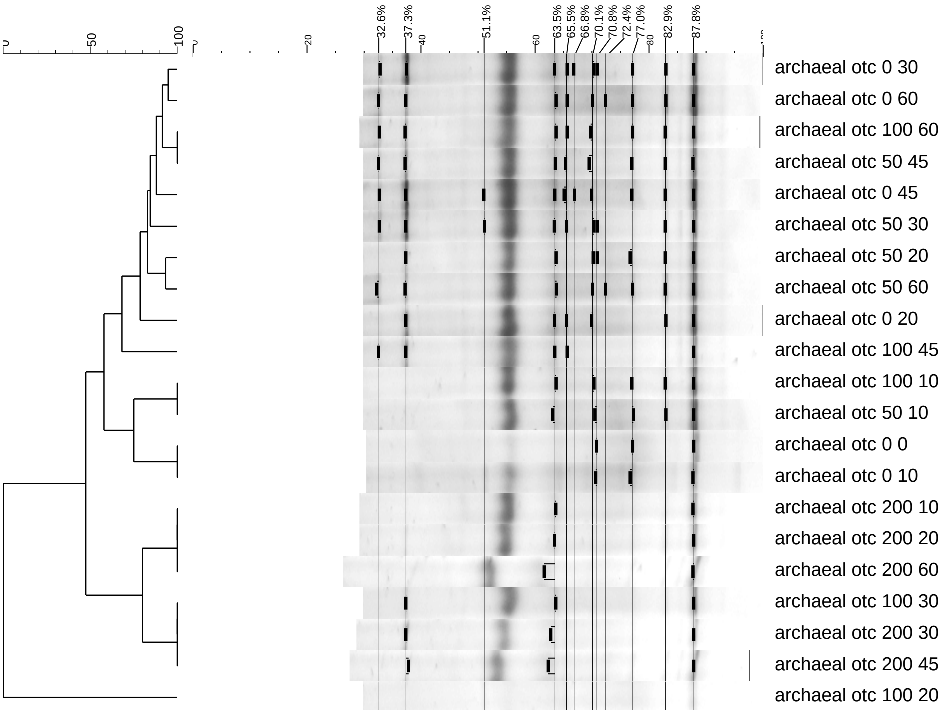


Figure 5.4. Archaeal community dendogram of OTC spiked Microcosms (OTC)

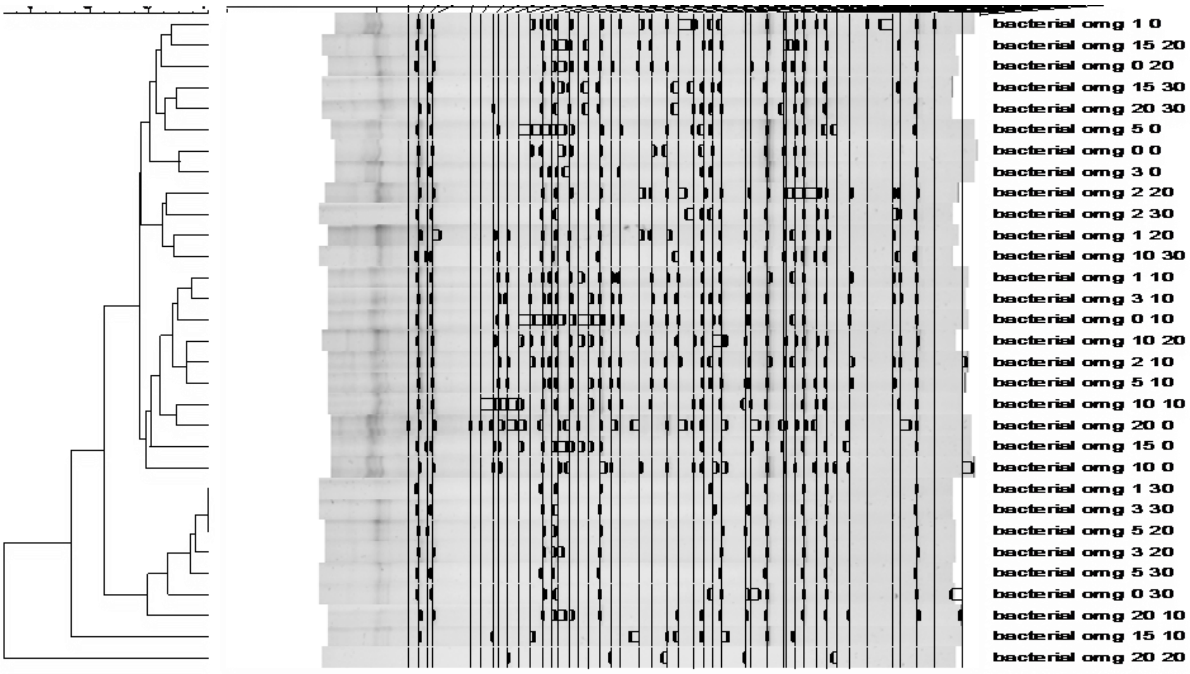


Figure 5.5. Bacterial community dendogram of OTC medicated Microcosms (OMG)

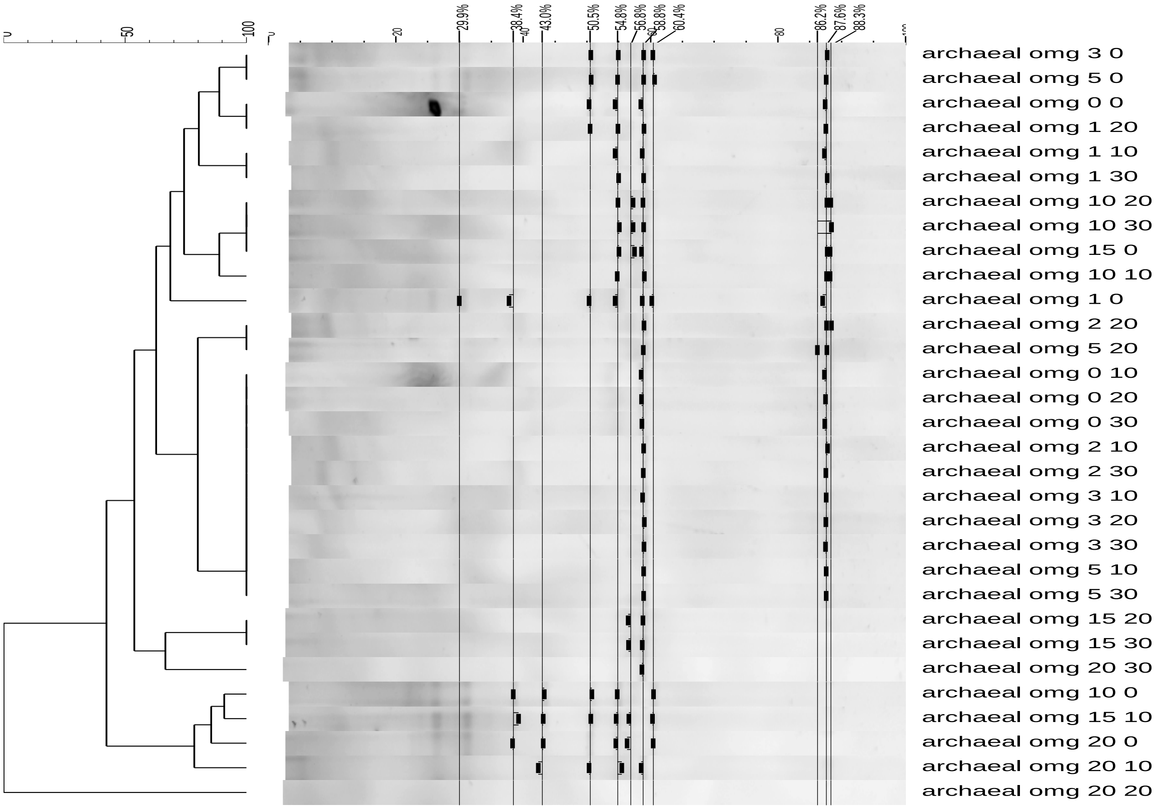


Figure 5.6. Archaeal community dendogram of OTC medicated Microcosms (OMG)

Analysis of community fingerprinting showed that bacterial diversity is more affected than archaeal diversity. Clustering pattern was according to antibiotic concentrations in first microcosm set (OTC) as second microcosm set (OMG) samples were clustered according to time.

5.2.3.2 16S rDNA Clone Library of microcosm sets. 16S rRNA clone library was formed to identify member of bacterial and archaeal communities. Analyzed sequences related to *Bacillus spp*., *Clostridium spp*. and *Acinetobacter spp*. for bacteria, *Methanosarcina spp.* and *Methanobacterium spp.* for archaea (Table 5.6.).

Table 5.6. Clone frequencies and similarities to known organisms

Domain	Clone amount (no.)	Clone percentage	**Highest similarity**		
			Organism	%	Accession no.
Bacteria	29	28.71	*Bacillales*	96.2	EM_PRO:AY837750
	28	27.72	*Clostridium lituseburense*	96.3	EM_PRO:EU887828
	11	10.89	*Acinetobacter*	99.2	EM_PRO:AF336348
	7	6.93	*Bacillus odyssei*	93.5	EM_PRO:AF526913
	5	4.95	*Bacillus psychrodurans*	99.1	EM_PRO:EF101552
	3	2.97	*Bacterium*	85.0	EM_PRO:DQ168648
	3	2.97	*Acinetobacter*	99.3	EM_PRO:AJ301674
	3	2.97	*Clostridium*	90.5	EM_PRO:AB207248
	3	2.97	*Clostridium*	84.0	EM_PRO:Y18176
	2	1.98	*Bacillus odyssei*	94.3	EM_PRO:AF526913
	2	1.98	*Bacillales*	96.7	EM_PRO:AY837750
	1	0.99	*Solibacillus silvertis*	96.0	EM_PRO:GU186125
	1	0.99	*Bacillus sp.*	97.0	EM_PRO:GU136567
	1	0.99	*Bacillus psychrodurans*	95.1	EM_PRO:AY822548
	1	0.99	*Bacillus sp.*	94.8	EM_PRO:GU124638
	1	0.99	*Clostridium mayombei*	97.9	EM_PRO:FR733682
Archaea	48	44.44	*Methanosarcina mazei*	99.8	EM_PRO:AE008384
	43	39.81	*Methanobacterium sp.*	89.1	EM_PRO:AB288281
	9	8.33	*Methanobacterium sp.*	99.6	EM_PRO:AJ550159
	4	3.70	*Methanosarcina mazei*	94.9	EM_PRO:AE008384
	2	1.85	*Methanoculleus marisnigri*	93.4	EM_PRO:CP000562
	2	1.85	*Methanosaeta sp.*	98.0	EM_PRO:AJ133791

5.2.3.3. Activity analysis by FISH technique. FISH analysis conducted to monitor active cells in the community. For the second microcosm set samples for FISH analysis were selected according to biogas yields. 3 samples were selected according to level of inhibition. Samples from Slurry Day 2, 10 and 15 were analyzed as representing high, moderate and light inhibition, respectively. Results were shown in Tables 5.7. and 5.8.; Figures 5.7.-5.10.

Table 5.7. Numbers of active bacterial and archeal cells in 1. microcosm set (cell/ml)

	Digestion Day 10		Digestion Day 20		Digestion Day 30	
Sample	**Bacteria**	***Archaea***	**Bacteria**	***Archaea***	**Bacteria**	***Archaea***
Slurry OTC 0	3.36E+07	1.54E+08	3.77E+07	6.42E+07	3.48E+07	2.49E+07
Slurry OTC 50	3.35E+07	1.53E+08	3.48E+07	1.03E+08	1.50E+07	2.46E+07
Slurry OTC 100	2.29E+07	1.44E+08	3.38E+07	6.31E+07	1.29E+07	1.95E+07
Slurry OTC 200	2.26E+07	1.09E+08	3.00E+07	3.25E+07	1.04E+07	1.94E+07

Table 5.8. Numbers of active bacterial and archeal cells in 2. microcosm set (cell/ml)

Sample	**Operation time (day)**	**Bacterial cell number**	**Archaeal cell number**
Slurry Day 0	0	2.69E+07	7.88E+07
Slurry Day 2	0	3.01E+07	3.21E+07
Slurry Day 10	0	3.90E+07	2.94E+07
Slurry Day 15	0	3.30E+07	4.11E+07
Slurry Day 0	10	6.83E+07	8.82E+07
Slurry Day 2	10	3.37E+07	4.90E+07
Slurry Day 10	10	3.83E+07	7.34E+07
Slurry Day 15	10	5.97E+07	7.61E+07
Slurry Day 0	20	9.22E+07	1.90E+08
Slurry Day 2	20	4.00E+07	6.36E+07
Slurry Day 10	20	6.24E+07	9.10E+07
Slurry Day 15	20	8.64E+07	1.92E+08
Slurry Day 0	30	6.73E+06	1.72E+07
Slurry Day 2	30	2.45E+06	1.38E+07
Slurry Day 10	30	6.22E+06	2.24E+07
Slurry Day 15	30	8.90E+06	1.07E+07

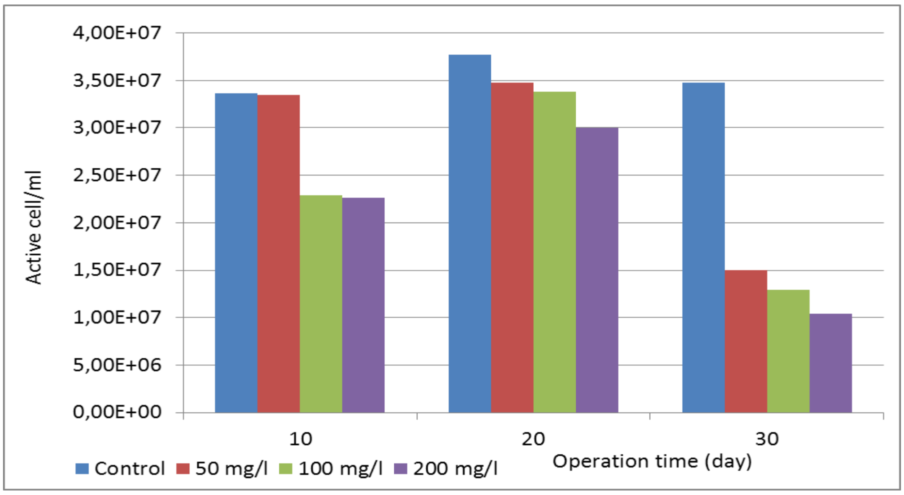


Figure 5.7. Changes in the total cell number of bacteria in 1. microcosm set.

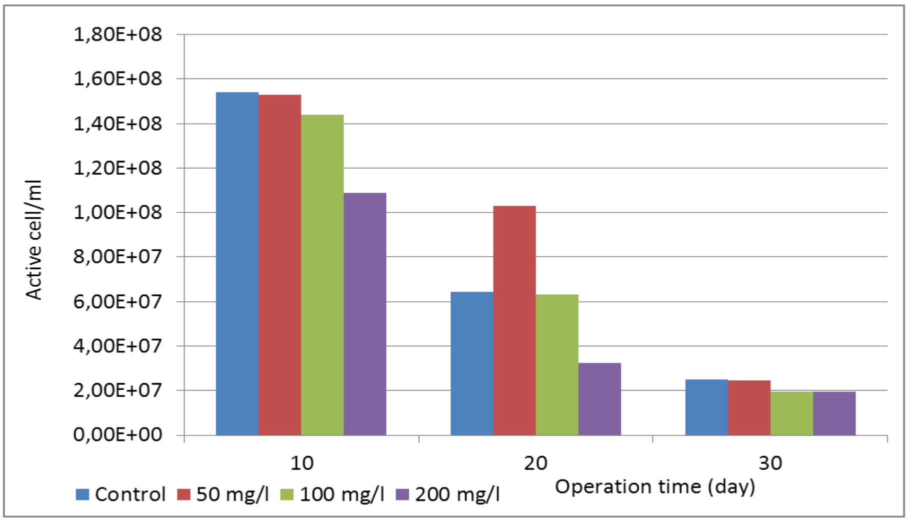


Figure 5.8. Changes in the total cell number of archaea in 1. microcosm set.

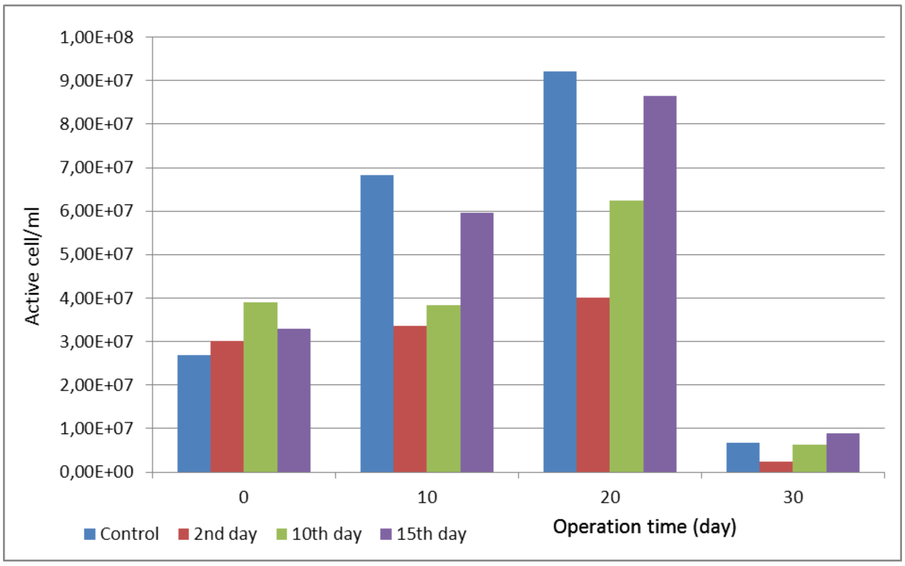


Figure 5.9. Changes in the total cell number of bacteria in 2. microcosm set.

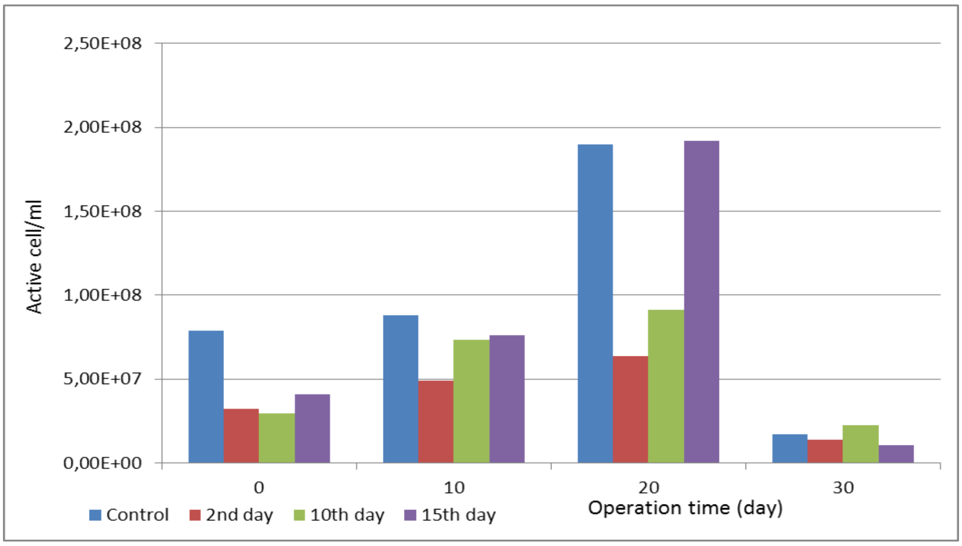


Figure 5.10. Changes in the total cell number of archaea in 2. microcosm set.

For both microcosm sets, number of active bacterial and archaeal cells increased until day 20. After that activity decreased rapidly. Also in both sets, activity was related to OTC concentration. Lower activity has been observed in high OTC concentration.

5.3. Effects of Environmental Parameters on Anaerobic Digestion

Anaerobic digesters were set up and operated with different operational parameters. Samples for physical, chemical, analytical and molecular analysis were taken to determine the relation between system performances with environmental factors. Changing operational parameters were given in Table 5.9.

Table 5.9. Operational parameters in digesters

Digester	**Temp.**	**Mixing rate (rpm)**	**TVS**
Set 1 S1 (Control)	37^{0}C	90	5-6%
Set 1 S3	37^{0}C	90	5-6%
Set 1 S4 (Control)	37^{0}C	90	8-9%
Set 1 S6	37^{0}C	90	8-9%
Set 2 S1 (Control	37^{0}C	120	5-6%
Set 2 S3	37^{0}C	120	5-6%
Set 2 S4 (Control)	37^{0}C	120	8-9%
Set 2 S6	37^{0}C	120	8-9%
Set 3 S1 (Control)	55^{0}C	90	4%
Set 3 S3	55^{0}C	90	4%
Set 3 S4 (Control)	55^{0}C	90	5-6%
Set 3 S6	55^{0}C	90	5-6%
Set 4 S1 (Control)	55^{0}C	120	4%
Set 4 S3	55^{0}C	120	4%
Set 4 S4 (Control)	55^{0}C	120	5-6%
Set 4 S6	55^{0}C	120	5-6%

5.3.1. Analysis of OTC and its Metabolites in Anaerobic Digesters

During digestion OTC concentrations in digesters were monitored by HPLC analysis. Analysis showed that OTC has a half-life of 24-26 days in mesophilic; 16-18 days in thermophilic digesters (Table 5.10).

Table 5.10. Change of OTC concentrations in digesters

Digester	OTC concentrations (mg/l)					Elimination (%)	Half-life (day)
	Day 0	Day 5	Day 10	Day 20	Day 30		
Set 1 S3	2.2		1.6	0.8	1.0	55	27
Set 1 S6	3.4		2.6	1.3	1.5	56	27
Set 2 S3	1.1		0.9	0.8	0.4	64	24
Set 2 S6	2.22		2.0	1.1	0.6	73	21
Set 3 S3	2.46	1.06	1.29	0.59		76	13
Set 3 S6	2.57	1.49	1.44	1.05		59	17
Set 4 S3	1.51	0.82	0.59	0.54		64	15
Set 4 S6	2.05	1.0	0.98	0.96		53	19

LC-MS/MS analysis has been done to monitor formation of OTC metabolites in anaerobic digestion (Table 5.11.). It is a common belief that in manure digesters, not only antibiotic itself, but also its metabolites can be inhibitors.

The results indicated similar half-lives for OTC both in mesophilic and thermophilic digesters (Table 5.12.). Transformation of OTC to its metabolites has been monitored. A relation between half-life and TVS has been deducted from results. Half-life of OTC was shorter in high TVS digesters at 37^0C where a longer half-life was observed in high TVS digester at 55^0C.

Table 5.11. OTC metabolites in the beginning (Day 0) and the end (Day 30 and Day 20 for mesophilic and thermophilic digesters, respectively) of digester operations

Digester	Concentrations at Day 0 (mg/kg)					Concentrations at Day 30 (mg/kg)*				
	OTC	4-epi	α-apo	β-apo	Total	OTC	4-epi	α-apo	β-apo	Total
Set 1 S3	2.30	0.10	0.14	0.44	**2.98**	0.94	0.20	0.09	0.17	**1.39**
Set 1 S6	3.35	0.14	0.17	0.65	**4.30**	1.51	0.36	0.10	0.22	**2.19**
Set 2 S3	1.21	0.09	0.07	0.14	**1.50**	0.35	0.08	0.04	0.05	**0.52**
Set 2 S6	1.92	0.14	0.10	0.20	**2.36**	0.66	0.14	0.06	0.08	**0.95**
Set 3 S3	2.38	0.20	0.35	0.73	**3.65**	0.75	0.24	0.17	0.22	**1.38**
Set 3 S6	2.85	0.23	0.20	0.38	**3.65**	0.94	0.29	0.20	0.24	**1.67**
Set 4 S3	1.49	0.13	0.13	0.32	**2.06**	0.54	0.20	0.16	0.13	**1.03**
Set 4 S6	2.02	0.15	0.15	0.44	**2.75**	0.97	0.29	0.22	0.22	**1.69**

*Day 20 for thermophilic digesters

Table 5.12. Elimination and half-lives of OTC in digesters

Digester	**Elimination (%)**		**Half-life (day)**	
	OTC	**Total**	**OTC**	**Total**
Set 1 S3	59	53	25	28
Set 1 S6	55	49	27	31
Set 2 S3	71	65	21	23
Set 2 S6	65	60	23	25
Set 3 S3	69	62	15	16
Set 3 S6	67	54	15	18
Set 4 S3	64	50	16	20
Set 4 S6	52	38	19	26

Half-life of OTC was found as 20-27 days in microcosms, 24-26 days in mesophilic digesters and 16-18 days in thermophilic digesters. In a study of Wang and Yates, (2005), it has been stated that two factors were crucial in half-life of OTC, temperature and humidity. Effect of temperature was observed easily by the half-life differences of OTC between mesophilic and thermophilic digesters. Effect of humidity was lesser than effect of temperature. It was seen that half-lives of low VS digesters were slightly less than half-lives of high VS digesters. Half-lives of antibiotic was also slightly higher than half-lives of OTC in digesters indicating that there was a transformation of parent compound occurred in digesters. A similar study was conducted by Arikan et al., (2006), where they found out the half-life of OTC as 56 days.

5.3.2. Biogas Yields in Anaerobic Digesters

Digesters fed with unmedicated and medicated manure were operated as described above.The process was monitored by taking samples. Produced biogas was recorded with miligas counters and biogas yield was calculated with the help of starting TVS. Methane yields were also calculated in terms of methane concentration in biogas (Table 5.13- 5.14.).

Table 5.13. Starting TS and TVS ratios of digesters

Digester	Total solid (TS) (%)	Total Volatile Solid (TVS) (%)
Set 1 S1 (Control)	6.9	5.8
Set 1 S3	7.4	6.2
Set 1 S4 (Control)	10.8	8.9
Set 1 S6	11.1	9.0
Set 2 S1 (Control)	6.4	5.4
Set 2 S3	6.1	5.0
Set 2 S4 (Control)	9.3	7.9
Set 2 S6	9.5	7.9
Set 3 S1 (Control)	5.5	4.4
Set 3 S3	5.1	4.1
Set 3 S4 (Control)	7.8	6.3
Set 3 S6	6.9	5.5
Set 4 S1 (Control)	4.7	3.8
Set 4 S3	4.8	3.9
Set 4 S4 (Control)	5.9	4.8
Set 4 S6	6.8	5.4

In digester studies, 1-3 mg/l OTC caused 15-35% inhibition in biogas production. The study of Arikan et al., (2006), also showed that 3.1 mg/l OTC caused 27% inhibition in biogas production. In their study, OTC medicated manure of oral fed calves were used.

In this chapter, biogas yields of 121 l/kgVS and 134 l/kgVS were obtained for mesophilic and thermophilic digesters, respectively. Seed was added as 10% of total working volume and incubation time was 30 and 20 days for mesophilic and thermophilic digesters, respectively. In a study of Alvarez and Lidden, (2009), biogas yields of 70-140 l/kgVS were monitored. They also monitored various OLR's which were changing from 4-6 kgVS/m^{3}day. Use of lower temperature, 25^{0}C, and mixing of various manure types possible caused these results. Both study of Arikan et al., (2006), and Amon, (2007), had reached similar biogas yields, 208-268 l/kgVS and 257 l/kgVS, respectively.

Table 5.14. Relation of OTC concentrations (mg/l) and operational parameters with biogas and methane yields (l/kgTVS)

Digester	**Day 20**			**Day 30**	
	OTC conc.	**Biogas yield**	**Methane yield**	**Biogas yield**	**Methane yield**
Set 1 S1 (Control)		109	53	121	75
Set 1 S3	0.83	75	40	92	55
Set 1 S4 (Control)		100	60	119	76
Set 1 S6	0.88	83	47	95	62
Set 2 S1 (Control)		92	39	118	70
Set 2 S3	1.18	85	39	102	60
Set 2 S4 (Control)		90	41	118	73
Set 2 S6	2.22	73	31	98	61
Set 3 S1 (Control)		124	87		
Set 3 S3	2.46	111	78		
Set 3 S4 (Control)		134	94		
Set 3 S6	2.57	117	80		
Set 4 S1 (Control)		132	92		
Set 4 S3	1.51	89	62		
Set 4 S4 (Control)		123	84		
Set 4 S6	2.05	106	72		

In literature, various biogas yields were obtained. Main reason for this was the use of different operational parameters. Solid content is an effective parameter in anaerobic digestion. Because it was affecting directly substrate and microorganism levels, lower or higher values will affect biogas production. High solid contents also increase viscosity of the digester, deteriorating an efficient mixing which may cause local inhibitions as well as a decrease substrate-microorganism contact.

5.3.3. Investigation of Physico-Chemical Parameters in Anaerobic Digesters

Total Solid and Total Volatile Solid ratios were recorded throughout the study. Fresh manure samples were also analyzed (Table 5.15.).

Table 5.15. TS and TVS values in fresh manure and anaerobic digesters

Sample	Digestion time (day)	TS (%)	TVS (%)	TVS/TS
Fresh Manure		16 -18	13 -16	78-85
Set 1 S1 Control	0	6.9	5.8	83
	10	4.9	3.9	80
	20	4.0	3.0	75
Set 1 S3	0	7.4	6.2	83
	10			
	20			
Set 1 S4 Control	0	10.8	8.9	83
	10	8.1	6.7	83
	20	6.8	5.4	79
Set 1 S6	0	11.1	9.0	81
	10	10.3	8.2	80
	20	8.6	6.5	75
Set 2 S1 Control	0	6.4	5.4	86
	10	5.6	4.7	84
	20	4.7	3.8	81
Set 2 S3	0	6.1	5.0	82
	10	5.5	4.5	82
	20	4.7	3.8	81
Set 2 S4 Control	0	9.3	7.9	85
	10	8.5	7.2	85
	20	7.0	5.9	84
Set 2 S6	0	9.5	7.9	83
	10	8.7	7.1	82
	20	7.3	6.0	82
Set 3 S1 Control	0	5.5	4.4	80
	10	4.1	3.3	82
	15	3.9	3.1	79
Set 3 S3	0	5.1	4.1	80
	10	4.3	3.6	82
	15	4.1	3.3	81
Set 3 S4 Control	0	7.8	6.3	81
	10	6.5	5.3	82
	15	6.1	4.9	81
Set 3 S6	0	6.9	5.5	80
	10	5.7	4.7	82
	15	5.7	4.6	80
Set 4 S1 Control	0	4.7	3.8	82
	10	4.1	3.3	81

Table 5.15. (continued)

	15	3.8	3.1	80
Set 4 S3	0	4.8	3.9	82
	10	4.6	3.6	79
	15	4.3	3.5	81
Set 4 S4 Control	0	5.9	4.8	81
	10	5.7	4.6	81
	15	5.1	4.1	80
Set 4 S6	0	6.8	5.4	80
	10	6.5	5.0	82
	15	6.2	4.6	82

In our study, single and multiple effects of selected operational parameters on biogas yield, microbial communities and elimination of OTC. Selected parameters were temperature, solid content and mixing rate. Most influential parameter was found to be temperature. In literature, there are many studies indicating advantages of thermophilic digesters. But there are also many disadvantages of thermophilic digesters. Firstly thermophilic digesters were more delicate than mesophilic digesters (Duran and Speece, 1997). Some microorganisms, especially pathogens, have very low survival rate in thermophilic digesters (Sahlstrom, 2003). This may be an important feature since manure contained a high number of pathogens which will be dangerous if manure was treated properly to eliminate these pathogens. One of the advantages of thermophilic digester over mesophilic ones are high biogas and methane yields. In our study, thermophilic digesters have a biogas yield nearly twice much biogas yield in 20th day of incubation. It also has been observed that thermophilic digesters had a higher methane yield due to higher methane content in biogas. OTC elimination was increased with temperature as discussed earlier. According to literature studies, methane content of biogas produced in thermophilic digesters is generally found more than in mesophilic digesters (Goberna et al., 2009).

Another parameter selected in this study was solid content. Literature was advising a continous operation at 2-3 kgVS/m^{3}day will be most efficient. Therefore, in our study, we used two VS concentration to represent low sand high solid contents. Although biogas yields were quite similar in control digesters for different VS contents, OTC containing digesters had different biogas yields for different VS concentrations. Biogas yield tended to increase in high VS digesters. In manure digesters, solid content was directly affecting number of introduced microorganisms. Therefore, a possible explanation for this phenomenon will be positive effect of these introduced microorganisms.

Mixing rate was third operational parameter that was used in this study. Mixing rate is another important parameter for biological systems. Lower mixing will end up in lower biogas yield. Mixing rate is important for an effective microorganism-substrate interaction as well as preventing local (acute) inhibitory conditions due to accumulation for VFA, ammonia and other inhibitors like antibiotics. In our study, we used two mixing rate, 90 rpm and 120 rpm. Biogas yields did not show a significant difference but effect of mixing on microbial communities was clearly seen in molecular studies. In another study, it has been stated that there was no effect of mixing on digesters performance when fed with 5% TS manure slurry (Karim et al., 2005).

But when digesters were fed with 10% TS manure slurry, mixing increased biogas production by 15%-29% in respect to unmixed digester (Karim et al., 2005). Also mixing had positive effect on start-up of the digester (Gomez et al., 2006; Kaparaju et al., 2008)

Every 10 day biogas composition was monitored by GC analysis. Results of analysis were given in Table 5.16.

Table 5.16. Biogas compositions during digester operation

Digester	Digestion time (day)	N_2 (%)	CH_4 (%)	CO_2 (%)
Set 1 S1 Control	20	19	49	31
	30		62	37
Set 1 S3	20	6	53	41
	30		60	40
Set 1 S4 Control	20	6	60	34
	30		64	36
Set 1 S6	20	6	56	38
	30		65	35
Set 2 S1 Control	20	7	42	50
	30		59	42
Set 2 S3	20	7	46	46

Table 5.16. continued

	30		59	41
Set 2 S4 Control	20	9	45	45
	30		64	36
Set 2 S6	20	9	42	46
	30		62	38
Set 3 S1 Control	10	6	64	30
	20		70	30
Set 3 S3	10	9	62	29
	20		70	30
Set 3 S4 Control	10		65	35
	20		70	30
Set 3 S6	10	4	66	31
	20		68	32
Set 4 S1 Control	10		62	38
	20		70	30
Set 4 S3	10		63	37
	20		70	30
Set 4 S4 Control	10	5	65	30
	20		68	32
Set 4 S6	10	9	63	28
	20		68	32

VFA concentrations were also recorded thoughout the study in each sampling time and results were given in Table 5.17.

Table 5.17. Volatile Fatty Acid (VFA) concentrations in digester studies (mg/l)

Digester	acetic	propionic	isobutyric	butyric	isovaleric	valeric	isocaproic	caproic	heptanoic
Set 1 S1 C. D0	1049	244	29	114	25	27	0	0	0
Set 1 S1 C. D10	61	999	37	0	74	11	9	0	0
Set 1 S1 C. D20	30	0	0	0	0	0	0	0	0
Set 1 S1 C. D30	27	0	0	0	0	0	0	0	0
Set 1 S3 D0	205	36	8	19	8	5	0	0	0
Set 1 S3 D10	38	0	0	0	0	0	0	0	0
Set 1 S3 D20	38	0	0	0	0	0	0	0	0
Set 1 S3 D30	30	0	0	0	0	0	0	0	0
Set 1 S4 C. D0	1811	394	47	184	41	44	0	0	0
Set 1 S4 C. D10	58	2241	18	0	119	17	16	0	0
Set 1 S4 C. D20	30	0	0	0	0	0	0	0	0
Set 1 S4 C. D30	28	0	0	0	0	0	0	0	0

Table 5.17 continued

Set 1 S6 D0	550	147	25	71	27	11	0	0	0
Set 1 S6 D10	108	571	0	0	0	7	0	0	0
Set 1 S6 D20	55	0	0	0	0	0	0	0	0
Set 1 S6 D30	48	0	0	0	0	0	0	0	0
Set 2 S1 C. D0	302	57	15	35	16	13	0	0	0
Set 2 S1 C. D10	31	343	0	0	15	0	0	0	0
Set 2 S1 C. D20	31	165	6	0	0	0	0	0	0
Set 2 S1 C. D30	50	0	0	0	0	0	0	0	0
Set 2 S3 D0	216	57	10	36	13	7	0	0	0
Set 2 S3 D10	35	637	0	0	0	0	13	0	0
Set 2 S3 D20	44	24	0	0	0	0	0	0	0
Set 2 S3 D30	52	0	0	0	0	0	0	0	0
Set 2 S4 C. D0	800	189	39	101	35	31	0	0	0
Set 2 S4 C. D10	57	1139	0	0	44	7	5	0	0
Set 2 S4 C. D20	72	423	0	0	0	0	0	0	0
Set 2 S4 C. D30	105	0	0	0	0	0	0	0	0
Set 2 S6 D0	623	167	25	135	32	22	5	0	0
Set 2 S6 D10	59	2096	97	0	177	37	13	4	0
Set 2 S6 D20	68	997	0	0	70	11	16	0	0
Set 2 S6 D30	68	0	0	0	0	0	0	0	0
Set 3 S1 C. D0	47	0	0	0	0	0	0	0	0
Set 3 S1 C. D5	55	164	0	0	11	0	0	0	13
Set 3 S1 C. D10	48	91	0	0	0	0	0	0	0
Set 3 S1 C. D15	76	12	0	0	0	0	0	0	0
Set 3 S1 C. D20	49	0	0	0	0	0	0	0	0
Set 3 S3 D0	370	88	12	28	13	11	0	0	0
Set 3 S3 D5	97	291	0	0	35	6	0	0	0
Set 3 S3 D10	56	0	0	0	0	0	0	0	0
Set 3 S3 D15	58	0	0	0	0	0	0	0	0
Set 3 S3 D20	58	0	0	0	0	0	0	0	0
Set 3 S4 C. D0	147	25	0	4	0	0	0	0	0
Set 3 S4 C. D5	87	281	0	0	15	0	63	0	0
Set 3 S4 C. D10	56	0	0	0	0	0	0	0	0
Set 3 S4 C. D15	49	0	0	0	0	0	0	0	0
Set 3 S4 C. D20	50	0	0	0	0	0	0	0	0
Set 3 S6 D0	546	136	19	43	21	17	0	0	0
Set 3 S6 D5	174	491	11	0	50	16	74	0	0
Set 3 S6 D10	70	0	0	0	0	0	0	0	0
Set 3 S6 D15	54	0	0	0	0	0	0	0	0
Set 3 S6 D20	52	0	0	0	0	0	0	0	0

Table 5.17 continued

Set 4 S1 C. D0	36	0	0	0	0	0	0	159	0
Set 4 S1 C. D5	37	179	30	15	25	7	0	7	0
Set 4 S1 C. D10	29	201	0	0	0	0	0	0	0
Set 4 S1 C. D15	34	110	0	0	0	0	0	140	0
Set 4 S1 C. D20	70	60	0	0	0	0	0	0	0
Set 4 S3 D0	170	42	0	13	3	0	0	0	0
Set 4 S3 D5	82	228	44	11	51	15	18	8	12
Set 4 S3 D10	39	239	0	0	0	0	0	0	0
Set 4 S3 D15	45	62	0	0	0	0	0	0	0
Set 4 S3 D20	50	9	0	0	0	0	0	0	0
Set 4 S4 C.D0	61	0	0	0	0	0	0	0	0
Set 4 S4 C. D5	65	245	0	0	20	0	43	0	15
Set 4 S4 C. D10	38	24	0	0	0	0	0	0	15
Set 4 S4 C. D15	44	35	0	0	0	0	0	0	0
Set 4 S4 C.D20	40	0	0	0	0	0	0	0	0
Set 4 S6 D0	279	87	12	29	13	9	0	0	0
Set 4 S6 D5	90	538	25	0	108	21	100	0	13
Set 4 S6 D10	53	217	0	0	0	0	0	0	0
Set 4 S6 D15	45	0	0	0	0	0	0	0	0
Set 4 S6 D20	38	0	0	0	0	0	0	0	0

COD analysis in anaerobic degradation was mainly suitable for wastewater treatment systems since homogeneous liquids in influent and effluent was easily diluted and analyzed. On the other hand, in manure digesters viscosity is low and slurry is not homogeneous. Also low solubility of organic part of manure cannot reflect total COD of samples. In our study, we observed that TS elimination was directly related to biogas production and therefore, can be used in monitoring system performance. In digesters which had higher biogas yields, higher elimination of TS was also observed. Another common parameter for monitoring system performance was VFA concentration and composition. An increase in VFA will be suspicious and need to be closely monitored. In our study, VFA concentrations were increased time to time but do not accumulated with time. Similarly, Arikan et al. (2006) reported that the presence of OTC in manure from medicated calves showed no significant effect on biogas composition or on reductions in volatile solids. Due to presence of high alkanity, manure digesters can tolerate higher VFA concentrations than wastewater treatment systems (Coats et al., 2011). VFA concentration up to 6000 mg/l was detected in operational manure digesters (Masse et al., 2000). This was probably due to the low hydrogen pressure in the system resulted in action of hydrogenotrophic methanogens (Coats et al., 2011). Also low concentrations of VFA like in our study can be interpreted as the system can tolerate higher organic loads (Ahring, 2003).

5.3.4. Microbial Community Structures in Anaerobic Digesters

Identification of microbial community structures were done by RNA and DNA targeting molecular techniques.

5.3.4.1. Genomic DNA and total RNA extraction. At each sampling time total RNA and Genomic DNA was extracted from samples collected. Total RNA was used then for cDNA synthesis. All nucleic acids were analyzed for its concentrations by Nanodrop 2000 (Thermo Scientific, Australia). Results were given in Table 5.18. and 5.19.

Table 5.18. GDNA concentration of samples collected from digesters

Set 1	**Operation Day**	**Concentration (ng/ml)**	**Set 2**	**Operation Day**	**Concentration (ng/ml)**
S1	D0	124	S1	D0	162
S3	D0	206	S3	D0	206
S4	D0	275	S4	D0	234
S6	D0	320	S6	D0	257
S1	D10	223	S1	D10	159
S3	D10	305	S3	D10	206
S4	D10	324	S4	D10	160
S6	D10	238	S6	D10	154
S1	D20	307	S1	D20	128
S3	D20	214	S3	D20	165
S4	D20	566	S4	D20	200
S6	D20	556	S6	D20	116
S1	D30		S1	D30	146
S3	D30	451	S3	D30	177
S4	D30	610	S4	D30	183
S6	D30	512	S6	D30	348
Set 3	**Operation Day**	**Concentration (ng/ml)**	**Set 4**	**Operation Day**	**Concentration (ng/ml)**
S1	D0	56	S1	D0	107
S3	D0	51	S3	D0	158

Table 5.18. continued

S4	D5	112	S4	D5	79
S6	D5	38	S6	D5	177
S1	D5	55	S1	D5	136
S3	D5	60	S3	D5	92
S4	D10	120	S4	D10	165
S6	D10	53	S6	D10	113
S1	D10	50	S1	D10	146
S3	D10	44	S3	D10	82
S4	D15	68	S4	D15	82
S6	D15	49	S6	D15	132
S1	D15	41	S1	D15	76
S3	D15	37	S3	D15	85
S4	D20	98	S4	D20	82
S6	D20	71	S6	D20	77
S1	D20	52	S1	D20	158
S3	D20	75	S3	D20	135
S4	D20	71	S4	D20	116
S6	D20	49	S6	D20	94

Table 5.19. cDNA concentrations of samples collected from digesters

Set 1	**Operation Day**	**Concentration (ng/ml)**	**Set 2**	**Operation Day**	**Concentration (ng/ml)**
S1	D0	2265	S1	D0	2616
S3	D0	2616	S3	D0	2229
S4	D0	2229	S4	D0	2272
S6	D0	2272	S6	D0	2140
S1	D10	2140	S1	D10	2130
S3	D10	2130	S3	D10	1930
S4	D10	1930	S4	D10	1900
S6	D10	1900	S6	D10	1990
S1	D20	1990	S1	D20	2170
S3	D20	2170	S3	D20	2130
S4	D20	2130	S4	D20	2250
S6	D20	2250	S6	D20	2000
S1	D30	2000	S1	D30	2313
S3	D30	2313	S3	D30	2174
S4	D30	2174	S4	D30	2186
S6	D30	2186	S6	D30	2405

Table 5.19. continued

Set 3	Operation Day	Concentration (ng/ml)	Set 4	Operation Day	Concentration (ng/ml)
S1	D0	2120	S1	D0	2326
S3	D0	2090	S3	D0	2265
S4	D5	2390	S4	D5	2300
S6	D5	2380	S6	D5	2250
S1	D5	2340	S1	D5	2305
S3	D5	2433	S3	D5	2312
S4	D10	3240	S4	D10	2420
S6	D10	2460	S6	D10	2470
S1	D10	2360	S1	D10	2451
S3	D10	2370	S3	D10	2365
S4	D15	2430	S4	D15	2311
S6	D15	2550	S6	D15	2430
S1	D15	2315	S1	D15	2400
S3	D15	2280	S3	D15	2350
S4	D20	2190	S4	D20	2470
S6	D20	2165	S6	D20	2390
S1	D20	2240	S1	D20	2546
S3	D20		S3	D20	
S4	D20	2160	S4	D20	2620
S6	D20	1960	S6	D20	2460

Concentrations of GDNA were high enough for downstream analysis. Concentrations of cDNAs were very high therefore; they were diluted to 100 ng/ml before Q-PCR analysis.

5.3.4.2. Community fingerprinting with DGGE. Community fingerprinting of each digester was performed with DGGE. Dice correlation which is based on presence-absence of bands was used to changes in community diversity. Digesters in each set were analyzed within the set and relation tree was constructed. Changes in diversity with time and relation trees were given through Figure 5.11-5.26.

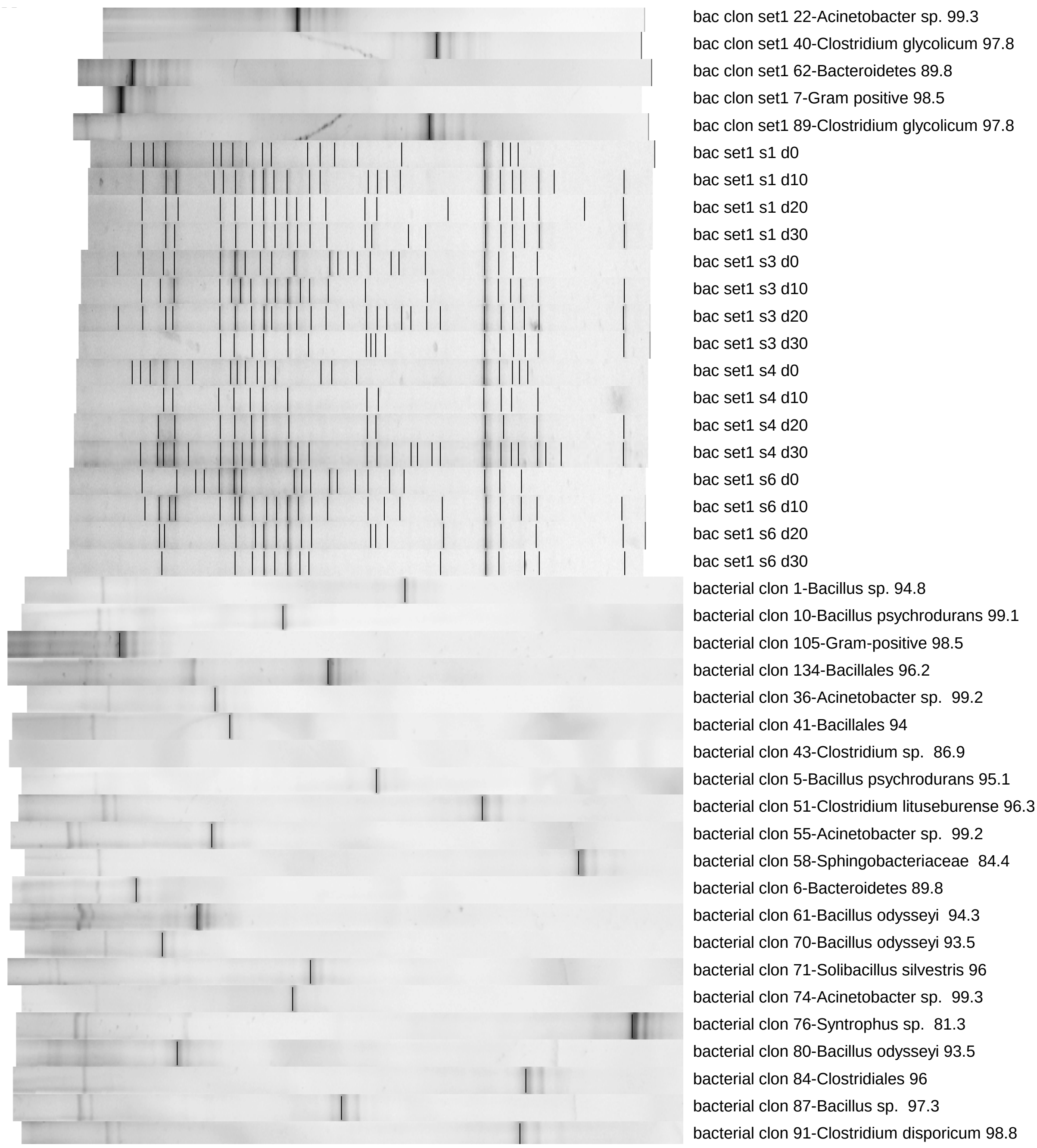


Figure 5.11. Change of bacterial diversity of Set 1 digesters according to time

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9%

Set 1: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

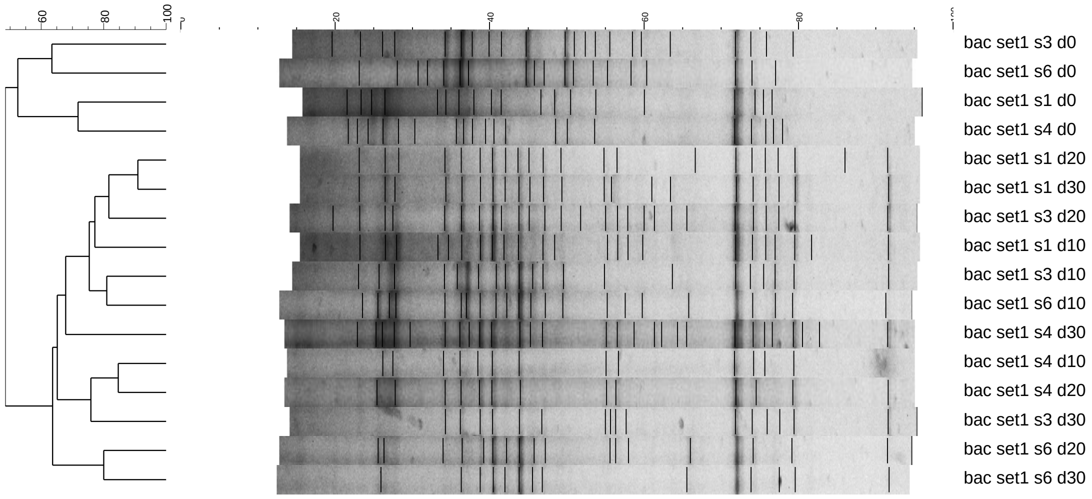


Figure 5.12. Bacterial community dendogram of Set 1 digesters according to Dice correlation

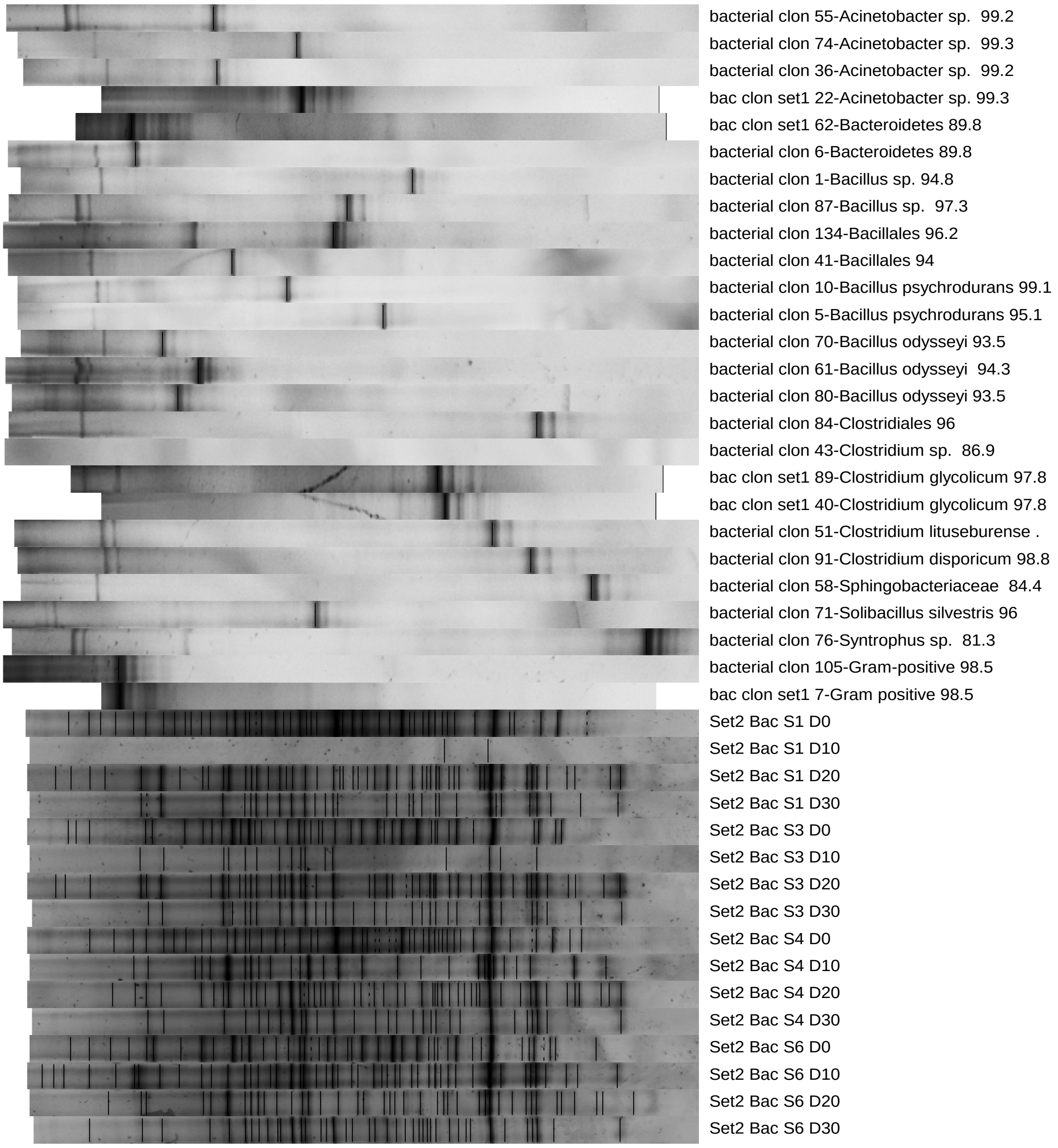


Figure 5.13. Change of bacterial diversity of Set 2 digesters according to time

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9%

Set 1: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

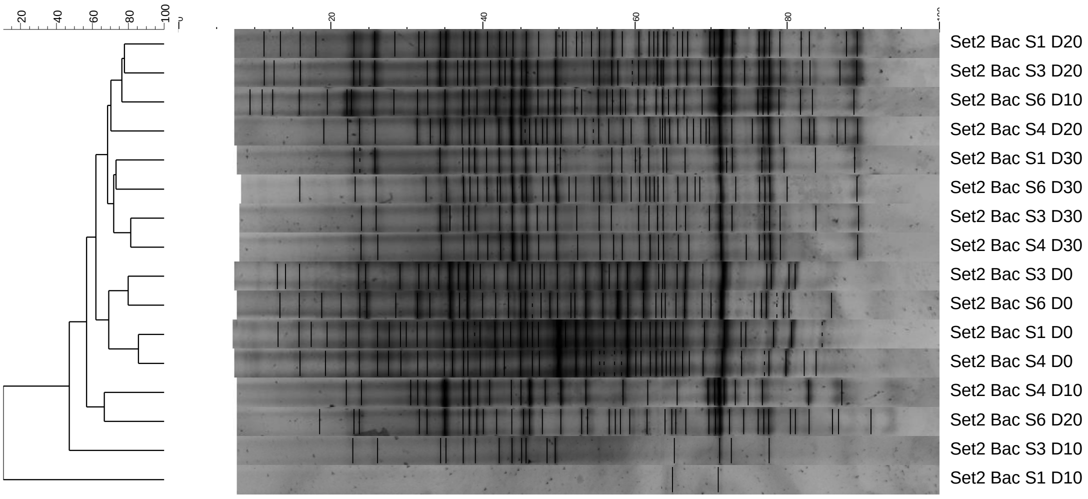


Figure 5.14. Bacterial community dendogram of Set 2 digesters according to Dice correlation

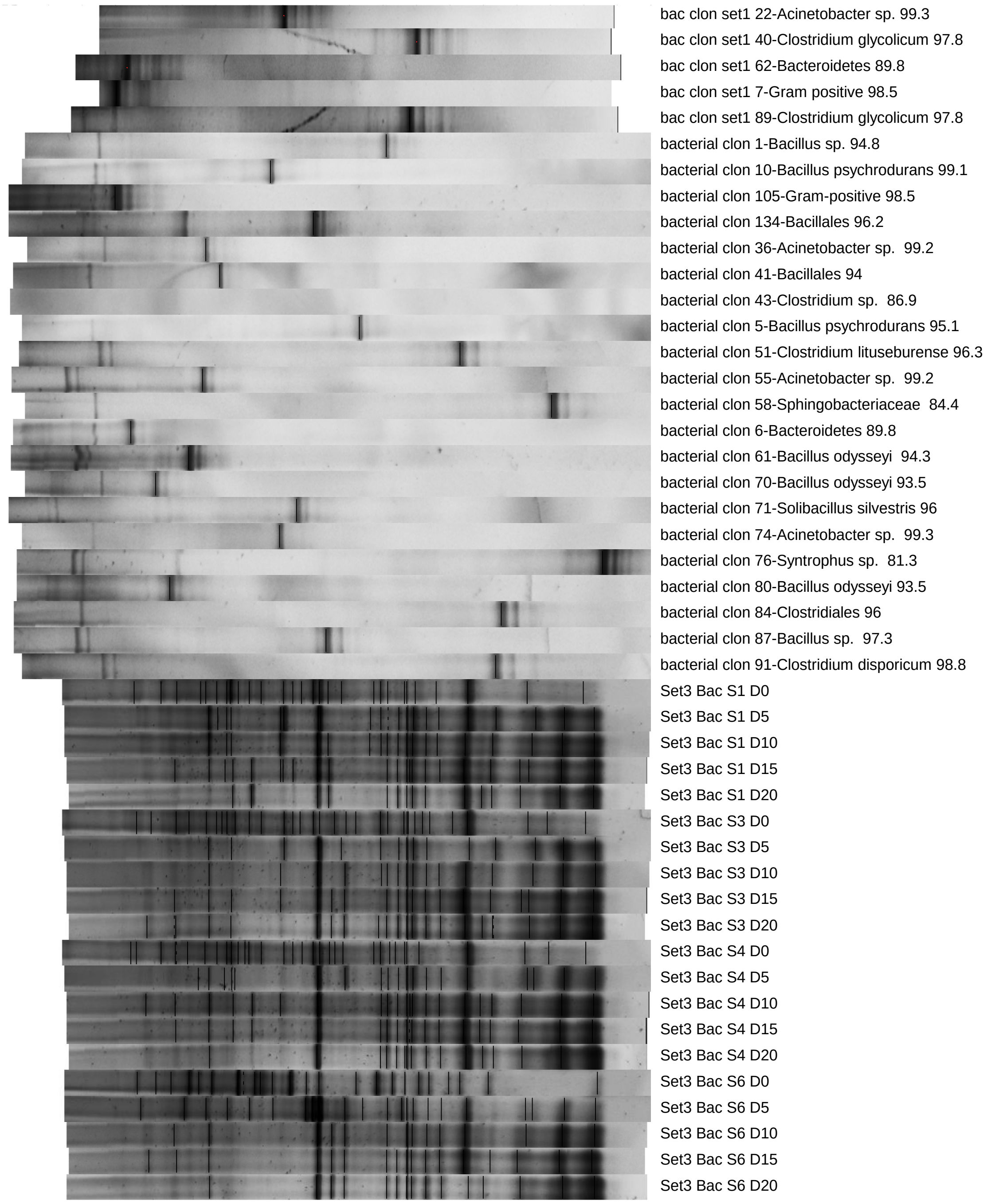


Figure 5.15. Change of bacterial diversity of Set 3 digesters according to time

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9%

Set 1: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

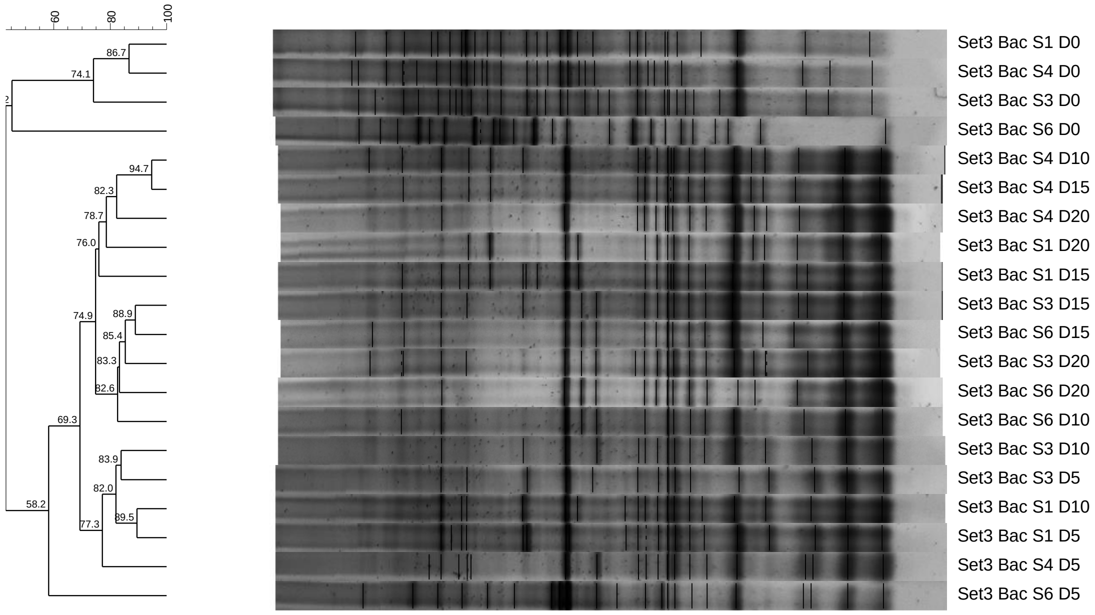


Figure 5.16. Bacterial community dendogram of Set 3 digesters according to Dice correlation

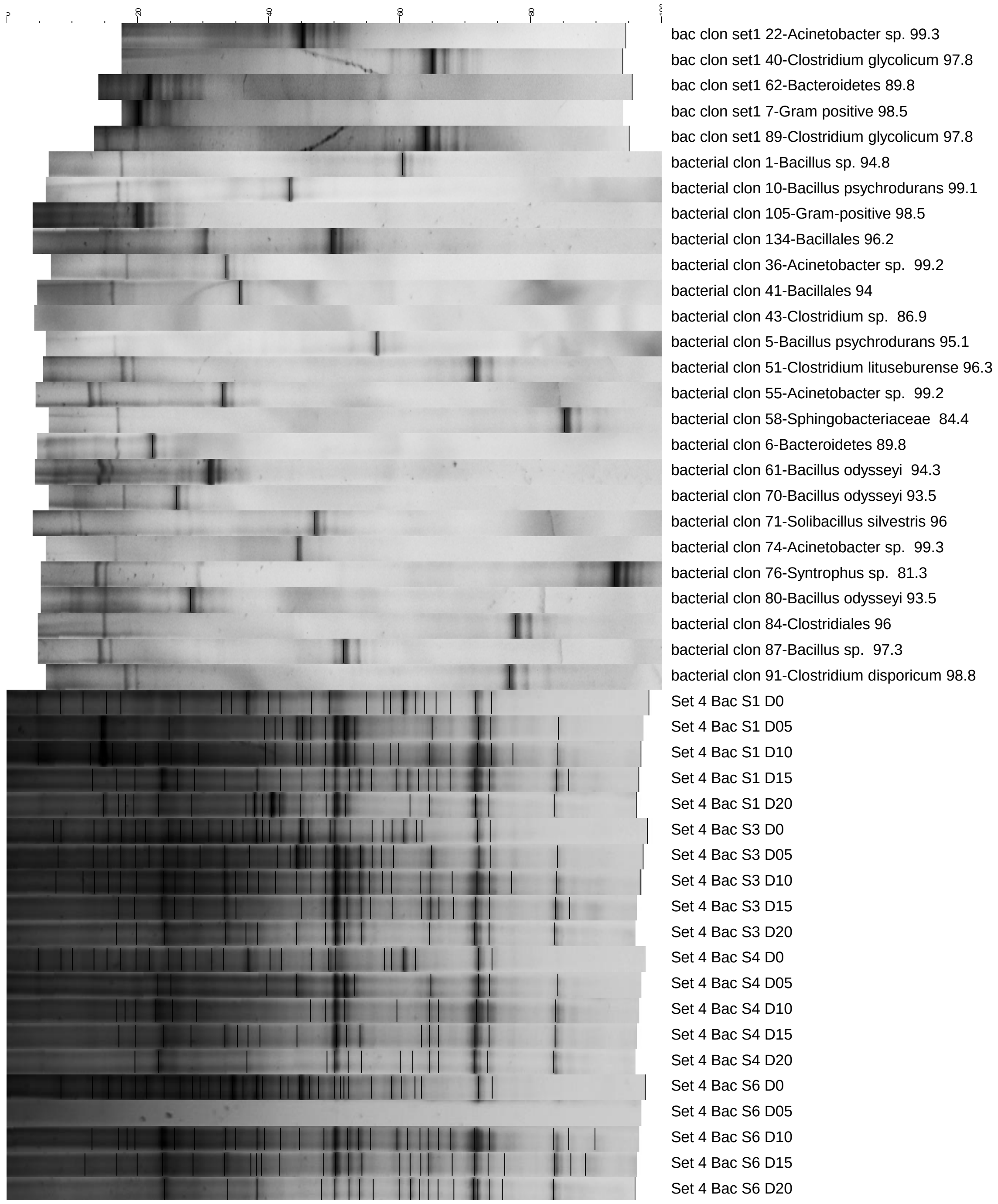


Figure 5.17. Change of bacterial diversity of Set 4 digesters according to time

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9%

Set 1: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

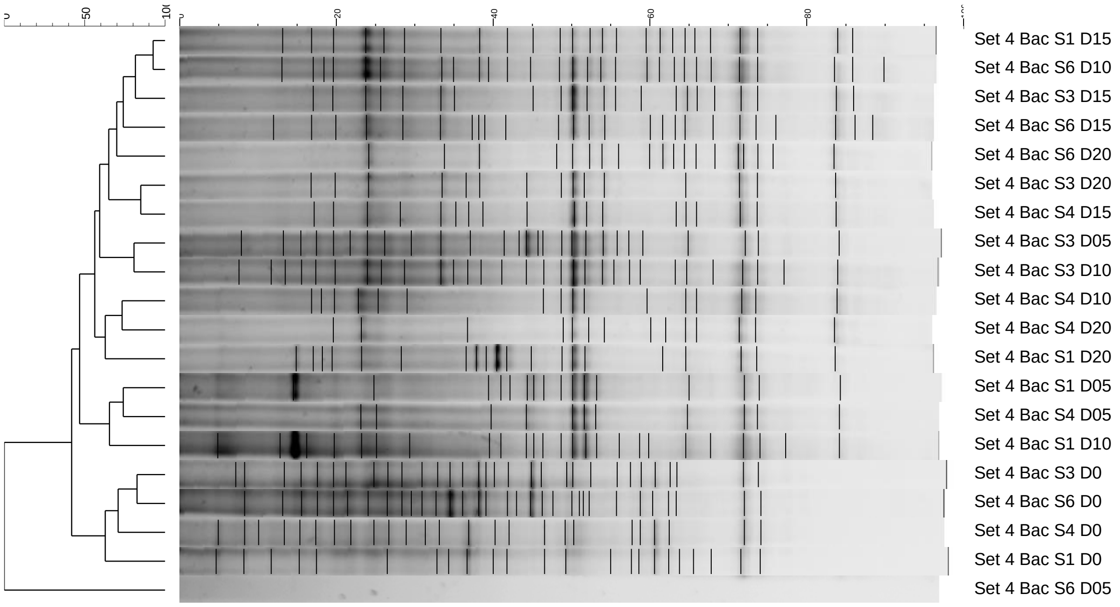


Figure 5.18. Bacterial community dendogram of Set 4 digesters according to Dice correlation

In DGGE analysis of samples collected from digesters it has been shown that bacterial diversity changed with time. Temperature has strong impact on diversity change. In thermophilic digesters 25-30 bands decreased in number with time to 11-16 bands. High temperature does not allow survival of all bacterial species; therefore, eliminated bands were supposedly belonging species that cannot survive in high temperatures. The decrease in bacterial band number was less in mesophilic digesters. In Set 1, from 20-22 different bands, some are eliminated, new bands appeared and 16-18 bands were present at day 30. Same situation was also observed in Set 2. 30+ bands in starting community were decreased to 24-26 bands during digestion. In all digesters, starting communities (Day 0) clustered differently than other samples. According dice correlation analysis of DGGE, in all digesters clustering was related to operation time rather than operational parameters. This finding indicated that although microbial diversity changed with time, effects of mixing rate and solid content do not have a significant impact on microbial diversity. In literature it has been stated that, especially bacterial species doesn’t change according to reactor type or operational parameters but to substrate type (Ince et al., 2011).

Many of species in bacterial diversity has been seen in bacterial clone library. *Clostridium glycolicum, Clostridium lituseburense and Acinetobacter* spp. has been seen in all digesters in all sampling times but *Bacteriodetes, Bacillales, Solibacillus silvestris, Bacillus odyssey* has seen only in starting communities and eliminated from the system with time. *Bacillus psychrodurans, Bacillus odysseyi, Clostridium disporicum* were seen only in mesophilic digesters. One *Bacillales* spp. was seen only in mesophilic digesters at starting communities but survived in thermophilic digesters throughout digestion time. In statistical analysis of bacterial species and OTC concentrations, a negative relation with OTC concentration and *Bacillales* group was monitored as *Clostridiales* group showed a positive relation. It is known that species of *Clostridium* is resistant to environmental conditions, therefore, they may possible resistant to OTC too (Kuesel et al., 2001). Microbial analysis of soil studies in this study also supported that theory.

In clone library analysis of our digesters, it has been showed that dominance of *Firmicutes* group which was composed of *Bacillus* and *Clostridium* spp. In a study, microbial communities of mammal gut were investigated along with species and specific diet of animal. In this study, 17 different species has been found. Microbial communities were made of *Firmicutes* by 66% and *Bacteriodetes, Proteobacteria* and *Acinetobacteria* by 16% (Ley et al., 2008). Another study monitored dominant members of microbial diversity in swine manure by DGGE and found that *Clostridium* spp. were related with dominant bands (Leung and Topp, 2001). A study identified microbial community structure of thermophilic cattle manure digester with SSCP where 80% of community was made of *Bacillus* and *Clostridium* spp. Remaining percentage was made up by *Bacteriodetes* and *Preoteobacteria* spp. (Chachkhiani et al., 2004). In general, diversity analysis of bacterial domain of digester can be interpreted as follows: *Bacillales* group was seen in starting communities but eliminated with time; *Clostridium* spp. survived during digestion.

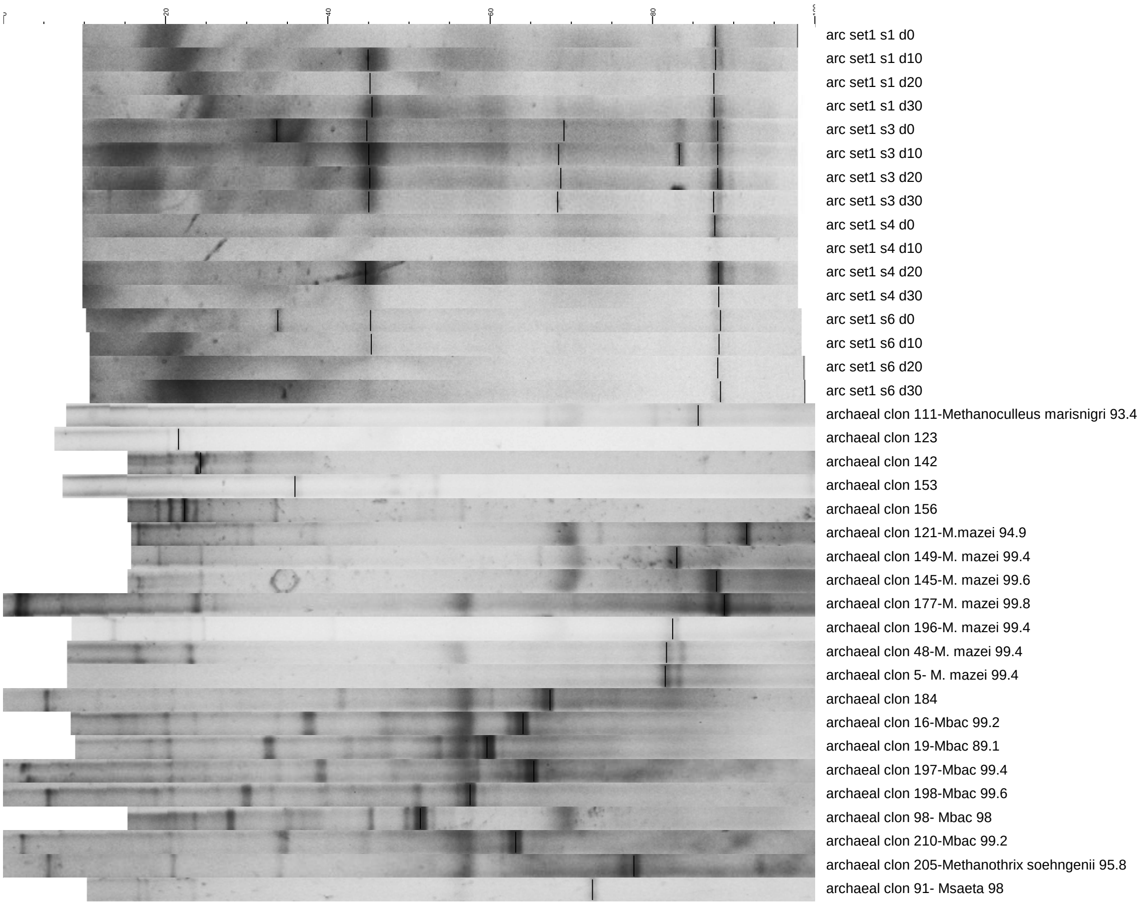


Figure 5.19. Change of archaeal diversity of Set 1 digesters according to time

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9%

Set 1: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

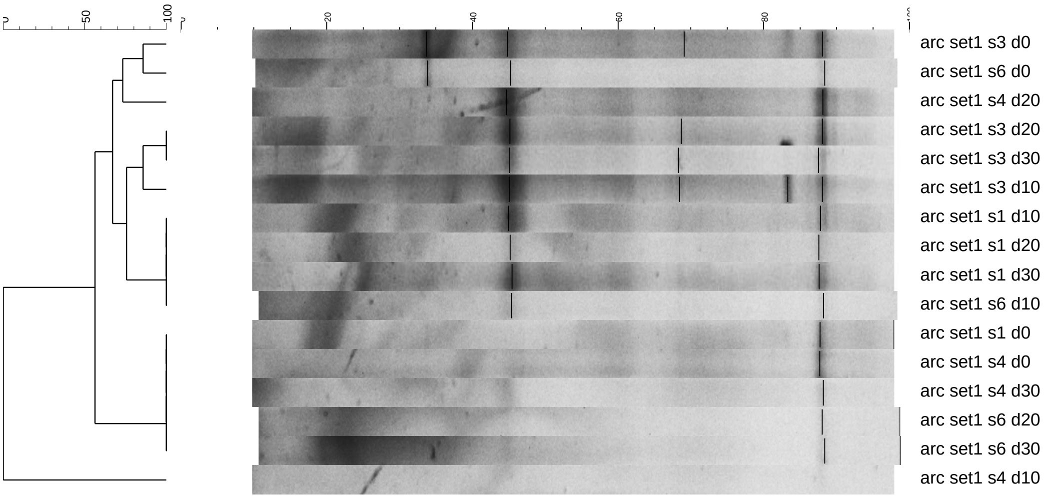


Figure 5.20. Archaeal community dendogram of Set 1 digesters according to Dice correlation

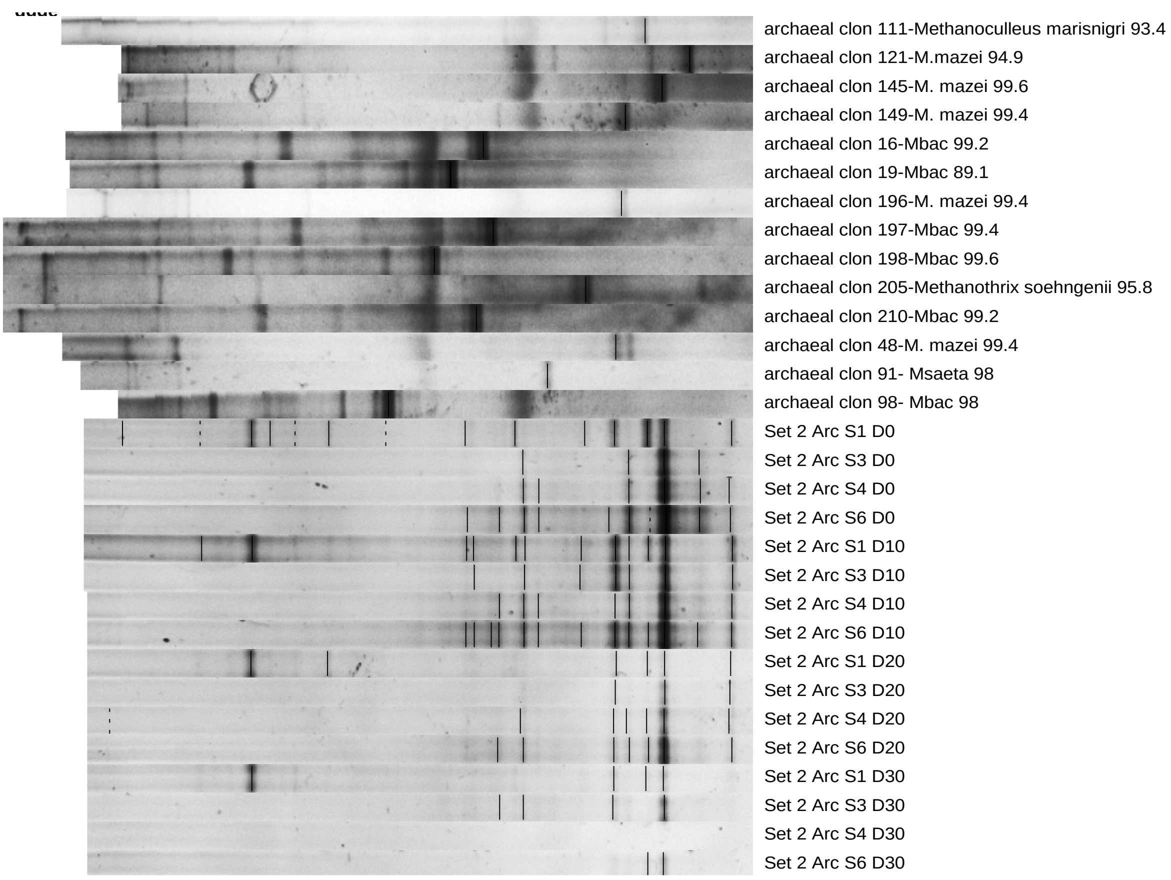


Figure 5.21. Change of archaeal diversity of Set 2 digesters according to time

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9%

Set 1: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

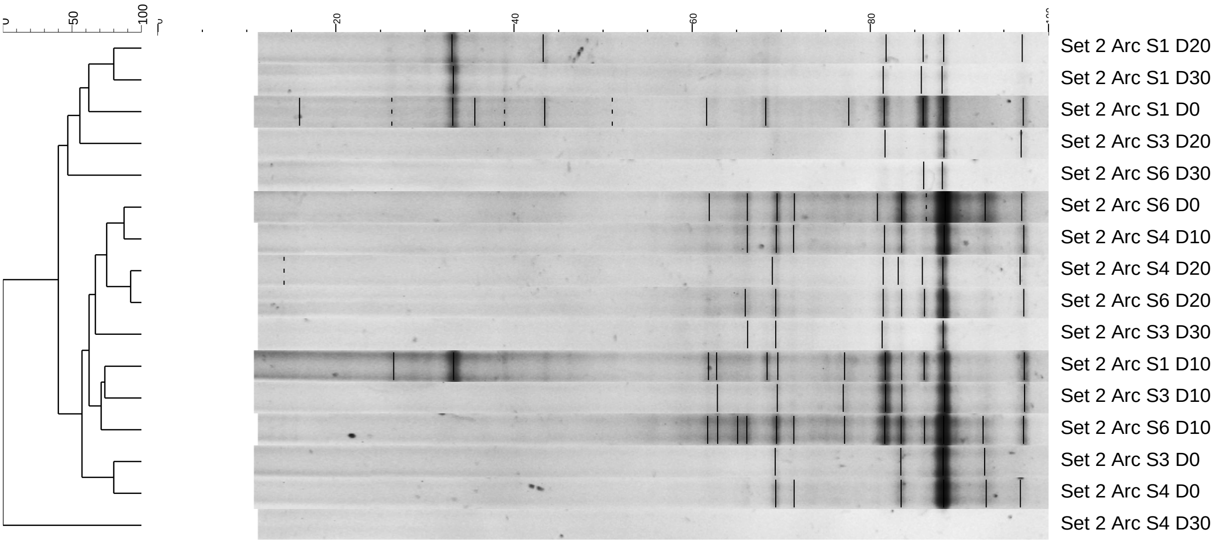


Figure 5.22. Archaeal community dendogram of Set 2 digesters according to Dice correlation

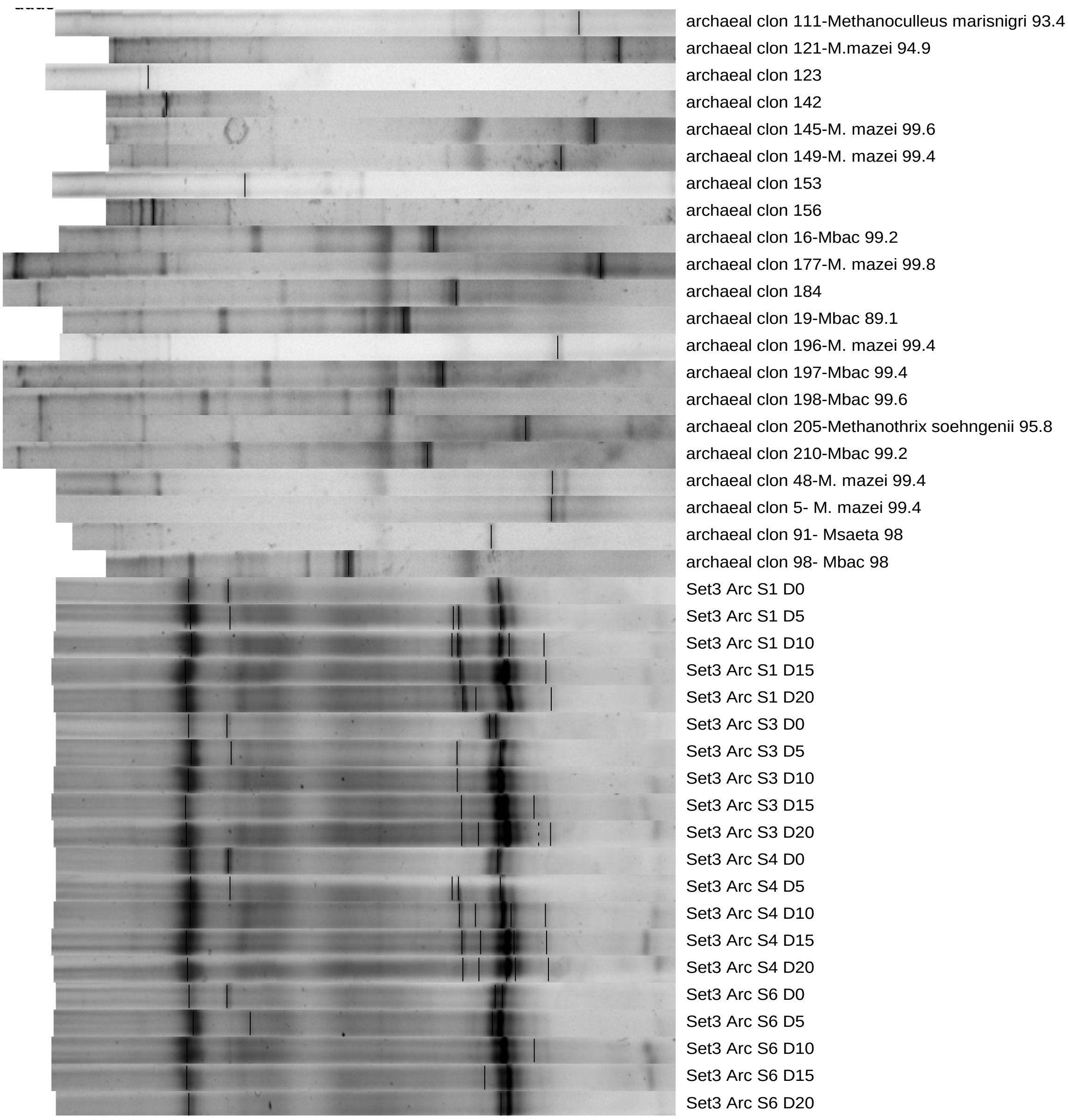


Figure 5.23. Change of archaeal diversity of Set 3 digesters according to time

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9%

Set 1: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

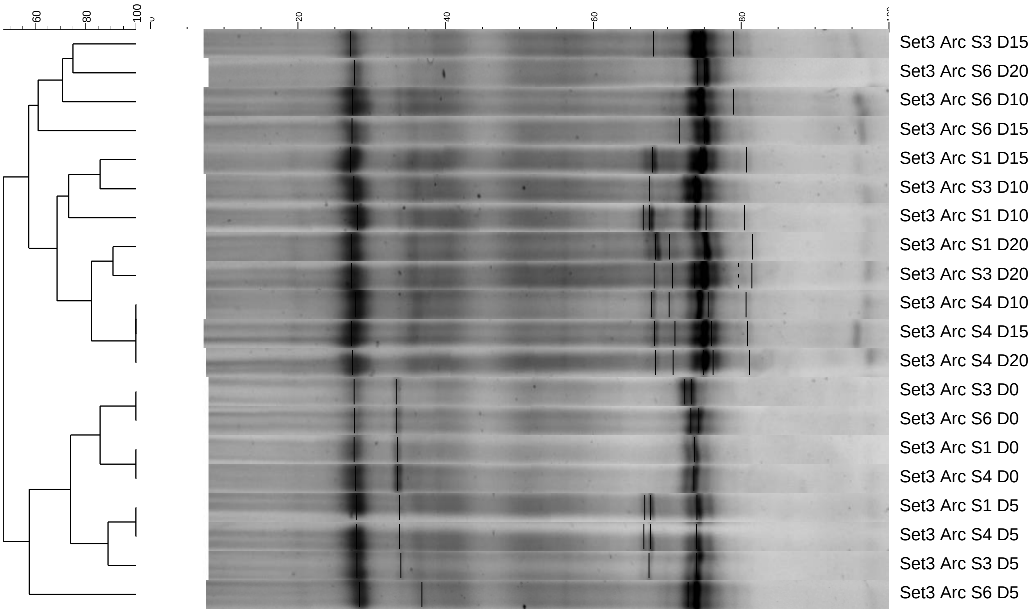


Figure 5.24. Archaeal community dendogram of Set 3 digesters according to Dice correlation

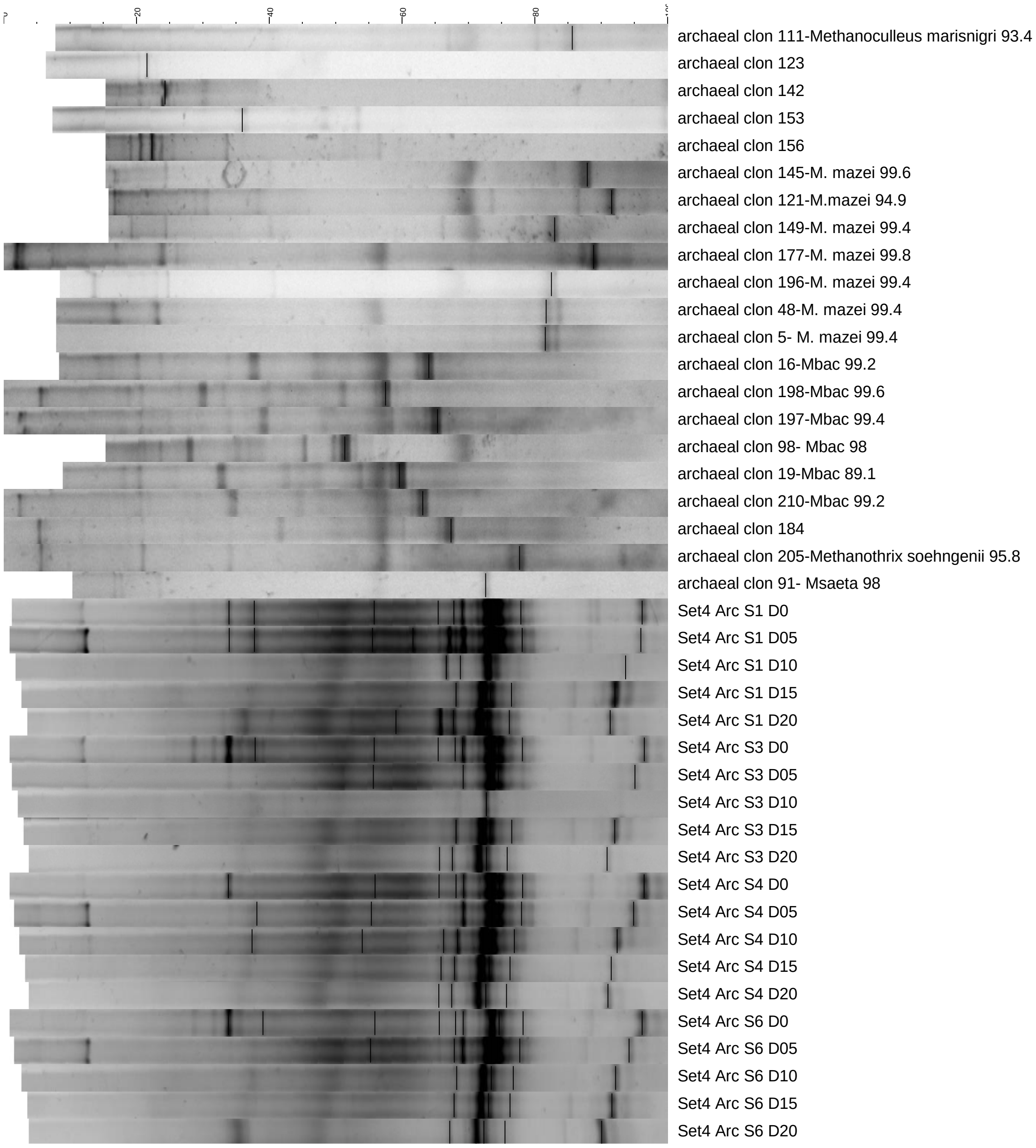


Figure 5.25. Change of archaeal diversity of Set 4 digesters according to time

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9%

Set 1: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

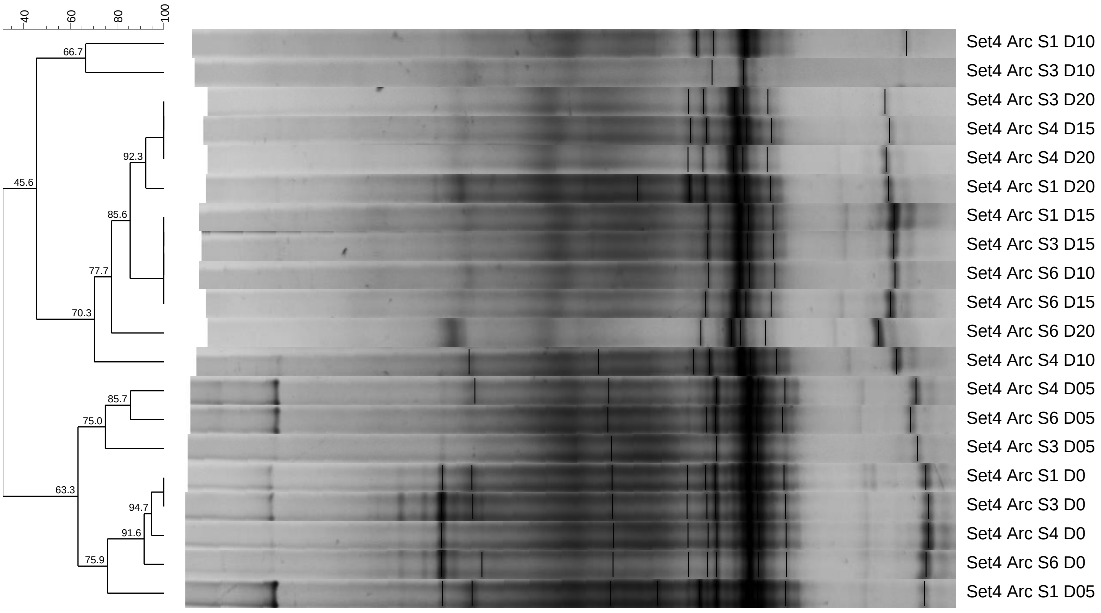


Figure 5.26. Archaeal community dendogram of Set 4 digesters according to Dice correlation

Archaeal diversity was low in digesters (5-10 bands). Like in bacterial diversity analysis, clustering in archaeal diversity was depended on time rather than digester type. Especially in thermophilic digesters, diversity was stabilized after 10 days. As Day 0 and Day 5 samples made a cluster, other samples (Day 10, Day 15 and Day 20) made another cluster. In Set 1, bands were related to *Methanosarcina mazei* and *Methanobacteriales* spp. In thermophilic digesters, diversity was low as mesophilic digesters but bands were related to *Methanosaeta* spp.*, Methanobacteriales* spp. *and Methanosarcina* spp. In Set 3 digesters, bands related to *Methanosaeta* spp. were not dominant, but they changed into dominant bands with time. These results showed that even in archaeal domain if the conditions were met, archaeal species may increase in number and dominate the system.

16S rDNA clone library analysis of archaeal community in our digesters showed dominance of hydrogenotrophic methanogens represented by *Methanobacteriales and Methanosarcina* spp. Genus *Methanosarcina* contains very versatile species which are able to use all methane pathways. In study of Chachkhiani et al., (2004), archaeal diversity of thermophilic cattle manure digesters was dominated by *Methanobacteriales* and *Methanosarcina* spp. Main pathway to methane follows route over acetate and mainly this reaction is performed by *Methanosaeta* spp. But in the absence of these species like in manure digesters, whole methane formation was done by hydrogenotrophic methanogens. This pathway was mainly supported by the activity of syntrophic bacterial species like *Clostridium spp.* and hydrogenotrophic methanogens like *Methanobacteriales* and *Methanomicrobiales* spp. (Schnurrer et al., 1997; Hattori et al., 2000).

5.3.4.3. Quantitative analysis of microbial communities with Q-PCR. Quantitative analysis of total bacteria, *Methanosaeta* spp., *Methanosarcinales, Methanomicrobiales* and *Methanobacteriales* groups were performed by Real Time PCR. Results were given through Tables 5.20-5.23. and Figures 5.27 – 5.44.

Table 5.20. Q-PCR analysis of Set 1 digester samples

Set 1		**Bac**	**M.Bac**	**M.Mic**	**M.Sar**
Day	**Digester**	**Gene copy**	**Gene copy**	**Gene copy**	**Gene copy**
D0	**S1**	3.51E+09	3.60E+08	7.96E+05	2.36E+08
D0	**S3**	4.84E+09	4.20E+08	9.56E+06	2.28E+08
D0	**S4**	5.94E+08	1.65E+08	4.54E+05	3.88E+06
D0	**S6**	9.53E+08	2.62E+08	1.60E+06	4.90E+07
D10	**S1**	7.12E+08	9.94E+07	2.78E+07	5.24E+08
D10	**S3**	1.26E+09	3.25E+08	9.90E+07	1.76E+09
D10	**S4**	4.06E+07	3.08E+07	1.22E+06	2.84E+07
D10	**S6**	2.32E+08	2.41E+08	5.00E+07	7.72E+08
D20	**S1**	2.82E+08	1.93E+08	1.33E+07	5.92E+08
D20	**S3**	5.91E+07	5.01E+07	1.90E+06	3.98E+07
D20	**S4**	8.26E+07	7.73E+07	3.12E+06	2.78E+08
D20	**S6**	9.14E+07	1.82E+08	9.86E+06	3.20E+08
D30	**S1**	7.64E+07	4.52E+07	1.95E+06	4.92E+07
D30	**S3**	4.29E+07	3.07E+07	1.07E+06	2.38E+07
D30	**S4**	1.86E+07	1.38E+07	2.20E+05	4.36E+06
D30	**S6**	1.05E+07	1.12E+07	1.03E+05	1.79E+06

Table 5.21. Q-PCR analysis of Set 2 digester samples

Set 2		**Bac**	**M.Bac**	**M.Mic**	**M.Sar**
Day	**Digester**	**Gene copy**	**Gene copy**	**Gene copy**	**Gene copy**
D0	**S1**	5.00E+08	1.57E+07	2.32E+06	2.92E+06
D0	**S3**	1.84E+09	2.32E+07	4.90E+06	2.24E+06
D0	**S4**	2.88E+08	3.38E+07	1.03E+06	2.00E+07
D0	**S6**	1.93E+09	3.02E+08	1.36E+06	1.84E+09
D10	**S1**	2.56E+08	8.52E+07	8.62E+05	1.23E+08
D10	**S3**	7.76E+08	2.14E+08	1.97E+06	3.66E+08
D10	**S4**	3.08E+08	9.04E+07	7.86E+05	1.32E+08
D10	**S6**	1.61E+09	5.12E+08	9.14E+06	1.41E+09
D20	**S1**	6.48E+08	2.10E+08	4.40E+06	3.02E+08
D20	**S3**	1.38E+09	6.54E+08	5.26E+06	1.04E+09
D20	**S4**	1.98E+08	4.16E+07	1.11E+06	1.40E+08
D20	**S6**	1.34E+09	6.46E+08	1.09E+07	9.68E+08
D30	**S1**	8.02E+08	2.00E+08	1.28E+06	4.54E+08
D30	**S3**	1.05E+09	1.97E+08	3.84E+06	5.82E+08

Table 5.22. Q-PCR analysis of Set 3 digester samples

Set 3		**Bac**	**M.Bac**	**M.Mic**	**M.Saeta**	**M.Sar**
Day	**Digester**	**Gene copy**	**Gene copy**	**Gene copy**	**Gene copy**	**Gene copy**
D0	**S1**	3.39E+10	2.52E+09	1.11E+08	3.10E+06	3.32E+08
D0	**S3**	2.96E+10	1.02E+09	1.65E+08	6.76E+06	6.02E+08
D0	**S4**	7.77E+09	2.41E+09	8.69E+07	4.60E+06	1.02E+08
D0	**S6**	1.58E+10	9.08E+08	9.50E+07	1.54E+06	1.74E+08
D5	**S1**	2.02E+09	1.18E+09	8.95E+06	5.56E+05	3.12E+08
D5	**S3**	3.24E+09	1.36E+09	1.25E+07	4.42E+06	1.81E+09
D5	**S4**	1.87E+09	1.45E+09	2.22E+07	3.56E+06	1.62E+09
D5	**S6**	2.46E+09	2.29E+09	7.96E+06	1.11E+06	1.41E+09
D10	**S1**	1.40E+09	1.60E+09	4.69E+06	2.04E+07	2.02E+09
D10	**S3**	2.96E+08	7.45E+08	3.97E+06	4.76E+05	1.32E+09
D10	**S4**	7.13E+08	1.38E+09	1.01E+07	1.03E+06	9.84E+08
D10	**S6**	1.07E+08	6.98E+08	7.04E+06	5.44E+05	5.62E+08
D15	**S1**	2.10E+08	1.06E+09	5.20E+06	1.34E+06	7.58E+08
D15	**S3**	1.37E+08	7.84E+08	7.20E+06	5.22E+05	5.10E+08
D15	**S4**	7.91E+06	4.49E+08	2.77E+05	2.46E+07	2.04E+08
D15	**S6**	3.59E+07	3.78E+08	3.18E+06	3.86E+05	6.18E+08
D20	**S1**	5.99E+07	3.22E+08	3.60E+06	8.38E+05	3.82E+08
D20	**S3**	9.68E+06	6.30E+08	2.03E+05	9.04E+05	4.10E+06

Table 5.22. continued

D20	**S4**	4.91E+06	1.25E+08	3.90E+05	2.40E+05	4.18E+07
D20	**S6**	1.21E+07	3.38E+08	3.33E+06	6.06E+05	4.06E+07

Table 5.23. Q-PCR analysis of Set 4 digester samples

Set		**Bac**	**M.Bac**	**M.Mic**	**M.Saeta**	**M.Sar**
Day	**Digester**	**Gene copy**	**Gene copy**	**Gene copy**	**Gene copy**	**Gene copy**
D0	**S1**	1.53E+09	6.08E+09	2.86E+07	5.24E+05	6.16E+07
D0	**S3**	1.13E+09	3.90E+09	1.00E+07	4.78E+05	1.31E+08
D0	**S4**	2.35E+09	2.88E+09	1.38E+06	2.06E+05	3.64E+06
D0	**S6**	5.51E+09	5.86E+09	9.48E+06	3.84E+06	1.52E+07
D5	**S1**	1.38E+08	2.66E+09	1.51E+05	2.56E+05	1.74E+07
D5	**S3**	6.65E+08	8.66E+09	3.75E+06	3.78E+05	1.46E+08
D5	**S4**	2.05E+08	6.04E+09	1.78E+06	5.00E+05	3.50E+08
D5	**S6**	3.42E+08	2.04E+10	4.49E+06	1.14E+06	1.36E+09
D10	**S1**	1.63E+08	1.18E+10	7.25E+05	7.40E+05	3.48E+08
D10	**S3**	5.90E+07	4.62E+09	1.26E+05	3.88E+05	6.40E+08
D10	**S4**	2.13E+08	1.19E+10	4.27E+06	3.62E+05	7.20E+07
D10	**S6**	1.71E+08	9.00E+09	9.87E+05	7.22E+05	5.86E+07
D15	**S1**	2.13E+08	1.98E+10	9.75E+05	1.03E+05	7.56E+07
D15	**S3**	2.17E+08	2.18E+10	3.13E+05	9.12E+05	1.75E+08
D15	**S4**	7.98E+07	2.04E+10	9.91E+05	9.04E+05	6.80E+06
D15	**S6**	7.93E+07	1.45E+10	1.61E+06	7.48E+05	4.84E+08
D20	**S1**	3.11E+08		2.53E+06	1.27E+06	2.18E+08
D20	**S3**	3.79E+08		2.24E+06	2.96E+05	6.86E+06
D20	**S4**	8.36E+07		3.91E+05	4.48E+04	5.34E+07
D20	**S6**	2.16E+08		1.22E+06	4.00E+05	2.80E+07

From samples of digesters, total RNA was extracted to monitor active genes. Active genes were counted with Q-PCR after cDNA synthesis from total RNA. Q-PCR analysis showed that main methanogenic groups were *Methanobacteriales* and *Methanosarcinales.* Tolerance of *Methanobacteriales* to toxic substances has been mentioned in the literature before (Gözdereliler, 2008). Low gene copy numbers were also obtained for *Methanomicrobiales* and *Methanosaeta*. This incident was also reported earlier in another study where effect of OTC was different for different methanogens (Sanz et al., 1996). The results of Set1 analysis showed a decrease in bacterial and *Methanobacteriales* gene copy numbers with time. *Methanomicrobiales* and *Methanosarcinales* gene copy numbers was increased with time but then decreased down to starting numbers. In Set 2, gene copy numbers of bacteria and *Methanomicrobiales* remained relatively unchanged, as gene copy numbers of *Methanobacteriales* and *Methanosarcinales* decreased. In Set 3, gene copy numbers of bacteria, *Methanobacteriales*, *Methanomicrobiales*, *Methanosaeta* and *Methanosarcinales* decreased. In Set 4, gene copy numbers of bacteria and *Methanomicrobiales* decreased while gene copy numbers of *Methanobacteriales* and *Methanosarcinales* increased. Gene copy number of *Methanosaeta* did not change with time significantly. In all digesters, generally gene copy numbers of bacteria and *Methanomicrobiales* tends to decline. Gene copy numbers of *Methanobacteriales* and *Methanosarcinales* increased in high mixing rate digesters and decreased in low mixing digesters.

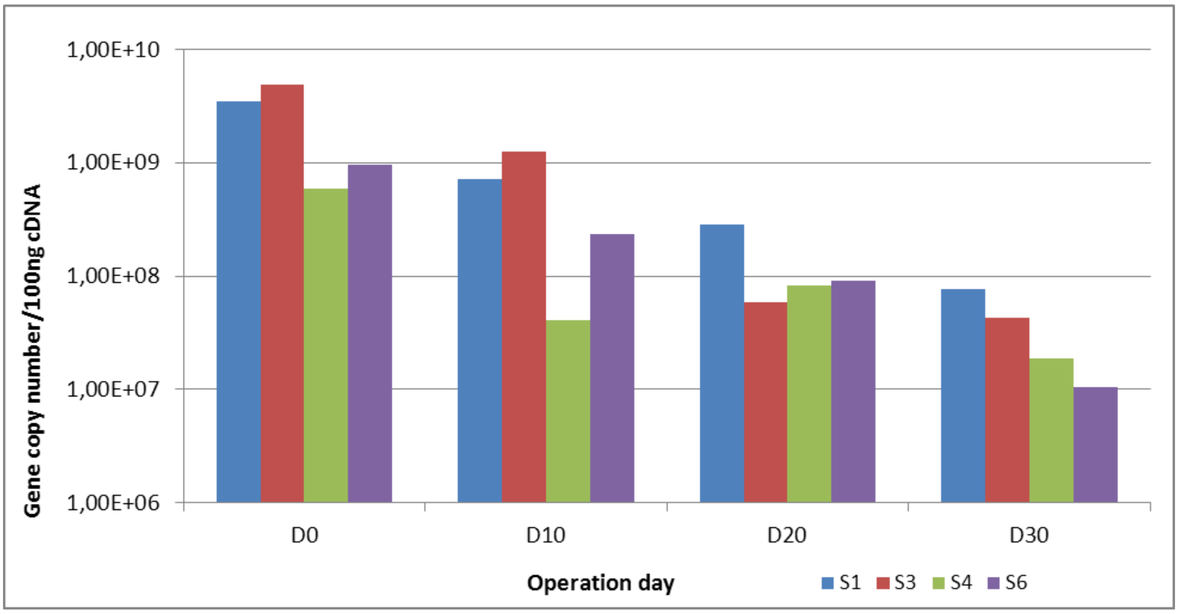


Figure 5.27. Change of bacterial gene copy number in Set 1 with time

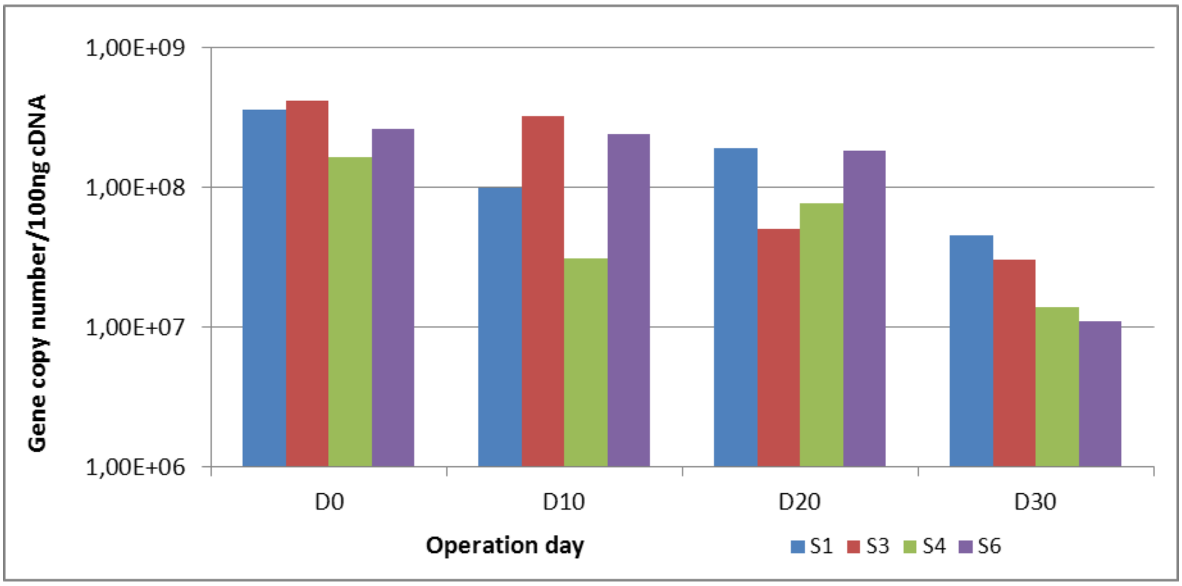


Figure 5.28. Change of *Methanobacteriales* gene copy number in Set 1 with time

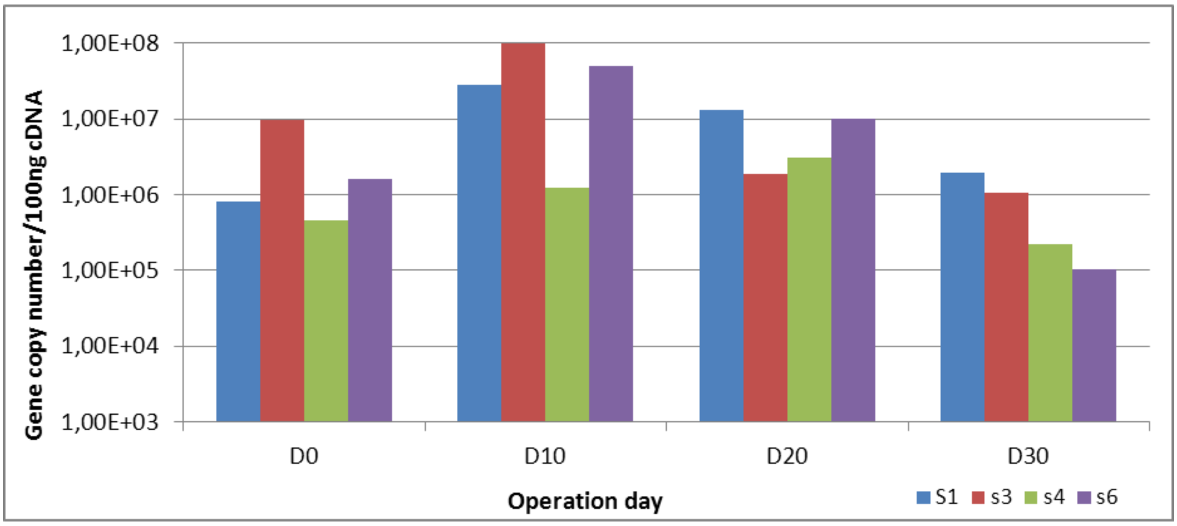


Figure 5.29. Change of *Methanomicrobiales* gene copy number in Set 1 with time

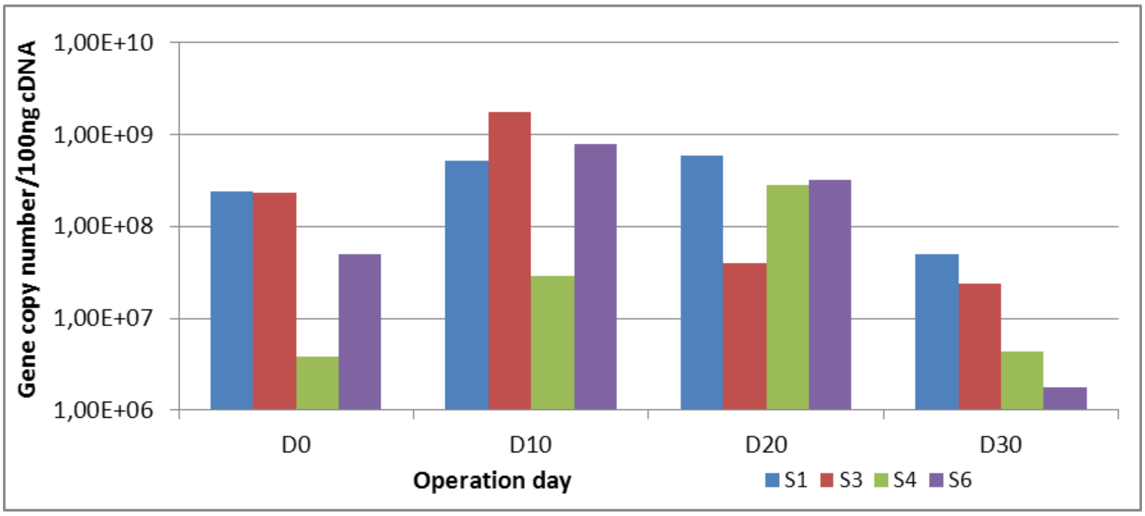


Figure 5.30. Change of *Methanosarcinales* gene copy number in Set 1 with time

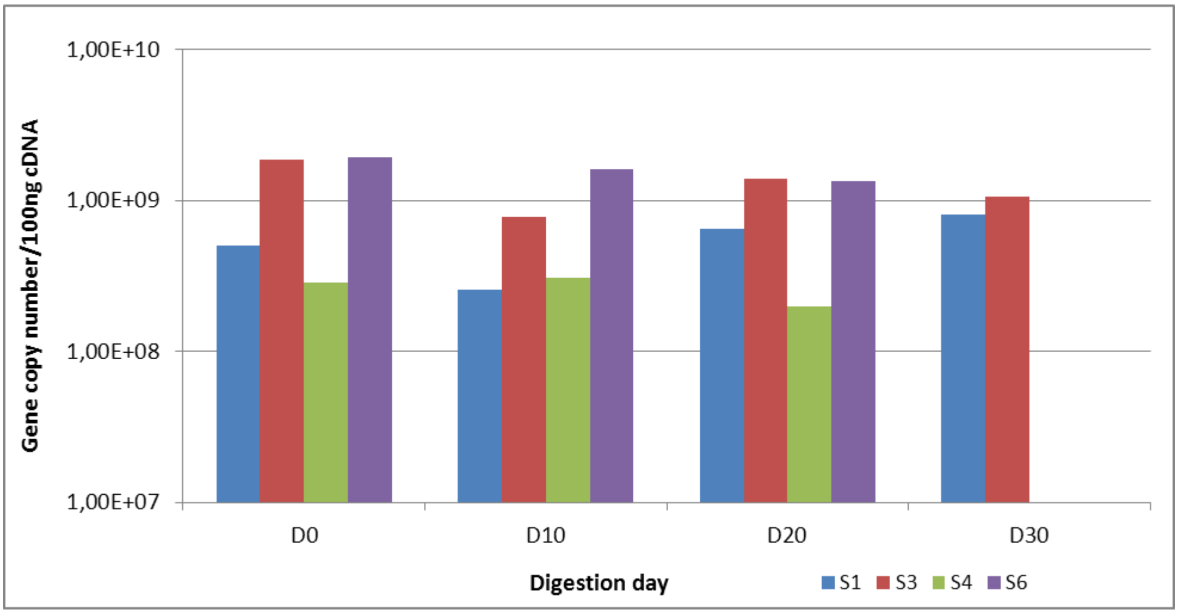


Figure 5.31. Change of bacterial gene copy number in Set 2 with time

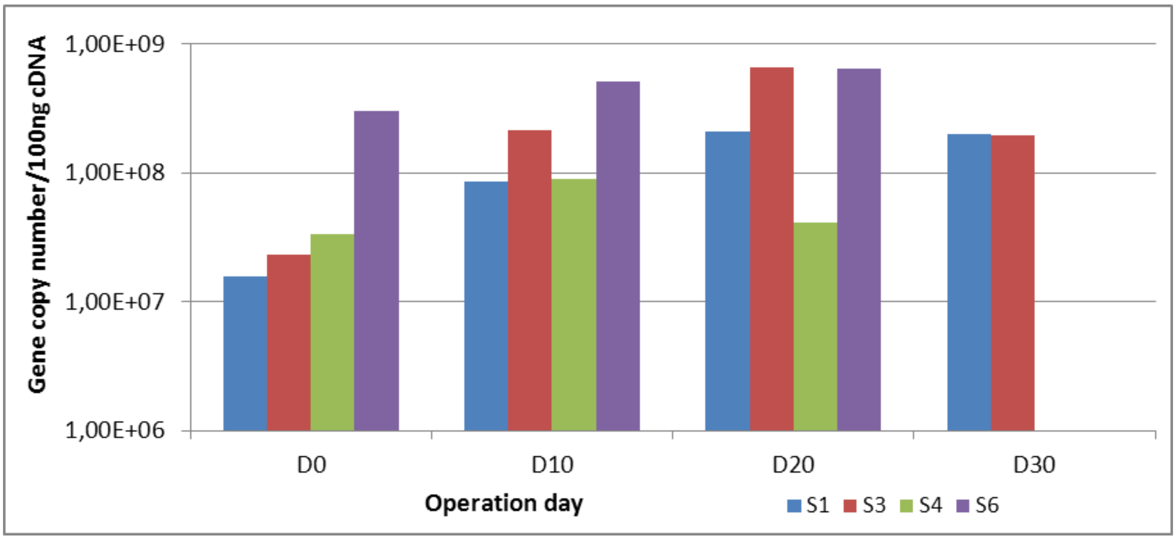


Figure 5.32. Change of *Methanobacteriales* gene copy number in Set 2 with time

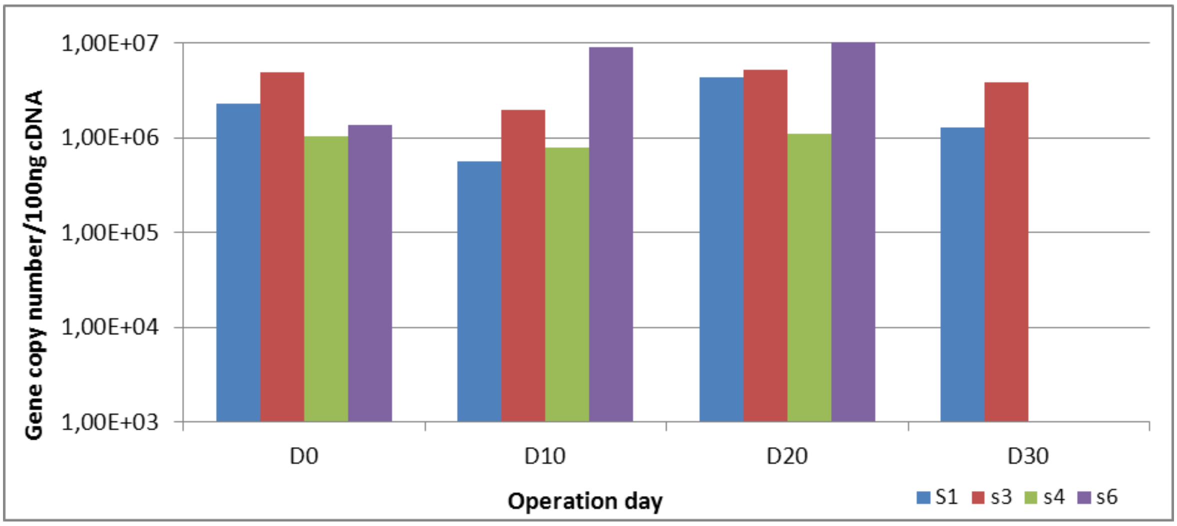


Figure 5.33. Change of *Methanomicrobiales* gene copy number in Set 2 with time

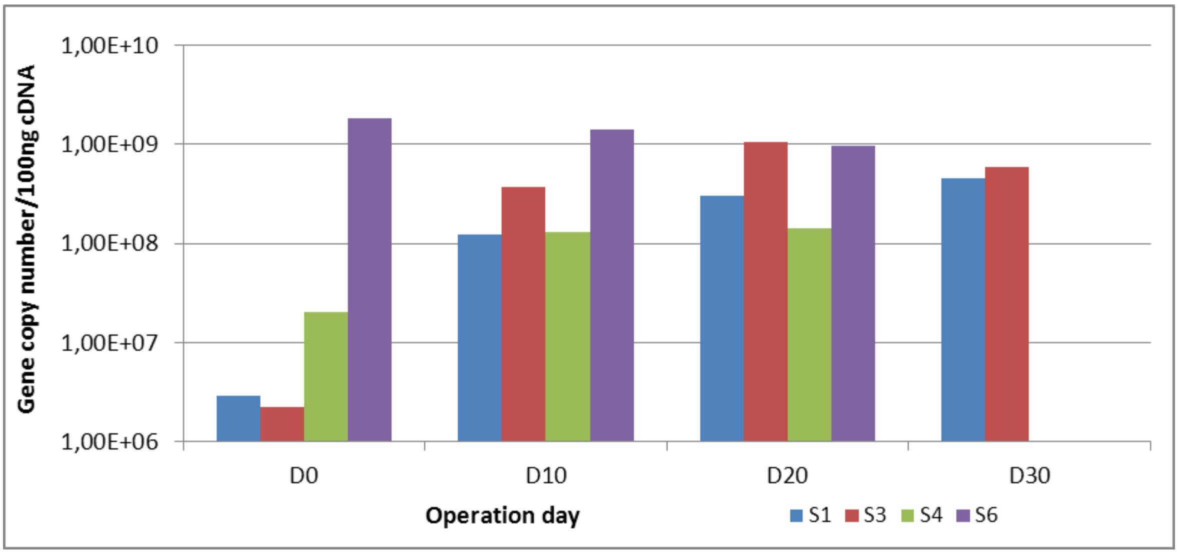


Figure 5.34. Change of *Methanosarcinales* gene copy number in Set 2 with time

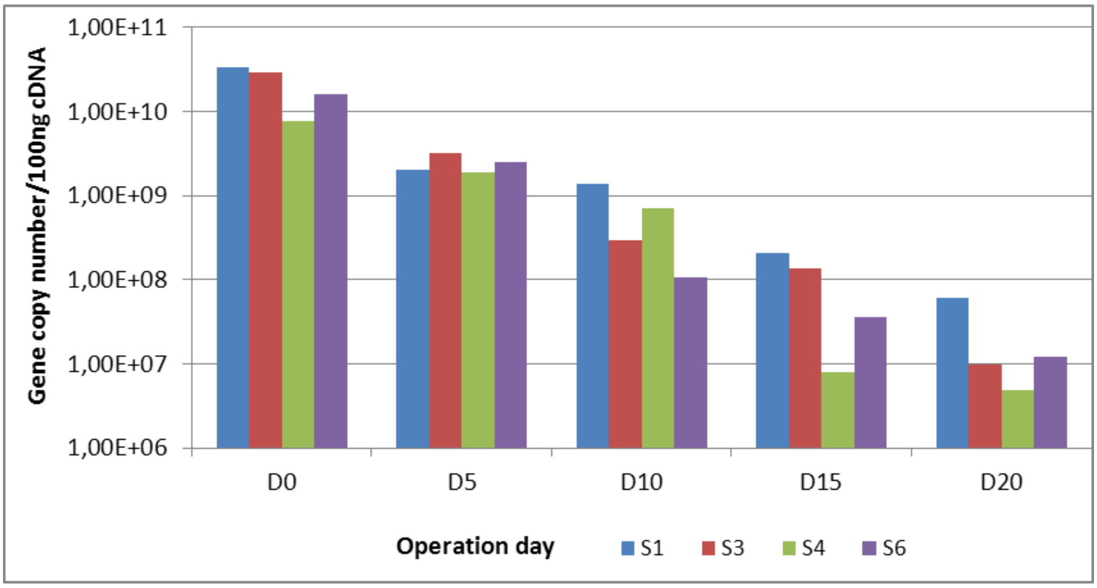


Figure 5.35. Change of bacterial gene copy number in Set 3 with time

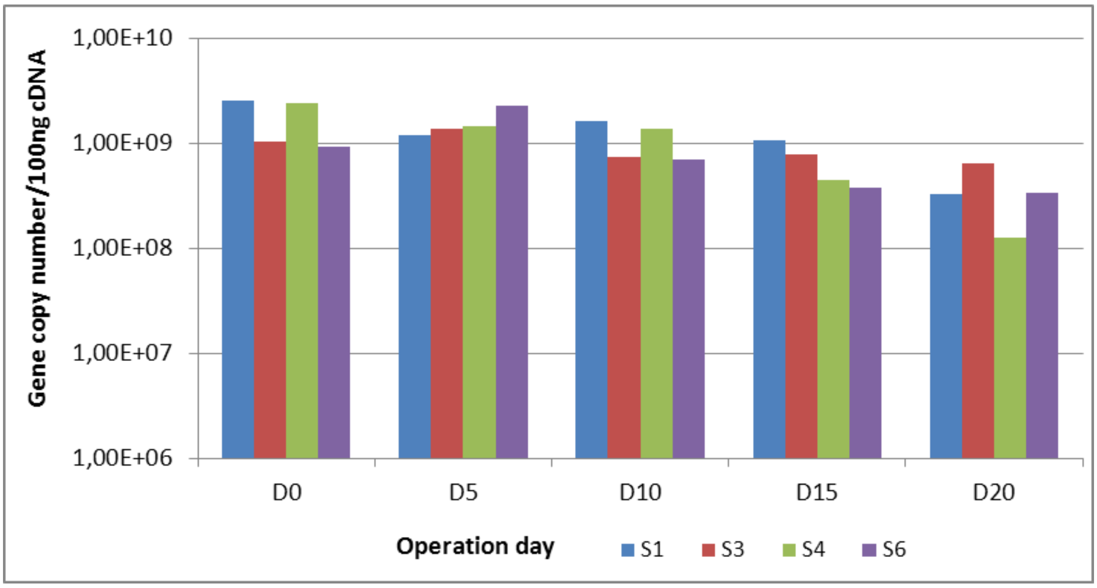


Figure 5.36. Change of *Methanobacteriales* gene copy number in Set 3 with time

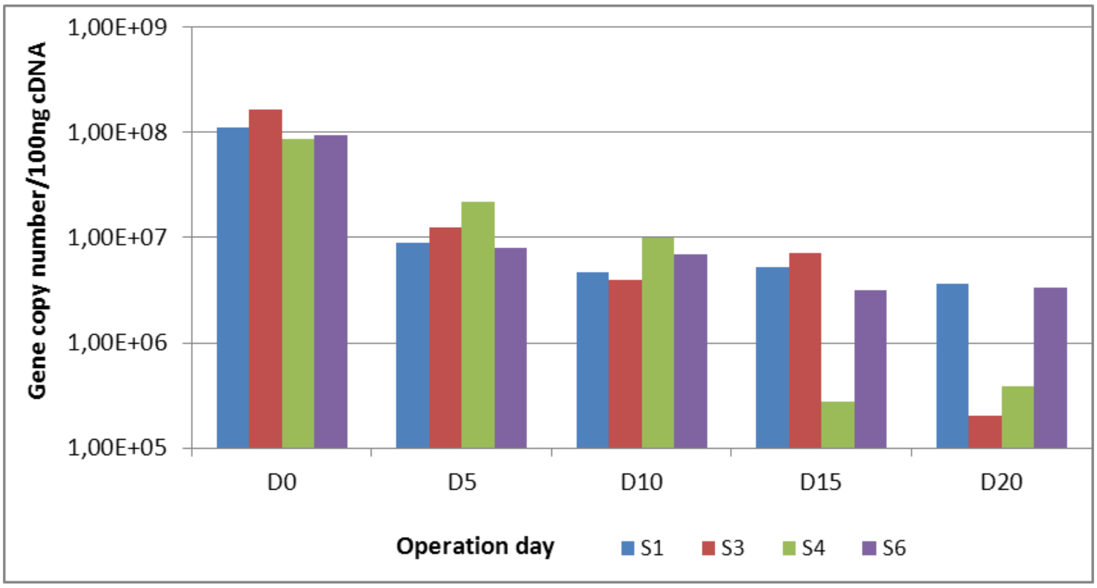


Figure 5.37. Change of *Methanomicrobiales* gene copy number in Set 3 with time

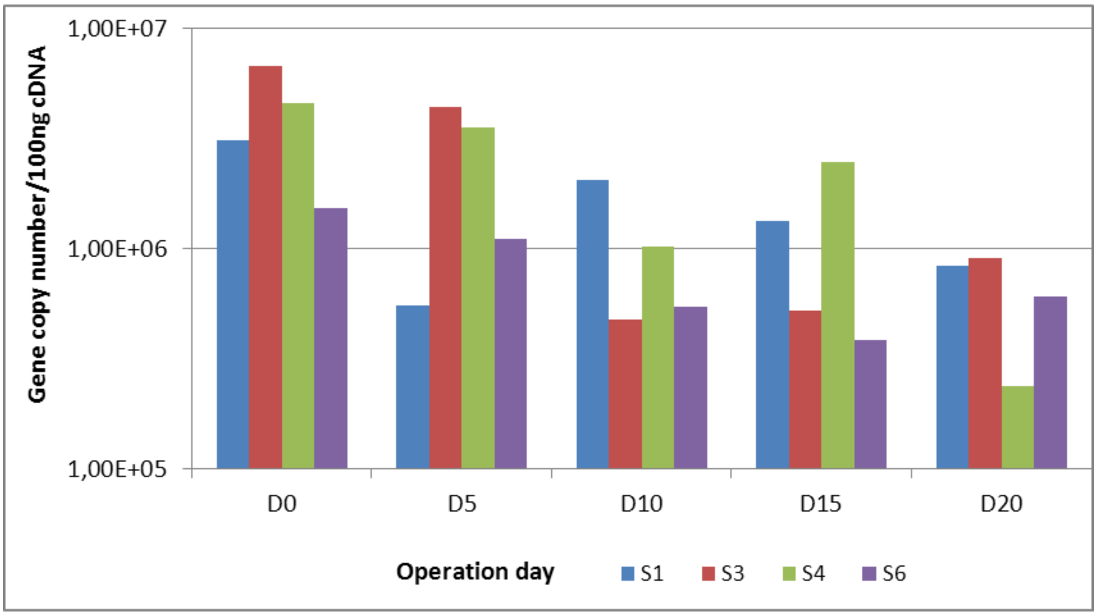


Figure 5.38. Change of *Methanosaeta* gene copy number in Set 3 with time

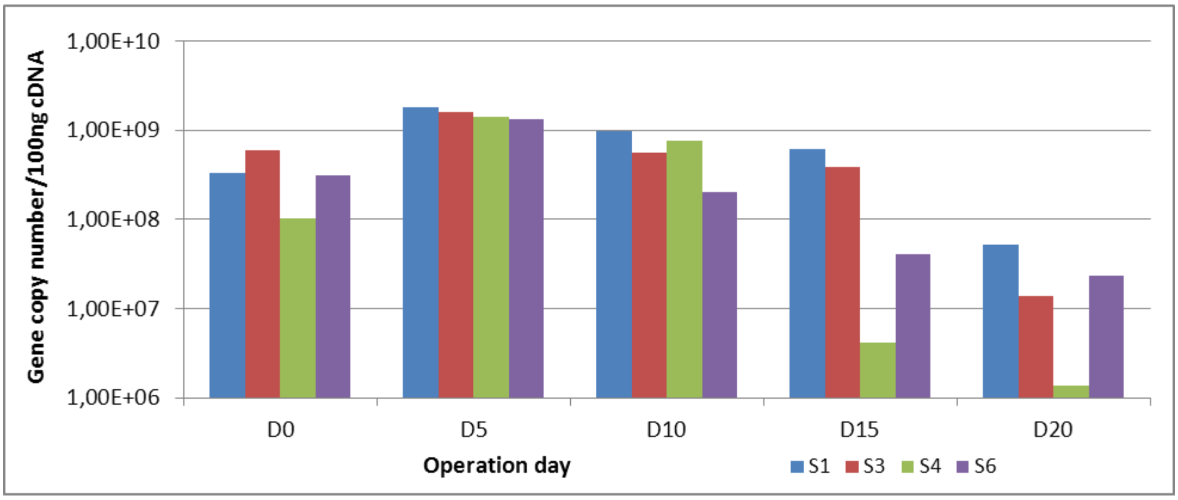


Figure 5.39. Change of *Methanosarcinales* gene copy number in Set 3 with time

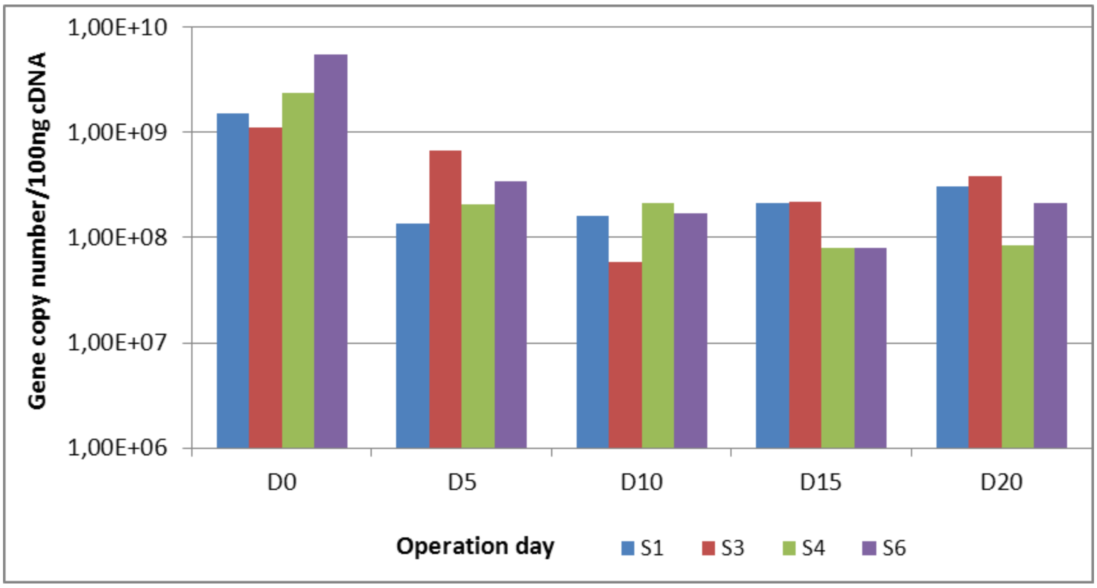


Figure 5.40. Change of bacterial gene copy number in Set 4 with time

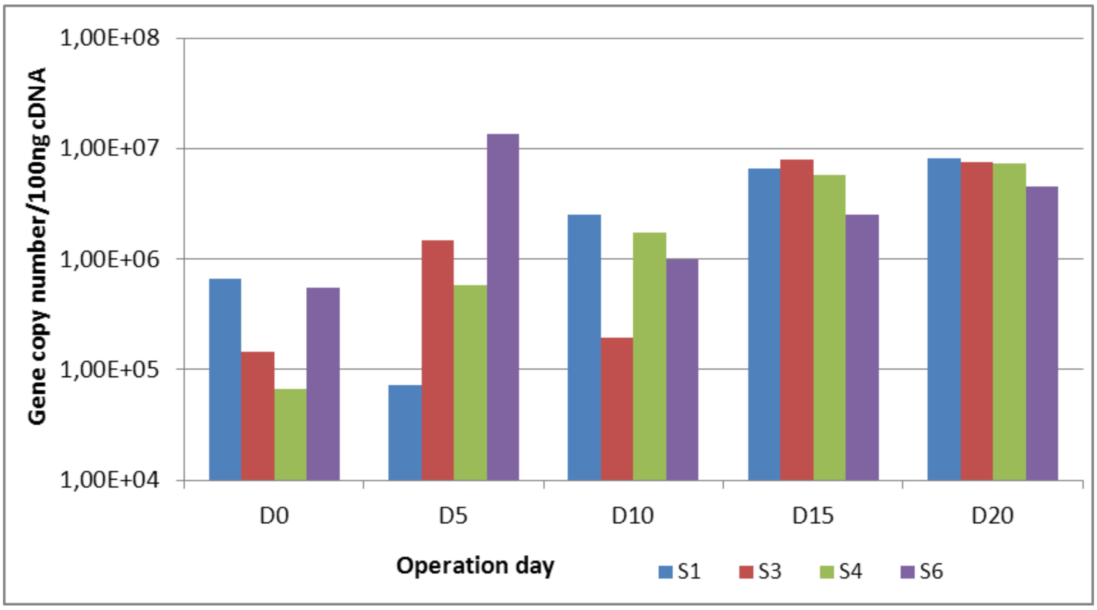


Figure 5.41. Change of *Methanobacteriales* gene copy number in Set 4 with time

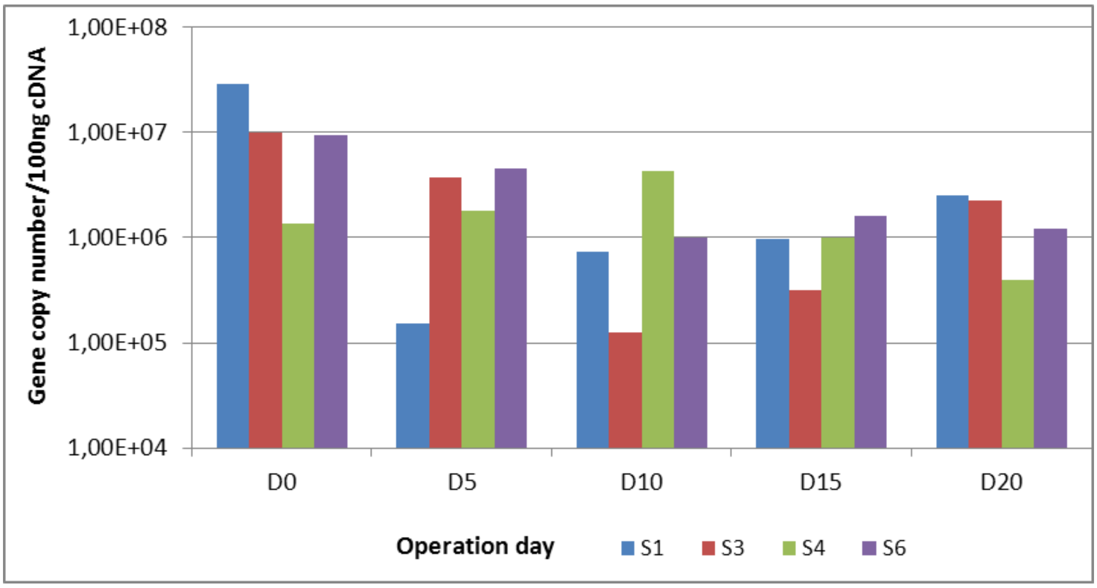


Figure 5.42. Change of *Methanomicrobiales* gene copy number in Set 4 with time

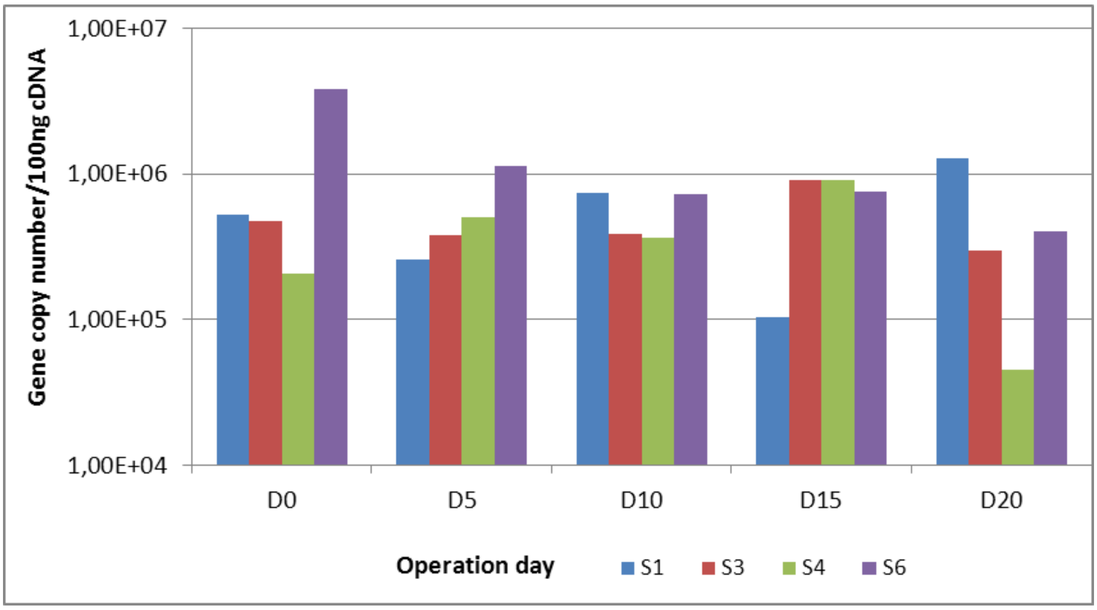


Figure 5.43. Change of *Methanosaeta* gene copy number in Set 4 with time

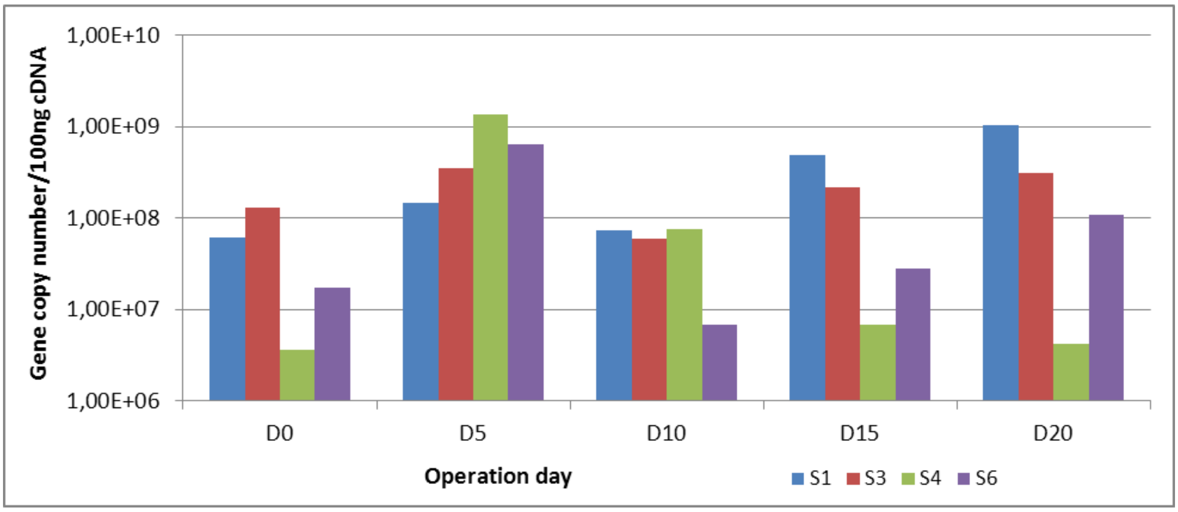


Figure 5.44. Change of *Methanosarcinales* gene copy number in Set 4 with time

<u>5.3.4.4. Determination of active microbial groups with FISH.</u> FISH analysis has been performed at Day 0 and Day 20 where activity made a peak. Results were given through Tables 5.24 -5.27.

In our study, microbial activity of anaerobic digestion was monitored by RNA targeting molecular techniques, FISH and RNA based Q-PCR. FISH analysis of samples showed activity of *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinacaea*. *Methanosaeta* spp. were found not active in digesters. These hydrogenotrophic methanogens were also dominant in cut microbiota (Dworkin et al., 2006). Absence of *Methanosaeta spp.* in manure digesters was reported previously in a study (Karakashev et al., 2005). In this study, it has been shown that abundance *Methanosaeta spp.* was high in seed of solid waste digesters but in manure digesters abundance of *Methanosarcina* spp. instead of *Methanosaeta* spp. was reported (Schmidt et al., 2000). In study of Karakashev et al., (2005), they monitored archaeal structure of 6 sludge digesters and 9 manure digesters with FISH. They showed two distinct archaeal structures for two digester types. Manure digesters were dominated by *Methanobacteriales* and *Methanosarcinacaea* while sludge digesters were dominated by *Methanosaeta spp.* It was suspected that it was because of seed communities but analysis of seed communities showed that it was not relevant. Chemical analysis of digesters indicated that in manure digesters high concentrations of VFA and ammonia present where sludge digesters have low concentration of these. In another study, it was stated that morphology of *Methanosarcina* helps to resist ammonia toxicity which is common in manure digesters (Demirel and Scherer, 2008). Different studies support that, methanogenesis uses hydrogenotrophic pathway in manure digesters (Angelidaki and Ahring, 1993; Karakashev et al., 2006).

Table 5.24. Ratios of active bacteria, archaeal and methanogens in respect to DAPI count in Set 1 digesters

S1 Probe	Total Cell /DAPI (%)	Digestion day	S3 Probe	Total Cell /DAPI (%)	Digestion day
Eubmix	16	0	**Eubmix**	16	0
Arc915	31	0	**Arc915**	12	0
MB310	11	0	**MB310**	05	0
MG1200	09	0	**MG1200**	01	0
MSMS1414	07	0	**MSMS1414**	04	0
MSMX	11	0	**MSMX**	06	0
Eubmix	08	20	**Eubmix**	20	20
Arc915	39	20	**Arc915**	17	20
MB310	13	20	**MB310**	11	20
MG1200	05	20	**MG1200**	02	20
MSMS1414	20	20	**MSMS1414**	01	20
MSMX	20	20	**MSMX**	01	20
S4 Probe	**Total Cell /DAPI (%)**	**Digestion day**	**S6 Probe**	**Total Cell /DAPI (%)**	**Digestion day**
Eubmix	19	0	**Eubmix**	10	0
Arc915	30	0	**Arc915**	13	0
MB310	10	0	**MB310**	07	0
MG1200	09	0	**MG1200**	03	0
MSMS1414	09	0	**MSMS1414**	02	0
MSMX	11	0	**MSMX**	03	0
Eubmix	12	20	**Eubmix**	15	20
Arc915	46	20	**Arc915**	25	20
MB310	15	20	**MB310**	12	20
MG1200	08	20	**MG1200**	08	20
MSMS1414	23	20	**MSMS1414**	03	20
MSMX	23	20	**MSMX**	03	20

Table 5.25. Ratios of active bacteria, archaeal and methanogens in respect to DAPI count in Set 2 digesters

S1 Probe	**Total Cell /DAPI (%)**	**Digestion day**	**S3 Probe**	**Total Cell /DAPI (%)**	**Digestion day**
Eubmix	10	0	**Eubmix**	40	0
Arc915	33	0	**Arc915**	80	0
MB310	24	0	**MB310**	49	0
MG1200	01	0	**MG1200**	12	0
MSMS1414	08	0	**MSMS1414**	17	0
MSMX	07	0	**MSMX**	20	0
Eubmix	16	20	**Eubmix**	21	20
Arc915	65	20	**Arc915**	62	20
MB310	27	20	**MB310**	33	20
MG1200	22	20	**MG1200**	12	20
MSMS1414	16	20	**MSMS1414**	15	20
MSMX	17	20	**MSMX**	17	20
S4 Probe	**Total Cell /DAPI (%)**	**Digestion day**	**S6 Probe**	**Total Cell /DAPI (%)**	**Digestion day**
Eubmix	12	0	**Eubmix**	01	0
Arc915	52	0	**Arc915**	2 4	0
MB310	35	0	**MB310**	15	0
MG1200	05	0	**MG1200**	-	0
MSMS1414	10	0	**MSMS1414**	02	0
MSMX	12	0	**MSMX**	07	0
Eubmix	33	20	**Eubmix**	05	20
Arc915	84	20	**Arc915**	20	20
MB310	66	20	**MB310**	07	20
MG1200	09	20	**MG1200**	03	20
MSMS1414	08	20	**MSMS1414**	09	20
MSMX	10	20	**MSMX**	10	20

Table 5.26. Ratios of active bacteria, archaeal and methanogens in respect to DAPI count in Set 3 digesters

S1 Probe	**Total Cell /DAPI (%)**	**Digestion day**	**S3 Probe**	**Total Cell /DAPI (%)**	**Digestion day**
Eubmix	10	0	**Eubmix**	15	0
Arc915	52	0	**Arc915**	41	0
MB310	24	0	**MB310**	23	0
MG1200	14	0	**MG1200**	13	0
MSMS1414	20	0	**MSMS1414**	12	0
MSMX	21	0	**MSMX**	13	0
Eubmix	6	20	**Eubmix**	7	20
Arc915	46	20	**Arc915**	38	20
MB310	23	20	**MB310**	13	20
MG1200	15	20	**MG1200**	11	20
MSMS1414	11	20	**MSMS1414**	16	20
MSMX	12	20	**MSMX**	17	20
S4 Probe	**Total Cell /DAPI (%)**	**Digestion day**	**S6 Probe**	**Total Cell /DAPI (%)**	**Digestion day**
Eubmix	23	0	**Eubmix**	23	0
Arc915	37	0	**Arc915**	33	0
MB310	15	0	**MB310**	20	0
MG1200	8	0	**MG1200**	10	0
MSMS1414	13	0	**MSMS1414**	11	0
MSMX	14	0	**MSMX**	12	0
Eubmix	6	20	**Eubmix**	15	20
Arc915	43	20	**Arc915**	55	20
MB310	16	20	**MB310**	21	20
MG1200	10	20	**MG1200**	20	20
MSMS1414	16	20	**MSMS1414**	13	20
MSMX	17	20	**MSMX**	14	20

Table 5.27. Ratios of active bacteria, archaeal and methanogens in respect to DAPI count in Set 4 digesters

S1 Probe	**Total Cell /DAPI (%)**	**Digestion day**	**S3 Probe**	**Total Cell /DAPI (%)**	**Digestion day**
Eubmix	21	0	**Eubmix**	20	0
Arc915	41	0	**Arc915**	22	0
MB310	29	0	**MB310**	13	0
MG1200	7	0	**MG1200**	1	0
MSMS1414	3	0	**MSMS1414**	6	0
MSMX	5	0	**MSMX**	8	0
Eubmix	2	20	**Eubmix**	16	20
Arc915	38	20	**Arc915**	75	20
MB310	16	20	**MB310**	48	20
MG1200	3	20	**MG1200**	14	20
MSMS1414	4	20	**MSMS1414**	10	20
MSMX	18	20	**MSMX**	12	20
S4 Probe	**Total Cell /DAPI (%)**	**Digestion day**	**S6 Probe**	**Total Cell /DAPI (%)**	**Digestion day**
Eubmix	7	0	**Eubmix**	7	0
Arc915	60	0	**Arc915**	5	0
MB310	30	0	**MB310**	1	0
MG1200	12	0	**MG1200**	1	0
MSMS1414	14	0	**MSMS1414**	2	0
MSMX	15	0	**MSMX**	2	0
Eubmix	28	20	**Eubmix**	8	20
Arc915	65	20	**Arc915**	8	20
MB310	24	20	**MB310**	3	20
MG1200	20	20	**MG1200**	1	20
MSMS1414	19	20	**MSMS1414**	5	20
MSMX	20	20	**MSMX**	6	20

FISH analysis showed that activity increased with time and decreased after 20th day. It was parallel to advised HRT of manure digesters which was 18-20 days. After these days production of biogas was decreased, therefore, longer incubation time became economically unacceptable. In this study, it has been shown that there was a negative relation with activity of *Methanomicrobiales* and OTC where no negative relation was found with *Methanosarcinales*. An opposite results has been found in another study where tetracycline was used instead of Oxytetracycline (Stone et al., 2009). Interpretation of FISH results with environmental parameters has been given in following chapter.

5.4. Investigation of Effects of Antibiotics in Soil

Elimination and effects of OTC in soil environments was monitored by medicated manure amendment to soil. Elimination of OTC and changes in microbial structures were monitored for 150 days.

5.4.1. Texture Analysis and Selection of Suitable Soil Types

In soil studies, soil types which were used in similar studies in literature were targeted. Different soil samples were collected from forest soil where activity and diversity tend to be high and analyzed for their texture. Among eight samples, two samples (Arboretum-Sandy Loam and Belgrad-Silt Loam) were selected for downstream analysis (Table 5.28.).

Table 5.28. Texture analysis of collected soil samples

	Sampling point	**Sand**	**Silt**	**Clay**	**Soil Type**	**pH**	**E.C.**
Moist	Belgrad Ormanı Çıkış	49	28	24	Silt Loam	4.6	40.9
	Geyve Meyvelik	30	22	49	Clay soil	6.2	
	Arboretum karşısı	63	19	19	Sandy loam	5.2	42.5
Dry	Meşe Ormanı	67	21	13	Loam	5.6	137
	Göknar Ormanı	62	26	13	Loam	5.5	173
	Ladin Ormanı	65	24	12	Loam	5.2	194
	Karaçam Ormanı	75	14	12	Sandy Loam	5.0	233
	Sarıçam Ormanı	75	16	10	Sandy Loam	5.1	484
	2 no'lu bölge	78	10	13	Sandy Loam	4.1	60.2

Also additionally to samples mentioned above, in November of 2011 some soil samples were collected from an orchard in Geyve county. But preliminary texture analysis resulted that this soil was not suitable for soil studies; therefore, this soil was used for preliminary soil studies where it was aimed to see problems like sampling, mixing etc. that we may encounter during soil studies. Microcosms were prepared and incubated for 60 days. This setup was very helpful to monitor problems during soil studies. With the help of this setup, optimization for sampling, analytical analysis and humidity level can be performed.

Acidity of the soil samples was high. It was a seasonal problem where in fall and winter the mobility of ions in soil increased with rainfall. Geyve soil was sampled before the rainy season therefore, it had a higher pH.

5.4.2. OTC Concentration in Soil Studies

OTC concentration and its eliminaion in soil were monitored after manure amendment to soil. In each sampling time HPLC analysis has been performed to determine OTC levels (Table 5.29 and Figure 5.45-5.46.) OTC levels in soil upon manure amendment were around 1 mg/kg. This finding was in agreement of study of Karci and Balcioglu, (2009), where they found 0.05-0.5 mg/kg TC in medicated manure amended soils. After 150 days of incubation, it has been seen that starting concentration were halved. A proposed half-life of OTC in soil was then 135 days. It has been known that tetracycline group antibiotics have a strong tendency to bind soil matrix especially clay part of soil. This binding possibly increased half-life of OTC in soil environments.

Table 5.29. OTC concentration, elimination and half-life in soil studies

Soil type	**Amendment***	**OTC concentrations (mg/kg)**							**Elimination (%)**	**Half-life (day)**
		Day 0	**Day 30**	**Day 45**	**Day 60**	**Day 90**	**Day 120**	**Day 150**		
Sandy Loam	**S**	1.8	2.1	1.9	1.7	1.4	1.2	1.1	39	192
	F	0.7	0.6	0.5	0.5	0.5	0.4	0.4	43	174
	D	0.3	0.5	0.3	0.1	0.1	0.1	0.1	67	112
Silt Loam	**S**	5.9	4.0	3.2	3.0	2.3	2.1	1.9	68	110
	F	0.7	0.4	0.5	0.5	0.4	0.4	0.3	57	132
	D	0.5	0.2	0.2	0.1	0.1	0.1	0.1	80	94

* S: Spiked manure, F: Fresh medicated manure, D:Digestate

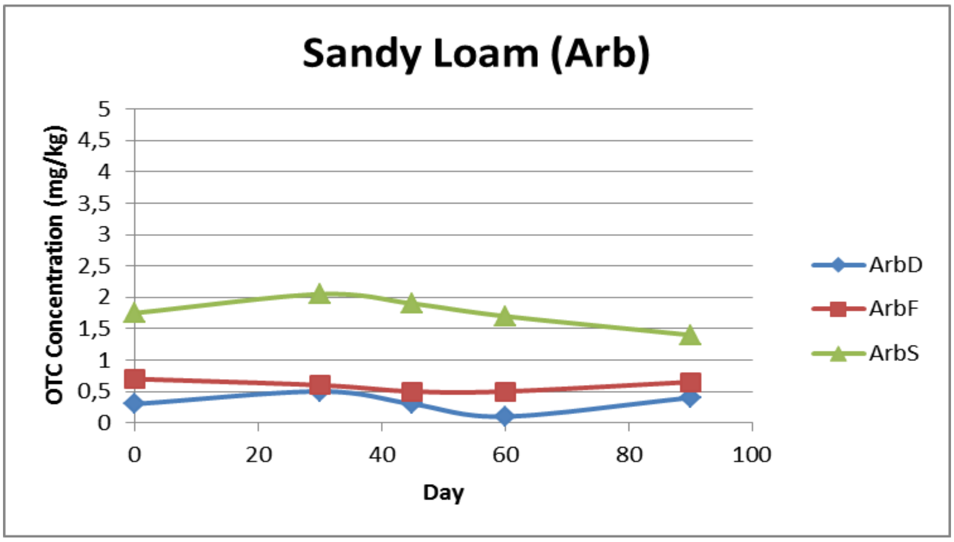


Figure 5.45. OTC levels in sandy loam (Arboretum)

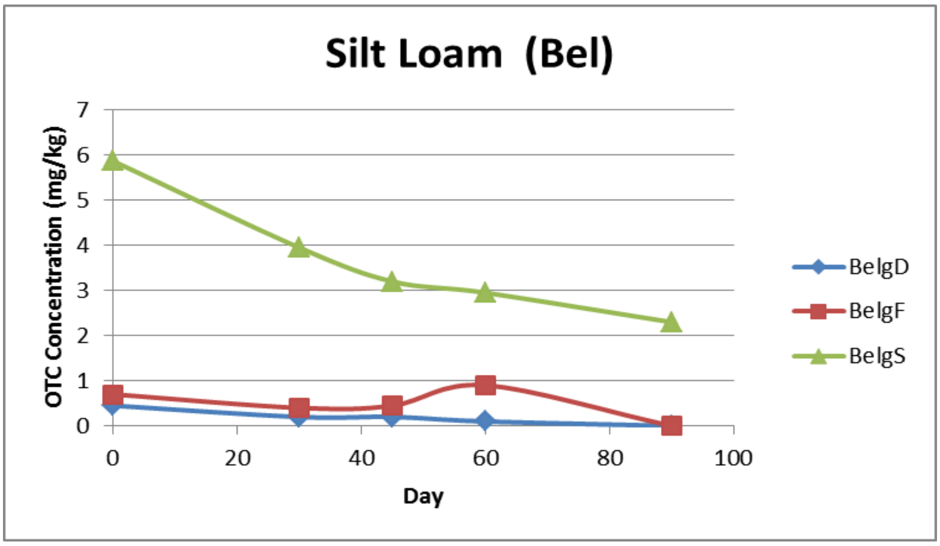


Figure 5.46. OTC levels in silt loam (Belgrad)

Manure amendment into soil is a common practice in rural regions. In developed countries, mostly digestate is used for amendment where fresh manure is used in less developed countries. In our study, it has been monitored that digestate had less than half of the concentration in fresh manure. Therefore, application of fresh manure as fertilizer introduces a higher amount of OTC to soil system which had high affinity for soil. Due to complex formation of tetracycline with soil matrix and lower humidity of soil, tetracyclines were persistent in soil (Halling-Sorenson et al., 2003). In our study, half-life of OTC in soil matrix was found to be 94-192 days. A similar study showed no decrease in

OTC concentration in soil after 180 days (van Gool, 1993). Yang et al., (2009) monitored half-life of OTC in anoxic and aerobic soil in presence and absence of microbial communities. In this study, half-lives were found to be 29-56 days in normal soil and 99-120 days in sterile soil under aerobic conditions where half-lives of 43-63 days in normal soil and 69-104 days in sterile soil were found under anoxic conditions. This study clearly showed that microorganisms in soil play a significant role in elimination of OTC.

5.4.3. Analysis of Microbial Diversity in Soil Microcosms

Microbial diversity of soil samples were monitored by DGGE. In this step microbial diversity of all three soil microcosms were monitored (Figure 5.47. and 5.48.). In clay soil diversity in 60 days, in other samples diversity in 150 days was observed.

There was high bacterial diversity in soil samples. DGGE analysis showed 36 bands in sandy loam, 30 bands in silt loam and 37 bands in clay soil. Analysis showed that microbial diversities of sandy loam and silt loam were more closely related than clay soil (70% and 65%, respectively). Bands in soil DGGE picture were matched up with members of clone library to find possible candidates for each band. Gram positive bacteria and *Bacteriodetes* groups cannot be found in soil samples while *Acinetobacter* spp. was present all soil samples except silt loam. *Clostridium* spp. (*lituseburense, glycolium and disporicum), Sphingobacteriacea* spp., *Bacillales* spp. (*Bacillus psychodurans and Bacillus odyssey)* were found in all soil samples. *Solibacillus silvestris* was found only in silt loam and clay soil.

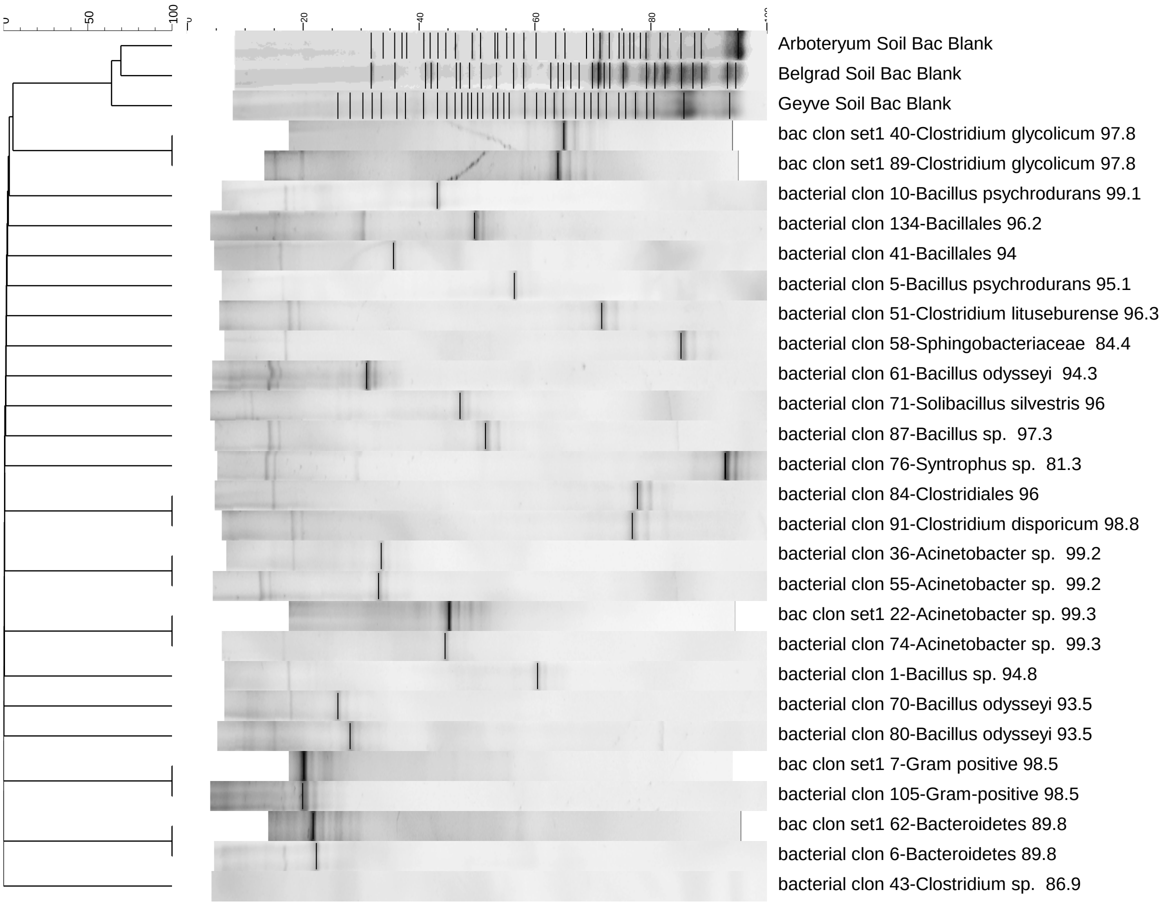


Figure 5.47. Bacterial diversity of blank soil samples and clone library match ups

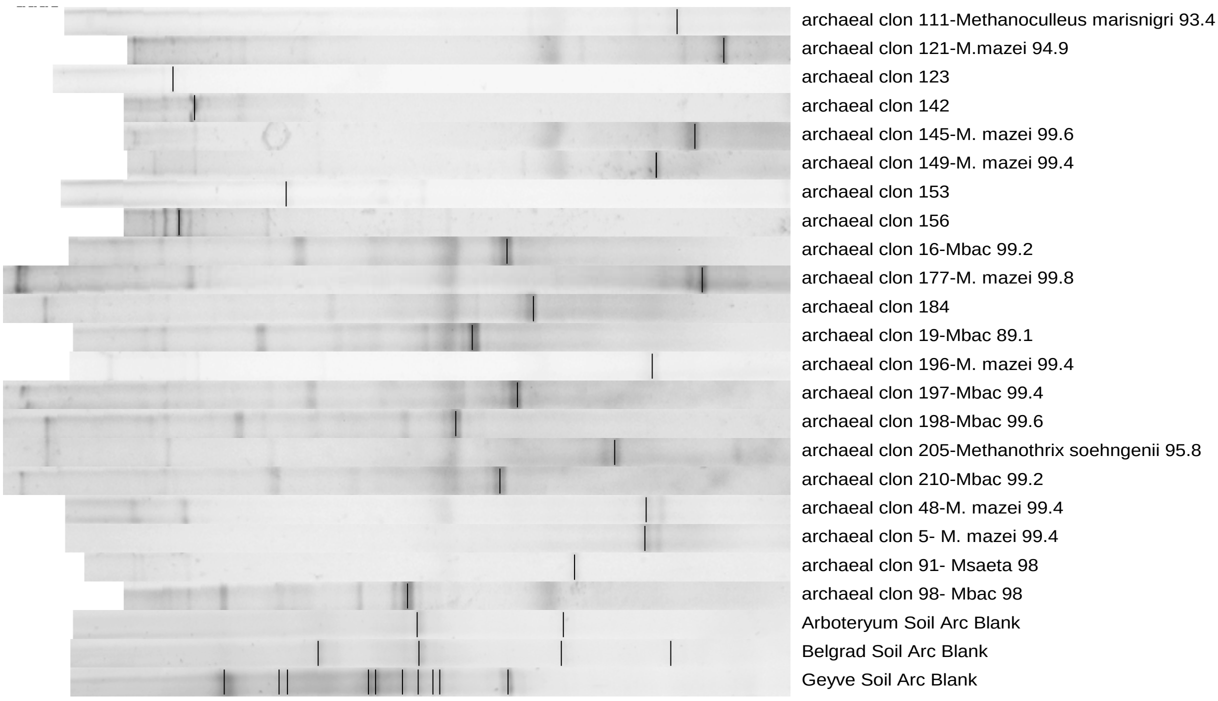


Figure 5.48. Archaeal diversity of blank soil samples and clone library match ups

Archaeal diversity in soil was low especially in winter collected samples. Clay soil had 10 bands where other soil samples contained 2-4 bands. Like bacterial diversity analysis, similarity of archaeal diversity of sandy loam and silt loam was higher than clay soil (67% and 15%, respectively). Only *Methanobacteriales* spp. was matched with bands in soil communities. This may suggested that archaeal diversity in soil was significantly different than diversity in digesters.

DGGE analysis of 60 day incubation of clay soil samples showed that although diversity changed the number of different species was preserved. Analysis showed that manure amendment had a strong influence on bacterial diversity. Cluster formation clay soil samples were according to amendment type rather than operation time. Digestate amended soil samples were made a distinct cluster than other samples. Manure amended soil samples made a big cluster where samples made sub clusters with time. Day 10 and 20 samples made a subcluster while Day 30, 45 and 60 made another (Figure 5.49. and 5.50)

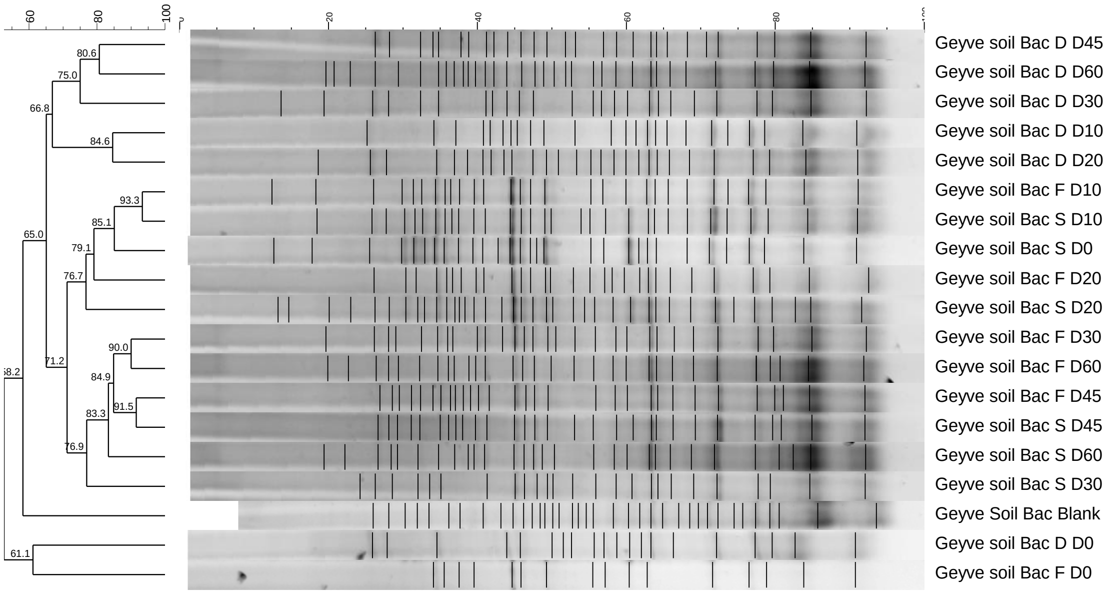


Figure 5.49.. Bacterial diversity dendogram of clay soil samples

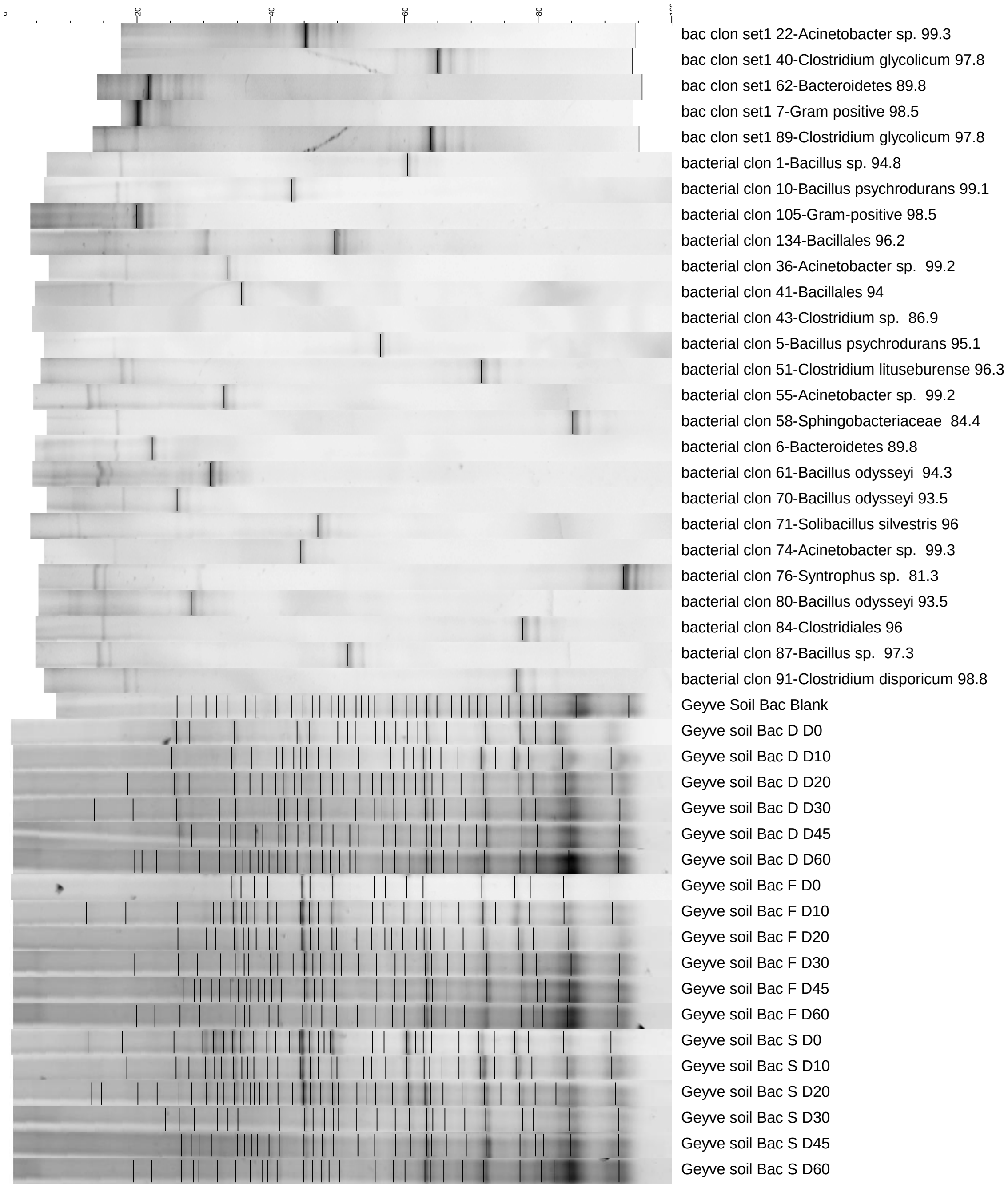


Figure 5.50. Change of bacterial diversity with time in clay soil and clone match ups.

In DGGE analysis of sandy loam, a large bacterial diversity has been found (45-50 bands). Diversity was decreased with incubation time. Cluster formation in sandy loam DGGE was according to amendment and in each cluster, sub clusters were formed according to time. Most dominant band was found to be *Clostridium lituseburense* and was introduced by manure addition. Clones related to *Bacillales* and *Clostridiales* groups were matched up with bands in sandy loam samples (Figure 5.51. and 5.52).

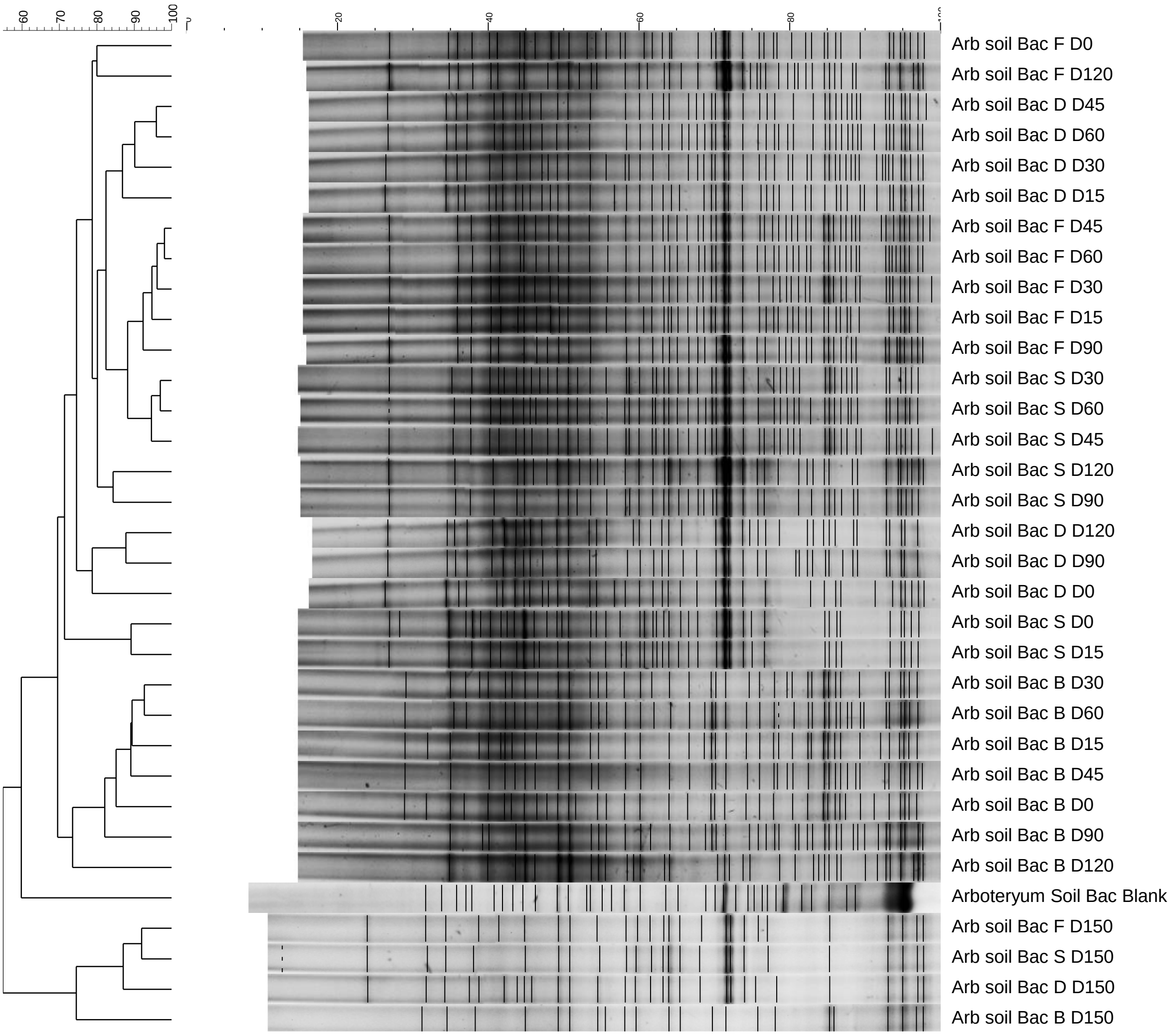


Figure 5.51. Bacterial diversity dendogram of sandy loam samples

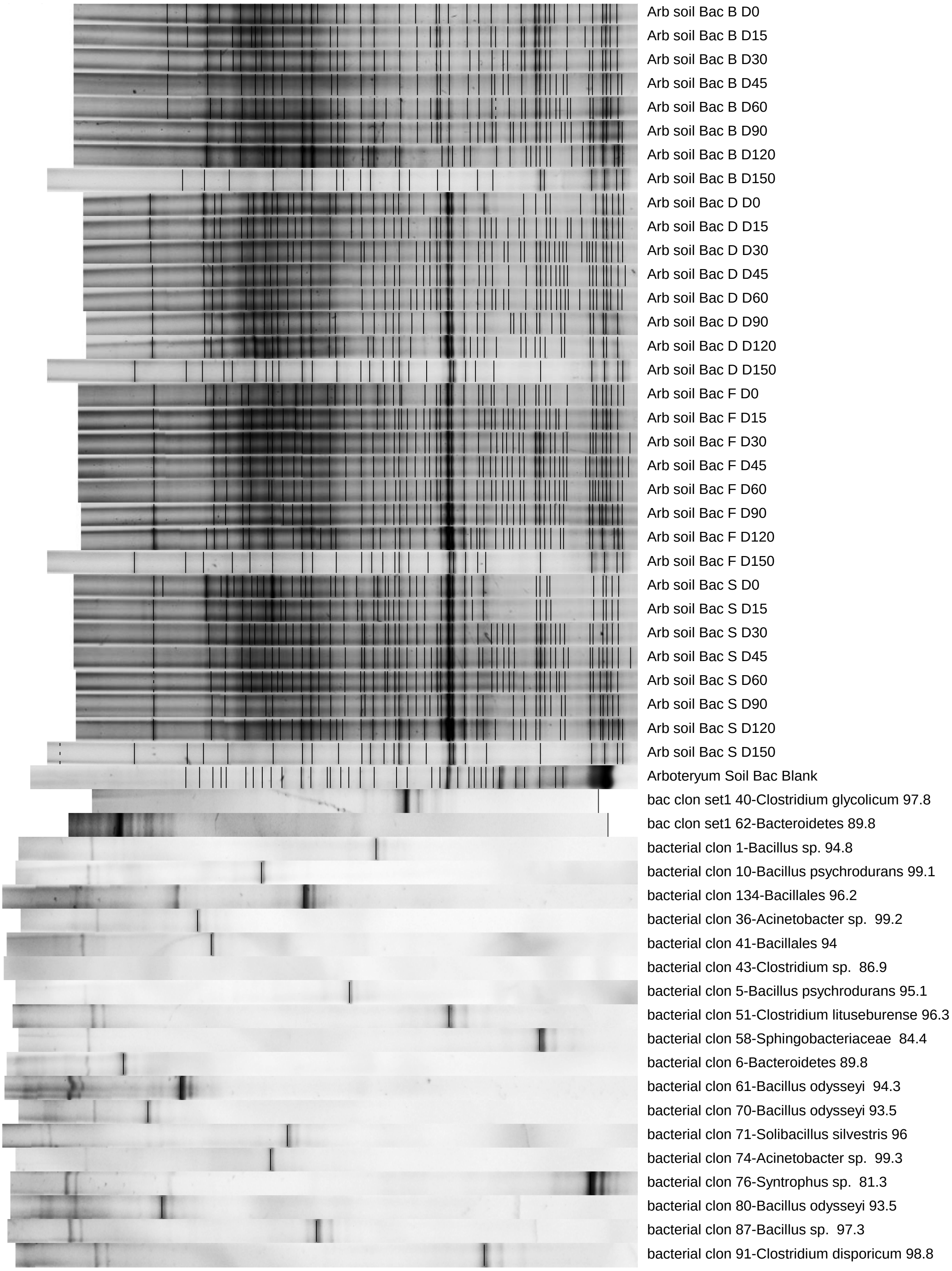


Figure 5.52. Change of bacterial diversity with time in sandy loam and clone match ups

In DGGE analysis of silt loam samples, a lower diversity has been observed (20-28 bands). Diversity was decreased with respect to time. Last samples (Day 120 and 150) has formed distinct cluster different from amendment. Otherwise cluster formation was again according to amendment and sub clusters were formed according to time. Again most dominant band was related to *Clostridium lituseburense* and it was introduced with manure amendment (Figure 5.53. and 5.54.).

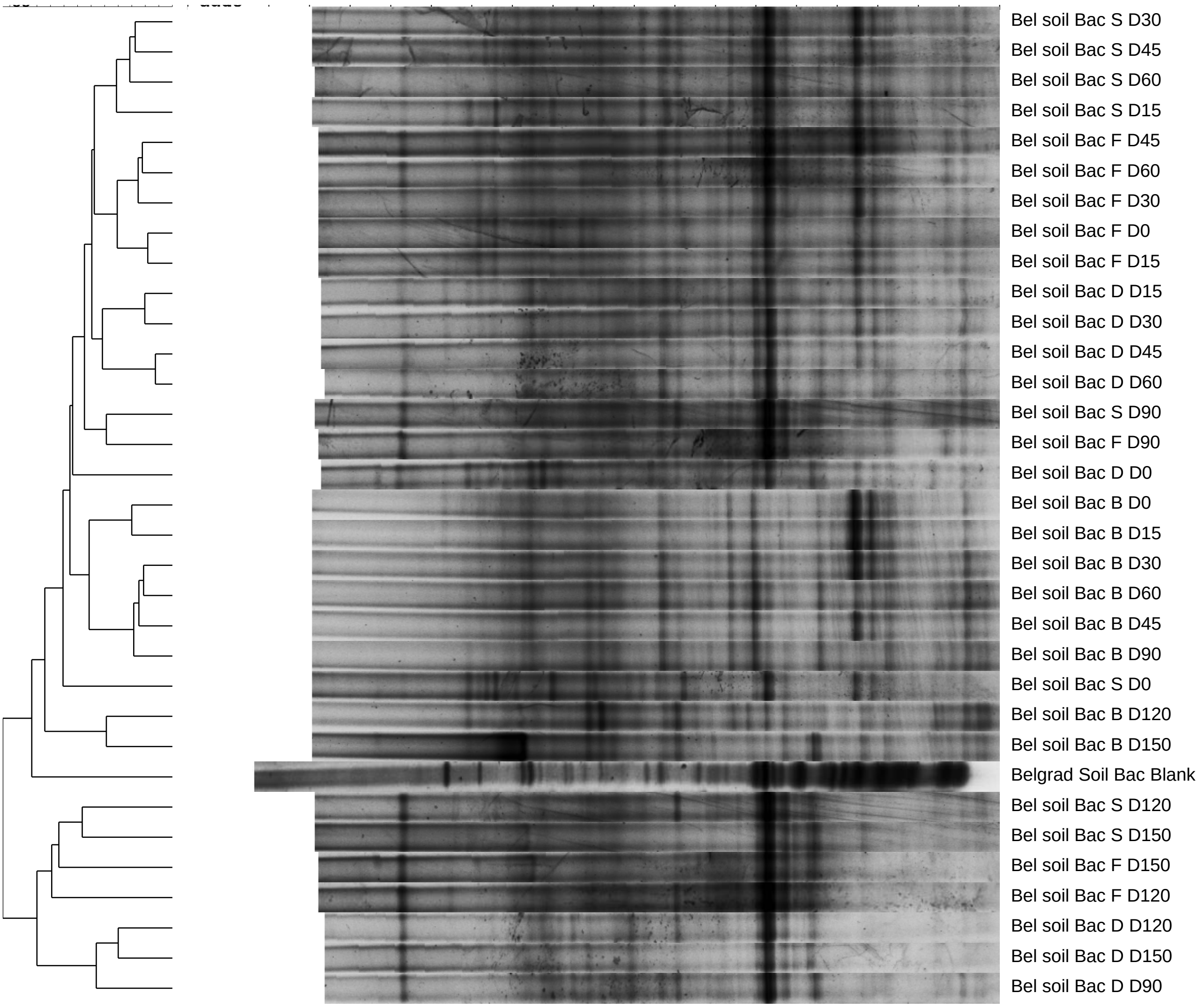


Figure 5.53. Bacterial diversity dendogram of silt loam samples

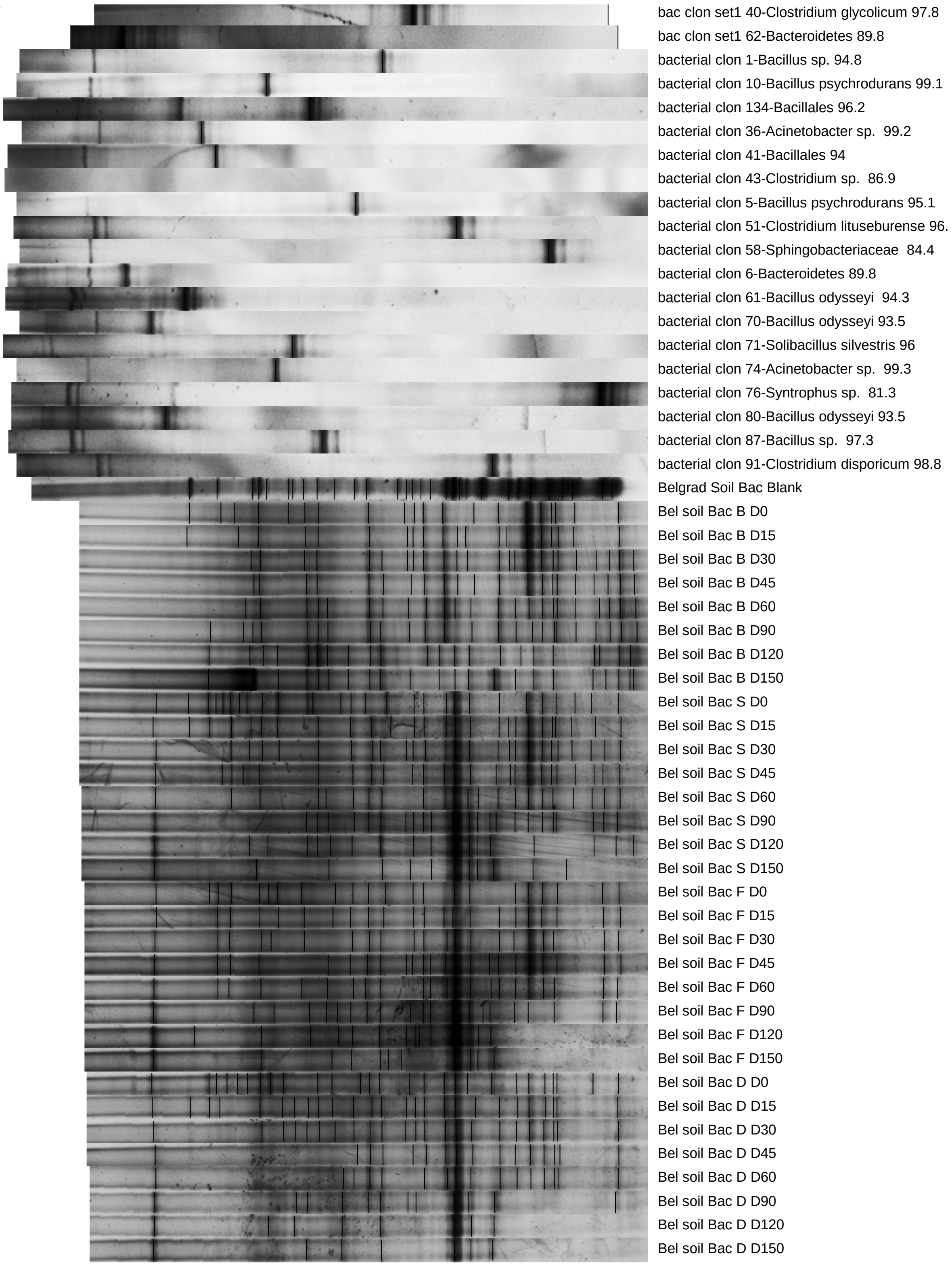


Figure 5.44. Change of bacterial diversity with time in silt loam and clone match ups

5.4.4. Investigation of Microbial Communities in Soil with Respect to Their Activity

Three different soil types were analyzed by FISH for active bacterial communities. 12 probes were used to analyze bacterial groups in samples (Table 5.30. and Figure 5.55.). In clay soil *alfa-proteobacteria* was most dominant group in sample while in sandy and silt loam *Spirochaetaceae* was most dominant group. In all samples, ratio of active archaea was low (5%). For nonspecific binding Non-Eub probe was used. Although the ratio of it was at 1% in sandy and silt loam; it was about 10% in clay soil. Possible the high non-spesific bonding was related to high clay content of the soil.

Table 5.30. Ratios of active bacterial communities to DAPI in blank soil samples

		Silt Loam	**Sandy Loam**	**Clay soil**
Probes	**Target**	**Total Cell/DAPI (%)**	**Total Cell/DAPI (%)**	**Total Cell/DAPI (%)**
EUBMix	Bacteria	52.1	32.0	64.0
ARC915	Archaea	4.9	2.7	1.0
ALF968	*Alpha-prot*	8.8	4.3	50.0
BET42a	*Beta-prot*	1.4	3.1	1.0
GAM42a	*Gamma-prot*	0	0	0
LGC354 Mix	*Firmicutes*	7.6	1.8	2.0
SPIRO1400	*Spirochaetaceae*	16.8	6.8	1.0
PLA46	*Planctomycetales*	3.9	5	2.0
CFB1082	*Bacterioidetes*	3.9	2.7	3.0
CFX1223	*Chloroflexi*	3.5	2.6	3.0
HGC69A	*Actinobacteria*	3.9	3	1.0

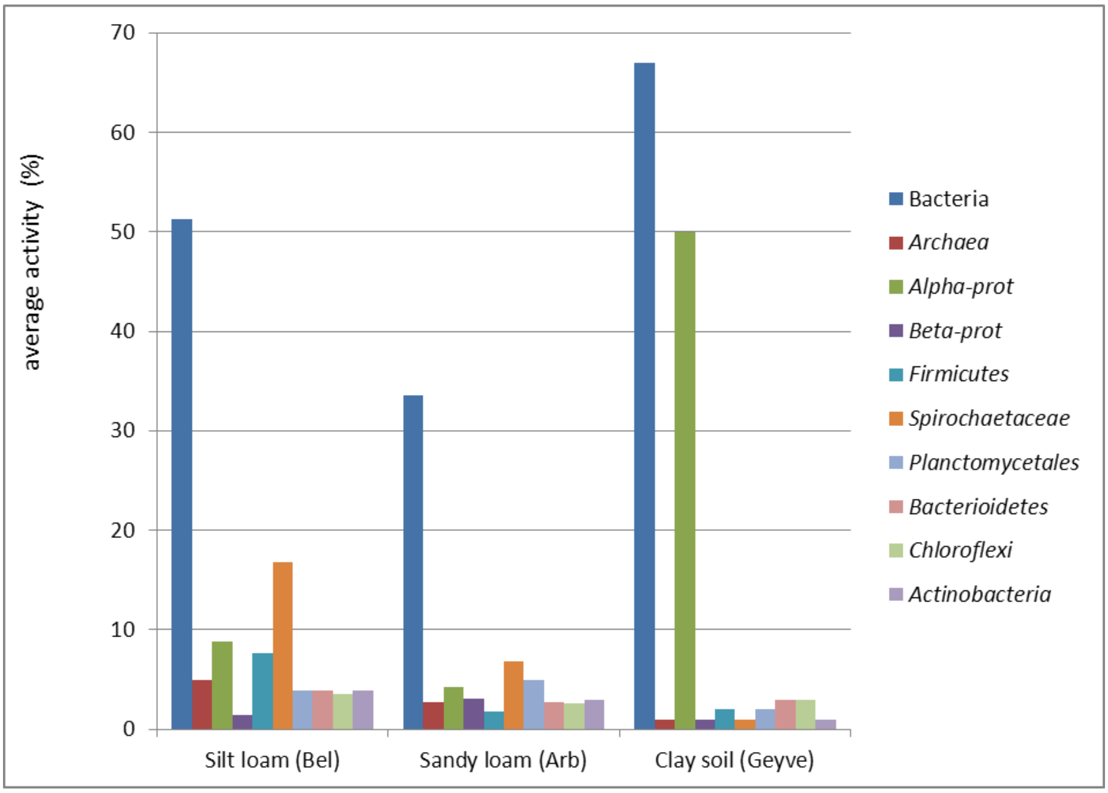


Figure 5.55. Active bacterial communities in blank soil samples

FISH analysis was also performed during incubation to monitor effect of amendment (Table 5.31.). From previous analysis, it has been seen that some bacterial groups were remained not active; therefore, FISH was performed only for groups (*Proteobacteria*, *Spirochaetacea* and *Actinobacteria*) which showed strong activity (Figure 5.56. and 5.57.).

Table 5.31. Changing bacterial groups in soil studies during incubation

	Sandy Loam (Arboretum)									
		Day 0	**Day 60**	**Day 150**	**Day 0**	**Day 60**	**Day 150**	**Day 0**	**Day 60**	**Day 150**
	Blank	**S**			**F**			**D**		
Proteobacteria	7.4	38	26	0	25	14	3	15	28	1
Actinobacteria	3	42	9	3	13	1	2	38	10	1
Spirochaetaceae	6,8	17	9	6	41	41	2	26	7	7
	Silt Loam (Belgrad)									
		Day 0	**Day 60**	**Day 150**	**Day 0**	**Day 60**	**Day 150**	**Day 0**	**Day 60**	**Day 150**
	Blank	**S**			**F**			**D**		
Proteobacteria	10.2	11	26	2	29	23	2	18	23	8
Actinobacteria	4	26	7	10	26	12	6	30	32	13
Spirochaetaceae	17	35	14	6	43	34	40	27	22	-

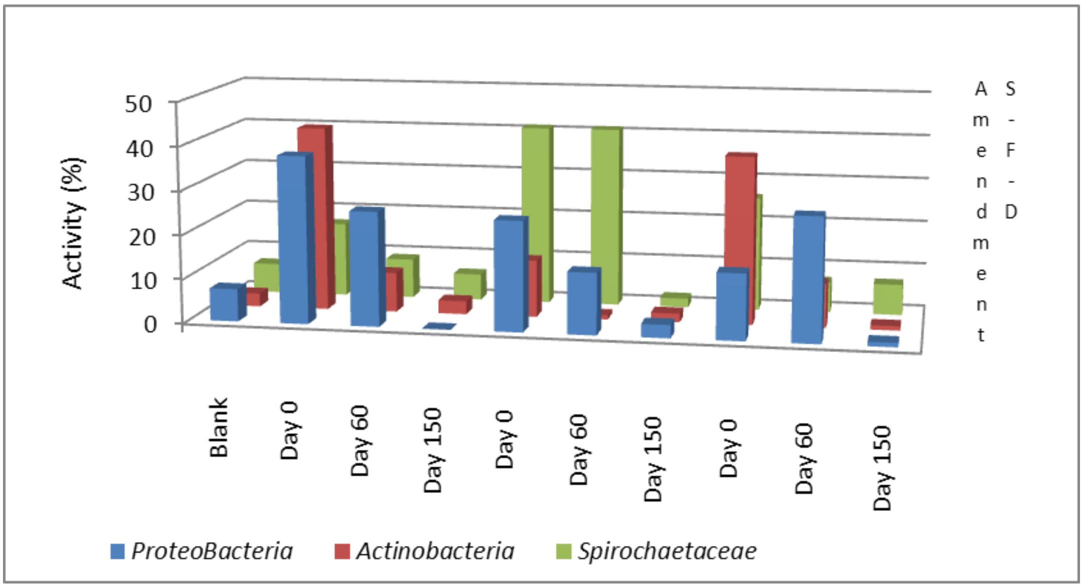


Figure 5.56. Changing bacterial groups in sandy loam during incubation

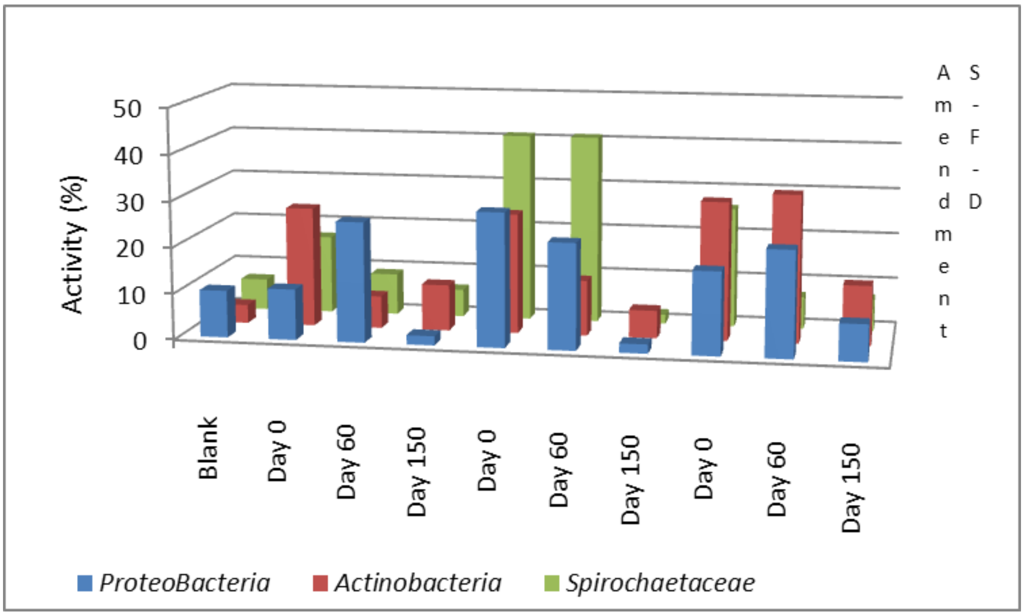


Figure 5.57. Changing bacterial groups in silt loam during incubation

Activity in soil samples was increased significantly (min 15%) after manure amendment. This result showed that there was introduction of bacterial cells into soil microbiota. In general, activity increased upon manure amendment but then decreased to starting level.

5.4.5. Quantitative Analysis of Soil Microbial Communities

During incubation, changes in active bacterial genes were monitored with QPCR analysis (Table 5.32.). Manure amendment increased gene copy number 5-10 times. Manure amendment had introduced more gene than digestate amendment. There was a decrease in number of active genes with time (Figure 5.58. and 5.59.). There was a higher gene copy number (100 fold) in sandy loam than silt loam which was expected due to lower diversity of silt loam in respect to sandy loam.

Table 5.32. Quantitative analysis of bacterial 16S rRNA gene copy number in soil samples

Soil sample	**Incubation time (day)**	**Gene copy number**	**Soil sample**	**Incubation time (day)**	**Gene copy number**
ArB	**0**	1.35E+07	**BelB**	**0**	1.77E+06
	15	1.44E+07		**15**	1.11E+06
	30	9.67E+06		**30**	1.20E+06
	45	4.84E+06		**45**	7.77E+05
	60	6.36E+06		**60**	9.01E+05
	90	5.72E+06		**90**	1.08E+06
	120	7.76E+06		**120**	2.11E+06
	150	4.15E+06		**150**	1.23E+06
ArS	**0**	1.01E+08	**BelS**	**0**	4.06E+06
	15	3.01E+08		**15**	6.68E+06
	30	6.55E+07		**30**	1.77E+06
	45	1.97E+07		**45**	1.16E+06
	60	4.11E+07		**60**	1.82E+06
	90	6.30E+07		**90**	2.07E+06
	120	5.44E+07		**120**	2.76E+06
	150	4.42E+07		**150**	2.12E+06
ArF	**0**	3.59E+08	**BelF**	**0**	8.63E+06
	15	3.82E+08		**15**	5.67E+06
	30	5.16E+07		**30**	1.56E+06
	45	3.75E+07		**45**	7.68E+05
	60	4.60E+07		**60**	1.26E+06
	90	4.69E+07		**90**	1.18E+06
	120	4.96E+07		**120**	9.23E+05
	150	1.64E+07		**150**	1.13E+06
ArD	**0**	6.22E+07	**BelD**	**0**	1.07E+06
	15	3.77E+07		**15**	6.44E+05
	30	2.14E+07		**30**	6.88E+05

Table 5.32 continued

	45	1.56E+07		**45**	5.95E+05
	60	2.51E+07		**60**	6.13E+05
	90	1.55E+07		**90**	6.05E+05
	120	2.14E+07		**120**	6.12E+05
	150	1.59E+07		**150**	4.14E+05

* S: Spiked manure, F: Medicated manure, D: digestate, B: Control, Ar: Sandy Loam, Bel: Silt Loam

Quantitative analysis of bacterial 16S rRNA genes showed that an increased number of genes upon manure amendment. After day 15 a decrease began until day 45. After that time, gene copy numbers do not show a significant change until end of incubation. Similar bacterial gene copy numbers were detected in another study (Munir and Xagoraraki, 2011).

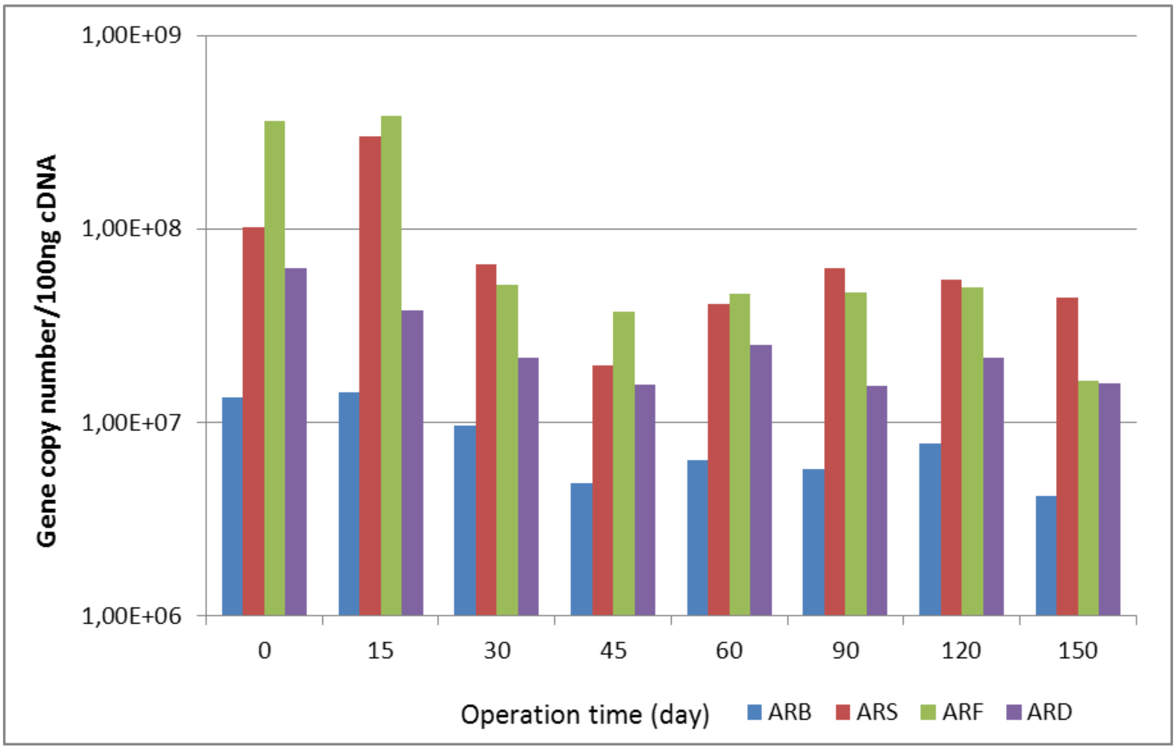


Figure 5.58. Change of bacterial 16S rRNA gene copy numbers in sandy loam

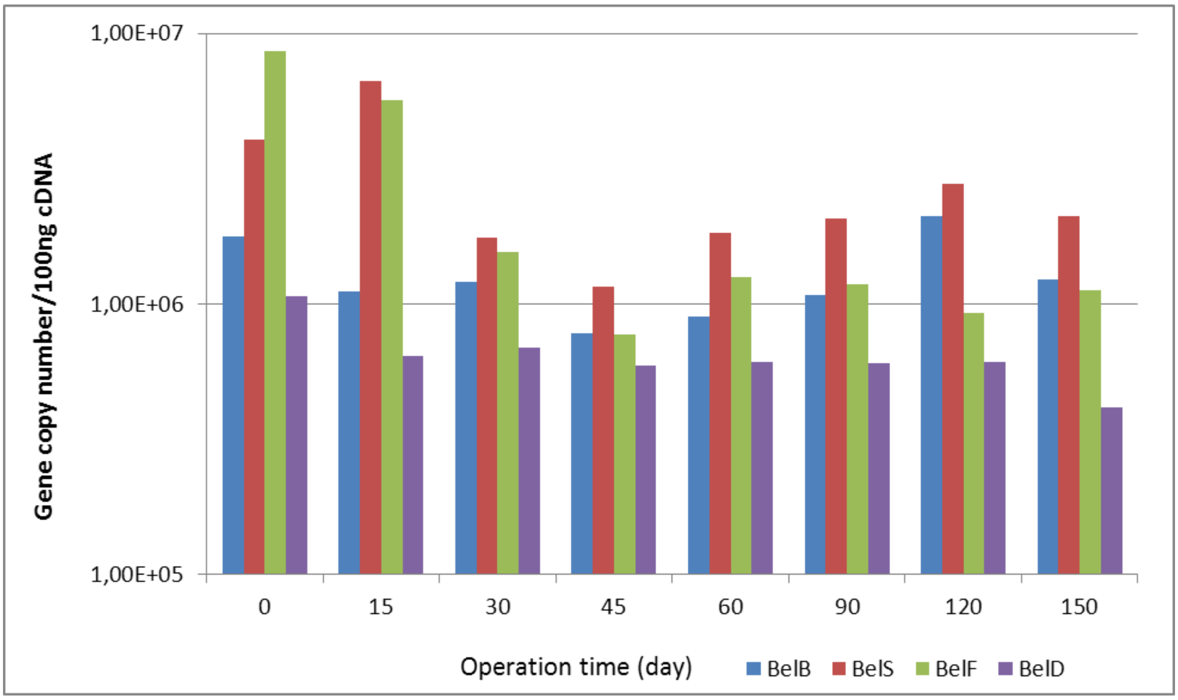


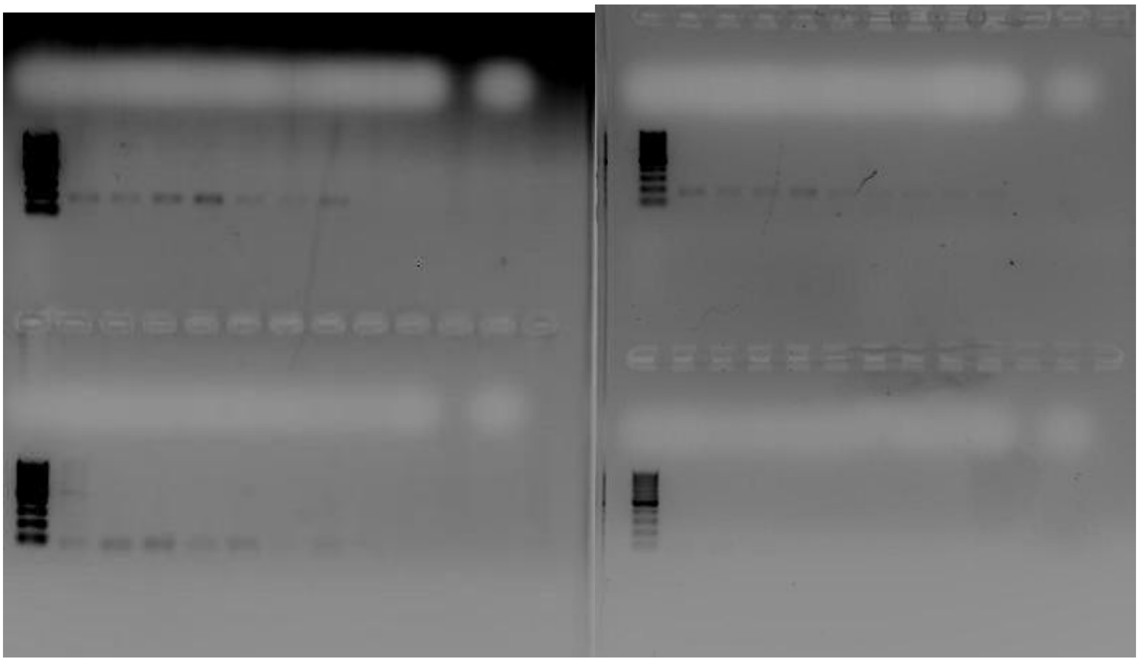
Figure 5.59. Change of bacterial 16S rRNA gene copy numbers in silt loam

5.5. Investigation of Formation of Antibiotic Resistance

Analyses of tetracycline resistance gene formation were performed by Q-PCR targeting specific resistance genes. The findings of analysis showed dynamics of resistance genes as its translocation from animal body to receiving environments.

5.5.1. Presence of ARG in Digester Microcosms

Samples taken from digesters were analyzed by specific primers coding for four different tetracycline resistance genes. These four genes were grouped into two, efflux protein coding and ribosomal protection protein coding. Out of four genes, only two coding for ribosomal protection protein, Tet M and Tet O, was found (Figure 5.60.).



a) b)

Figure 5.60. PCR analysis of resistance gene in samples collected from microcosm samples a) Tet (O) and Tet (B) b) Tet (M) and Tet (K) 100 bp marker was applied. 200 bp product was expected.

Positive amplicons were amplified again and cleaned by High Pure PCR purification Kit (Roche, Germany) according to manufacturer's protocol. Cleaned products were sequenced to verify their identification. Sequences were analyzed in databank at www.ebi.ac.uk. Sequence analysis showed that sequences were resembled to Tet M and Tet O genes by 99% and 100%, respectively (EM_ENV:HE580607 and TR:F2X9A4_9BACT). Sequences were given below:

>18-tet_M

GTAGATCCCTTCAGTAACATCGGCCACCGTGGACAGAGGTACAACGAAAACGGATAATACGCTTTTAGAACGTCAGAGAGGAATTACAATTCAGACGGCGATAACCTCTTTTCAGTGGAAAAATACTAAGGTGAACATCATAGACACGCCAAGA

>15-tet_O
GGGAGCGTAGATGAAGGCACAACAAGGACAGATACAATGAATTTGGAGCGTC
AAAGGGGAATCCACTATCCAGACAGCAGTGACATCTTTTCAGTGGGAGGATGT
AAAAGTCAACATTATAGATACGCCAAAGATTTCCATTAACCCTCCCTAAT

5.5.2. Quantitative Analysis of ARG in Anaerobic Digesters

Dynamics of resistance gene in digesters were monitored by quantitative analysis. Ratio of resistance genes per 16S rRNA gene was also calculated to monitor trend of resistance gene in community. In digester studies, quantitative analysis of Tet M and Tet O was performed (Table 5.33. and 5.34., Figure 5.61- 5.68.).

Table 5.33. Quantitative analysis of Tet M resistance gene in digesters

		Set 1	**Set 2**			**Set 3**	**Set 4**
Digester*	**Day**	**Gene copy number**	**Gene copy number**	**Digester***	**Day**	**Gene copy number**	**Gene copy number**
S1	**D0**	1.20E+06	1.27E+04	**S1**	**D0**	6.60E+05	1.20E+05
S3	**D0**	2.18E+05	1.26E+04	**S3**	**D0**	1.26E+06	1.28E+05
S4	**D0**	6.20E+05	4.68E+03	**S4**	**D0**	5.33E+05	1.07E+05
S6	**D0**	6.42E+05	6.82E+03	**S6**	**D0**	7.54E+05	9.97E+04
S1	**D10**	3.40E+05	1.60E+04	**S1**	**D5**	3.23E+05	2.38E+05
S3	**D10**	2.02E+06	6.44E+04	**S3**	**D5**	6.46E+05	9.04E+04
S4	**D10**	5.26E+05	1.86E+04	**S4**	**D5**	6.69E+05	1.20E+05
S6	**D10**	2.32E+06	2.20E+03	**S6**	**D5**	2.57E+05	1.29E+05
S1	**D20**	2.14E+05	5.34E+03	**S1**	**D10**	4.87E+05	1.19E+05
S3	**D20**	1.61E+05	2.84E+03	**S3**	**D10**	4.04E+05	1.28E+05
S4	**D20**	1.92E+05	1.45E+05	**S4**	**D10**	1.90E+05	1.33E+05
S6	**D20**	7.18E+05	8.50E+04	**S6**	**D10**	1.74E+05	9.89E+04
S1	**D30**	1.24E+05	1.69E+03	**S1**	**D15**	8.94E+04	9.89E+04
S3	**D30**	3.54E+05	2.28E+04	**S3**	**D15**	1.09E+05	7.69E+04
S4	**D30**	3.82E+04	3.32E+04	**S4**	**D15**	1.30E+05	8.71E+04
S6	**D30**	6.32E+04	1.08E+04	**S6**	**D15**	1.13E+05	1.00E+05
				S1	**D20**	1.10E+05	7.00E+04
				S3	**D20**	2.37E+05	6.07E+04
				S4	**D20**	1.27E+05	9.23E+04
				S6	**D20**	1.54E+05	6.13E+04

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9%
Set 1: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

Table 5.34. Quantitative analysis of Tet O resistance gene in digesters

		Set 1	Set 2			Set 3	Set 4
Digester*	**Day**	**Gene copy number**	**Gene copy number**	**Digester***	**Day**	**Gene copy number**	**Gene copy number**
S1	**D0**	1.55E+05	4.28E+04	**S1**	**D0**	2.91E+05	9.97E+04
S3	**D0**	1.03E+05	4.26E+04	**S3**	**D0**	7.06E+05	5.57E+05
S4	**D0**	1.51E+05	6.28E+04	**S4**	**D0**	6.57E+05	2.75E+05
S6	**D0**	6.90E+05	7.00E+04	**S6**	**D0**	9.18E+05	9.44E+05
S1	**D10**	2.12E+05	8.88E+04	**S1**	**D5**	1.03E+05	4.30E+04
S3	**D10**	9.66E+04	3.88E+04	**S3**	**D5**	6.56E+04	6.21E+04
S4	**D10**	2.02E+05	2.24E+05	**S4**	**D5**	1.12E+05	3.96E+04
S6	**D10**	1.02E+05	8.70E+04	**S6**	**D5**	3.68E+05	1.21E+05
S1	**D20**	9.72E+04	7.96E+04	**S1**	**D10**	3.60E+05	4.47E+04
S3	**D20**	1.53E+05	8.58E+04	**S3**	**D10**	1.07E+05	7.37E+04
S4	**D20**	7.28E+04	9.68E+04	**S4**	**D10**	1.40E+05	6.40E+04
S6	**D20**	1.23E+05	1.65E+05	**S6**	**D10**	1.01E+05	7.03E+04
S1	**D30**	1.61E+05	2.86E+04	**S1**	**D15**	5.41E+04	3.68E+04
S3	**D30**	5.48E+04	4.68E+04	**S3**	**D15**	8.97E+04	4.56E+04
S4	**D30**	3.32E+04		**S4**	**D15**	8.53E+04	2.55E+05
S6	**D30**	5.36E+04		**S6**	**D15**	1.19E+05	1.00E+05
				S1	**D20**	6.45E+04	3.24E+04
				S3	**D20**	1.95E+05	3.25E+04
				S4	**D20**	9.16E+04	7.79E+04
				S6	**D20**	9.88E+04	6.10E+04

***S1:**Control, TVS 5-6% **S3**: OTC, TVS 5-6% **S4**:Control, TVS 8-9% **S6**: OTC, TVS 8-9% **Set 1**: 37^0 C,90 rpm **Set 2**: 37^0 C,120 rpm **Set 3**: 55^0 C,90 rpm **Set 4**: 55^0 C,120 rpm

In this study, four tetracycline resistance genes were selected to monitor resistance gene promotion in anaerobic digestion and in soil. Two of the resistance genes were efflux protein coding genes; the other two were coding for ribosomal protection proteins. PCR analysis done on microcosm samples showed only ribosomal protection type resistance was present in digesters. Change of resistance genes in respect to time showed that for both resistance genes starting number of genes were 1.00+E5 -1.00+E6 in all sets except Set 2 which was in parallel to the results obtained in another study (Storteboom et al., 2007; Munir and Xagoraraki, 2011; Barkovski et al., 2012). In those sets, number of gene copies decreased with time and halved in the end of digestion. In Set 2 digesters, resistance genes were started with less gene copy numbers and increased with time. As resistance gene numbers were monitored according to environmental parameters, it has been found that in low mixing rate digesters gene copy number of resistance genes were higher than the numbers high mixing rate digesters. Although there was a tendency of decrease in gene copy numbers, the decrease was not at significant level.

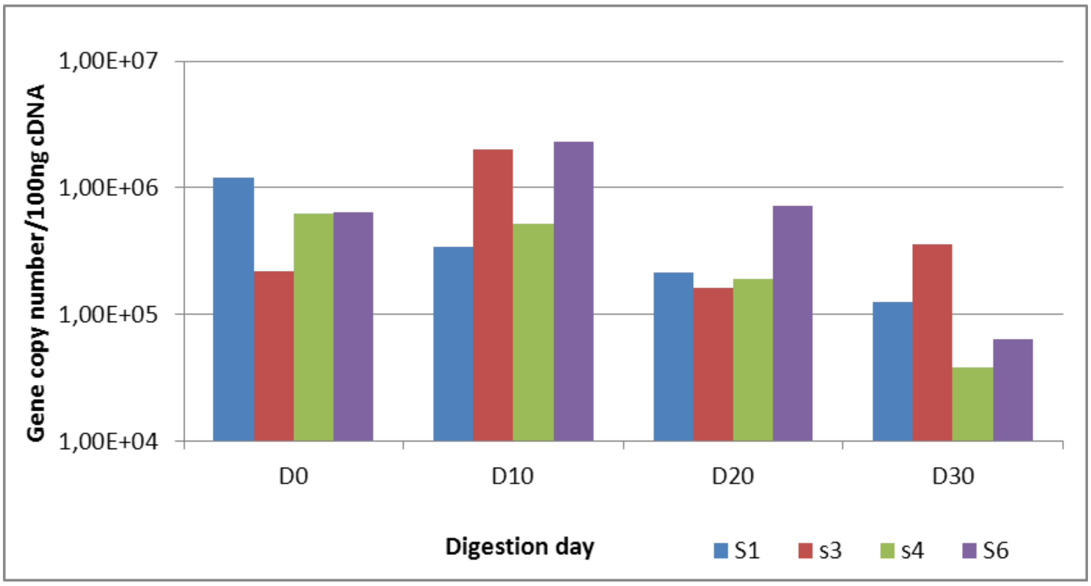


Figure 5.61. Changes in gene copy number of Tet M gene in Set 1 digesters

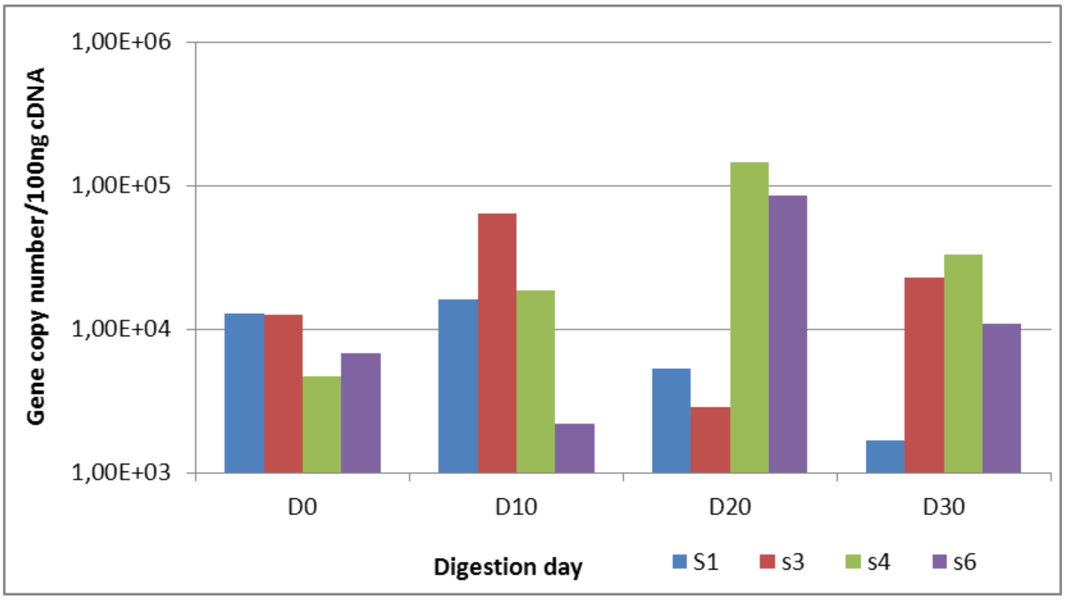


Figure 5.62. Changes in gene copy number of Tet M gene in Set 2 digesters

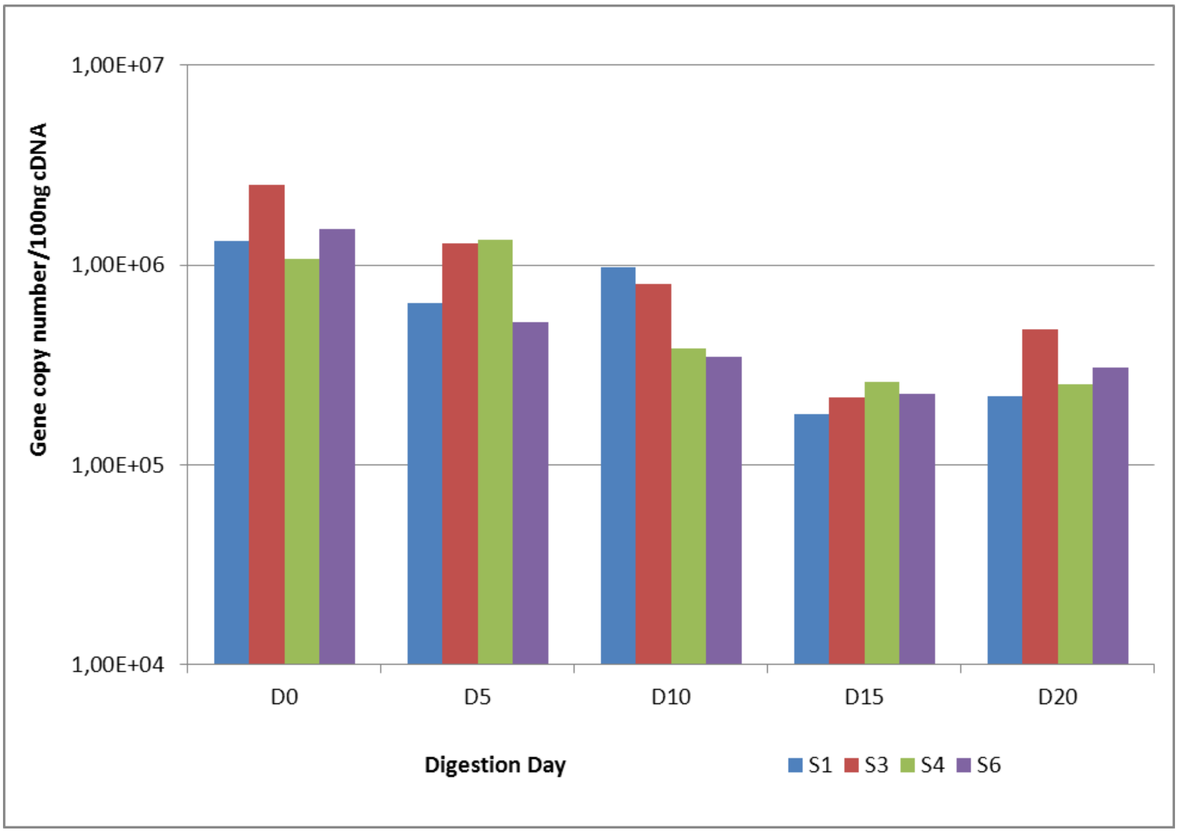


Figure 5.63. Changes in gene copy number of Tet M gene in Set 3 digesters

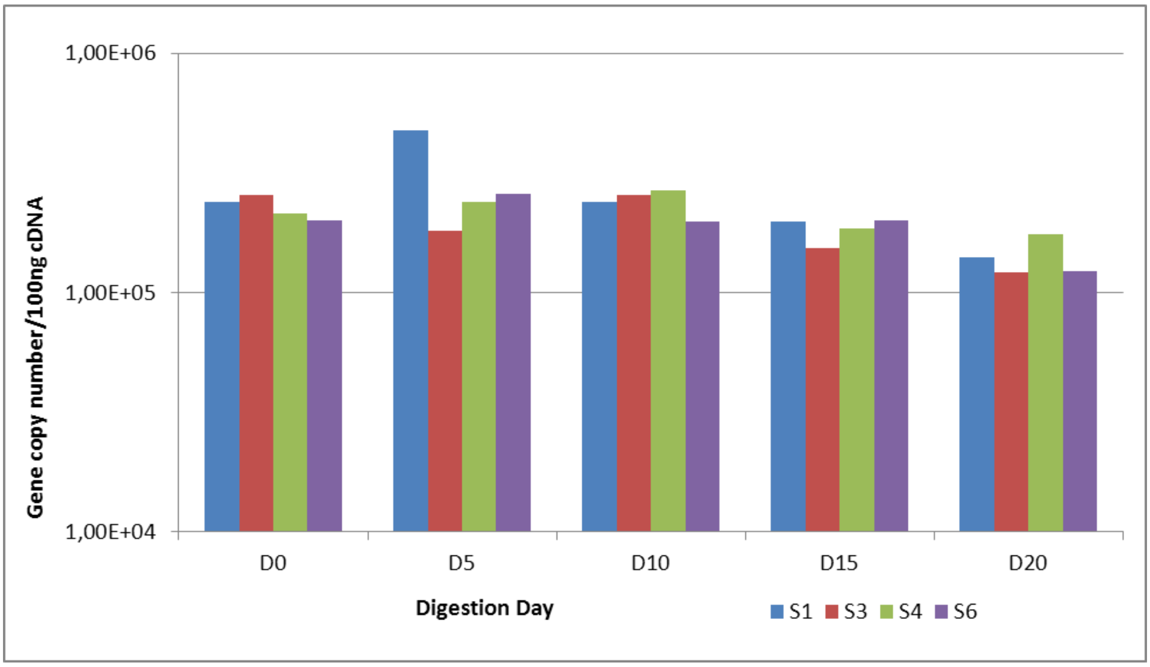


Figure 5.64. Changes in gene copy number of Tet M gene in Set 4 digesters

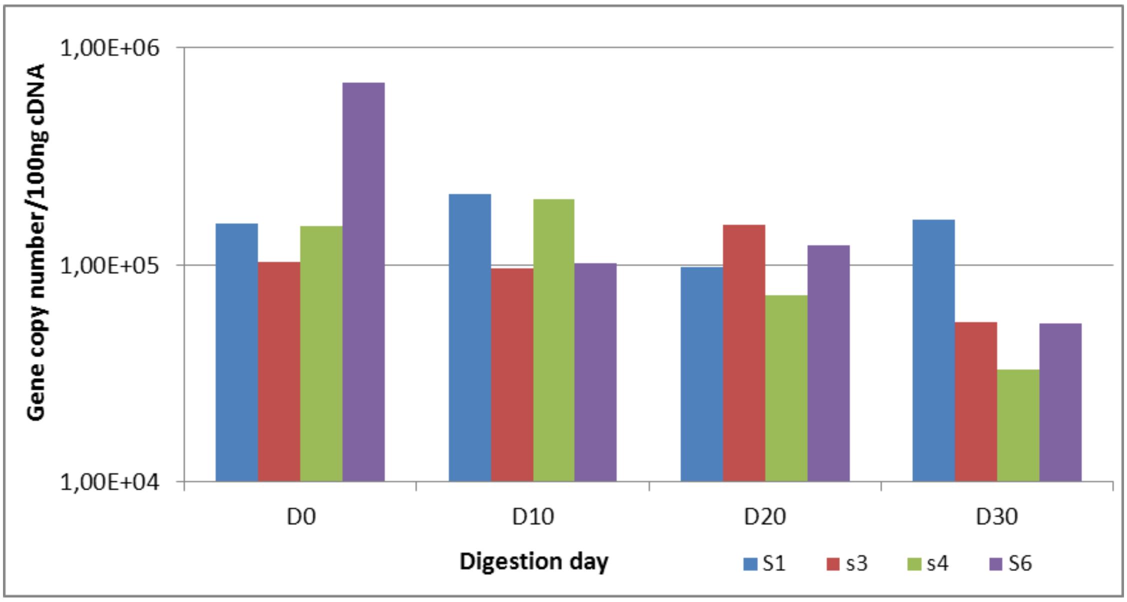


Figure 5.65. Changes in gene copy number of Tet O gene in Set 1 digesters

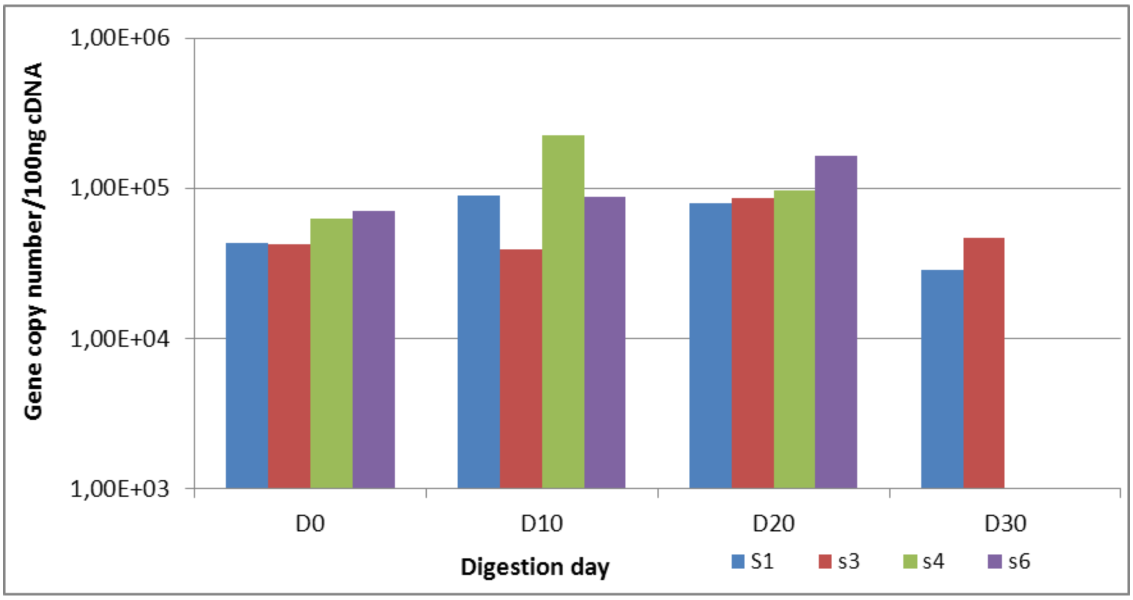


Figure 5.66. Changes in gene copy number of Tet O gene in Set 2 digesters

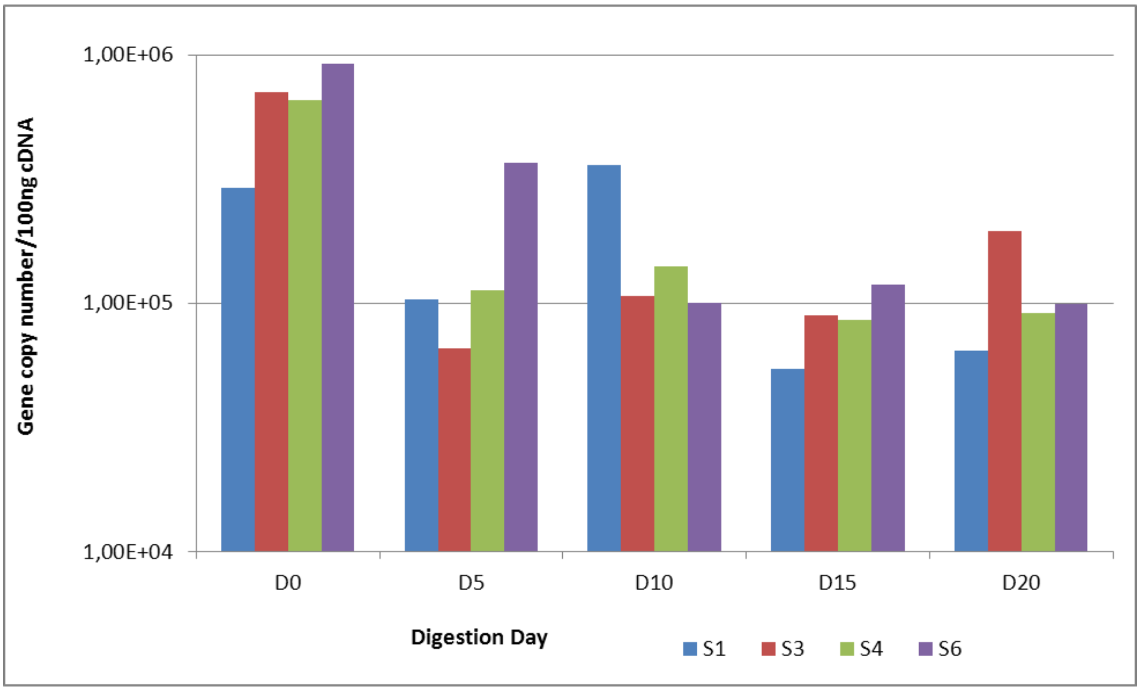


Figure 5.67. Changes in gene copy number of Tet O gene in Set 3 digesters

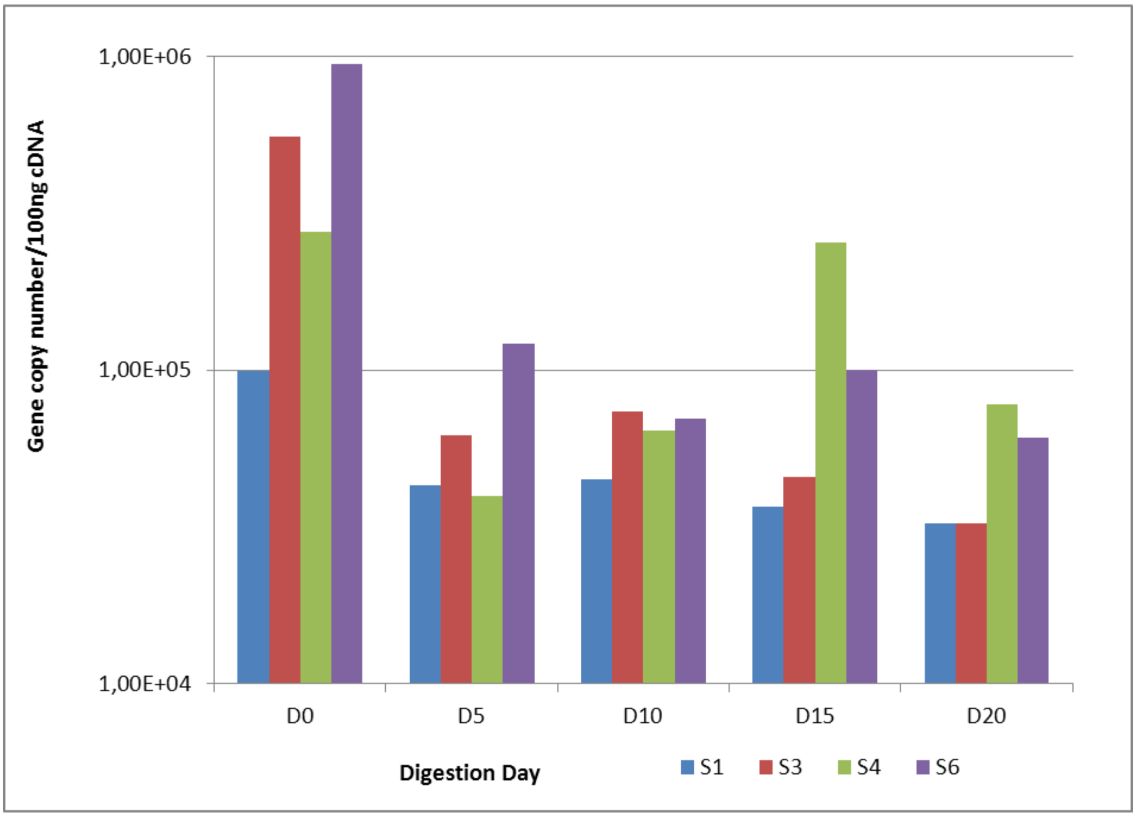


Figure 5.68. Changes in gene copy number of Tet O gene in Set 4 digesters

Ratios of resistance genes to bacterial 16S rRNA genes were used to interpret dynamics of resistance gene in respective environments throughout the study (Figure 5.69.-5.76.). Analysis of ratio of Tet genes to bacterial 16S rRNA genes showed that for both resistance genes the ratio was increased. As results were analyzed in respect to digesters, we saw an increasing trend of ratio in low mixing digesters and at the end of digestion the ratio was increased 50-100 times. In high mixing digesters, no clear trend can be seen. Ratio was first increased and then decreased and at the end of digestion ratio was 10-50 times higher than starting ratio. Main reason of these trends were copy number of bacterial 16s rRNA genes. The decrease of bacterial genes has led to an increase in ratio. In low mixing digesters, decrease of bacterial gene copy numbers was high and clear; this decrease was more erratic in high mixing rate digesters. This finding may suggest that eliminated bacteria did not carry resistance genes, so ratio increased although Tet resistance gene copy numbers do not change significantly. Similar results were found in literature regarding ratio of Tet genes to Bacterial 16S rRNA genes in various environments. Ratio of Tet O gene to bacterial 16S rRNA gene was around 10^{-4} and 10^{-6} in samples collected from anaerobic manure digesters and activated sludge (Ghosh et al., 2009; Munir and Xagoraraki, 2011; Negreanu et al., 2012). Ratio of Tet M gene to bacterial 16S rRNA gene was around 10^{-5} and 10^{-6} in samples collected from anaerobic lagoons (Barkovski et al., 2012).

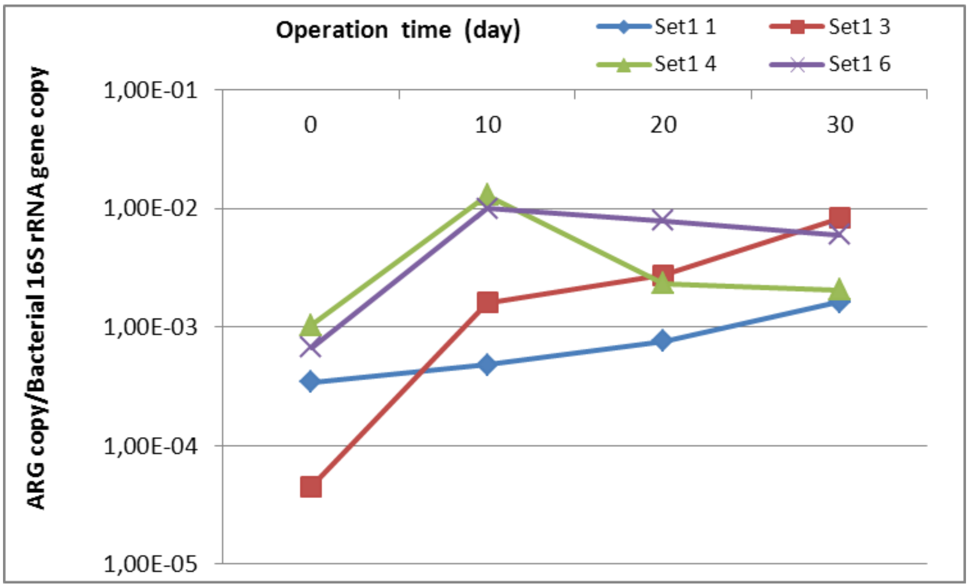


Figure 5.69. Ratio of Tet M gene to bacterial 16S rRNA gene in Set 1 digesters

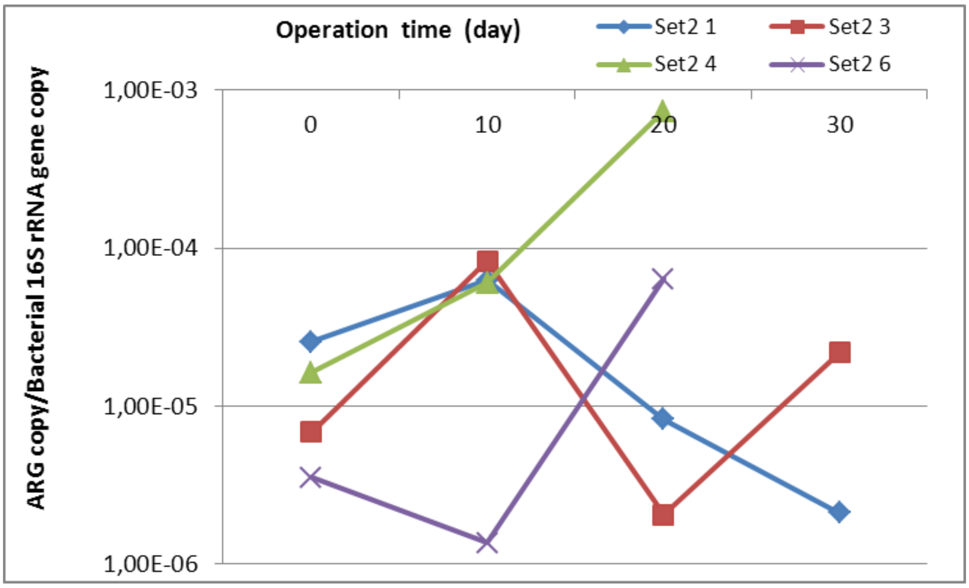


Figure 5.70. Ratio of Tet M gene to bacterial 16S rRNA gene in Set 2 digesters

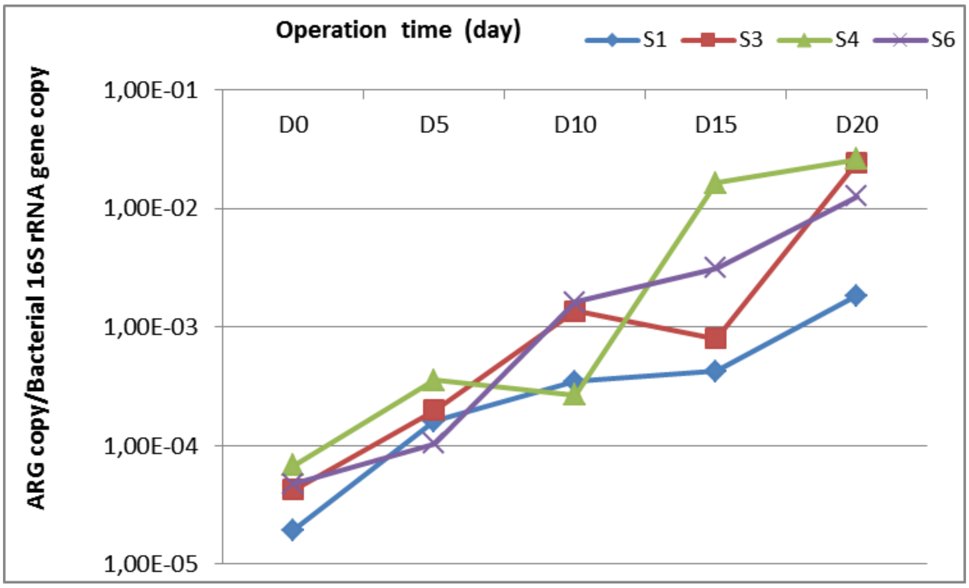


Figure 5.71. Ratio of Tet M gene to bacterial 16S rRNA gene in Set 3 digesters

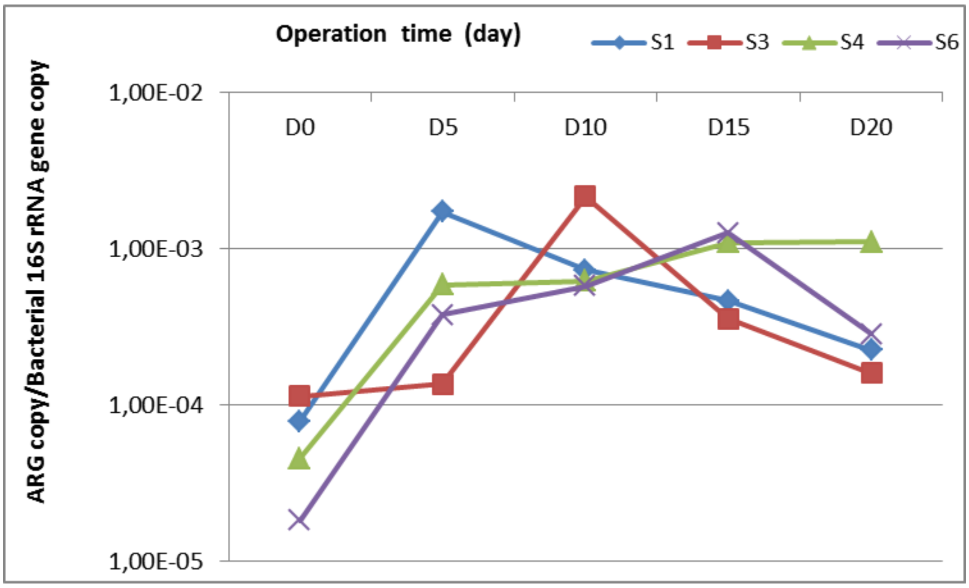


Figure 5.72. Ratio of Tet M gene to bacterial 16S rRNA gene in Set 4 digesters

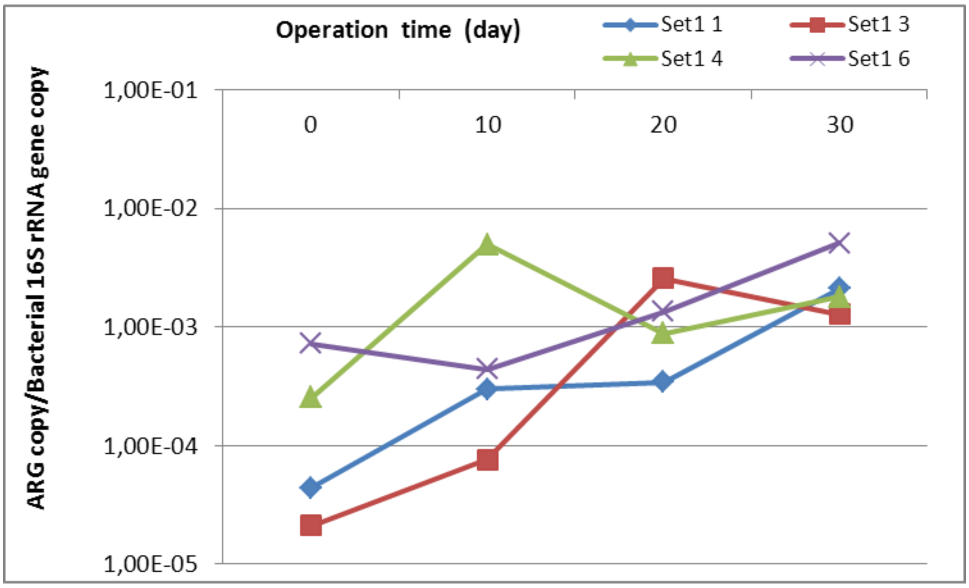


Figure 5.73. Ratio of Tet O gene to bacterial 16S rRNA gene in Set 1 digesters

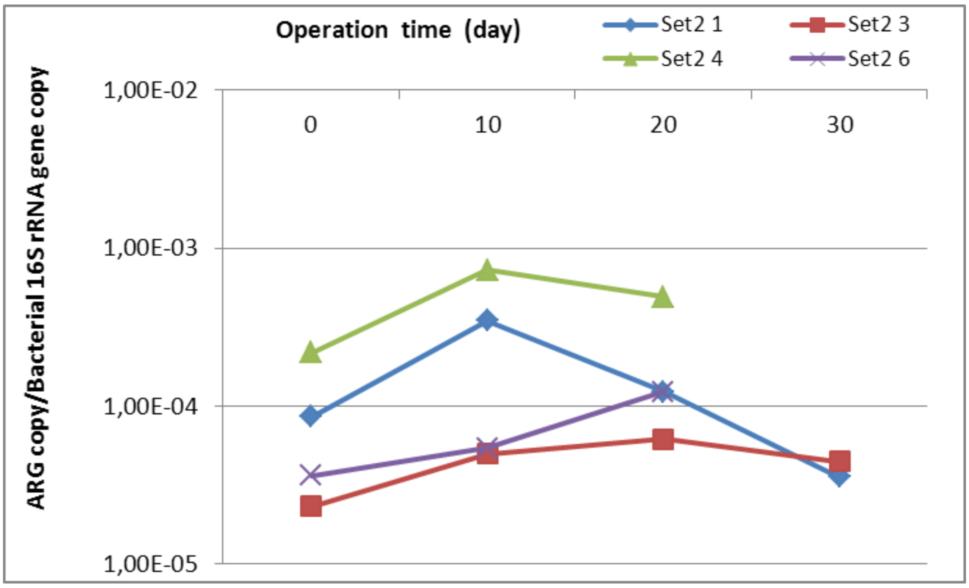


Figure 5.74. Ratio of Tet O gene to bacterial 16S rRNA gene in Set 2 digesters

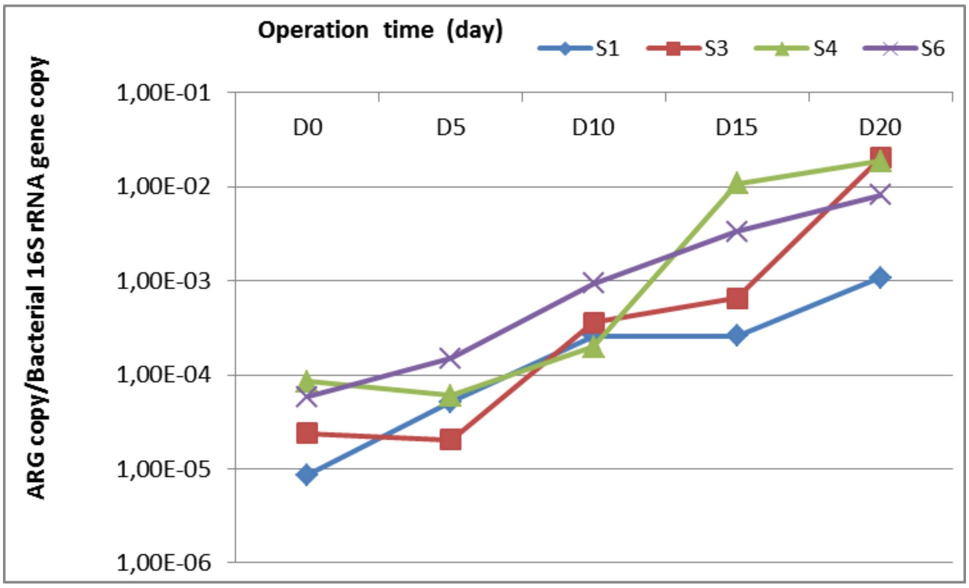


Figure 5.75. Ratio of Tet O gene to bacterial 16S rRNA gene in Set 3 digesters

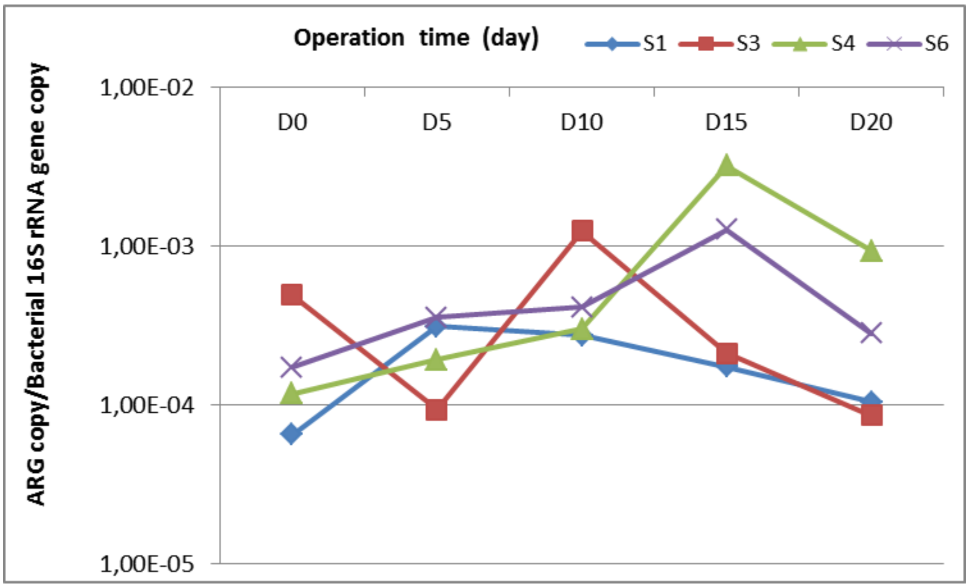


Figure 5.76. Ratio of Tet O gene to bacterial 16S rRNA gene in Set 4 digesters

5.5.3. Quantitative Analysis of ARG in Soil Microcosms

Application of manure or digestate as amendment introduces antibiotics to soil and may promote antibiotic resistance gene proliferation. This phenomenon was investigated by resistance gene quantification in soil microcosms (Table 5.35 & 5.36, Figure 5.77-5.80.).

Table 5.35. Change of Tet M resistance gene in soil microcosms

Soil Sample*	Incubation day	Gene copy number	Soil Sample*	Incubation day	Gene copy number
ArB	**0**	7.85E+02	**BelB**	**0**	1.30E+03
	15	2.03E+03		**15**	8.80E+01
	30	5.42E+02		**30**	2.83E+02
	45	7.13E+01		**45**	2.01E+03
	60	2.76E+02		**60**	1.07E+03
	90	1.10E+03		**90**	1.89E+02
	120	1.36E+03		**120**	9.92E+02
	150	2.76E+03		**150**	1.04E+04
ArS	**0**	4.66E+03	**BelS**	**0**	3.10E+03
	15	8.51E+02		**15**	3.99E+02
	30	1.46E+02		**30**	5.09E+03
	45	7.24E+02		**45**	1.86E+03
	60	1.16E+02		**60**	1.50E+02
	90	1.44E+02		**90**	2.26E+03
	120	2.83E+02		**120**	3.17E+03
	150	3.74E+02		**150**	7.13E+03
ArF	**0**	1.61E+03	**BelF**	**0**	6.08E+02
	15	1.07E+03		**15**	5.51E+03
	30	3.78E+01		**30**	1.90E+04
	45	2.79E+02		**45**	2.49E+03
	60	1.89E+03		**60**	2.76E+02
	90	9.41E+02		**90**	1.20E+03
	120	1.04E+03		**120**	4.58E+03
	150	2.22E+03		**150**	1.37E+02
ArD	**0**	2.13E+03	**BelD**	**0**	9.92E+02
	15	1.35E+03		**15**	4.43E+02
	30	1.70E+03		**30**	4.31E+02
	45	1.47E+03		**45**	1.62E+02
	60	7.89E+02		**60**	2.66E+02
	90	6.78E+01		**90**	7.03E+02
	120	1.86E+02		**120**	1.84E+03
	150	1.47E+03		**150**	3.21E+02

* S: Spiked manure, F:Medicated manure, D: digestate, B: Control, Ar:Sandy Loam, Bel: Silt Loam

Table 5.36. Change of Tet O resistance gene in soil microcosms

Soil Sample*	Incubation day	Gene copy number	Soil Sample*	Incubation day	Gene copy number
ArB	**0**	1.35E+05	**BelB**	**0**	8.12E+04
	15	8.88E+04		**15**	8.76E+04
	30	1.41E+05		**30**	7.47E+04
	45	8.23E+04		**45**	1.10E+05
	60	1.07E+05		**60**	7.88E+04
	90	1.58E+05		**90**	1.03E+05
	120	1.62E+05		**120**	8.24E+04
	150	9.96E+04		**150**	9.16E+04
ArS	**0**	9.05E+04	**BelS**	**0**	8.83E+04
	15	9.82E+04		**15**	1.16E+05
	30	1.43E+05		**30**	6.89E+04
	45	9.20E+04		**45**	1.66E+05
	60	9.19E+04		**60**	8.69E+04
	90	7.98E+04		**90**	8.08E+04
	120	1.02E+05		**120**	7.87E+04
	150	9.62E+04		**150**	9.54E+04
ArF	**0**	1.05E+05	**BelF**	**0**	7.38E+04
	15	8.87E+04		**15**	7.20E+04
	30	9.17E+04		**30**	6.78E+04
	45	8.07E+04		**45**	7.99E+04
	60	7.50E+04		**60**	7.42E+04
	90	6.42E+04		**90**	5.94E+04
	120	8.21E+04		**120**	5.26E+04
	150	7.80E+04		**150**	3.49E+04
ArD	**0**	7.72E+04	**BelD**	**0**	6.51E+04
	15	9.11E+04		**15**	6.62E+04
	30	8.65E+04		**30**	7.34E+04
	45	6.57E+04		**45**	6.52E+04
	60	7.14E+04		**60**	5.22E+04
	90	5.03E+04		**90**	5.88E+04
	120	6.98E+04		**120**	4.55E+04
	150	1.05E+05		**150**	2.80E+04

* S: Spiked manure, F: Medicated manure, D: Digestate, B: Control, Ar:Sandy Loam, Bel: Silt Loam

Tet gene copy numbers obtained in soil studies were lower than numbers obtained in digester studies. Also opposite to digesters, in soil studies Tet O gene was significantly higher (started at 1.00+E5 gene copy numbers) than Tet M gene (started at 1.00+E3 gene copy numbers). This also created a higher ratio of resistance gene to bacterial 16S rRNA gene. Copy numbers of Tet M gene did not show a clear tendency while Tet O gene copy numbers were stable and conserving their numbers. Tet resistance gene copy numbers were increased upon manure amendment but then decreased with time and after day 45 they were stable around starting level. In this study, it was then found that increase in resistance gene copy number was directly related to manure amendment but no increase in number of resistant bacteria was found in soil. This finding may suggest that resistant bacteria species were natural inhabitants of soil microbiota which was previously mentioned in another study (Agerso et al., 2006).

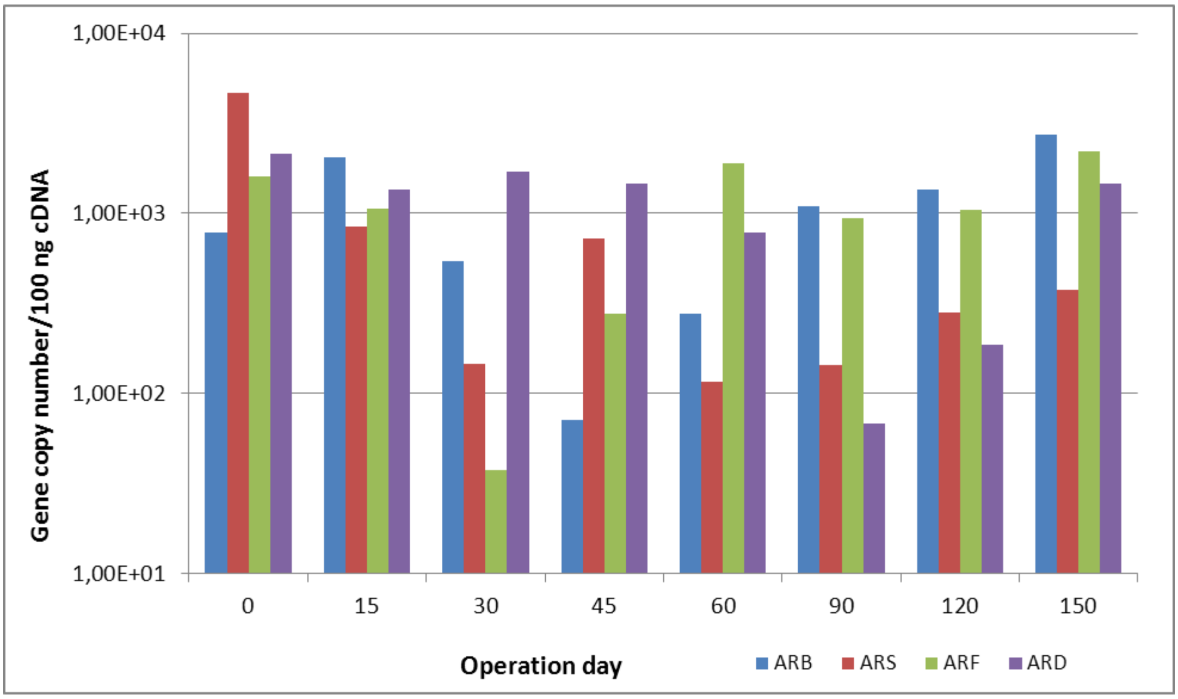


Figure 5.77. Change of Tet M resistance gene in sandy loam

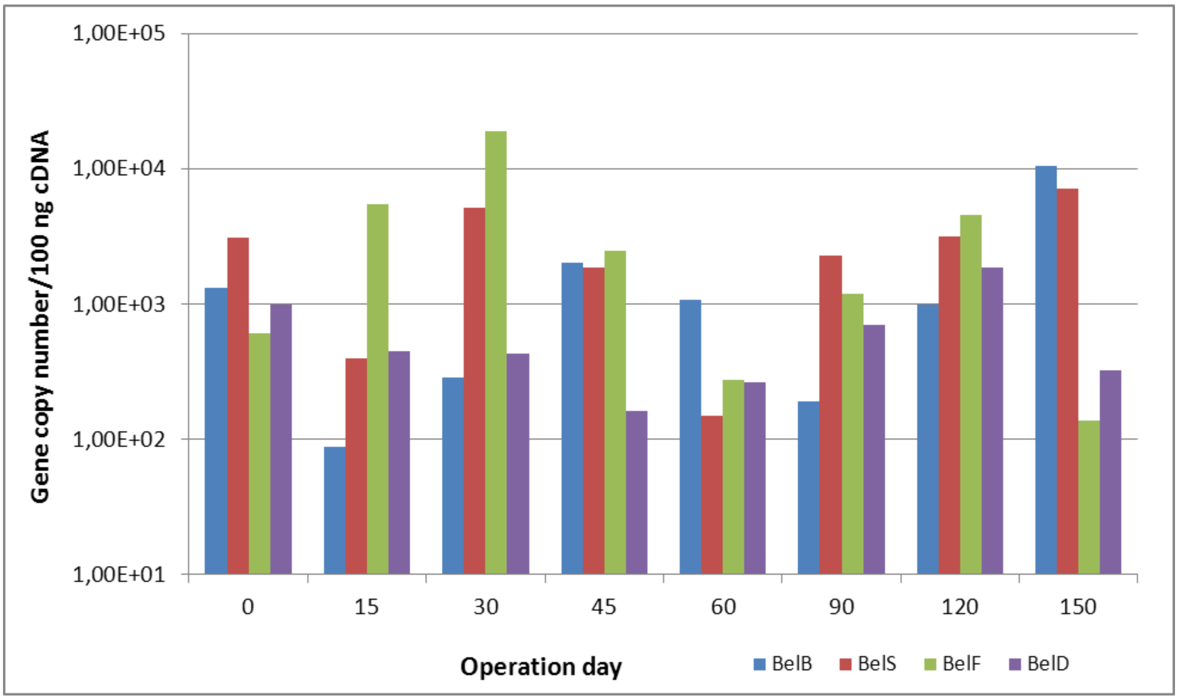


Figure 5.78. Change of Tet M resistance gene in silt loam

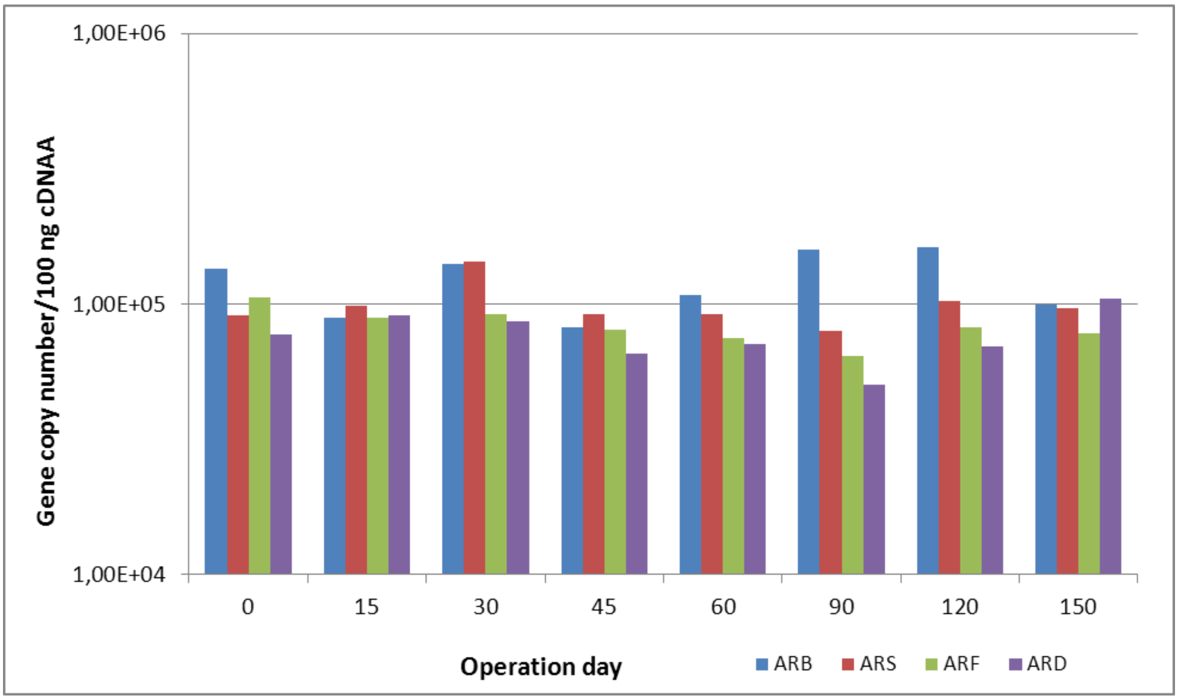


Figure 5.79. Change of Tet O resistance gene in sandy loam

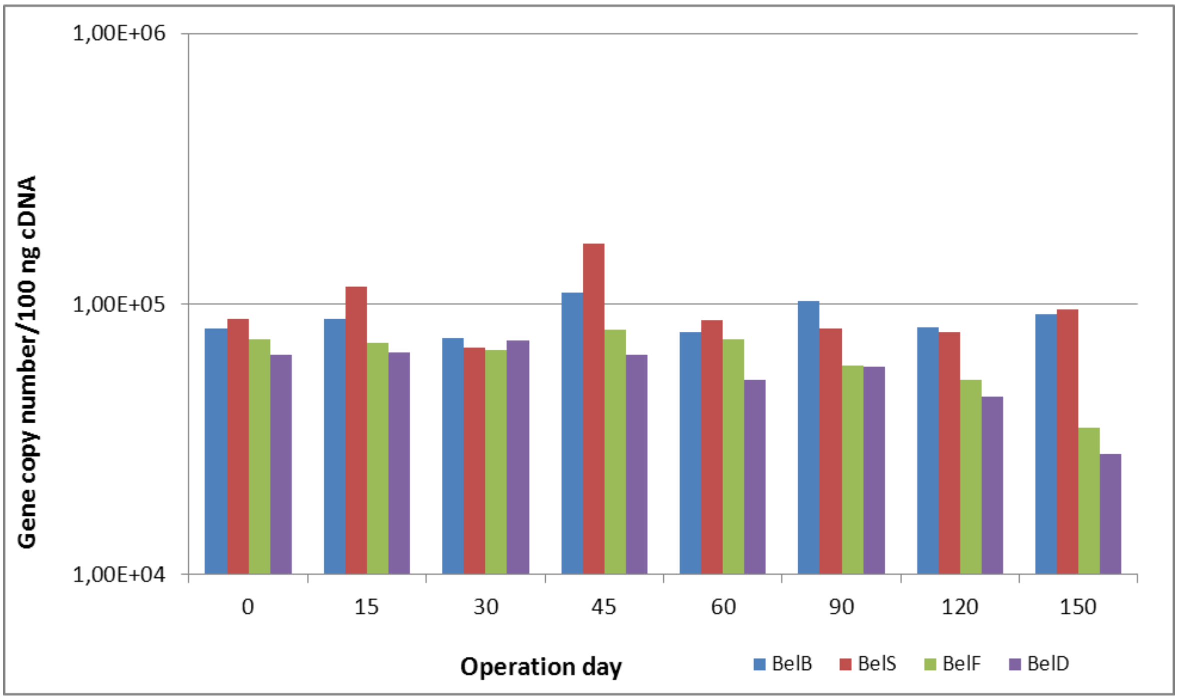


Figure 5.80. Change of Tet O resistance gene in silt loam

Monitoring of the ratio of resistance gens to bacterial 16S rRNA genes in soil operations, no significant change was observed (Figure 5.81-5.84.). But starting number of ratio was 100-1000 times higher than the ratio in digesters. Especially Tet O gene copy numbers were monitored as 100 times high than the Tet M gene copy numbers. As a derivation of it, ratio of Tet O genes to bacterial 16S rRNA genes was 100 fold higher than ratio of Tet M genes to bacterial 16S rRNA genes. In a study, ratio of Tet O to bacterial 16S rRNA was much lower than in our study, 10^{-4} and 10^{-2}, respectively (Munir and Xagoraraki, 2011).

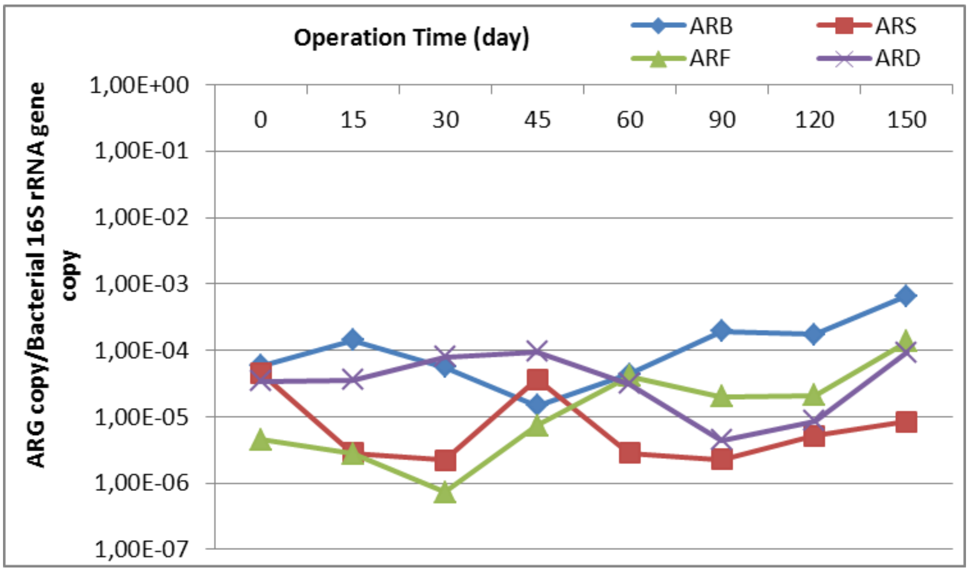


Figure 5.81. Ratio of Tet M resistance gene to bacterial 16S rRNA gene in sandy loam

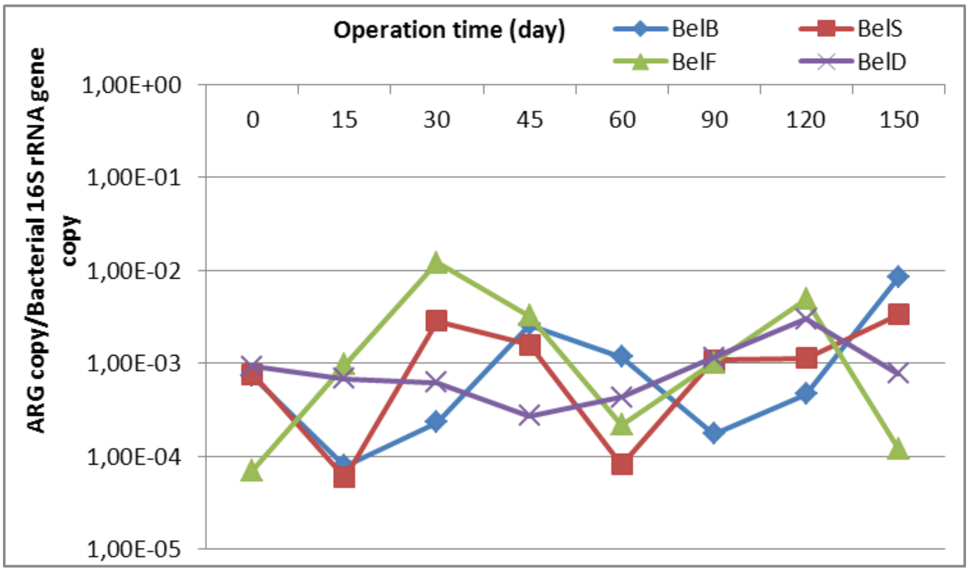


Figure 5.82. Ratio of Tet M resistance gene to bacterial 16S rRNA gene in silt loam

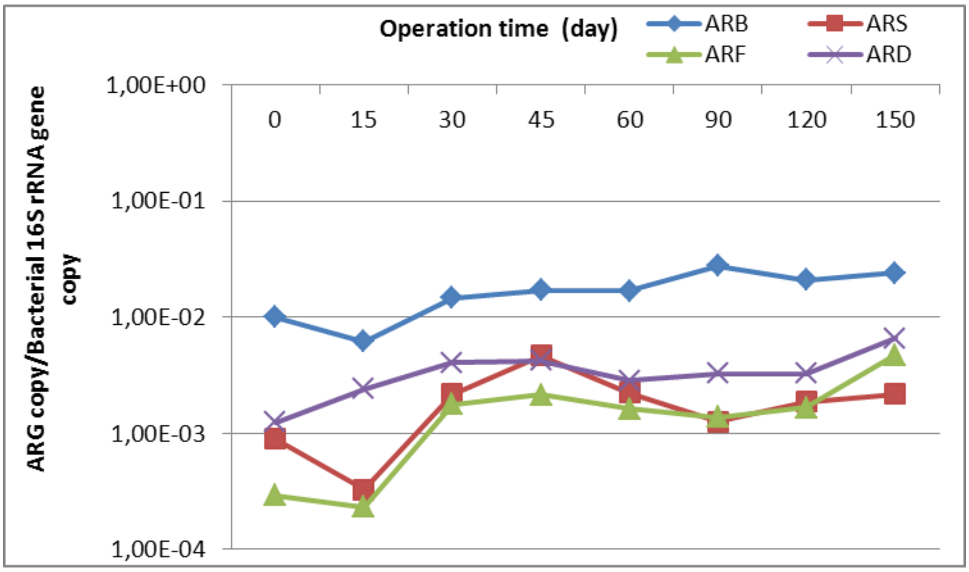


Figure 5.83. Ratio of Tet O resistance gene to bacterial 16S rRNA gene in sandy loam

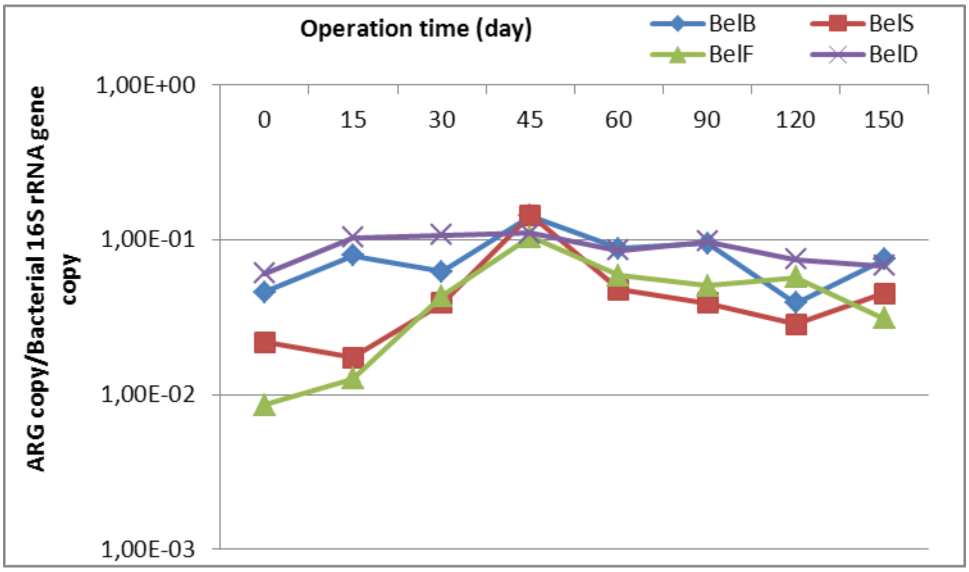


Figure 5.84. Ratio of Tet O resistance gene to bacterial 16S rRNA gene in silt loam

The investigation of resistance genes and their ratio to bacterial genes under the topic of public health showed that animal body is a suitable environment for survival and proliferation of resistant bacteria. In anaerobic digesters, this selective environment also played a crucial role which end up an increase of ratio of resistance genes to bacterial genes by 10-100 times. Although Tet gene copy numbers decreased during anaerobic digestion, the decrease in bacterial gene copy numbers were much higher. Then the ratio of Tet genes to bacterial 16S rRNA genes changes in favor of resistance genes. In soil, ratio of resistance genes to bacterial 16S rRNA genes was 10-100 times higher than the ratio in digesters. It was again due to low bacterial gene copy numbers. But opposite to ratio in digesters, no increase was observed during incubation time. This may suggest that manure amendment do not have a significant impact on resistance gene level in soil. Studies in literature mainly focused on the presence of resistance genes not the quantification of it. Even studies which gave results in number basis do not mentioned about the ratio of resistance gens to bacterial genes. Therefore, it cannot be decided that trends in ratios seen in our study was specific to our study. However, studies in literature and findings of this study are on the same opinion that there is an introduction of resistant bacteria to environment by human beings. The best method to prevent resistant bacteria in environment is prevention of formation of resistant bacteria at first place. It can only be achieved by decreasing the usage of agents like antibiotics and disinfectants which promote resistance gene formation.

6. CONCLUSIONS AND RECOMMENDATIONS

In the scope of this dissertation, fate and effects of OTC was investigated in manure, in anaerobic digestion, in soil. During this study dynamics of tetracycline resistance genes were also investigated. A brief summary of the main findings is highlighted in the following sections:

Excretion of pattern after OTC injection and excreted amount were determined to monitor the presence of OTC in animal manure.

-Injected OTC to animal was excreted from the animal in 13 days where main excretion was performed in first 5 days (4-13 mg/kg).

-At least 25% of injected amount of OTC was excreted in solid manure where this amount will further increase due to liquid manure and repetitive injections.

-Most important metabolite of OTC was ß-apo-OTC which was excreted more than OTC itself. Metabolite analysis of samples also showed that HPLC analysis targeting only parent compound will likely to underestimate concentration of antibiotic in the system.

Anaerobic digestion microcosms were set to monitor inhibition due to OTC and its metabolites.

-Microcosms digesters showed that metabolites of OTC played a significant role in inhibition. Since IC_{50} value of OTC was 68 mg/l where same inhibition was achieved by much lower concentration of OTC+metabolites. In anaerobic digestion OTC mainly transformed to 4-epi-OTC while ß-apo-OTC was transformed to ά-apo-OTC.

-Main microbial communities in anaerobic digestion cattle manure were *Clostridium* spp. and *Bacillus* spp. for bacteria; *Methanosarcina* spp. and *Methanobacteriales* spp. for

Archaea. Activity of microorganisms was directly related to OTC and was increased until 20th day of operation then decreased rapidly.

Effects of selected operarational parameters on system performance, microbial communities and elimination of OTC were investigated during this study.

-Most influential operational parameter was temperature and has positive effect both on the system performance and elimination of OTC. Other important parameters followed by solid content and mixing rate.

-OTC had a half-life of 24-26 days inn mesophilic digesters and 16-18 days in thermophilic digesters. Average activity of cells was increased in all digesters with time and mixing rate. OTC decreased the activity of cells in mesophilic digesters but had no effect on the activity in thermophilic digesters.

-Quantitative analyses of active genes don’t show a significant change in gene copy numbers. *Methanomicrobiales* spp. were low in number and *Methanosaeta* spp. were in negligible numbers.

Manure and digestate was amended to soil to simulate fertilization of agricultural lands with manure.

-The half-life of OTC in soil was greater than in the digesters (112 days in Silt Loam and 160 days in Sandy Loam). Manure amendment changed microbial diveristy of soil by introducing new species. *Clostridium* spp. was introduced by manure addition and became dominant in microbiota. Both FISH and Q-PCR studies showed that activity of cells increased upon manure amendment but return to initial state with time.

Antibiotic resistance gene levels and its dynamics were also investigated during the study.

-Only ribosomal protection type resistance genes (Tet M & Tet O) were found.

-Ratio of ARG genes to bacterial 16S rRNA genes increased significantly during anaerobic digestion.

-This increase was very clear in low mixing digesters where 10-100 fold increase was observed in mesophilic digesters and 100-1000 fold increase in thermophilic digesters. Ratio of ARG genes to bacterial 16S rRNA genes increased only 10 folds in high mixing digesters.

-Ratio of ARG genes to bacterial 16S rRNA genes did not change significantly in soil studies. Ratio of ARG genes to bacterial 16S rRNA genes in soil was higher than the ratio in digesters indicating that Tet genes were readily present in soil microbiota.

Recommendations

Digestate can be used directly on the fields but most of the time due to high volume of digestate will be processed and used as organic fertilizer. During this processing, digestate will go through specific processes like dewatering and homogenization. There was no study of antibiotic concentrations and resistance genes on finalized organic fertilizer. It needed to be investigated to finalize the fate of OTC in manure

It has been noticed that some topics remained unclear during in this study and studies in the literature. Resistance gene formation and its dynamics in the system can be understood better if more information on these topics has been obtained. One of the topics is bacterial species carrying resistance genes. It should be determined which species or genus is carrying resistance genes. This information will provide us enough data to determine which species are eliminated during process and which species survive. Also acquiring of resistance gene by bacterial species will be better undertood with this data.

Another topic needed to be investigated is multiple drug resistance how it is formed and how it is acquired. Resistance gene type is directly linked to multiple drug resistance, since an antibiotic resistance gene can trigger multiple drug resistance or act as one. Therefore, this topic also carries a great importance.

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