SINGLE AND MULTIPLE EFFECTS OF ORGANIC SOLVENTS ON THE EXPRESSION LEVEL OF ACETYL-CoA SYNTHETASE GENE AND ACTIVE METHANOGENIC POPULATION

by

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Integrated MS. and BS. in Teaching Chemistry, Boğaziçi University, 2008

Submitted to the Institute of Environmental Sciences in partial fulfillment of
the requirements for the degree of
Master of Science
in
Environmental Sciences

Boğaziçi University
2010

ACKNOWLEDGEMENT

I would like to thank my thesis supervisor Prof. Dr. Bahar İnce for her guidance, valuable advices, patience, understanding and support throughout the study. I am very grateful to her for giving me the chance to participate in her research group and experience the academic environment along with friends & colleagues.

I would also like to kindly thank Prof. Dr. Orhan İnce, the chair of the Molecular Ecology Group at Istanbul Technical University, who has opened the doors of working with such a nice group in Istanbul Technical University.

Special thanks are offered to Assist Prof. Nilgün Ayman Öz who shared all her knowledge and laboratory experience with me. I am deeply grateful to her for being incredibly helpful, tolerant and friendly with me throughout the study.

I want to thank to my colleagues Halil Çoban, Gökhan Türker and Şükriye Çelikkol for their great support and friendship. I also offer my special thanks to Res. Assist. Zeynep Çetecioğlu for her personal and academic support whenever I was in need of any help.

This study was supported by Bogazici University Scientific Research Fund (Project Coded 06Y102D) and TUBITAK Project No: 106Y241.

On a personal note, I want to say thanks to my parents for their patience, love and understanding.

ABSTRACT

Organic chemicals which are used as solvent in most of the industries may have inhibitory effects on microorganisms. Since acetoclastic methanogens play an important role in stabilizing the pollution load of wastewater by participating in the terminal methanogenesis step, defining effects of solvents on both microbial community structure and acetoclastic pathway in anaerobic reactors can lead to improvements in understanding of interactions in the bioreactors, thereby obtaining better reactor performance in terms of higher degradation capacity and biogas production.

In this study, effects of solvents such as methanol (0.1-1.0 M), toluene (0.5- 4.0 mM), iso-propanol (0.1-2.0 M) and toluene + methanol (0.5 mM + 1.0 M, 1.5 mM + 1.0 M) on methane production, expression level of acetly-CoA synthetase gene and viable microbial populations were studied in sludges taken from batch reactors. Within the scope of this study, it was found that biogas productions of the reactors decreased with increasing concentrations of the solvents. The most severe effects of solvents were observed for the iso-propanol and toluene + methanol added reactors, respectively. It was also found that the expression of acetyl-CoA synthetase genes of *Methanosaeta* remained quite unchanged after exposures to methanol and methanol + toluene added reactors whereas no expression was detected for toluene and iso-propanol added reactors.

The percentage of active microbial populations generally increased by the increasing concentrations of solvents. *Methanosaeta* usually dominated in the reactors. The dominance of *Methanosaeta* was followed by *Methanobacteriales* which was usually resistant to higher concentrations and observed to be dominating genus in toluene added reactor.

ÖZET

Endüstriyel atık sularda bulunabilen, sentetik olarak üretilen ve varolan enzim sistemleri tarafından tanınmayan solventler gibi organik maddeler çevre sorunlarına yol açarlar. Organik çözücüler ilaç, petrol, boya vb. endüstrilerin atıksularında yoğun olarak bulunurlar ve biyolojik arıtma sistemlerine inhibitör etki yapabilirler. Asetoklastik metanojenler anaerobik arıtımın son basamağı olan metanojenesise katılarak atık su arıtımında önemli bir rol oynadıklarından, solventlerin mikrobiyal topluluğun yapısına ve asetoklastik metabolik yola etkilerinin tanımlanması ile solvent gideren anaerobik biyoreaktörlerdeki mikrobiyal etkileşimlerin anlaşılması ve bu reaktörlerden daha iyi performans ve biyogaz elde edilmesi mümkün olacaktır.

Bu çalışmada, laboratuvar ölçekli kesikli reaktörlerden alınan anaerobik çamurda metanol (0.1- 1.5 M), toluen (0.5 - 4.0 mM), iso-propanol (0.1- 2.0 M) ve toluen + metanol (0.5 mM + 1.0 M, 1.5 mM + 1.0 M)'in metan üretimine, asetil-KoA sentetaz geninin ekspresyon seviyesine ve mikrobiyal kommüniteye inhibitör etkisi incelenmiştir. Reaktörlerde artan konsantrasyonların biogaz üretimlerinde düşüşe sebep olduğu görülmüştür. En yüksek oranda inhibisyon etkisi sırasıyla iso-propanol ve toluen + metanol ile beslenmiş reaktörlerde görülmüştür. *Methanosaeta*'nın asetil-KoA sentetaz geni ekspresyon seviyesi metanol ve metanol + toluen eklenmiş çamurlarda aynı kalırken, toluen ve iso-propanol eklenmiş çamurlarda gen ekspresyonu tespit edilememiştir.

Artan konsantrasyonlar aktif mikrobiyal populasyon yüzdesinde ise artışlara sebep olmuştur. Reaktörlerde genellikle baskın türün *Methanosaeta* olduğu tespit edilmiştir. *Methanosaeta* populasyonunu yüksek konsantrasyonlu solventlere dayanıklı olduğu görülen ve toluen eklenen reaktörde en baskın tür olduğu gözlenen *Methanobacteriales* izlemiştir.

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Symbol	Explanation	Units used
AK	Acetate Kinase	
AMP	Adenosine Monophosphate	
ATP	Adenosine Triphosphate	
DNA	Deoxyribonucleic acid	
DGGE	Denaturating Gradient Gel Electrophoresis	
EDTA	Ethylenediaminetetraacetic Acid	
EGSB	Expanded Granular Sludge Bed	
FISH	Fluorescent *in situ* Hybridization	
HRT	Hydraulic Retention Time	hour
LC_{50}	Lethal Concentration	mgL^{-1}
NRB	Nitrogen Reducing Bacteria	
PCR	Polymerase Chain Reaction	
PBS	Phosphate Buffer Solution	
PFA	Paraformaldehyde	
PTA	Phosphotransacetylase	
Q-PCR	Quantative Polymerase Chain Reaction	
RFLP	Restriction Fragment Length Polymorphism	
RNA	Riboxynucleic Acid	
rRNA	Ribosomal RNA	
rDNA	Ribosomal DNA	
SMA	Specific Methanogenic Activity	$mLCH_4/gVSS.d$
SRB	Sulphate Reducing Bacteria	
SSCP	Single Strand Conformation Polymorphism	
TS	Total Solids	mgL^{-1}
TVS	Total Volatile Solids	mgL^{-1}
UASB	Upflow Anaerobic Sludge Blanket	
VFA	Volatile Fatty Acids	
UAF	Upflow Anaerobic Filter	
CSTR	Continious Stirred Tank Reactor	

1. INTRODUCTION

Anaerobic processes have been widely used over the past decades for the treatment of especially high-strength industrial wastewaters at mesophilic temperatures due to several advantages of the processes over aerobic ones. In recent years, the processes have also been reported to be an option for treatment of complex wastewaters such as chemical synthesis-based pharmaceuticals (Terzis, 1994; Henry et al., 1996; Mohan et al., 2001; Enright et al., 2005). Anaerobic process has many benefits, including reducing the quantity of solids to be land applied or landfilled, decreasing the pathogen content and odor of the sludge and producing methane gas which can be used as an alternative energy source. Although the general processes occuring in anaerobic biological wastewater treatment plants, such as hydrolysis, acidogenesis, acetogenesis, methanogenesis, are well understood, the complex microbial ecology of the biological sludge, symbiotic relationships, the effect of microbial diversity on performance of anaerobic digestion systems were poorly understood.

The wastewaters can present difficulties for biological treatment due to heterogeneous wastewater composition containing refractory and inhibitory organic materials (Myabhate et al., 1988; Oz et al., 2003; Enright et al., 2005). A wide range of inhibitors are responsible for the upset of anaerobic reactor systems. Organic solvents such as methanol, toluene and iso-propanol are extensively used to dissolve compounds required for certain processes in industries. In recent years, discharges of the compounds have been subjected to stringent environmental regulations because of their undesirable effect on living organisms in aquatic environments. Increased application of anaerobic digestion to a broader range of wastewaters including organic solvents would provide significant environmental and economic benefits for the industries (Ince et al., 2002; Oz et al., 2003; 2004). Organic solvent containing inhibitory wastewaters may affect activity and composition of methanogens, since the most sensitive step to inhibitory substances through anaerobic digestion process is the methanogenesis (Speece and Parkin, 1983).

Recent developments with the integration of microbial ecology and molecular biology are rapidly evolving and provide a new insight into the interrelations between

microorganisms and their environment in bioreactors (Amann et al., 1990; Hugenholtz et al., 1998; McHugh et al., 2004; Roest, 2007). More recently, the microbial ecology of anaerobic reactor systems has been investigated in detail using several molecular techniques such as FISH, DGGE etc. (Delbes et al., 2001; Collins et al., 2003; Gerardi, 2003). Despite the gained experience on the matters in recent years, much more study should be carried out to define microbial community interactions inside the reactors treating specific pollutants such as solvent-containing wastewaters inside the bioreactors. In this manner, investigations should also include changes in quantification of different group of microorganisms under specific compounds in anaerobic systems. Only a limited number of studies cover the microbial ecology of anaerobic reactors solvent-containing wastewaters (Enright et al., 2005; 2007a; 2007b). Also none of these studies reveal inhibition effects of the compounds on metabolic pathway of anaerobic microorganisms (Terzis, 1994; Henry et al., 1996).

Defining effect of organic solvents on both microbial community structure, activity changes and understanding which metabolic step is highly affected can lead to improvements in the understanding of bioreactors treating wastewaters containing organic solvents, thereby obtaining better reactor performance in terms of higher degradation capacity with higher biogas production. Therefore, in this study, the inhibitory effect of methanol, toluene, iso-propanol and methanol + toluene, which are organic solvents and main pollutants in some specific wastewaters, were evaluated in terms of their effects on expression level of acetly-CoA synthetase gene, methane production and microbial population dynamics.

2. THEORETICAL BACKGROUND

2.1. Fundamentals of Anaerobic Degredation

Anaerobic wastewater treatment is considered the most cost-effective solution for organically polluted industrial waste streams (Van Lier et al., 2001) and has gained interest due to increasing energy prices and more stringent legislation for the discharge of industrial wastewater since 1970's (Lettinga et al., 1995). Anaerobic wastewater treatment systems can operate at different temperatures and convert a broad variety of wastes, such as food and beverage, pharmaceutical, pulp and paper, petrochemical (Macarie, 2000), alcohol distilleries, dairy, textile and leachates. Anaerobic digestion is also used for municipal wastewaters, solid wastes, agricultural wastes and manures. Anaerobic treatment processes are known for the unique ability to convert highly objectionable wastes into useful products (McCarty, 2001).

The process of anaerobic digestion results in lower energy release compared to other terminal electron accepting processes and therefore lower sludge yields. This feature of anaerobic digestion is a significant advantage, since sludge management is an expensive component of biological treatment systems. Also low energy and sludge release imply that most of the energy in the original substrates is stored in the biological fuel, energy rich biogas. These features reduce operation costs of this process significantly and makes it a net energy producer (Lettinga, 1995). Although large reactor volumes and long retention times are needed in order to achieve high treatment efficieny in the system (McCarty, 1971) with the recent developments in our knowledge on anaerobic digestion and the quality of the equipments used in the system, much cost-effective reactor configurations and operations are being achieved.

2.1.1. Biochemistry and Microbiology of Anaerobic Digestion

The biochemistry and microbiology of anaerobic digestion is a complex biogenic process which includes a number of microbial populations, linked by their individual substrate and product specifities (Hutnan et al., 1999). In the first two phases of anaerobic digestion, organic pollutants are hydrolyzed and/or fermented into intermediate short-chain

fatty acids (e.g., lactate, butyrate and propionate). Then they are degraded to acetate and H_2/CO_2. In the last phase, acetate and H_2/CO_2 are converted into methane (Liu et al., 2001) (Figure 2.1).

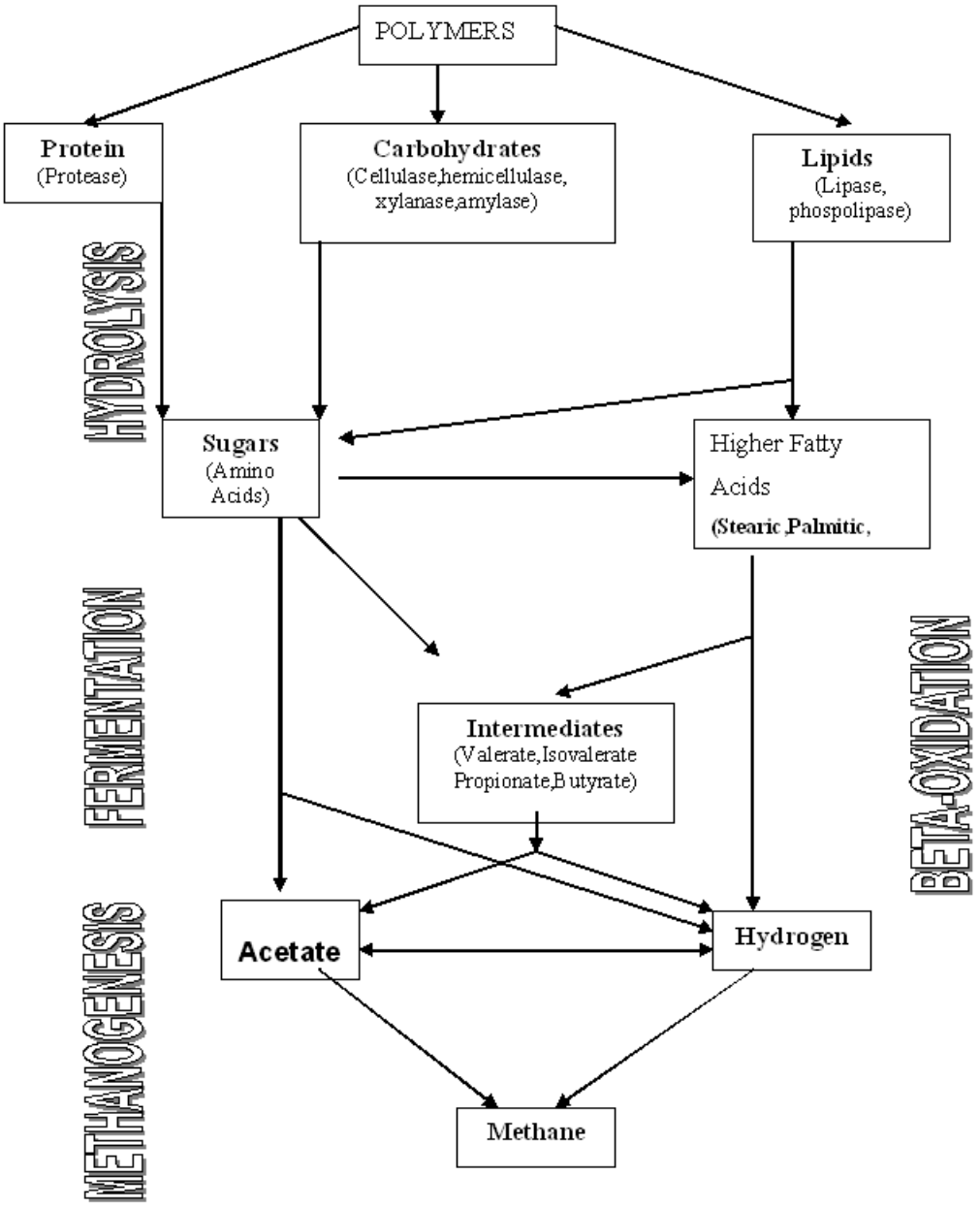

Figure 2.1. The breakdown of organic polymers (Stronach et al., 1986).

Several models have been developed to explain the biochemical steps in anaerobic digestion such as Three-stage Model (Gerardi, 2003), Six-stage Model (Lester et al., 1986)

and Nine-stage Model (Harper and Pohland, 1986).

Anaerobic degradation process was reported by some authors as a Nine-stage Model (Harper and Pohland, 1986) which have been listed as follows and shown diagrammatically in Figure 2.3.

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.

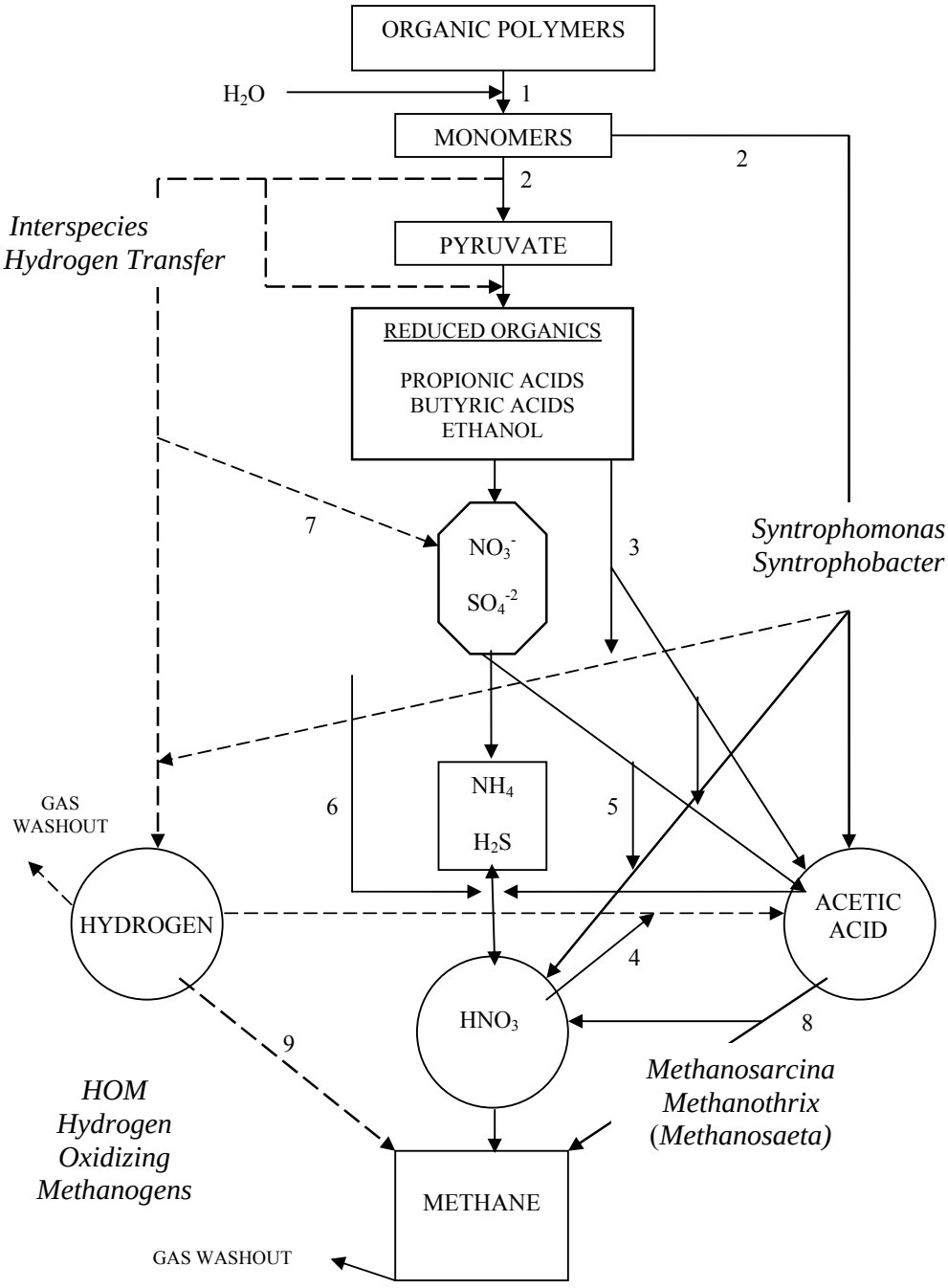

Figure 2.2. Substrate conversion patterns associated with the anaerobic digestion (Harper and Pohland, 1986).

In anaerobic digestion process there are numerous interactions between four major metabolic groups that are generally accepted as present in anaerobic digesters; hydrolytic-fermentative bacteria, proton-reducing acetogenic bacteria, hydrogenotophic methanogens, and acetolastic methanogens (Zinder et al., 1984). These microorganisms have a distinctive

biochemistry which enables them to gain metabolic energy from the methanogenic pathway (Whitman et al., 1982). Most of the described species of methanogens have different metabolisms than each other. *Methanobrevibacter spp.* is only able to use H_2 + CO_2 for growth, whereas *Methanosaeta spp*. only uses acetate as their energy substrate. *Methanosarcina sp*p. are more versatile; they can use H_2+CO_2, acetate, methanol, methylated amines and pyruvate for growth and methane production (Whitman et al., 1982; Jetten et al., 1992). Limited range of substrates are utilised by methanogens so the anaerobic breakdown of organic matter is carried out by communities of different physiological types of anaerobic bacteria (Stams, 1994; Schink, 1997). Figure 2.3. illustrates the different phases of the anaerobic digestion process.

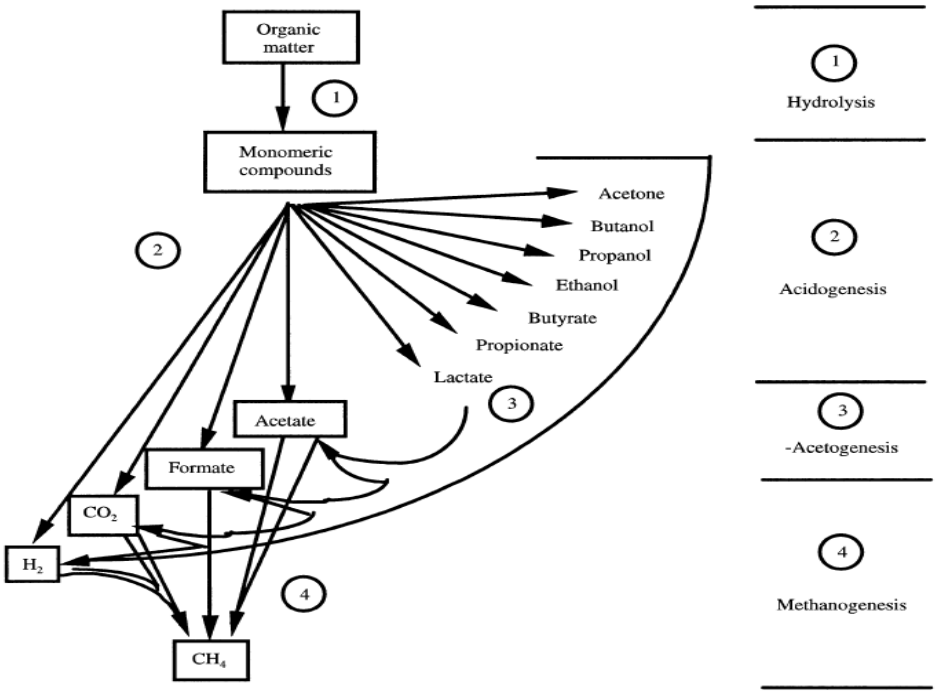

Figure 2.3. Schematic diagram showing anaerobic degradation of organic matter (Garcia et al., 2000).

2.1.1.1. Hydrolysis

Complex wastes are required to be hydrolyzed into units as a first step to be taken up by the microbial cells. The hydrolysis of macromolecules such as lipids, proteins and

carbohydrates under anaerobic conditions is carried out by specific extracellular enzymes, the reaction rates of which are influenced by pH, cell residence time and the waste constituents in the digester produced by hydrolytic bacteria.

In an anaerobic digestion process where a substantial portion of the waste stream contains complex organic compounds, the hydrolytic bacteria and their enzymes are of paramount importance since their activity produces the simpler substrates for the succeeding steps in the degradation sequence (Stronach et al., 1986). In the anaerobic digestion process, the hydrolytic activity relevant to each polymer is of paramount significance, since their activity produces simpler substrates for the succeeding steps in the degradation sequence (Stronach et al., 1986). It was stated that *Clostridium* is responsible for degradation of compounds containing cellulose and starch while *Bacillus* play role in the degradation of proteins and fats (Noike et al., 1985; Lema et al., 1991). The types of hydrolytic microorganisms are reported namely as, the cellulytic (*Clostridium thermocellum*), proteoytic (*Clostridium bifermentas, Peptococcus*), lipolytic (genera of clostridia and micrococci) and aminolytic (*Clostridium butyricum*, *Bacillus subtilis*) bacteria (Hungate, 1982; Payton and Haddock, 1986). The hydrolytic microorganisms are also capable of breaking down some intermediate products to simple volatile fatty acids (VFAs), carbon dioxide, hydrogen and ethanol (Eastman and Ferguson, 1981).

2.1.1.2. Acidogenesis

Amino acids, sugars and long chain fatty acids of the hydrolysis phase are converted to acetate, carbon dioxide and hydrogen by acid forming bacteria. It was reported that acetate is the most vital compound produced in the fermentation of organic substrates with propionate production of secondary consequence (Sorensen et al., 1981).

Two groups of acid forming bacteria are known. The first group is acidogens or fermentative bacteria which are capable of metabolizing amino acids and sugars to the intermediary products, acetate and hydrogen. The catabolism of these organic compounds is carried out by a large number of both obligatory and facultatively anaerobic microorganisms and the process utilizes single amino acids, pairs of amino acids or a single amino acid with a non-nitrogenous compound. Single amino acids are converted by

Clostridia, Mycoplasmas and *Streptococci* while butanol, butyric acid, acetone and iso-propanol are generally produced by the bacteria of the genera *Clostridum* and *Butyribacterium* under anaerobic conditions (i.e *Clostridium butyricum* produces butyrate, *Costridium acetobutylicum* mainly produces acetone and butanol and *Clostridium butylicum* produces butanol in addition to hydrogen, carbondioxide and iso-propanol).

2.1.1.3. Acetogenesis

The obligate hydrogen producing acetogenic bacteria is the second group of acid forming bacteria. They produce acetic acid, carbondioxide and hydrogen from propionate, butyrate and other higher fatty acids by the β-oxidation process. Fatty acids having more than two carbons lose one molecule at each reaction till all fatty acids are converted to acetate molecules. Acetic acid producing bacteria are *Methanobacterium bryantii*, *Desulfovibrio Syntrophobacter wolinii* (responsible for acetic acid production from propionic acid) (Stronach et al., 1986; Malina et al., 1992), *Syntrophomonas wofei* (responsible for acetic acid production from butyric, caproic and valeric acids) and *Syntrophus buswellii* (Gujer et al., 1983; Malina et al., 1992).

2.1.1.4. Methanogenesis

The final step of anaerobic degredation; Methanogenesis, is a common and important process in many natural and engineered anaerobic environments, such as, anaerobic digesters (Raskin et al., 1994), cattle rumen (Miller et al., 1986), rice fields (Joulian et al., 1998), oil wells (Ollivier et al., 1997), landfills (Fielding et al., 1988) and a range of extreme habitats (Garcia et al., 2000). It plays an important role in anaerobic treatment of organic wastes, formation of biogas as an alternative source of energy (Cicerone and Oremland, 1988). Methanogenesis is the rate-limiting step in the whole anaerobic digestion process due to the slow growth rate of the methanogens comparing with acidogens (Speece, 1983; Noike et al., 1985; Malina et al., 1992). Therefore, the performance of anaerobic reactors and the quality of the effluent depend on the activity of methanogens.

Methanogens which are group of strictly anaerobic *Archaea*, carry out the methane production in methanogenesis. Methanogens convert the end products of the fermentation into methane and carbon dioxide via two conversion mechanisms including decarboxylation of acetic acid and reduction of carbon dioxide in the absence of other electron acceptors such as oxygen, nitrate, and sulfate and only bicarbonate and protons act as terminal electron acceptors (Garcia et al., 2000; De Bok et al., 2004; Stams et al., 2006).

It has been reported that at least ten substrates can be converted to methane by pure cultures of methanogens. Three classes of compounds including CO_2-type substrates, methyl substrates and acetate are listed in Table 2.1.

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

I. CO_2-type substrates
Carbon dioxide (with electrons derived from H_2, certain alcohols, or pyruvate)
Formate
Carbon monoxide
II. Methyl substrates
Methanol
Methylamine
Dimethylamine
Trimethylamine
Methylmercaptan
Dimethylsulfide
III. Acetotrophic substrate
Acetate

CO_2-type substrates including CO_2, formate and carbon monoxide are reduced to methane by bacteria. Although the reduction of carbondioxide to methane is generally hydrogen dependent, other substrates in this class can provide the electrons for CO_2 reduction.

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

Methyl group substances which are listed above as the second class of methanogenic substrates are converted to methane by two mechanisms. The formation of methane by reducing methyl group substances using an external electron donor such as H_2 is the first mechanism. In the conversion equations methanol (CH_3OH) is used as a model methyl substrate.

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

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

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

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

$CH_3COO^-+H_2O \rightarrow CH_4+HCO_3^-$ $\Delta G°= -31$ kJ (2.4)

Each of the above reactions is exergonic and can be used to synthesize ATP. Concerning carbon for cellular biosynthesis, CO_2 is the precursor for all cellular components when growing on CO_2 + H_2. If methanogenic substrates are acetate or methylated compounds, these compounds are also used in the organic cell components with the fixation of some CO_2.

Characteristics and Taxonomy of Methanogens

Methanogens are microorganisms that produce methane as the end-product of their anaerobic respiration. All methanogens are strictly anaerobic *Archaea* belonging to

Euryarchaeota. They are a large and diverse group, all of which are obligate methane producers that obtain most of their energy from methanogenesis. (Table 2.2.)

Methanogens have been cultivated from a wide variety of anaerobic environments. In addition to temperate habitats, they are also common in environments of extreme temperatures, salinity and pH. The common methanogenic habitats include marine sediments, freshwater sediments, flooded soils, human and animal gastrointestinal tracts, termites, anaerobic digestors, landfill, geothermal systems and heartwood of trees.

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

Order	**Morphology**	**Substrate for methanogenesis**
Methanobacteriales		
Methanobacterium	Long rods	H_2+CO_2, formate
Methanobrevibacter	Short rods	H_2+CO_2, formate
Methanosphaera	Cocci	Methanol+H_2
Methanothermus	Rods	H_2+CO_2, can also reduce S^0; hyperthermophile
Methanococcales		
Methanococcus	Irregular cocci	H_2+CO_2, 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 Methanol, methylamines
Methanococcoides	Irregular cocci	Methanol, methylamines, methyl sulfides; halophile
Methanohalophilus	Irregular cocci	Acetate
Methanosaeta	Long rods to filaments	
Methanopyrales		
Methanopyrus	Rods in chains	CO_2, hyperthermophile, growth at 110°C

Morphologically, the methanogens exhibit a wide variety of shapes and sizes, including rods, regular and irregular cocci, long-chained rods, spirilla, sarcina and irregular unusual flattened plates. Motility is sometimes present. Some species can aggregate in clusters. Several species of *Methanosarcina* and *Methanosaeta* contain gas vacuoles. The gram reaction can be positive or negative even within members of the same genus (Garcia et al., 2000).

Methanogens have unique cell membrane lipid and lack a rigid cell wall. They are capable of degrading substrates such as organic wastes and produce methane by their specialized coenzymes. Coenzymes that are unique to methane forming microorganisms are coenzyme M and the nickel containing coenzymes. Coenzyme M is used to reduce CO_2 to methane. The nickel-containing coenzymes are important hydrogen carriers in methanogens (Thauer and Shima, 2006).

Even though, methanogens are very diverse, they are only capable of utilizing a small number of substrates. The substrates are limited to three major types including CO_2, methyl-group containing compounds, and acetate. Most organic substances, i.e, carbohydrates and long-chain fatty acids and alcohols, are not appropriate substrates for methanogens (Table 2.3.). These compounds must first be processed by anaerobic bacteria or eukaryotes to produce the substrates used by methanogens. Thus, in most methanogenic environments, most of the energy available for growth is utilized by these nonmethanogenic organisms (Thauer and Shima, 2006).

Table 2.3. Methanogenic orders (Karakashev et al., 2005).

Order	**Physiology**
Methanopyrales	Hydrogenotrophic; hyperthermophilic
Methanobacteriales	Hydrogenotrophic; mesophilic or thermophilic
Methanococcales	Hydrogenotrophic; mesophilic or thermophilic
Methanomicrobiales	Hydrogenotrophic; mesophilic
Methanosarcinales	Strict aceticlastic (*Methanosaetaceae*), aceticlastic or hydrogenotrophic (*Methanosarcinaceae*); mesophilic or thermophilic

Most methanogens are hydrogenotrophs that can reduce CO_2 to methane with H_2 as the primary electron donor. Many hydrogenotrophic methanogens are also able to use formate as the major electron donor. Besides, some hydrogenotrophic methanogens can also use secondary alcohols, such as 2-propanol, 2-butanol, and cyclopentanol, as electron donors. A small number of methanogens can also use ethanol.

Methyl-group containing compounds, including methanol, methylated amines (monomethylamine, dimethylamine, trimethylamine, and tetramethylammonium), and methylated sulfides (methanethiol and dimethylsulfide) are other types of substrates that are used by methanogenic *Archaeae*. Methanogens that are able to use methylated compounds, or methylotrophic methanogens, are limited to the order *Methanosarcinales*, except for *Methanosphaera* species, which belong to the order *Methanobacteriales* (Fricke et al, 2006).

The third type of substrate that is used by methanogens is acetate. Acetate is a major intermediate in the anaerobic food chain, and as much as 70% of the biologically generated methane is derived from acetate. Surprisingly, only two genera are known to use acetate for methanogenesis: *Methanosarcina* and *Methanosaeta*. They carry out an aceticlastic reaction that splits acetate, oxidizing the carboxyl-group to CO_2 and reducing the methyl group to CH_4. *Methanosarcina* is a relative generalist that prefers methanol and methylamine to acetate, and many species also utilize H_2. *Methanosaeta*, which is thought to use only acetate although recent studies revealed that *Methanosaeta* might be metabolicly more diverse than previusly thought (Smith and Smith, 2007), is a superior acetate utilizer. It can use acetate at concentrations as low as 5–20 μM, while *Methanosarcina* requires a minimum concentration of about 1.0 mM. The difference of acetate affinity is probably due to differences in the first step of acetate metabolism. *Methanosarcina* uses the low-affinity acetate kinase (AK)-phosphotransacetylase (PTA) system to activate acetate to acetyl-CoA, while *Methanosaeta* uses the high-affinity adenosine monophosphate (AMP) – forming acetyl-CoA synthetase. Moreover, based on their genome sequences, these two genera probably have different modes of electron transfer and energy conservation, even though the main steps in the methanogenesis pathway are likely to be similar (Liu and Whitman, 2008).

Table 2.4. Typical organisms of methanogenesis reactions (Zinder et al., 1990; Liu and Whitman, 2008).

Reaction	Organisms
I. CO_2-type	
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	Most methanogens
$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	Many hydrogenotrophic methanogens
CO_2 + 4 isopropanol$\rightarrow$CH4 + 4 acetone + $2\ H_2O$	Some hydrogenotrophic methanogens
$4\ CO + 2H_2O \rightarrow CH_4 + 3\ CO_2$	*Methanothermobacter* and *Methanosarcina*
II. Methylated C1 compounds	
$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	*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
$4\ CH_3NH_3Cl + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4Cl$	Some methylotrophic methanogens
III. Acetate	
$CH_3COOH \rightarrow CH_4 + CO_2$	*Methanosarcina* and *Methanosaeta*

Taxonomy of Methanogens

Woese et al. (1990) proposed a new classification for living organisms, dividing life on earth into three major domains: bacteria, *Archaea* and eukarya (Figure 2.4.).

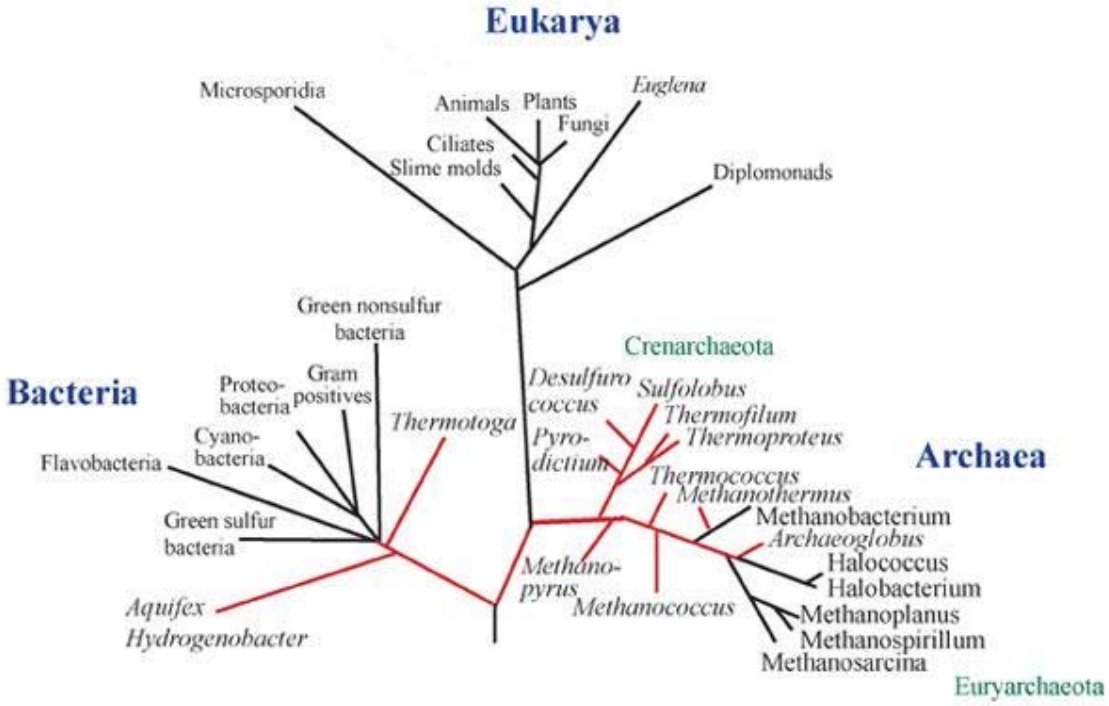

Figure 2.4. Universal phylogenetic tree (Woese et al., 1990).

The unique phylogenetic status and evolutinary divergence of *Archaea* suggest that they should exhibit wide physiological diversity. However, traditional culture-based studies have led to belief that opposite was the case. Two major lineages of *Archaea* are Crenarchaeota and Euryarchaeota (Figure 2.5.). The first kingdom, Crenarchaeota derived from being phylogenetically close to ancestor or source of *Archaea* (Woese et al., 1990). It was believed to include only sulphur-dependent extreme thermophiles. Euryarchaeota is a heterogenous group comprimising a broad spectrum of organisms with varied patterns of metabolism from different habitats. It includes extreme halophiles, methanogens and some extreme thermophiles so far. Moreover, a third archaeal kingdom has been discovered which is reported isolation of several archaeal sequences evolutinary distant from all *Archaea* known to date by Barns et al. in 1994 and then in 1996. The new group was placed on phyologenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota.

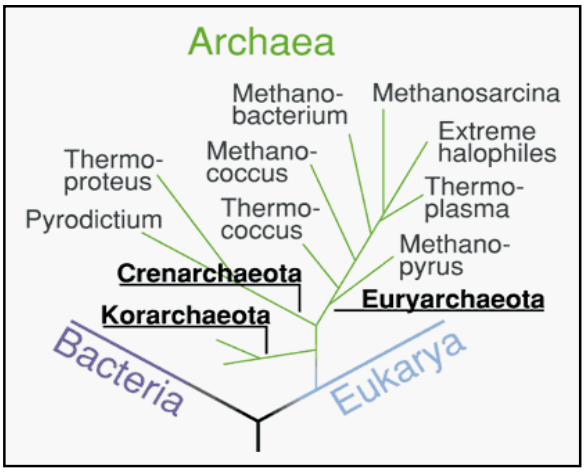

Figure 2.5. Major lineages of *Archaea*: Crenarchaeota, Euryarchaeota and Korarchaeota (Madigan et al., 2002).

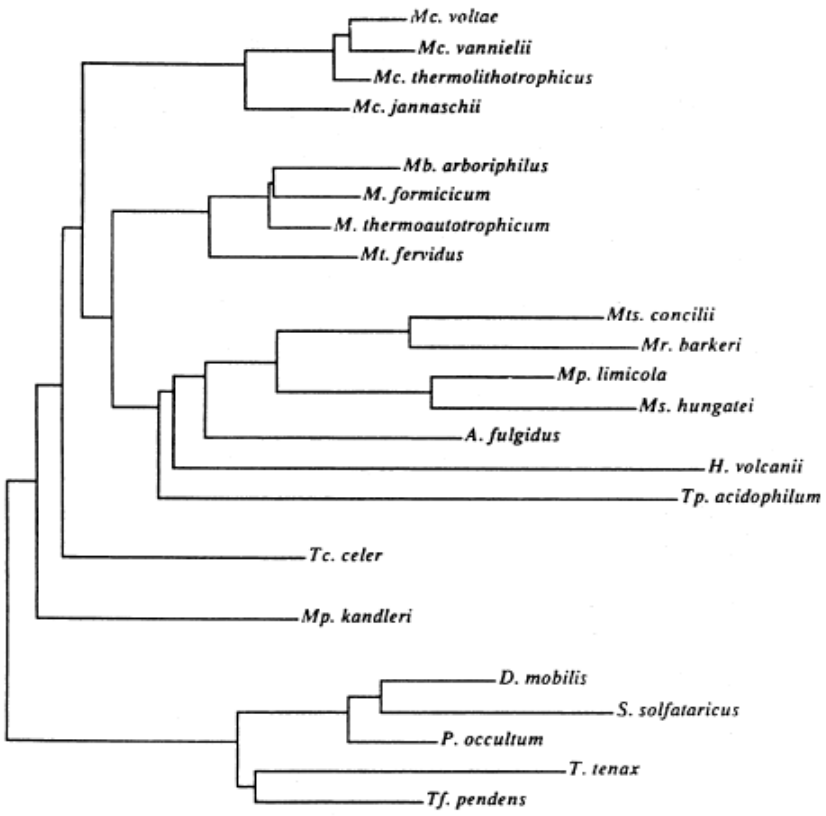

Figure 2.6. Representative phylogenetic tree for *Archaea*, based upon 16S rRNA sequences. The scale bar measures five nucleotides changes per 100 residues.

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

Methanogens are classified into five orders within the kingdom *Archaeobacteria*: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales,* and *Methanopyrales* (Figure 2.7.). Organisms from different orders have less than 82% 16S rRNA sequence similarity. Methanogens belonging to different orders also possess different cell envelope structure, lipid composition, substrate range, and other biological properties.

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

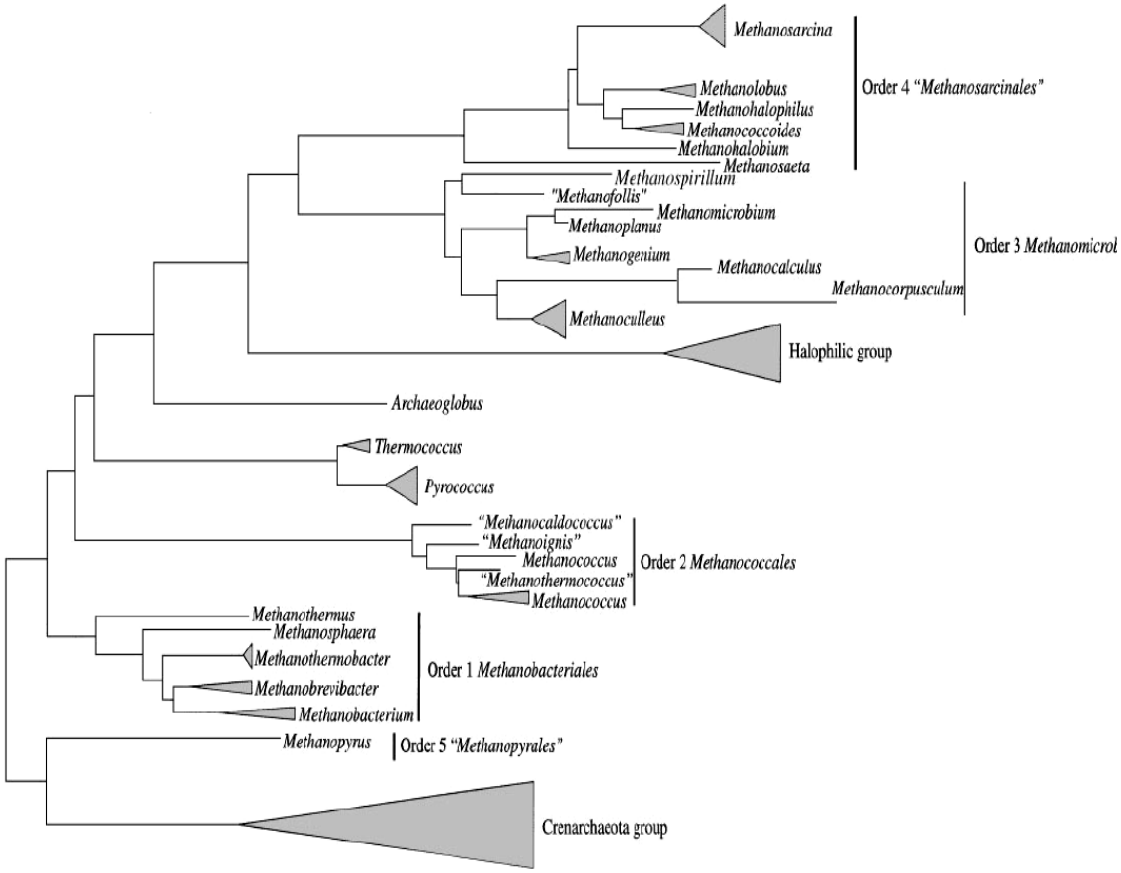

Figure 2.7. Updated phylogeny of methanogens, domain *Archaea* (Garcia et al., 2000).

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

Members of the order *Methanomicrobiales* are order of Methanogens that use CO_2 as the electron acceptor and H_2 as electron donor. Most species can use formate, and many species also use secondary alcohols as alternative electron donors. Their morphology is diverse, including cocci, rods, and sheathed rods. They are widely distributed in anaerobic habitats, including marine and freshwater sediments, anaerobic sewage digestors, and

animal gastrointestinal tracts. The order of *Methanomicrobiales* is divided into three families, *Methanomicrobiaceae, Methanospirillaceae* and *Methanocorpusculaceae*.

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

The order of *Methanopyrales* is represented by only one species, *Methanopyrus kandleri*. Cells reduce CO_2 with H_2 for methanogenesis. They are rod-shaped. *M. kandleri* is hyperthermophilic with a growth temperature range of 84–110^0C. It inhabits marine hydrothermal system.

Acetyl- CoA Pathway

To be able to catabolize acetate to methane, acetate must first be activated to acetyl coenzyme A (acetyl-CoA). *Methanosaeta*, contains high levels of acetyl-CoA synthetase which catalyzes the activation to acetyl-CoA. For this activation process, different pathways have been described in species of *Methanosarcina* and *Methanosaeta*, the two genera of the archaeal domain that are able to convert acetate to methane. In *Methanosaeta*, acetate is activated in one step by the enzyme acetyl-CoA synthetase (Acs) Equation 1. Then the acetyl-CoA is converted into CO_2 and CH_4 in a series of reactions, the first of which is catalyzed by carbon monoxide dehydrogenase (Cdh) (Eggen et al., 1991). The formation of acetyl-CoA (AcCoA) which is catalyzed by decarboxylase enzyme is a key reaction step which contributes the formation of methane. It could be calculated that up to 4% of the soluable cell protein of *Methanosaeta* is acetely-CoA synthetase from the increase in specific activity and from the 6% recovery in a acetyl–CoA synthetase isolation study (Jetten et al., 1989). Acetyl-CoA synthetase from *Methanosaeta* has been purified and characterized. This enzyme, with a native molecular mass of 148 kDa, is composed of

two identical subunits of 73 kDa. It is not sensitive to oxygen. The enzyme is capable of activating some other fatty acids, like propionate (Aceti and Ferry, 1988). Half maximal rates for the synthetase were obtained at 0.86 mM acetate consistent with the ability of *Methanosaeta* to outcompete *Methanosarcina* for low concentrations of acetate in the environment (Jetten et al., 1989). Utilization of reactions of the acetyl-CoA pathway during growth on acetate is shown in Figure 2.8.

acetate+ATP+ coenzyme A→ acetyl-CoA + AMP + PPi. (2.5)

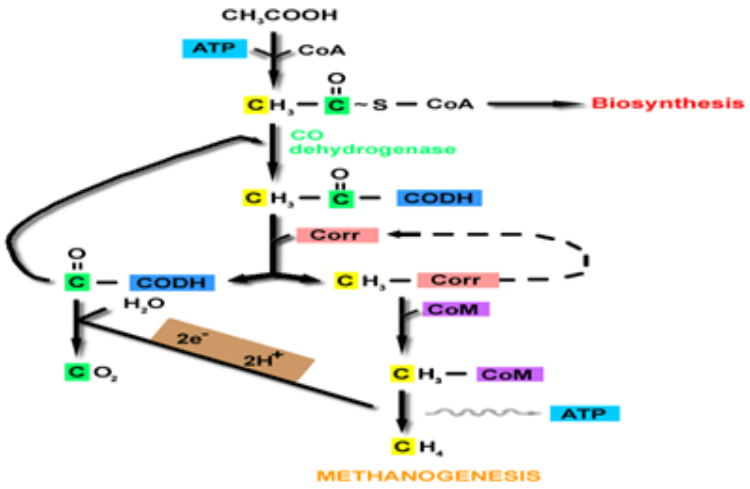

Figure 2.8. Acetyl-CoA pathway during growth on acetate (Madigan et al.,2002).

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2.2. Molecular Methods used in Microbial Ecology

Traditional enrichment techniques and the pure culture approach have offered only a narrow portal into the microbial world (Rochelle, 2001 as cited in Youngseob et al., 2005). Recent applications of molecular techniques to environmental samples have shown the remarkable possibility of studying microbial populations and metabolic pathways in the absence of culturing (Akarsubasi et al., 2005). Microbial diversity studies were limited in the past by the lack of methodological tools, but the availability of the new molecular methods, such as 16S rRNA gene cloning and sequencing (Urakawa et al., 2000), fluorescent *in situ* hybridization (FISH) (Amann, 1995; Amann et al., 1995), and denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; Muyzer and Smalla,

1998), has made it possible to investigate the dynamics of the composition and structure of microbial populations and communities in natural and engineered ecosystems, the phylogenetic relationships and the impact of environmental or specific factors such as pollution by xenobiotics on microbial diversity (Morris et al., 2002). Furthermore, molecular phylogeny which employs nucleic acid sequences to document the history of evolution, has provided a new basis for the direct identification and quantification of microorganisms (Olsen and Woese, 1993).

The classical method for identification of viable microorganisms in environmental samples is plate counting on agar medium (Edlund and Jansson, 2006). Only between 0,5% and 10% prokaryote diversity has been identified due to the small size and the absence of distinguishing phenotypic characters of prokaryotic organisms. Also most of these organisms cannot be cultured (Torsvik et al., 2002). Almost 99% of all microorganisms in nature can not be isolated and classified based on physiological and biochemical features mainly due to the previously mentioned limitations of cultivation (Muyzer, 1999). Therefore, studies based on cultivation methods could not reveal the appropriate microbial diversity. Such cultivation based approaches have restrictions and biases leading to a distorted representation of the true community composition (Amann et al., 1995).

However, nucleic-acid based methods are capable of characterizing microbial community without cultivation (Hofman-Bang et al., 2003). Techniques such as reassociation analysis of DNA (Torsvik et al., 1996), denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; Teske et al., 1996), and restriction fragment length polymorphism (Moyer et al., 1994) have yielded insight into bacterial diversity and community composition. Moreover, phylogenetically based oligonucleotide hybridization techniques allow monitoring of individual and quantification of phylogenetic groups their abundance in natural habitats (Amann et al., 1995). *In situ* hybridization with rRNA-targeted fluorescent oligonucleotide probes permits identification and quantification of individual cells and also characterizes bacterial community composition in several environments (Llobet-Brossa et al., 1998).

The analysis of rRNA gene was a revolution in microbial ecology and enlarged our knowledge of microbial phylogeny. This concept was developed 30 years ago (Woese and

Fox, 1977). Since by the pioneering work of Carl Woose, the rRNA has become the most commonly used molecule for phylogenetic analysis. rRNA or the corresponding rDNA are particularly suitable as evolutionary chronometers (Stahl et al., 1988). Using 16s rDNA or rRNA is currently the most common method for community analysis (Dahllöf, 2002). Studies based on the rRNA gene (rDNA) or the rRNA has become common to investigate community diversity. rDNA sequence data bank which is accessible via internet is rapidly growing and now makes it possible to compare sequences from across the world (Dahllöf, 2002).

Molecular methods for studying microbial diversity can be grouped as PCR based methods and non PCR based methods. All PCR based methods use PCR amplification products. Some of these methods are random sequencing in clone libraries, methods based on separation by electrophoresis like DGGE/TGGE, SSCP and T-RFLP. Flourescence *in situ* hybridization (FISH) is one of the most commonly used technique in non PCR based methods.

(PCR) is the first step for these methods. The PCR products can be analyzed by techniques such as DGGE (denaturation gradient gel electrophoresis), TGGE (temperature gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism), or SSCP (single stranded conformation polymorphism), which can separate the PCR products originating from different DNA sequences representing populations in the original samples. It is possible to clone the PCR products and subsequently sequence them to allow identification of population (Hofman-Bang et al., 2003). However, PCR method has biases, especially in the amplification step. Therefore, all techniques that are based on PCR (cloning, pattern analysis and sequencing) will be affected by the biases introduced by PCR (Dahllöf, 2002).

Table 2.5. Overview of molecular approaches used in microbial ecology (Roest, 2007).

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

Real Time PCR (Q-PCR)

Real-time- or quantitative PCR is based on the continuous monitoring of changes of fluorescence in the PCR tube during PCR. In contrast to the conventional endpoint detection PCR, quantification occurs during the exponential phase of amplification (Malinen et al., 2003). Thus, the bias often observed in the PCR template-to-product ratios can be largely avoided (Suzuki and Giovannoni, 1996). This is most commonly achieved through the use of fluorescence-based technologies, including: (i) probe sequences that fluoresce upon hydrolysis (TaqMan; Applied Biosystems, Foster City, CA, USA) or hybridization (LightCycler; Roche, Indianapolis, IN, USA); (ii) fluorescent hairpins; or (iii) intercalating dyes (SYBR Green). 1) Q-PCR Analysis can be done to observe absolute levels (i.e., numbers of copies of a specific RNA per sample) or relative levels (i.e., sample 1 has twice as much mRNA of a specific gene as sample. 2) For absolute

quantification, a RNA standard curve of the gene of interest is prepared in order to calculate the number of copies. In this case, a serial dilution of a known amount (number of copies) of pure RNA is made and subjected to amplification. The unknown signal is compared with the standard curves so as to calculate the starting concentration. Alternatively, a computation method for absolute quantitation has been proposed that does not use standard curves (Jia, 2009).

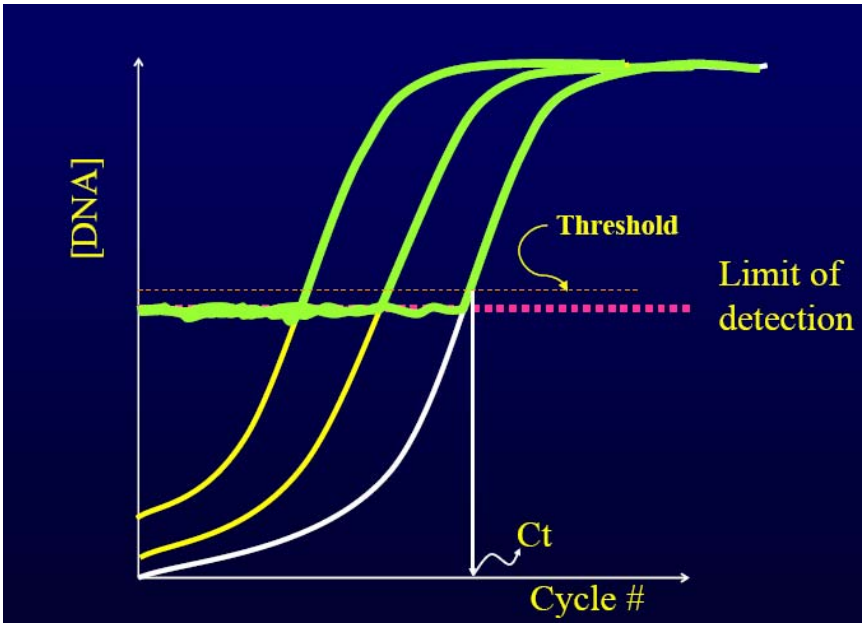


Figure 2.9. Monitoring of changes of fluorescence in Q-PCR reaction (Jia, 2009).

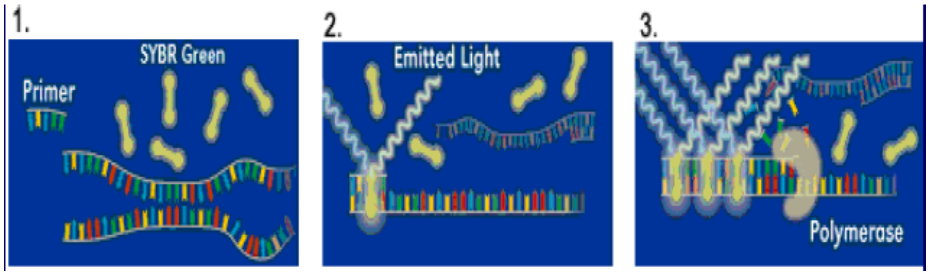


Figure 2.10. SYBR Green (Jia, 2009).

The real-time machine not only monitors DNA synthesis during the PCR, it also determines the melting point of the product at the end of the PCR. The melting temperature of a DNA double helix depends on its base composition (and its length if it is very short). All PCR products for a particular primer pair should have the same melting temperature -

unless there is contamination, mispriming primer-dimer artifacts, or some other problem. Since SYBR green does not distinguish between one DNA and another, an important means of quality control is to check all samples have a similar melting peak. After real time PCR amplification, the machine can be programmed to do a melt curve, in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence will rapidly decrease. The software plots the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis, and this will peak at the melting temperature (Tm). If the peaks are not similar, this might suggest contamination, mispriming[1], primer-dimer artefact[2] etc.

[1] Mispriming: cDNAs made due to annealing of the primers to complementary, or partially complementary sequences on non-target DNAs.

[2] Primer-dimer artefacts: the primers can sometimes anneal to themselves and create small templates for PCR amplification. These are the so-called primer-dimer artifacts (Jia, 2009).

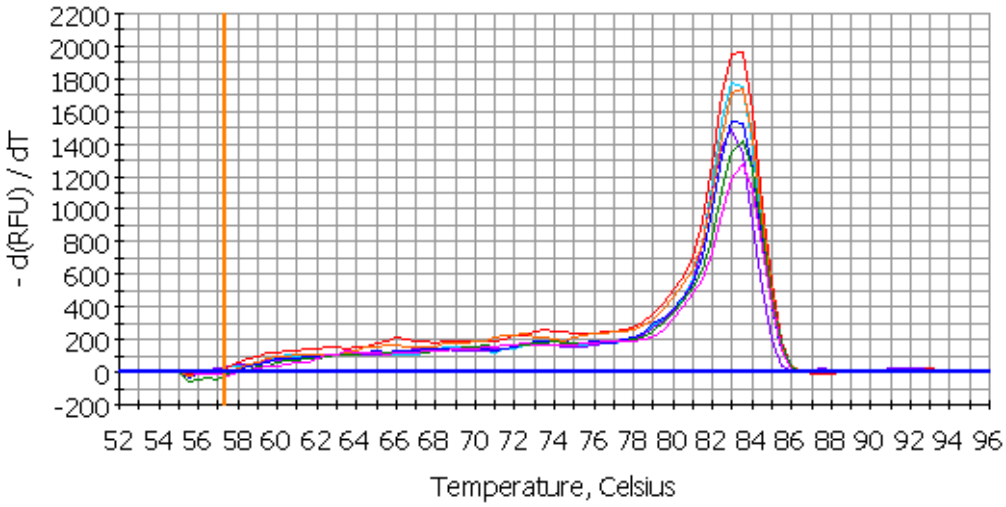


Figure 2.11. Melting point grafics in Q-PCR (Jia, 2009).

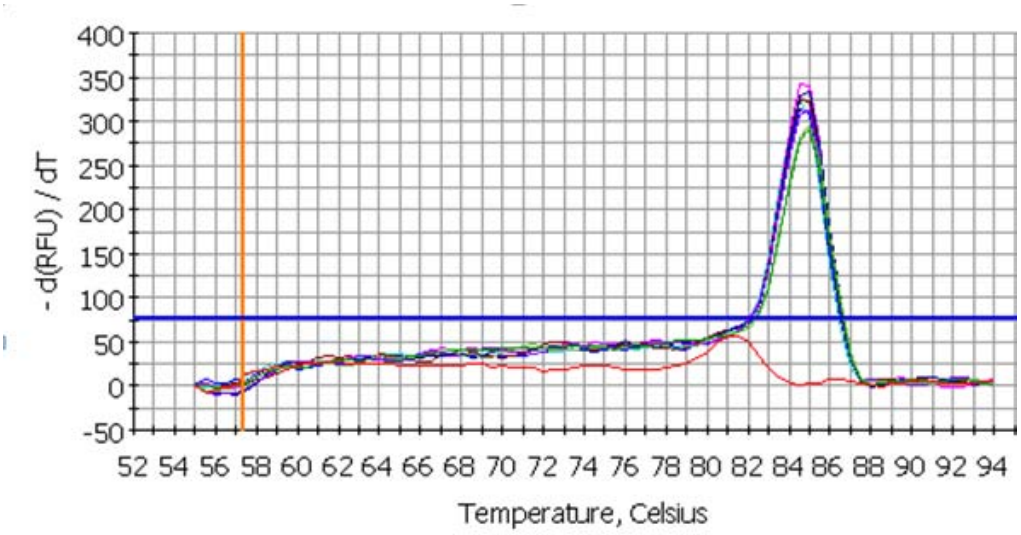


Figure 2.12. Primer dimer in Q-PCR (Jia, 2009).

Pattern Analysis and Denaturing Gradient Gel Electrophoresis (DGGE)

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

DGGE is nowadays a routine technique that is used to assess the diversity of microbial communities, to monitor their dynamics (Muyzer and Smalla, 1998; Muyzer, 1999) and to screen clone libraries. This method can be used to obtain qualitative and semi-quantitative estimations of biodiversity. Also, the DGGE pattern obtained provides a rapid identification of the predominant species in the microbial community.

DGGE is a gel electrophoresis method that separates genes/ DNA fragments of the same size (obtained after PCR of DNA extracted from an environmental sample) that differ in base sequence, at least by one nucleotide into distinct bands on a chemical denaturing gradient polyacrylamide gel.

Fluorescence in situ hybridization (FISH)

FISH is based on the microscopic analysis of already defined (at least its SSU rRNA gene sequence) groups of bacteria by a fluorogenic oligonucleotide (or probe) targeting SSU rRNA molecules inside cells (Giovannoni et al., 1988; Amann et al., 1990). Microbial cells are first fixed with appropriate chemical fixatives and then hybridised under appropriate conditions on a glass slide or in solution with oligonucleotide probes. These probes are generally 15–25 nucleotides in length and are labelled covalently at the 5'end with a fluorescent dye. After washing steps, specifically stained cells are detected by epifluorescence microscopy or flowcytometry. The determination of composition and number of bacteria can be achieved by rRNA-targeted oligonucleotide probes without cultivation, directly in their natural environment. rRNA gene fragments were used as phylogenetic stains firstly in 1989 (De Long et al., 1989). Since the pioneering study of De Long, fluorescence *in situ* hybridization technique has become a common tool for identification of microorganisms in environmental samples (Amann et al., 2001). Several hundred rRNA-targeted oligonucleotide probes suitable for FISH have been described, together with a large online database providing an encompassing overview of over 700 published probes and their characteristics (Loy et al., 2003). Such probes can be readily developed and tested to detect lineages of uncultured microbes in environmental samples (Pernthaler et al., 1997; Ravenschlag et al., 2001). The signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. This allows a quantification of rRNA concentrations both in single cells and in the environment (Poulsen et al., 1993; Pernthaler et al., 2001). Raskin et al. (1994a) evaluated the methanogenic group composition in anaerobic digesters by oligonucleotide probe hybridization. Several studies (Merkel et al., 1999; Sekiguchi et al., 1999; Araujo et al., 2000; Imachi et al., 2000; Tagawa et al., 2000; Upton et al., 2000; Wu et al., 2001) include FISH results using these same oligonucleotides but experimental conditions are variable. These probes are still reasonably accurate to target most of the defined phylogenetic groups of methanogenic *Archaea.*

In addition, the microbial community dynamics could be analyzed by FISH (Fernandez et al., 1999). By chancing the environmental factors, dominant members of the community could be monitored by FISH. Harmsen et al. (1996) applied FISH to identify

syntrophic propionate-oxidizing bacteria, and this study revealed the distribution of bacteria and methanogens in anaerobic granular sludge systems resolved the phylogenetic affiliation and localization of important microbial populations in a full-scale UASB reactor treating brewery wastewater.

The main advantage of FISH that it does not need any DNA or RNA amplification and allows microscopic inspection of intact cells in the samples. The other important advantages of FISH technique are listed as follows:

- it is an easy and fast technique;
- it allows direct visualization of organisms without cultivation;
- it is generally quantative;
- it also allows quantification of specific microbial groups, in contrast to traditional methods and other molecular methods;
- it is possible to detect active microorganisms in the sample (Sanz and Kohling, 2006).

Despite the advantages above, FISH technique has its limitations and disadvantages like any other technique. One of the most important limitations of FISH is that not all bacterial and archaeal cells can be permeabilisied by oligonucleotide probes using standart fixation protocols (Amann et al., 1995). Besides, the use of rRNA targeted oligonucleotide probes, which are covalently mono-labelled with fluorescent dye molecules, limits the sensitivity of the method and aggravates the use of FISH for identification of prokaryotes with low ribosome content per cell. The other disadvantages of FISH are as follows:

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

- quantification of microorganisms can be tedious and subjective (manual counting) or complex (image analysis).

2.3. Environmental Factors Affecting Anaerobic Treatment Processes

Temperature

Temperature is an important parameter for microbial systems. It effects the system in several ways including ionization equilibrium, solubility of substrates, substrate removal rate and other constants such as specific growth rate, decay biomass yield, and half saturation constant. Anaerobic processes are proven to be strongly affected by the temperature variations. Especially methane conversion of acetate to CH_4 is known as more sensitive to temperature than the acetate forming process (Stover et al., 1994). Methane production has been documented under a wide range of temperatures. In municipal wastewater treatment plants, anaerobic digestion is carried out in the mesophilic range at temperatures from 25°C to up to 40°C with the optimum at approximately 35°C (95°F). Thermophilic digestion operates at temperature ranges of 50–65°C. It allows higher loading rates and is also conductive to greater destruction of pathogens. One drawback of thermophilic digestion is its higher sensitivity to toxicants. Because of their slower growth as compared with acidogenic bacteria, methanogenic bacteria are very sensitive to small changes in temperature, which leads to a decrease of the maximum specific growth rate while the half-saturation constant increases (Noike et al., 1985; Speece, 1983).

Retention Time

The hydraulic retention time (HRT) depends on wastewater characteristics and environmental conditions. It must be long enough to permit sustaining anaerobic bacteria in digesters. The retention times of mesophilic and thermophilic digesters range between 25 - 35 days, since anaerobic treatment based on attached growth have a lower HRT (1-10 days).

pH

pH is also a significant parameter that affects the solubility of substances and the reaction behavior of microorganisms. As a consequence it influences performance of anaerobic digestion. Most methanogenic bacteria function in a pH range between 6.1 and 7.5. Optimum pH values for some methanogens are given in Table 2.6. Deviations from this optimum may result in excess production and accumulation of acidic or basic conversion products such as organic fatty acids or ammonia respectively. It has been shown that pH below 6.0 are inhibitory to methanogenic bacteria while acid forming bacteria can live at this pH and keep producing volatile fatty acids despite low pH, therefore making the environmental conditions worser (Pohland and Suidan, 1987). Acidogenic bacteria produce organic acids, which tend to lower the pH of the bioreactor (Malina and Pohland, 1992). Under normal conditions, this pH reduction by the acidogenic bacteria is buffered by the bicarbonate which is produced by methanogens. Under adverse environmental conditions, the buffering capacity of the system can be upset, eventually stopping the production of methane. Acidity is inhibitory to methanogens than of acidogenic bacteria. An increase in volatile acid level thus serves as an early indicator of system upset.

Table 2.6. Optimum pH for some methanogenic *Archaea* (Gerardi, 2003).

Methanogenic *Archaea*	**Optimum pH Range**
Methanosphaera	6.8
Methanothermus	6.5
Methanomicrobiales	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

Mixing

Mixing allows the complete contact between the reactor contents and the biomass. It also reduces the inhibitory effects of local build-up of VFAs and other digestion products. Moreover, mixing prevents settling which could cause reduction of substrate and microorganism contact.

Nutrients

Trace amounts of elements called micronutrients besides nitrogen and phosphorus are required for methanogen's fundamental bacterial metabolism (Speece and Parkin, 1983). Iron, nickel, magnesium, calcium, sodium, barium, tungstate, molybdate, selenium and cobalt are considered as necessary for various conditions of active methanogenesis (Henze and Harremoes, 1983). Some of the elements such as selenium, tungsten and nickel are significant in the enzyme systems of acetogenic and methanogenic bacteria (Stronach, 1986).

Inhibitors

Inhibitory substances are usually the main cause of anaerobic reactor failures since they are found in substantial consantrations in wastewaters and sludges (Chen et al., 2007). A wide range of inhibitors cause the occasional failure of anaerobic digesters. A substance may be called inhibitory when it causes an adverse shift in the microbial community or inhibition of bacterial growth. A decrease of the steady-state rate of methane gas production and accumulation of organic acids usually point out the inhibition (Kroeker et al., 1979).

The inhibition levels reported for most substances on anaerobic digestion vary a lot in the literature. These variations are caused by the complexity of the anaerobic digestion process where mechanisms such as antagonism, synergism, acclimation and complexing may affect the phenomenon of inhibition (Chen et al., 2007).

Even though, all groups involved in process can be affected, methanogenesis is generally the most sensitive step to inhibitory or toxic material. Bacteria are affected by increasing undesirable environmental conditions. However, methanogens can be acclimatized to these compounds (Speece and Parkin, 1983).

Ammonia Inhibition

Although ammonia is an important buffer in an anaerobic treatment, high concentrations of ammonia may cause failure in the system. Ammonia can be present in the form of ammonium ion (NH_4^+) or dissolved ammonium gas (NH_3). Although these forms are in equilibrium with each other at constant pH, at high pH levels the equilibrium shifts the ammonia gas. Ammonia nitrogen concentrations up to 1000 mg/l have no adverse effect on methanogens, whereas in the rage of 1500 and 3000 mg/l may have inhibitory effect on methanogens at higher pH values.

The methanogens are the least tolerant to ammonia inhibition among the four types of anaerobic microorganisms (Kayhanian, 1994). As ammonia concentrations were increased in the range of 4000–5700 mg NH_3–N/l, acidogenic populations in the granular sludge were hardly affected while the methanogenic population lost 56.5% of its activity (Koster and Lettinga, 1988). There are two different aspects in the literature about the sensitivity of aceticlastic and hydrogenotrophic methanogens to ammonia. Some research based on the comparison of methane production and growth rate indicated that the inhibitory effect was in general stronger for the aceticlastic than for the hydrogenotrophic methanogens (Koster and Lettinga, 1984; Zeeman et al., 1985; Sprott and Patel, 1986; Bhattacharya and Saffermann, 1989; Robbins et al., 1989; Angelidaki et al., 1993; Borja et al., 1996), while others observed the relatively high resistance of acetate consuming methanogens to high total ammonia nitrogen levels as compared to hydrogen utilizing methanogens (Zeeman et al., 1985; Wiegant and Zeeman, 1986).

Sulfide Inhibition

Sulfate is a common constituent of many industrial wastewaters (O'Flaherty et al., 1998). In anaerobic reactors, sulfate is reduced to sulfide by the sulfate reducing bacteria

(SRB) (Koster et al., 1986; Hilton and Oleszkiewicz, 1988). Introduction of the waste streams and/or the biological production in the anaerobic digestion may cause the sulfides via reduction of sulfates or other sulphure-containing inorganic compounds. Anderson et al. (1986) found that sulfate in the influent of an anaerobic digester could inhibit methanogenesis due to both the competition for acetate and hydrogen by SRBs and the production of sulfide from sulfate reduction by SRBs. While soluble sulfide concentrations between 50 and 100 mg/l can be tolerated in anaerobic treatment with slightly or no acclimation, higher than 200 mg/l soluble sulfides does not show a significant inhibitory effect after acclimation. Stronache et al. (1986) stated that sulfate concentrations in excess of 200 mg/l had a direct toxic effect on anaerobic systems.

Volatile Fatty Acids (VFA) Inhibition

Anaerobic reactor effluent contains low concentrations of higher fatty acids however it contains higher concentrations of mainly acetic acid, propionic and butyric acids. Studies show that two important fermentation types occur complementary to each other; butyric and propionic acid. During butyric acid fermentation butyrate, acetate, hydrogen and CO_2 are produced, while propionic acid type fermentation produces propionate, acetate and some valerate, with no significant gas production (Dinopolou et al., 1988). The most common inhibition in anaerobic processes is the accumulation of VFA produced by acidogenic bacteria. Inhibition is identified by its high accumulation of VFA is the system which is an indicator of failure of methanogenic population. This failure might be caused by negative impact of bad environment conditions including shock loading, nutrient depletion or infiltration of inhibitory substances. High concentrations of VFA (i.e.; butyric and propionic acid) in a system is making toxic impact on the microorganisms in the reactor. It is reported that inhibition of microbial growth was observed at 35 mg/l acetic acid and excess of 3000 mg/l propionic acid concentrations (Ionnati and Fisher, 1983). The same researchers indicated that butyrate has a toxic effect at 1000 mg/l concentrations minimum. The inhibition of VFA at acidic medium can be attributed to the existence of unionized VFA in significant quantities in the system.When the pH value drops, the equilibrium goes to the left causing the increasing of unionized VFAs. Krocker et al. (1979) reported that reactor failure can be generally expected at the concentrations above 10 mg/l of unionized acids.

Light Metal Ions Inhibition

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

Heavy Metal Inhibition

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

Organic Inhibitors

Many organic chemicals that are sources of food for anaerobic microorganisms at low concentrations can show inhibitory effects at higher concentrations. A wide range of organic compounds can inhibit anaerobic degradation. Organic chemicals which are poorly soluble in water or adsorbed to surfaces of sludge solids may accumulate to high levels in anaerobic digesters. The accumulation of apolar pollutants in bacterial membranes causes the membrane to swell and leak, disrupting ion gradients and eventually causing cell lysis (Heipieper et al., 1994; Sikkema et al., 1994). The parameters that affect the toxicity of organic compounds include toxicant concentration, biomass concentration, toxicant exposure time, cell age, feeding pattern, acclimation, and temperature (Yang and Speece, 1986). The inhibition concentration ranges vary widely for specific toxicants. Blum and Speece (1991) conducted a comparative analysis of the toxicity of a large number of organic compounds to unacclimated mixed cultures. Since the cultures were not

acclimated, meaning they are not given time to adapt to inhibition, the compounds probably were not degraded following addition. Acetate-utilizing methanogenic cultures were used in the study for the analyses and found concentrations that resulted in a 50% reduction in gas production. Their results for selected compounds are summarized in Table 2.7. The study indicates concentrations that could cause problems in anaerobic treatment systems.

Table 2.7. Concentrations of organic compounds that reduce gas production by 50% (LC_{50}) with nonacclimated acetate-utilizing methanogens (Blum and Speece, 1991).

Toxicant	mg/l	**Toxicant**	mg/l	**Toxicant**	mg/l	**Toxicant**	mg/l
Hydrocarbons		1-Pentanol	4700	**Halogenated Alkanes**		Trichloroethane	13
Alkanes		1-Hexanol	1500	Chloromethane	50	Tetrachloroethane	22
Cyclohexane	150	1-Octanol	370	Methylene Chloride	7	1,3-Dichloropropene	0.6
Octane	2	1-Decanol	41	Chloroform	1	5-Chloro-1-pentyne	44
Decane	0.35	1-Dodecanol	22	Carbon tetrachloride	6	**Halogenated Aromatics**	
Undecane	0.61	**Ketones**		1-1-Dichloroethane	6	Chlorobenzene	270
Dodecane	0.23	Acetone	50000	1,2-Dichloroethane	25	1,2-Dichlorobenzene	150
Pentadecane	0.09	2-Butanone	28000	1,1,1-Trichloroethane	0.5	1,3-Dichlorobenzene	260
Heptadecane	0.03	2-Hexanone	6100	1,1,2-Trichloroethane	1	1,4-Dichlorobenzene	86
Nonadecane	0.01	**Miscellaneous**		1,1,1,2-Tetrachloroethane	2	1,2,3-Trichlorobenzene	24
Aromatics		Cateschol	1400	1,1,2,2-Tetrachloroethane	4	1,2,3,4-Tetrachlorobenzene	20
Benzene	1200	Resorcinol	1600	Pentachloroethane	11	2-Chlorotoluene	53
Toluene	580	Hydroquinone	2800	Hexachloroethane	22	2-Chloro-p-xylene	89
Xylene	250	2-Aminophenol	6	1-Chloropropane	60	2-Chlorophenol	160
Ethylbenzene	160	Isopropylether	4200	2-Chloropropane	620	3-Chlorophenol	230
Phenols		Ethylacrylate	130	1,2-Dichloropropane	180	4-Chlorophenol	270
Phenol	2100	Butylacrylate	150	1,2,3-Trichloropropane	0.6	2,3-Dichlorophneol	58
o-Cresol	890	Acetonitrile	28000	1-Chlorobutane	110	3,5-Dichlorophenol	14
p-Cresol	91	Acrylonitrile	90	1-Chloropentane	150	2,3,4-Trichlorophenol	8
2,3-Dimethylphenol	71	Carbon disulfide	340	Bromomethane	4	2,3,5,6-Tetrachlorophenol	0.1
4-Ethyphenol	240	2-Aminosulfide	6	Bromodichloromethane	2	Pentachlorophenol	0.04
Alcohols		4-Aminophenol	25	1,1,2-Trichlorotrifluoroethane	4	2,2-Dichloroethanol	18
Methanol	22000	2-Nitrophenol	12	**Halogenated Alkanes**		2,2,2-Trichloroethanol	0.3
Ethanol	43000	3-Nitrophenol	18	1,1-Dichloroethane	8	3-Chloro-1,2-propanediol	630
1-Propanol	34000	4-Nitrophenol	4	1,2-Dichloroethane	19	2-Chloropropionic Acid	0.01
1-Butanol	11000	2,4-Dinitrophenol	0.01	t-1,2-Dichloroethane	48	Trichloroacetic Acid	‹0.001

Methanol Degredation and Inhibition in Anaerobic Processes

Methanol, is a chemical compound with chemical formula CH_3OH. At room temperature it is a polar liquid. It is the simplest alcohol and is used as an antifreeze, solvent, fuel, and as a denaturant for ethyl alcohol. It is a light, volatile, colourless, flammable, poisonous liquid with a distinctive odor that is somewhat milder and sweeter than ethanol. Basic properties of methanol. Basic properties of methanol are given in Table 2.8.

Table 2.8. Basic properties of methanol.

Molecular formula	CH_3OH
Molar mass	32.04 g/mol
Appearance	Colorless liquid
Density	0.7918 g/cm³, liquid
Melting point	–97°C (176 K)
Boiling point	64.7°C (337.8 K)
Solubility in water	Fully miscible
Acidity (pK_a)	~ 15.5
Viscosity	0.59 mPas at 20 °C
Dipole moment	1.69 D (gas)

Methanol is a main pollutant in some specific wastewaters like pharmaceutical industry, the evaporator condensate of pulp and paper industries, coal-gasification plants, potato-starch producing gactories, and landfill leachates. Such wastewaters can be treated anaerobically (Minami et al., 1991; Paulo et al., 2001; Yamaguchi et al., 2001).

Methanol can be directly metabolised by methanogens, homoacetogens and sulfate reducers under anaerobic conditions. It can also be converted to H_2 and CO_2, provided a low hydrogen partial pressure is sustained by hydrogenotrophic methanogens. The degradation of methanol and its final fate in an anaerobic system depend on specific environmental conditions and the history of the anaerobic consortium. However,

acetogenesis of methanol to acetate is also an important fate under mesophilic conditions, especially when methylotrophic methanogenesis is disturbed (Lettinga et al., 1981; Florencio et al., 1994). A syntrophic route via the intermediates H_2 and CO_2 followed by hydrogenotrophic methanogenesis does not appear to be an important route during methanol degradation under mesophilic conditions (Florencio et al., 1994; Gonzalez-Gil et al., 1999). By using 13C-labelled substrates and specific inhibitors it was shown that in an anaerobic methanol-fed thermophilic bioreactor, about 50% of the methanol, at a concentration of 37 mM in the anaerobic reactor, was directly converted to methane by methylotrophic methanogens, and about 50% via the intermediates H_2/CO_2 and acetate (Paulo et al., 2001, Paulo et al., 2003).

In mixed cultures methanol potentially supports a complex food chain as shown in Figure 2.13. The important groups that plays key a role in degradation of methanol in anaerobic reactors are as follows:

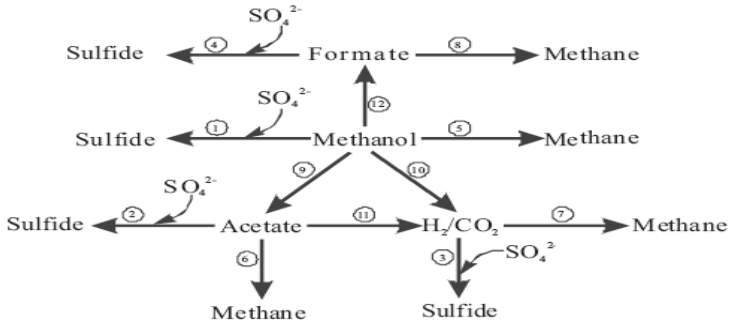


Figure 2.13. Anaerobic methanol mineralization (Weijma et al., 2000).

Methanogens

All methanol-utilizing methanogens isolated from anaerobic digesters are *Methanosarcina* relatives. *Methanosarcina* spp. converts methanol to methyl-coenzyme M by methyltransferases which are enzymes that have a cobalt-containing corrinoid as catalytic group (Vogels et al., 1988). In the presence of hydrogen methyl-coenzyme M is completely converted to methane. However when methanol is the sole substrate, part of the methanol has to be oxidized to CO_2 to provide reducing equivalents for reduction of

methanol to methane. This oxidation of the methyl-group likely proceeds via a reversed pathway which methanogens use to reduce CO_2 to methane. In this pathway the methanogenic C1-carrier tetrahydromethanopterin is involved (Weijma et al., 2000).

Sulfate Reducing Bacteria

Only a few mesophilic sulfate reducing bacteria (SRB) are reported to grow on methanol, but acetate is always needed as carbon source (Braun and Stolp, 1985; Nanninga and Gottschal, 1986). Growth rates of mesophilic SRB on methanol are very low compared to that of methanogens and acetogens.

Homoacetogens

Methanol is an excellent homoacetogenic substrate. The mesophiles *Acetobacterium woodii*, *Eubacterium limosum*, *Butyribacterium methylotrophicum* and the thermophiles *Moorella thermoautotrophicum* and *M. thermoaceticum* show very fast growth on methanol. For growth of homoacetogens on methanol, bicarbonate must be present as electron acceptor. Bicarbonate is inevitably present when methylotropic methanogens or SRB are also active.

Table 2.9. Selected methanol-utilizing methanogens, SRB and homoacetogens and some growth kinetic properties and physiological characteristics (Florencio, 1994; Weijma, 2000; Driessen et al., 2000).

Organism	T_{opt} °C	pH_{opt}	μ_{max} Day^{-1}	Yield	other growth substrates		
					Ac	H_2/CO_2	formate
Methanogens							
Methanosarcina acetivorans	35–40		3.20		+	+	–
Methanosarcina barkeri strain MS	30–40	7.0	2.35	3.5a	+	+	–
Methanosarcina mazei	37–40	6.0–7.0	3.24		+	+	–
thermophilic *Methanosarcina* species	50–58	6.5–7.0			+	+	–
Sulfate reducers							
Desulfovibrio carbinolicus	35a		0.22		–	+	+
Desulfotomaculum kuznetsovii	60–65		0.72		+	+	–
Acetogens							
Acetobacterium woodii	30	7.5		5.3–8.2	–	+	+
Butyribacterium methylotrophicum	39	7.5	1.85	8.2b	–	+	+
Eubacterium limosum	39	7.2	2.38	7.1b	–	+	
Moorella thermoautotrophicum	56–60	5.8	1.8	6–9c	–	+	+
Moorella thermoaceticum	55–60		1.85		–	+	+

[a]cultivation temperature; [b]g dry cell/mol methanol; [c]g dry cell/mol acetate

T_{opt}: optimum growth temperature; pH_{opt}: optimum growth pH; Ac: acetate.

Mixed Cultures

In mixed cultures methanogens, homoacetogens and SRB compete for methanol. In addition, SRB and methanogens may also compete for hydrogen and acetate, the product of methanol catabolism by homoacetogens.

Competition for Methanol

Florencio (1994) studied the competition between methanogens and homoacetogens for methanol in mesophilic UASB reactors in detail. The *K*s value of methanogens for methanol is 0.25 mM, while that of the homoacetogens is much higher (16 mM). This shows that at low concentrations methanol is mainly used by methanogens. The opposite is not necessarily true because substantial homoacetogenesis from methanol only occurs when in addition to a high methanol concentration, also sufficient bicarbonate and cobalt is available. The digestion process even may completely collapse when acetate accumulation leads to further reduction of methanogenesis due to toxicity of undissociated acetic acid towards methanogens.

LC_{50} concentrations for methanol have been found to be 950 mM for granular sludge from a citric acid production plant, 1350 mM for a sludge treating alcohol distillery wastewater and 400 mM for a non-granular sludge from a cattle manure (Enright et al., 2005). Parallel results were obtained in a previous study in which SMA tests for single phase anaerobic reactor were carried out with the seed sludge from the EGSB reactor used in the anaerobic stage of a two stage anaerobic-aerobic biological treatment system at a brewery. IC_{50} concentration of methanol was found to be 0.4 M (Oz, 2008). Besides, inhibitory concentration of methanol for bacteria was stated to be 90 mg/l in some sources (Vance, 1997).

Toluene Degredation and Inhibition in Anaerobic Processes

Toluene which is a relatively water-soluble aromatic hydrocarbon is used as a solvent in the production of paints, thinners, adhesives, inks and many pharmaceutical products. Toluene concentrations in industrial wastewaters have been reported to be

approximately between 7–753 mg/l depending on the manufacture type (De Witt, 1999). Several treatment methods including chemical oxidation and combustion, activated carbon adsorption and biological stabilization may be used for the conversion of toluene to a non-toxic substance. Aerobic biological treatment methods have been also previously reported. However, partially or substantially volatilization of the compounds to air due to agitation and aeration of the wastewaters limits its use. Therefore, anaerobic digestion can be preferred to aerobic treatment for the treatment of volatile compounds, such as toluene. Losses to the atmosphere due to agitation and aeration of the wastewaters are avoided in anaerobic digestion. Although there are valuable studies investigating the effects of some aromatic hydrocarbons such as benzene, toluene, ethyl acetate on pure or binary cultures, little attempt has been made to assess effects of particular solvents on anaerobic wastewater treatment reactor sludges in terms of qualitative and quantitative measures of methanogenic species and their activities (Rogers et al., 2000; Alagappan and Cowan, 2001; Alagappan and Cowan, 2003; Hwang et al., 2003; Meckenstock et al., 2004). Treatment of toluene-containing waste streams has been studied by using a variety of anaerobic bioreactor types including anaerobic horizontal-flow anaerobic immobilized biomass (Cattony et al., 2005), expanded granular sludge bed reactor (Enright et al., 2007a) and completely stirred tank reactor (CSTR) (Oz et al., 2003). Basic properties of toluene is given in Table 2.10.

Table 2.10. Basic properties of toluene.

Molecular Formula	C_7H_8 ($C_6H_5CH_3$)
Molecular weight	92.14 g/mol
Boiling point	110.62°C
Vapor pressure	28.5 Torr at 20°C
Solubility in water	0.47 g/L (20-25°C)
Density	0.8669 g/mL (7.234 lb/gal) at 20°C

While much is known about aerobic toluene degradation pathways and the many aerobic species that mineralize toluene, comparatively little is known about anaerobic

degradation of toluene. Toluene degradation occurs under all of the major anaerobic electron-accepting conditions, including nitrate-reducing (Fries et al., 1994), sulfate-reducing (Beller et al., 1996), iron(III)- reducing (Lovley et al., 1990), and methanogenic (Edwards and Grbic-Galic, 1994; Ficker et al., 1999) conditions, and pure cultures of nitrate-reducing, sulfate-reducing, and iron-reducing bacteria that degrade toluene have been isolated. In contrast, toluene degradation to methane and CO_2 requires more than one species because of the limited substrate range of methanogenic bacteria. It was thought that fermentative or acetogenic bacteria first transform toluene to methanogenic precursors, such as acetate and hydrogen; methanogenic bacteria then convert these substrates to methane and CO_2. Since transformation of toluene to acetate and hydrogen is energetically favorable only when the concentrations of hydrogen and acetate are kept low by the activity of methanogenic bacteria, toluene degradation is necessarily dependent on syntrophic relationships between species in a consortium. Anaerobic toluene degradation under methanogenic conditions was first reported in 1986. Several lines of evidence suggest that the activation of toluene via benzylsuccinate synthetase is the first step of anaerobic toluene mineralization, and subsequent steps in the mineralization pathway have been proposed based on biochemical and genetic studies (Beller et al.,1996).

Research on the anaerobic biodegradation of monoaromatic hydrocarbons, like toluene, and detailed biochemical analysis has been hampered by difficulties associated with studying anaerobic microorganisms such as low growth rates and need for specialized equipment and low substrate concentrations (Edwards et al., 1994). Strict anaerobes found in sulfate-reducing and methanogenic cultures are inhibited by high substrate concentrations (for toluene, typically less than 400 μM), and thus only low amounts of substrate can be used to sustain growth. As a result of these low substrate concentrations, together with the low rates of growth and degradation, and possibly the relatively small amount of energy available from the reaction (Edwards et al., 1992; Edwards and Grbic-Galic, 1994), intermediates in the catabolic pathways do not appear to accumulate and have proven to be very difficult to detect. Radioactive tracing and isotope trapping are very effective techniques for determining metabolic pathways, especially when the concentrations are very low, because these low concentrations can be overcome by using a radioactive substrate with high specific activity (Edwards et al., 1994). Labelled substrates also provide an indisputable link between the substrate and any labelled products detected.

In the more recent studies, it was stated that toluene biodegredation starts in the methanogenesis stage with a pH 7.0-7.2 of anaerobic digestion, especially faster in the beginning of the methanogenesis. The degredation rate decreases after the stabilization of methanogenesis stage (Mrowiec et al., 2005) and toluene degradation by methanogenic culture proceeded via methyl hydroxylation to benzyl alcohol, followed by further oxidation steps to benzaldehyde and benzoate, with perhaps a parallel pathway via ring hydroxylation to p-cresol (Edwards et al., 1994). The proposed compounds as intermediates in toluene degradation in the study namely benzoic acid, benzyl alcohol, benzaldehyde, and p-cresol, have previously been implicated in anaerobic toluene degradation (Vogel and Grbic-Galic, 1986; Grbic-Galic and Vogel, 1987; Livley and Lonergan, 1990; Evans et al., 1992). Figure 2.14 shows the proposed toluene degredation pathway.

Different inhibitory concentrations for toluene were reported in different studies which might be caused by differences in starting microbial composition, operational and nutritional factors. In the study of Edwards and Galic (1994), a creosote-contaminated sediment from Pensacola, Fla. was used as source inoculum in 250 ml microcosm bottles and it is stated that degradation of toluene in stable mixed methanogenic cultures, inhibition noted at substrate concentrations above about 1,800 mM whereas Ghosh et al. (1996) found no evidence of inhibition of the anaerobic digestion process by 50 mg/l of toluene for continuous-flow, mesophilic (35°C), plug-flow digester to cometabolic degradation of toluene by an anaerobic microbial consortium in the presence of conventional pollutants serving as the major carbon and energy sources.

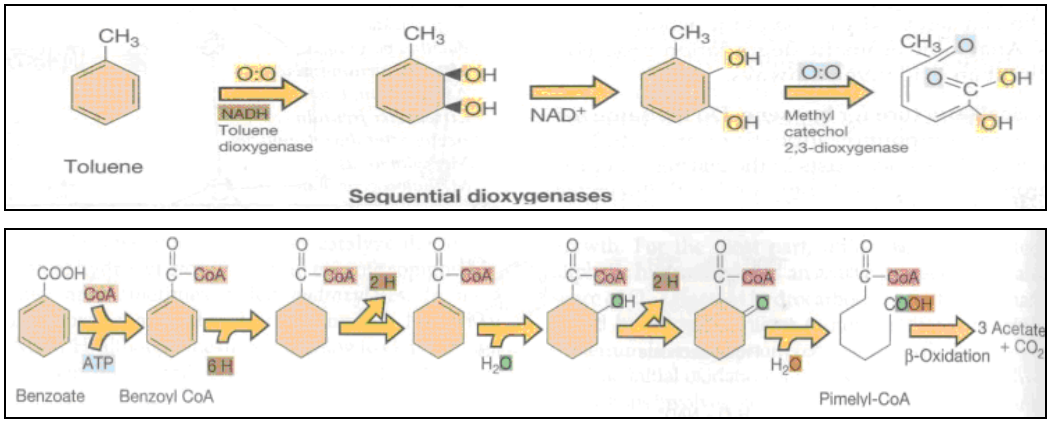


Figure 2.14 Toluene degredation pathway (Madigan et al, 2000).

Iso –propanol Degredation and Inhibition in Anaerobic Systems

Isopropyl alcohol (IPA) whose production worldwide exceeds 1 million tonnes per annum through its many industrial applications including rubber, cosmetics, textiles, pharmaceuticals, and fine chemicals industries is the most widely used volatile organic compound. Basic properties of iso-propanol are given in Table 2.11. The anaerobic degradation of 2-propanol in anoxic paddy soil was studied with soil cultures and a 2-propanol-utilizing methanogen. Acetone has been reported to be the first and the major intermediate involved in the methanogenic degradation of 2-propanol. Analysis with a methanogenesis inhibitor, bacteria antibiotics, and the addition of H_2 to the gas phase revealed that 2-propanol oxidation to acetone directly occurred using 2-propanol-utilizing methanogens, but not with H_2-producing syntrophic bacteria, for which the removal of acetone is required for complete 2-propanol oxidation. The 2-propanol-utilizing strain IIE1,which is phylogenetically closely related to *Methanoculleus palmolei,* was isolated from paddy soil, and the potential role of the strain in 2-propanol degradation was investigated. 2-propanol is one of the representative fermentation intermediates in anaerobic environments. 2-propanol is used as a hydrogen donor for methanogenesis in the first step of anaerobic degradation in anoxic paddy soil. It is then mineralized to methane and carbondioxide via acetone followed by acetate production. At least three organisms are concerned in the anaerobic 2-propanol degradation in anoxic paddy soil: 2-propanol-utilizing methanogens, acetone-degrading bacteria, and acetotrophic methanogens. Previous studies have shown that alcohols, such as isopropanol can be oxidised by hydrogenophilic methanogens to acetone during growth on H_2/CO_2 (Widdel, 1986; Widdel et al., 1988). Homoacetogenic bacteria capable of metabolising iso-propanol to acetate and higher fatty acids have also been reported (Eichler and Schink, 1984). Co-metabolism with glucose of the compound has been reported in a mesophilic anaerobic study (Fox and Ketha, 1996). However, the studies on effects of the solvents on anaerobic reactors have been limited.

Table 2.11. Basic properties of iso-propanol.

Molecular Formula	C_3H_8O
Molecular weight	60.10 g/mol
Boiling point	82.3°C, 355 K, 180°F
Melting point	−89°C, 184 K, -128°F
Solubility in water	miscible

Various inhibitory concentrations were stated for iso-propanol. IC_{50} concentration of iso-propanol for bacteria is 5000 mg/l according to union carbide corporation material safety sheet and iso-propanol inhibition concentration was stated as 55 mg/l in some sources (Vance, 1997). Oz (2008) carried out SMA tests for single phase anaerobic reactor with the seed sludge from the EGSB reactor used in the anaerobic stage of a two stage anaerobic-aerobic biological treatment system at a brewery. and found IC_{50} concentration of iso-propanol to be 0.4 M.

There are many studies about degradation of organic solvents in anaerobic processes in the literature, however there are limited studies regarding inhibition. The point is that organic solvent concentrations in wastewaters are generally above the limit dose that can be degraded by microorganisms and that cause serious problems in the systems. Therefore, evaluating inhibitory effects of organic solvents in these systems keeps an important place in terms of better reactor performance.

3. AIM OF THE STUDY

The aim was to investigate effects of selected solvents such as methanol, toluene and iso-propanol on biogas production in anaerobic reactor and acetyl-CoA synthetase enzyme of *Methanosaeta*, which has an important role in the methane production from acetate in anaerobic degredation. The inhibitory effects of these solvents on the key enzyme of methane production was detected by quantitative PCR (Q-PCR). A new designed primer pair which is specific for acetyl-CoA synthetase gene was used for this purpose. Also effects of solvent/solvent mixtures on microbial diversity were analyzed using FISH to determine mixed culture interactions at various single and multiple solvent concentrations.

4. MATERIALS AND METHODS

4.1. Seed Sludge Characteristics

The inoculum sludge was taken from a a full scale upflow anaerobic sludge blanket (UASB) reactor (435 m^3) treating alcohol (raki) distillery wastewater. Total solid (TS) and total volatile solid (TVS) concentration of the anaerobic granular sludge was 58,000 mg/l and 48,000 mg/l respectively.

4.2. Serum Bottle Tests

An anarobic sludge with the concentration of 2000 mg/l was added to the 100 ml serum bottles (Figure 4.1.) with the prepared dilution solution. To prepare the dilution solution OECD protocol no: 311 was followed. According to the OECD protocol, the chemicals on Table 4.1. and 4.2. were used. pH was set to 7.0 by using HCl and KOH. Anaerobic condition was provided by flushing the bottles with nitrogen gas for 3 minutes. Sludge was incubated at 37^0C for 24 hours. After all feeding procedures, bottles were covered with parafilm to hinder oxygen uptake and escape of gases. Then, the bottles were kept in stirred water baths at 37^0C for 7-10 days.

Table 4.1. Dilution solution (OECD, 1993).

Name	Amount
anhydrous potassium dihydrogen phosphate	0.27g
disodium hyrogen phosphate dedocahyrate	1.12g
ammonium chloride	0.53g
Calcium chloride dihydrate	0.075g
magnesium chloride hexahydrate	0.10g
Iron(II)chloride tetrahydrate	0.02g
Resazurin (oxygen indicator)	0.001g
Sodium sulphidenonahydrate	0.10g
Stock solution of trace elements	10 ml
de-oxygenated water	to 1 L

Table 4.2. Stock solution (OECD, 1993).

magnesium chloride	50 mg
Boric acid	5 mg
zinc chloride	5 mg
Copper(II) chloride	3 mg
disodium molybdate dihydrate	1 mg
cobalt chloride hexahydrate	100 mg
nickel chloride hexahydrate	10 mg
disodium selenite	5 mg
de-oxygenated water	to 1L

Figure 4.1. Serum bottle.

4.3. Experimental feeding procedure

Anaerobic sludge was fed with acetate which has concentration of 2000 mg/l. Acetate concentrations ranging from 1000 to 4000 mg/l have been tested in order to determine maximum acetoclastic activity and 2000 mg/l acetate concentration was found to be optimum. In a previous study, Şimşek (2007) also found 2000 mg/l to be optimum concentration for acetoclastic activity.

After the incubation period the anaerobic sludge was fed with different concentrations of methanol, toluene, iso-propanol and methanol + toluene. Methanol molarities used were 0.1 M, 0.3 M, 0.5 M, 0.7 M, 1.0 M (ranging between 3200 - 32,000 mg/l). Toluene molarities were 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM and 4.0 mM (ranging between 46- 386 mg/l). Iso-propanol molarities were 0.1 M, 0.5 M, 1.0 M, 2.0 M (6000-120,000 mg/l). Also in order to observe the multiple effects of toluene and methanol, 1.0

M methanol + 0.5 mM toluene (46 + 32,000 mg/l) and 1.0 M methanol + 1.5 mM toluene (115 + 32,000 mg/l) mixtures were used in the experimental procedure. A control reactor was only fed with acetate while other reactors were fed with acetate + solvents. Each of the reactors had a parallel to obtain more reliable and precise results. These solvents were fed to the anaerobic reactors after mixing with acetate. Toluene was dissolved in ethanol because of its low solubility before being given to the anaerobic reactors. All of the solvents were injected to the anaerobic sludge using 10 ml injectors. Three exposures to solvents were done. In each exposure same amounts of solvents were fed to the anaerobic reactors. For the second and third exposures, before feeding, to not to exceed the volume of the bottles some amount of dilution solution which is equal to the amount that will be added, was extracted from the bottle. Thereafter, same amount of solvents with the first exposure were added to the bottles.

4.4. Analytical Techniques

After the anaerobic sludge in the serum bottles were fed with acetate + solvents, the gas pressure in the bottles was measured with a 7000 mbar manometer once in three days.

Gas compositions were measured using HP Agilent 6850 gas chromatograph (GC) with a thermal conductivity detector (HP Plot Q column 30 m x 530 μm). Due to the granular characteristics of the reactor sludge, total solids and total volatile solids (TS/TVS) were measured. All analyses were carried out according to Standard Methods (APHA, 1997). When methane percentages were multiplied with gas pressures of the serum bottles, methane production values were obtained.

4.5. Molecular Techniques

4.5.1. RNA-based methods

After the gas composition and gas pressure values were obtained, samples were taken from the serum bottles for molecular analysis. 1.5 ml samples were taken into appendorf tubes for RNA extraction and further molecular analysis following RNA extraction. The tubes were kept in ice bath and immediately RNAs were extracted. To

extract the RNAs, Charge Switch RNA extraction kit (Invitrogen) and a magna rack (Invitrogen) shown in Figure 4.2. were used and the recommended procedure by the isolation kit was followed.

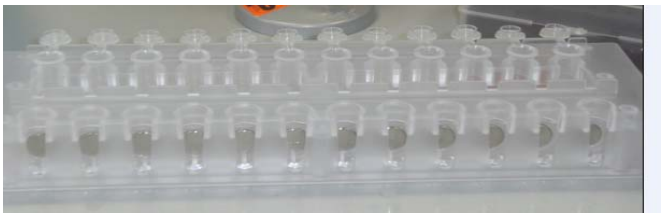


Figure 4.2. Magna rack.

Complementary DNA (cDNA) Synthesis

Superscript Vilo cDNA sythesis kit (Invitrogen) was used to sythesize cDNA from the extracted RNAs. Extracted RNAs were converted into cDNAs by the Reverse Transcription Polymerase Chain Reaction (RT-PCR). cDNA synthesis reaction had one cycle consists of 10 minutes at 25^0C, one hour at 42^0C, 5 minutes at 85^0C.

Real time Polymerase Chain Reaction (Q-PCR)

To investigate the amount of the sythesized mRNA that is responsible for the production of acetyl-CoA sythetase enzyme a Q-PCR reaction was set up by using a primer pair that is specific for *Methanosaeta concilii*'s acetyl-CoA sythetase enzyme gene.

Methanosaeta concilii's acetyl- CoA synthetase gene sequence:

"gtgttgaaattggctggcaaagaagacaagaagttgaagacgacagtatttcaggacgagaccagaatctttaatccgccaaaag
agttggtagaaaagtcaatcgtgatgcagtggatgaagaagaaagggttcaagactgagaaggagatgcgtgcttggtgctcctct
gatgagcattacctcgagttctgggacgagatggcaaagacgtacgttgactggcacaagccctatactaaggtgatggacgactc
ggagatgccctacttccactggttcactggaggtgagatcaacatcacatacaacgccgtggacaggcacgccaagggcgcaaa
gaaggacaaagttgcatacatatggataccagagcctacagaccagccagtccagaagatcacctacggcgacctctataagga
ggtcaataagttcgcaaacggcctcaagagcctcggcttgaagaaggggatcgggtcagcatctacatgcccatgataccccag
cttcccatcgccatgctcgcctgcgccaagctcggcgtcagccacatcgtggttttctccggattcagctccaaaggcctgatggac
agggctgctcactgcggttccagagccatcatcaccgtggatggattctacaggcgcggaaagccggtgcctctgaagccgaac
gccgacgaggcagctggcggcgctccgtcagttgagaagattatcgtctacaagcgtgcgggcgttgacgtttccatgaaagagg

gcagggacgtctggtggcatgatctggtcaagggccagtccgaagagtgtgagccggtatgggttgacccggagcacaggctgt
atatcctctacacctccggcacaaccggcaagcccaagggtatcgagcacgcaacgggcggaaacgccgtcggaccggctca
gacgcttcactgggtcttcgacttgaaggacgacgatgtctggtggtgtaccgccgatattggatgggtcaccggtcactcctacat
cgtctacgctccgctcattctgggcatgaccagcctcatgtacgagggcgctgcagactatccagacttcggtaggtggtggaaga
acatccaggaccacaaggtcaccgtcctttacactgcccccacggcggtaaggatgttcatgaagcagggcgcagaatggcctg
ataagtacgacctctcaagcctgaggctactgggatctgtgggcgagccgatcaatccggaggcctggatgtggtatcgtgagca
cattggacgaggcgagctccagatcatggacacctggtggcagaccgagaccggaaccttcctcaactctccgctgcccatcac
gccactgaagcccggatcgtgcacattcccgctccccggatatgacatatccatcctggatgaggaagggaatgaggttcctctgg
gatccggaggcaacatagtggcattgaagccctatccttcgatgctcagggcgttctggggcgacaaagagaggttcatgaagga
gtactggcagttctactgggatgttcccggccgccgcggcgtctatctcgctggagacaaggcgcagagggacaaggacggcta
cttcttcatccagggcagaatcgatgatgttctcagcgtcgcaggccacaggatagccaacgccgaggtcgagtctgccctggtg
gctcaccccaagatagccgaggcggcagtagttggaaagcccgacgaggttaagggcgagtcgatcgtcgccttcgtcattctga
gggtcggaaatgaaccgtcccctgagctggcaaaggatgcgatcgctttcgtcaggaagacccttgggccggtggctgcgccca
cggaggtccacttcgtcaacgacctcccgaagacgaggagcggcaagataatgcgccgtgtcgtcaaggcgagggcccttgga
aacccggttggggacatatcgaccctgatgaatccagaagcagtggatgggatccccaagatcgtctga”

Primer pair specific for this gene: MSaeta_Aco-A f: taatccgccaaaagagttgg and MSaeta_Aco-A r: tcttctggactggctggtct

To prepare a stok standard for Q-PCR reaction a PCR reaction was set up using an anaerobic sludge containing high *Methanosaeta concilii* population with designed primers. The results indicated PCR product was 99% acetyl-CoA sythetase gene of *Methanosaeta concilii*. Ten standards were prepared to quantify the number of acetyl-CoA expression genes by serial dilutions. A calibration curve was drawn by using these standards by the programme used..

The procedure recommended by Roche was followed and Light Cycler Master Kit (Roche) was used to set up the reaction (2.0 µl master mix, 1.6 µl $MgCl_2$ 1.0 µl Primer F and R, 13.4 µl H_2O, 1 µl sample). To observe the results of the reaction, Light Cycler Software 4.05 program provided by Roche was used. The program consists of 4 sections; denaturation (95^0C), amplification (95^0C, 56^0C, 72^0C), melting (95^0C, 53^0C, 95^0C) and cooling (40^0C). Computer view of the programme was given in Figure 4.3.

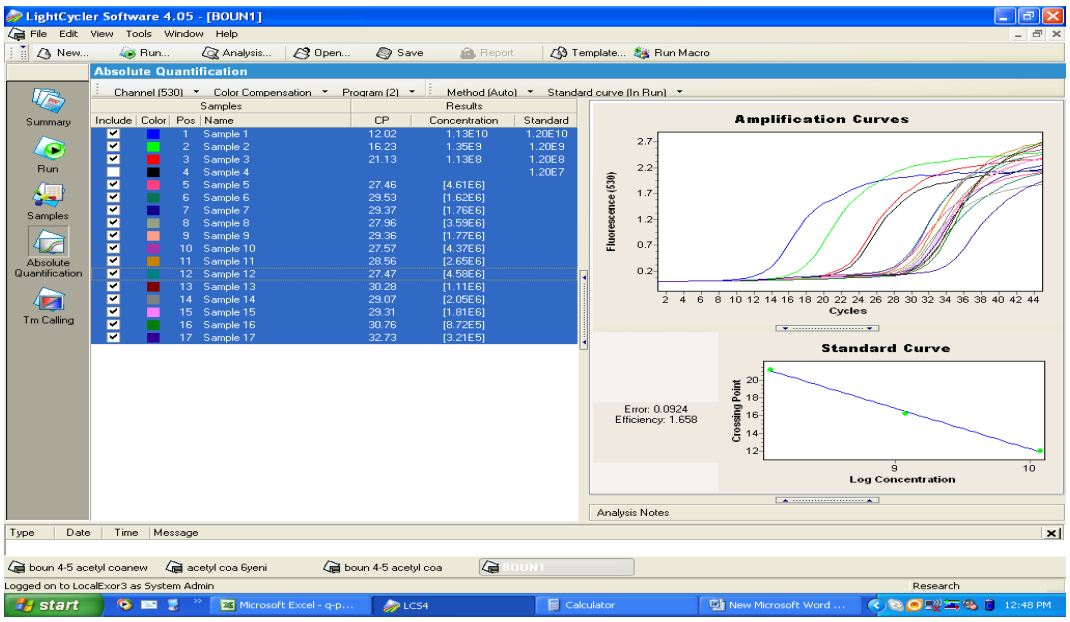


Figure 4.3. View of Light Cycler Software 4.05.

Fluorescence In situ Hybridization (FISH)

After the third exposure, 5 ml samples were taken from each of the serum bottles, mixed with equal amount of ethanol and put into 50 ml falcons for the FISH analysis to observe active microbial community. The samples were kept at -20^0C and standard paraformaldehyde fixation carried out within 3 days.

For the Standard Paraformaldehyde (PFA) Fixation 1000 µl of granular sludge-ethanol mix (1:1, (v/v)) was washed once with phosphate-buffered saline (PBS) [130 mM NaCl, 10 mM sodium phosphate, pH 7.2] and resuspended in 0.5 ml of PBS. 0.75 ml of freshly prepared 4% PFA in PBS (pH 7.2) was added to the suspension and incubated for at least 3 hours, or overnight, at 4^0C. After fixation, cells were washed once with PBS, resuspended in 0.5 ml of PBS-absolute ethanol (1:1(v/v))and stored at -20^0C.

For the hybridization part, 16S rRNA-targeted oligonucleotide probes used in this study and their target microbial groups nucleotide sequences are listed in Table 4.3 and Figure 4.4. Optimal hybridization conditions for each probe are also given in Table 4.4. All probes were obtained commercially (Qiagen Corp.).

Table 4.3. 16S rRNA-targeted oligonucleotide probes used in this study.

Probe	Target Group	Probe sequence (5'-3')	Labelling (5')	Reference
MC1109	*Methanococcales*	GCAACATAGGGCACGGGTCT	CY3	Raskin et al., 1994a
MB310	*Methanobacteriales*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin et al.,1994a
MG1200	*Methanomicrobiales* relatives	CGGATAATTCGGGGCATGCTG	CY3	Raskin et al., 1994a
MS1414	*Methanosarcina* + relatives	CTCACCCATACCTCACTCGGG	CY3	Raskin et al.,1994a
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	CY3	Stahl et al., 1988
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	Fluorescein	Amman et al.,1990a
UNIV1392	Virtualy all known organisms	ACGGGCGGTGTGTAC	TAMRA	Pace et al., 1986
NON338	Non sense probe	ACTCCTACGGCAGGCAGC	TAMRA	Manz et al., 1992

Table 4.4. Optimum hybridization conditions for oligonucleotide probes (Kolukirik, 2004).

Probe	Formamide concentration	Hybridization temperature	Washing temperature	NaCl Concentration
MC1109	20%	46 °C	48 °C	225 mM
MB310	20%	46 °C	48 °C	225 mM
MG1200	30%	46 °C	48 °C	112 mM
MS1414	35%	46 °C	48 °C	84 mM
ARC915	35%	46 °C	48 °C	84 mM
EUB338	10%	46 °C	46 °C	450 mM
UNIV1392	10%	37 °C	37 °C	450 mM

For each sample hybridization, two negative controls were prepared; one of the controls was used to assess non-specific binding (with Non338 probe), 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 UASB sludge samples were also stained using DAPI staining to visualize intact cells in the samples. 200 µl of the fixed samples were washed twice with PBS and once with MilliQ water. Then the fixed samples dehydrated at room temperature in increasing concentrations of ethanol (50, 80, and 100%). 3 µl of probe (50 ng/µl) and 17 µl hybridization buffer (4.5 M NaCl, 2 mg/ml Ficoll, 2 mg/ml Bovine serum albumen, 2 mg/ml polyvinyl pyrolidone, 5 mM EDTA,Tris HCl, pH 7.2, 25 mM NaH_2PO_4, pH 7.0, 0.1% SDS) were added and incubated at the optimal hybridization temperature for the given probe for at least 4 hours

or overnight. 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.

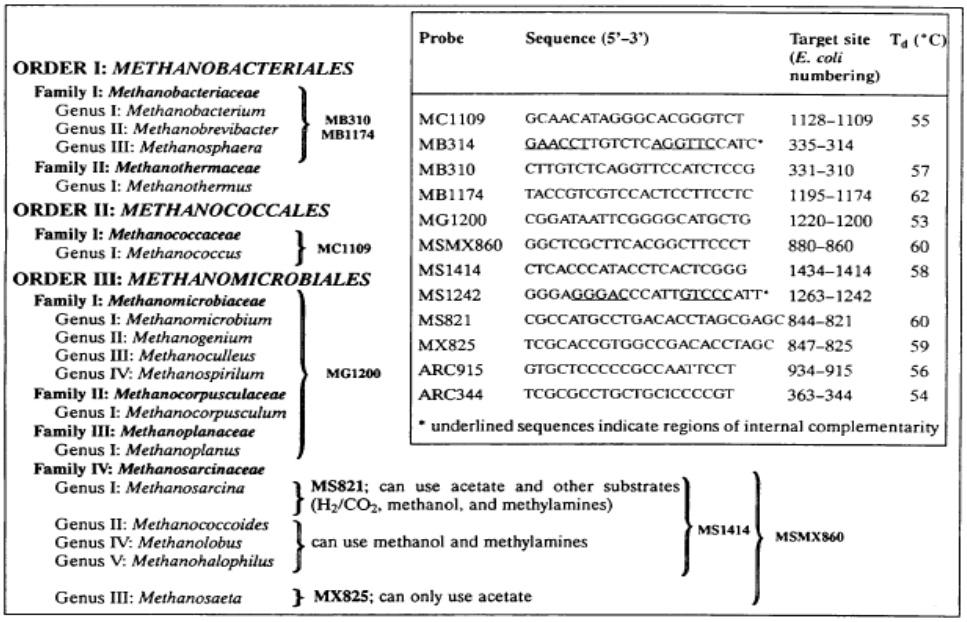


Figure 4.4. Classification of methanogens in relationship to the oligonucleotide probes characterized (Raskin et al., 1994).

In DAPI staining, the total cells present in the samples were previously determined by counting 4, 6-diamine phenylindol (DAPI) stained cells. Hybridization procedure of a regular sample was followed except the hybridization time in incubator. Hybridization time needed for DAPI is 15-20 minutes at 46^0C. Slides were examined under Olympus BX 50 epifluorescence microscope equipped with a 100 W high-pressure mercury lamp, U-MWIB and U-MWG filter cubes. Images were captured using a Spot RT charged coupled device (CCD) camera having special software supplied by the camera manufacturer (Diagnostic Instruments Ltd., UK). The images were processed and analyzed using Image-Pro Plus version 6.3 image analysis software (Media Cybernetics, U.S.A.). The dilution percent needed is determined by counting DAPI added cells. Optimal number of counts in

a FISH photo was accepted as 150-300. 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.

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 4.5.

Table 4.5. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used.

Fluorochrome	**Color of Fluorescence**	**Maximum excitation wavelength (nm)**	**Maximum emission wavelength (nm)**	**Filter cube used**
FLUOS	Green	494	518	U-MWIB
TAMRA	Orange	555	580	U-MWG
CY3	Red	552	565	U-MWG
DAPI	Blue	365	397	U-MWG

4.5.2. DNA based Molecular Methods

DNA Extraction

Approximately 0.5 g sediment was added up to lysing matrix tubes provided by the kit. The tube contains mixture of ceramic and silica particles to lyse all microorganisms in sample. Then lysing matrix tubes were spinned in Ribolyser (Fast Prep TM FP120 Bio 101 Thermo Electron Corporation) for 30 seconds at speed of 5.5 m/s. The tubes were then centrifuged at 14000xg for 30 seconds. After centrifugation supernatants were transferred to clean 1,5 ml appendorf tubes and added 250 µl PPS reagent. To mix the composition tubes were shaked by hands for 30 seconds. After mixing the tubes centrifuged again at 14000xg for 5 minutes to pellet the precipitate. Supernatants were transferred to 2 ml eppendorf tubes and 1 ml of binding matrix suspension was added to supernatant. Tubes

were inverted by hand for 2 minutes to allow binding of DNA to matrix. To settle the silica matrix tubes were incubated 3 minutes at room temperature. 500 µl of supernatant was removed carefully without disturbing settled silica matrix. Then the binding matrix was resuspended in the remaining supernatant. All mixture was filtered and filter was placed to a new tube. Filter was washed by 500 µl SEWS-M wash solution. After washing, filter was dried by centrifugation at 14000xg for 2 minutes. Filter was removed to a new tube and 50 µl DES (DNase/Pyrogen free water) was added. The filter with DES was vortexed and then centrifuged at 14000xg for 1 minute. Application-ready DNA was obtained in the tube. 1/100 diluted genomic DNA was run on the 1% (w/v) agarose gel, prestained with ethidium bromide (EtBr) in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8). Gel was visualized by using a gel documentation system, Mitsubishi 91.

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. Bac8f-Bac1541r and Arch07f-Arch1384r primers were used for the amplification 16S rDNA of bacteria and *Archaea* respectively. Extracted gDNAs were used as a template for these primers. Bac341f-Bac534r and Arch344f-Univ522r primers were used to amplify V3 region of 16S rDNA (approximately 200 bp long) of bacteria and *Archaea*, respectively.

PCR reactions were performed in a 30 µl (total volume) mixture containing 0.6 µM forward primer, 0.6 µM reverse primer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1U of Taq polymerase enzyme and the buffer supplied with the enzyme (Fermentas Life Sciences), and 0.6 µl of template. Amplification was performed with a thermal cycler (TECHNE-TC 412). Products of all reactions were screened for the amplification of correct band size. All PCR products were run on the %1 (w/v) agarose gel prestained with ethidium bromide (EtBr) in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8). Gels were visualized by using a gel documentation system, Mitsubishi 91.

Table 4.6. Bacterial and archaeal oligonucleotide primers used for PCR amplification.

Primer	Experimental Stage	Annealing (°C)	Position	Reference
Bact341f_GC[2]	DGGE	55	341-357	Muyzer et al., 1993
Bact534r			534-518	
Bact8f	First round of nested PCR		8-27	Edwards et al., 1988
Bact1541r			1541-1522	
Arch07f	First round of nested PCR	52	07-24	Lueders et al., 2004
Arch1384r			1384-1368	
Arch344f_GC[2]	DGGE	53	344-358	Raskin et al., 1994
Univ522r			522-504	Amann et al., 1995

Denaturing Gradient Gel Electrophoresis (DGGE)

The first step was the assembly of the perpendicular gradient gel sandwich. The thickness of the sandwich was established by using 1 mm spacers between two glass plates which are in size of 16x20 and 18x20 cm. Before assembly, glass plates were cleaned carefully 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. For bacterial DGGE, 10% (w/v) acrylamide:bisacrylamide 30% denaturant solution was prepared by mixing 33.3 ml of 30% acrylamide:bisacrylamide with 2 ml 50xTAE (2.0 M Tris, 50 mM EDTA, and 1.0 M acetic acid) and 12 ml formamide and 12.6 g urea. 60% of denaturant concentration was reached by adding 24 ml formamide and 25.2 g urea to 33.3 ml of %30 acrylamide: bisacrylamide and 2 ml 50xTAE (2.0 M Tris, 50 mM EDTA, and 1.0 M acetic acid). Both solutions were added distilled water up to 100 ml. For archaeal DGGE, 10% (w/v) acrylamide:bisacrylamide 40% denaturant solution was prepared by mixing 33.3 ml of 30% acrylamide:bisacrylamide with 2 ml 50xTAE (2 M Tris, 50 mM EDTA, and 1.0 M acetic acid) and 16 ml formamide and 16.8 g urea. 70% of denaturant concentration was reached by adding 28 ml formamide and 29.4 g urea to 33.3 ml of 30% acrylamide:bisacrylamide and 2 ml 50xTAE (2.0 M Tris, 50 mM EDTA, and 1.0 M acetic acid). 100% denaturant solution is defined as 40% (v/v) formamide and 7 M urea. Both solutions were added distilled water up to final volume of 100 ml. After solutions were prepared, they were filtered with 0.45 µm filter and sonicated for 10 minutes. The bottles

were wrapped with foil paper to avoid sunlight and stored at 40^0C for further uses. Into two beakers, 20 ml of 10% (w/v) acrylamide:bisacrylamide solutions containing 30% and 60% (40% and 70% for archaeal samples) denaturants were poured. The lower denaturant containing solution was then stained with bromophenol/xylene loading dye. Gradient former was set to 16 ml. To both solutions, 200 µl freshly prepare ammonium per sulfate (APS) and 10 µl TEMED was added and immediately 16 ml of it sucked with syringes of gradient former. The syringes were placed to their corresponding positions and the solutions were poured into the sandwich by turning the wheel of gradient former at a slow constant speed. When the gel sandwich was filled, 16 wells comb was placed carefully to avoid any bubble formation. The syringes were cleaned immediately with distilled water to prevent any polymerization in the syringes or capillaries. The polymerization was depending directly to the amount of APS and TEMED in solutions; usually took 60-90 minutes at room temp. During polymerization, electrophoresis tank was filled with 1xTAE until marked level and temperature was set to 650^0C. 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. The DGGE was conducted at a constant voltage of 200 V at 60^0C for 300 minutes 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. 20 µl of 1:100000 diluted SYBR Gold DNA staining dye was added to 300 ml 1xTAE washing buffer and gels were incubated for 30 minutes. Gels were distained and washed three times with distilled water to remove background impurity. Gels were visualized by using a gel documentation system, Mitsubishi 91.

For diversity analysis, DGGE images were converted, normalized and analyzed by using the Bionumerics 5.0 Software (Applied Maths, Kortrijk, 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 a band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at 100%.

5. RESULTS AND DISCUSSION

5.1. Serum Bottle Tests: Effects of Solvents on Biogas Production

Serum bottles which were fed with acetate and different solvents were operated for 40 days. In this time period three exposures were done. After each exposure when gas production reached a peak value, a gas sample was taken and next exposure of the same concentration was applied For each exposure, gas compositions and gas pressure values were recorded. Thereafter samples were taken from the batch reactors for RNA extraction. After the final exposure, samples for FISH were taken from the reactors.

Effect of Methanol on Methane Percentage and Methane Production

Various molarities of methanol were added to serum bottles and following each exposure maximum methane percentages were recorded by using gas chromotography. The decrease in the percent methane might be observed from Figure 5.1. by the addition of methanol comparing to the control reactor which produced 89% methane. Some findings which were not in accordance with the projected pattern were most probably caused by an experimental error which is fairly common while dealing with very small volumes. 15% decrease in percent methane were observed for 1.0 M methanol.

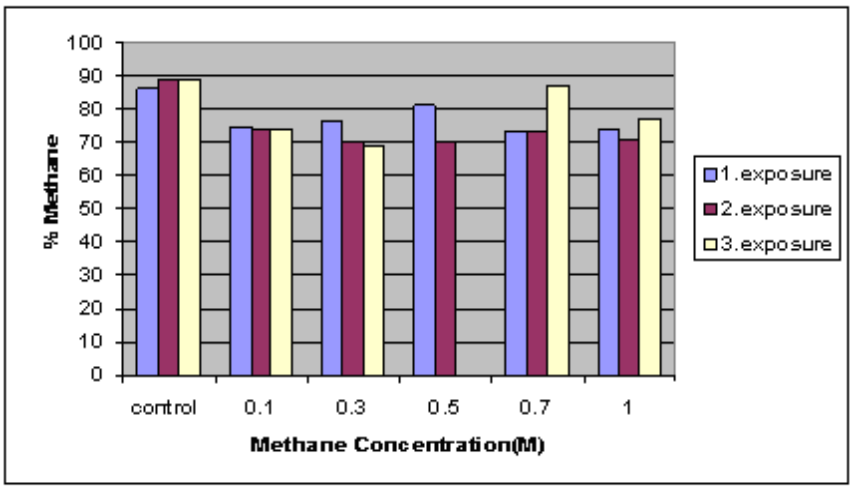


Figure 5.1. Effect of methanol on methane percentage.

In this study, successful degredation was observed for 0.1 M and 0.3 M methanol. It was found that methane production was higher than the control reactor due to the degradation of methanol. 48% and 79% inhibition were observed for 0.7 M and 1.0 M respectively. Figure 5.2 shows methane production of methanol added reactor.

Increase in methane production for lower concentrations was due to degredation of methanol which is degraded by methanogens, sulfate reducing bacteria and homoacetogens and supports a complex food chain in mixed cultures as stated in the literature. All methanol-utilizing methanogens isolated from anaerobic digesters are *Methanosarcina.* *Methanosarcina* converts methanol to methyl-coenzyme M by methyltransferases which are enzymes that have a cobalt-containing corrinoid as catalytic group (Vogels et al., 1988). In a previous study, by using 13C-labelled substrates and specific inhibitors it was shown that in an anaerobic methanol-fed thermophilic bioreactor, about 50% of the methanol, at a concentration of 37 mM in the anaerobic reactor, was directly converted to methane by methylotrophic methanogens, and about 50% via the intermediates H_2/CO_2 and acetate (Paulo et al., 2001, Paulo et al., 2003).

Beyond some limits, degradation tends to decrease as a result of inhibition to bacterial growth due to toxicity caused by high concentration of substrate (Bordel et al., 2007). In this study, 0.7 M and 1.0 M were beyond degradation limits for methanol.

In previous studies, IC_{50} value for methanol has been reported for a non granular sludge from a cattle rumen (Enright et al., 2005) and for a seed sludge from the EGSB reactor used at a brewery (Oz, 2007) to be 0.4 M. Enright et al. (2005) also investigated IC_{50} values for methanol in different anaerobic sludge types, including granular and non-granular sludges from different industries. IC_{50} concentrations for acetoclastic methane production have been found to be 0.95 M for methanol citric acid production wastewater and 1.35 M for a sludge treating alcohol distillery wastewater. The differences in IC_{50} concentrations of methanol most probably due to the use of different sources of inoculum sludge (Andrade and Buitrón, 2004). It has been already reported that the acclimation of an anaerobic sludge to a specific substrate may lead to a change in population that may be

quite different from that of the inoculum sludge due to several operational and nutritional factors (Anderson et al., 1994; Zhang and Noike, 1994).

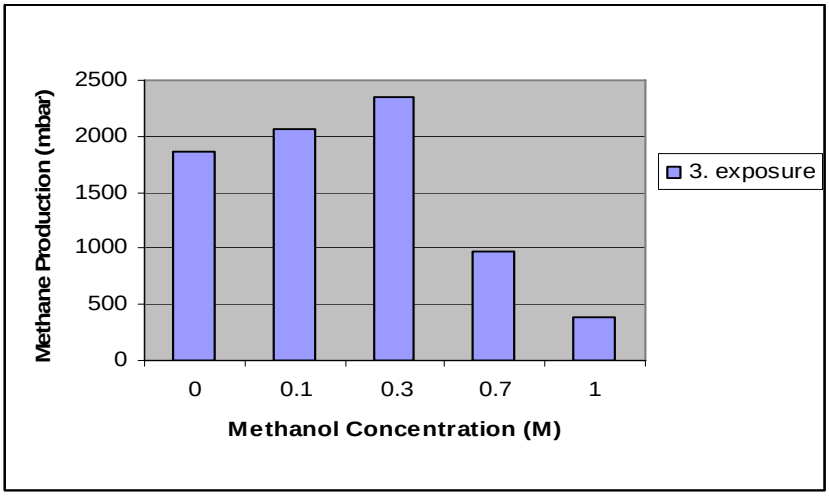


Figure 5.2. The effect of methanol on methane production.

Effect of Toluene on Methane Percentage and Methane Production:

As it can be observed from Figure 5.3., there is a stimulative effect of 0.5 mM toluene on methane percentage after the first exposure. Various concentrations of toluene didn't differ significantly from each other. In addition a decrease in %methane was reported after the first exposure and % methane decreased by 10% compared to control reactor.

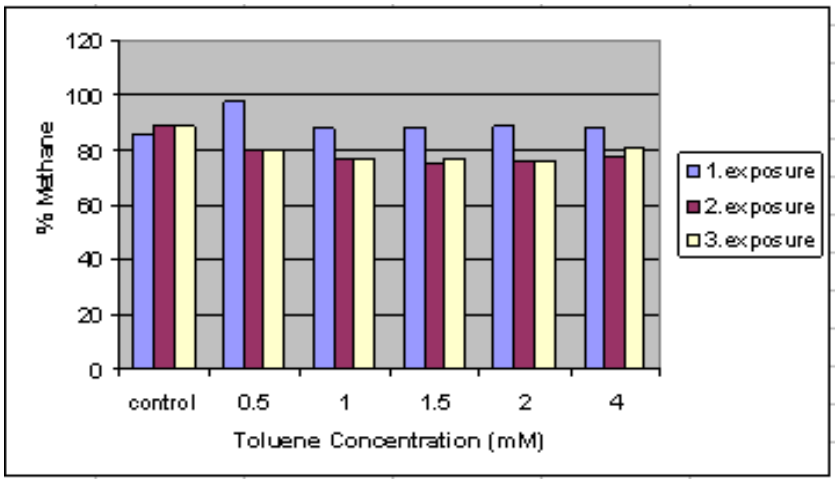


Figure 5.3. Effect of toluene on methane percentage.

Comparing to methanol added reactors, inhibition in toluene added reactors was very slight as shown in Figure 5.4. 4.0 mM toluene which corresponded to 386 mg/l gave rise to 50% inhibition. 1.0 mM toluene showed an unprojected pattern which might be caused by an experimental error. In a previous study, it was aimed to determine the effect of toluene on an anaerobic sludge taken from a full-scale upflow anaerobic sludge blanket (UASB) reactor in terms of potential activity and composition of acetoclastic methanogens. Specific methanogenic activity (SMA) test results indicated that 5%, 9.5%, 14%, 24%, 29%, 38% and 62% inhibition occurred in the potential methane production rate of the sludge at toluene concentrations of 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM and 1.0 mM, respectively (Ince et al., 2007). Similarly in the study of Oz (2008) SMA tests were carried out with the seed sludge from the EGSB reactor used in the anaerobic stage of a two stage anaerobic-aerobic biological treatment system at a brewery to determine IC_{50} concentration of toluene. 2000 mg/l acetate was used as substrate in the SMA test reactors and IC_{50} concentration of toluene was found to be 1.2 mM. In addition, in the study of Enright et al. (2005) a mesophilic anaerobic sludge was obtained from a full-scale (1500 m^3) internal circulation (IC) bioreactor, operated at 37°C for the treatment of citric acid production wastewater and IC_{50} concentrations were found to be 250 mg/l and 1350 mg/l for acetoclastic and hydrogenotropic methanogenic activity in the seed sludge respectively. The differences in inhibitory concentrations correlated with the literature saying that anaerobic biodegradability tests even for an easy-to-degrade substrates such as glucose shows a great variability due to the use of different sources of inoculum sludge

(Andrade and Buitrón, 2004) and also different operational and nutritional factors (Anderson et al., 1994; Zhang and Noike, 1994).

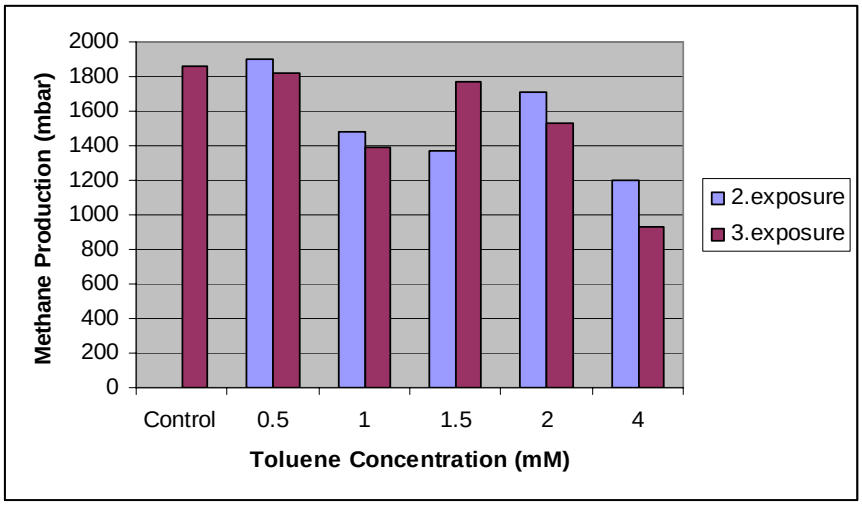


Figure 5.4. Effect of toluene on methane production.

Effect of Iso-propanol on Methane Percentage and Methane Production

Iso-propanol had the most pronounced effect on methane percentage compared to other solvents. Stimulative effect of less concentrated iso-propanol in the first exposure can be observed from Figure 5.5. There was a significant inhibitory effect for higher molarities of iso-propanol. The inhibitory effect became sharper after each exposure. 74% decrease in methane percentage was observed for 0.1 M iso-propanol addition and methane percentage could not be detected by the additon of 2.0 M iso-propanol after the third exposure.

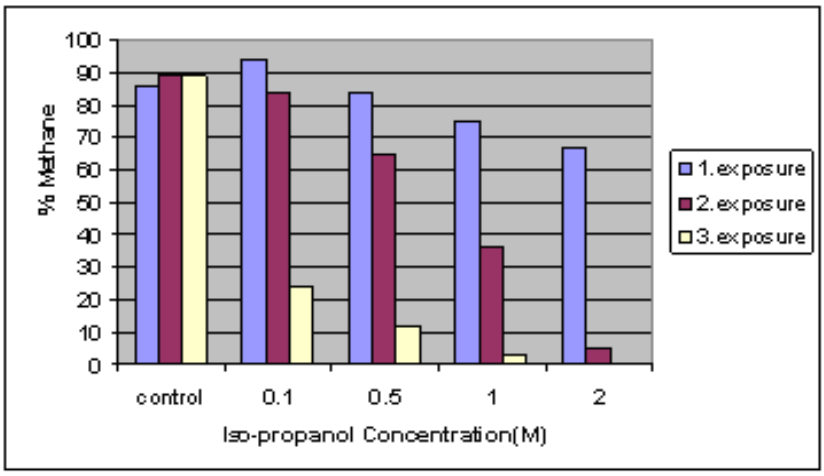


Figure 5.5. Effect of iso- propanol on methane percentage.

Iso-propanol had the most significant inhibition effect on methane production as shown Figure 5.6. Methane production couldn't be observed for 2.0 M iso-propanol. Decrease in methane production was much more drastic in the third exposure. 0.5 M iso-propanol gave rise to 50% inhibition after the second exposure. Parallel results were obtained in a previous study in which SMA tests for single phase anaerobic reactor were carried out with the seed sludge from the EGSB reactor used in the anaerobic stage of a two stage anaerobic-aerobic biological treatment system at a brewery. IC_{50} concentration of iso-propanol was found to be 0.4 M using 2000 mg/ L acetate as a substrate (Oz, 2008).

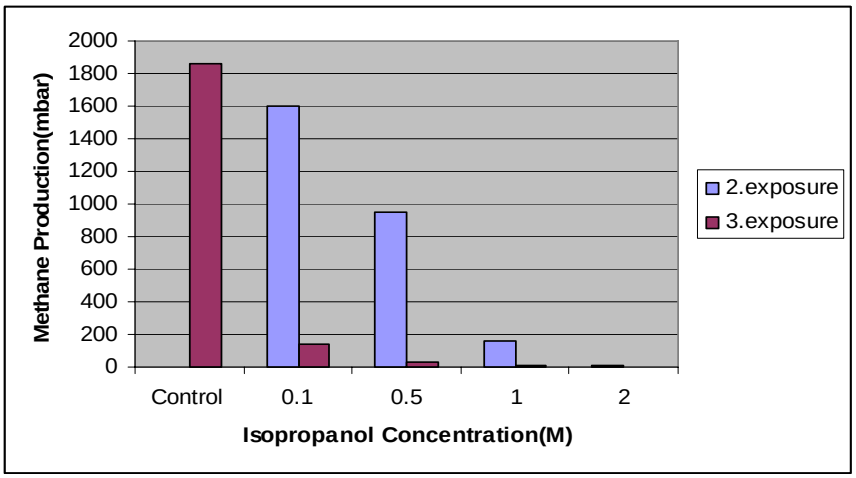


Figure 5.6. Effect of iso-propanol on methane production.

Effect of Methanol + Toluene on Methane Percentage and Methane Production

It was found that methane percentage of methanol + toluene added reactors decreased after each exposure as shown in Figure 5.7. A sudden decline in the methane percentage was observed after the third exposure. Lower concentration of solvents showed higher methane percentage. In the third exposure methane percentage of the more concentrated toluene added reactor was higher. This unexpected finding was most probably caused by an experimental error which is fairly common while dealing with very small volumes.

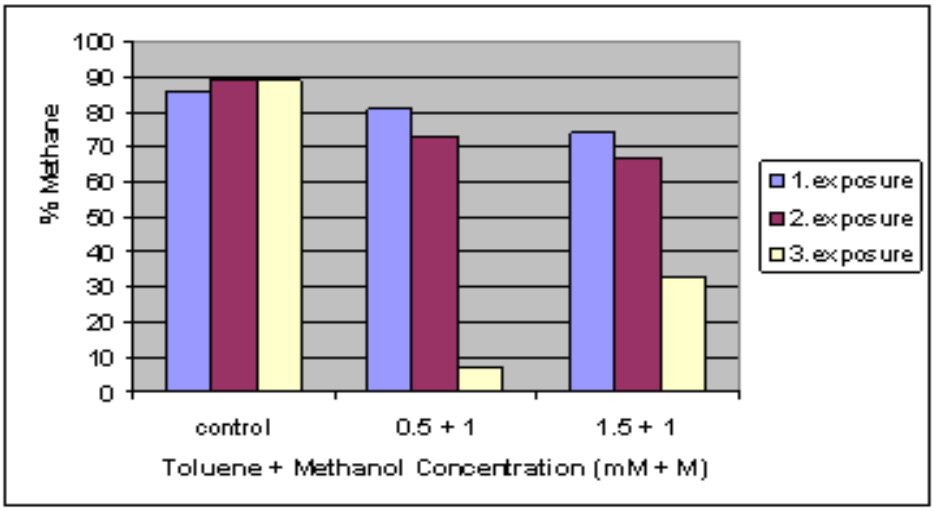


Figure 5.7. Effect of toluene + methanol on methane composition.

99% inhibition was observed after the third exposure for both concentrations of two mixtures (0.5 mM toluene +1.0 M methanol and 1.5 mM toluene + 1.0 M methanol). The results indicated that multiple efffect of solvents were more pronounced than individual effects of them**.** The multiple effects of toluene and methanol on methane production was given in Figure 5.8.

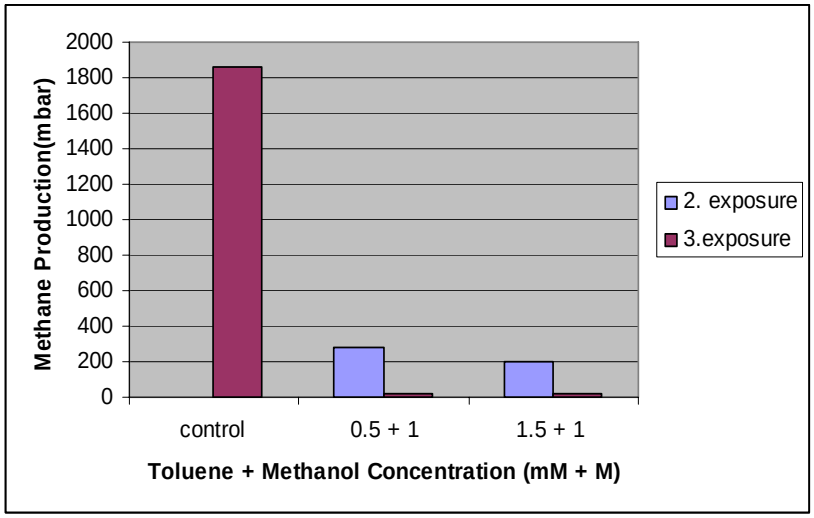


Figure 5.8. Effect of toluene + methanol on methane production.

5.2. Effects of Solvents on the Expression Level of Acetyl-coA Synthetase Gene

Before investigating the expression level of acetyl-CoA synthetase gene of *Methanosaeta concilii*, the presence of *Methanosaeta concilii* in the microbial community was initially confirmed by comparing DGGE band of a *Methanosaeta concilii* clone and DGGE bands of the sludge used in this study (Figure 5.9.). Bionumerics 5.0 was used to analyze the data obtained by DGGE.

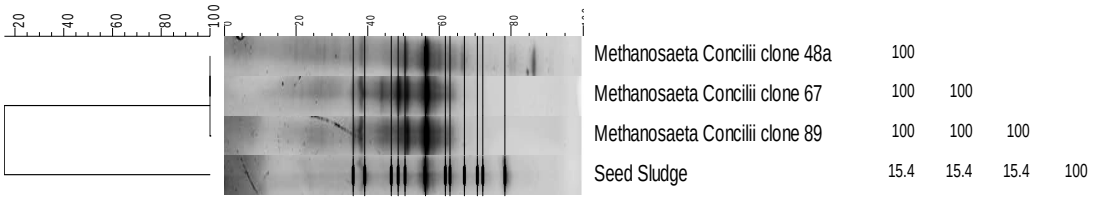


Figure 5.9. DGGE band of *Methanosaeta concilii.*

Q-PCR Results of Control and Solvent Added Reactors

Following each exposure, RNA of samples were extracted, RT-PCRs were run and acetyl-CoA synthetase genes were quantified by Q-PCR reaction. For control and methanol added reactors, it was found that there was an increasing pattern in number of genes after the second exposure. Number of acetyl-CoA synthetase gene for methanol added reactors were observed to be higher than the control reactor. The number of genes didn't change significantly at the end of the exposures compared to initial ones. The number of acetly-CoA synthetase genes counted were given in Table 5.1.

Table 5.1. Number of acetly-CoA synthetase genes for methanol added reactor.

Methanol (M)	1.exposure	2.exposure	3.exposure
Control	1.31 E+06	2.53 E+06	0.96 E+06
0.1	3.09 E+06	2.82 E+07	3.76 E+06
0.3	2.63 E+06	2.76 E+07	4.16 E+06
0.5	3.04 E+06	4.23 E+07	5.15 E+06
1	3.58 E+06	2.00 E+07	6.47 E+06

In toluene added reactors, in accordance with the results of methanol added reactor, again an increase in the number of acetyl-CoA synthetase gene was reported after the second exposure. However, after the third exposure, acetyl-CoA synthetase genes could not be detected. The number of acetly-CoA synthetase genes are given in Table 5.2.

Table 5.2. Number of acetly-CoA synthetase genes for toluene added reactor.

Toluene(mM)	1.exposure	2.exposure	3.exposure
Control	1.31 E+06	2.53 E+06	0.96 E+06
0.5	0.90 E+06	1.81 E+07	N. D.
1	0.30 E+06	3.11 E+07	N. D.
1.5	0.90 E+06	2.09 E+07	N. D.
2	0.33 E+06	1.16 E+06	N. D.
4	0.35 E+06	1.47 E+06	N. D.

N.D: not detected

The findings on the quantification of acetyl-CoA synthetase gene for iso-propanol were paralel with toluene added reactor. An increase in the number of genes was recorded after the second exposure and acetyl-CoA synthetase gene could not be detected after the third exposure as in the case of toluene added reactor. The number of acetly-CoA synthetase genes are given in Table 5.3.

Table 5.3. Number of acetly-CoA synthetase genes for iso-propanol added reactor.

Iso-propanol (M)	1.exposure	2.exposure	3.exposure
Control	1.31 E+06	2.53 E+06	0.96 E+06
0.1	0.50 E+06	2.37 E+06	N. D.
0.5	0.23 E+06	0.85 E+06	N. D.
1	0.43 E+06	0.63 E+06	N. D.
2	0.76 E+06	1.58 E+06	N. D.

N.D: not detected

The number acetly-CoA synthetase genes for toluene + methanol added reactor did not change significantly after the exposures. The number of genes were still detectable after the final exposure as in the case of methanol added reactor. The results were given in Table 5.4.

Table 5.4. Number of acetly-CoA synthetase genes for methanol + toluene added reactor.

Toluene (mM) + Methanol (M)	1.exposure	2.exposure	3.exposure
Control	1.31 E+06	2.53 E+06	0.96 E+06
0.5 + 1	1.35 E+06	3.55 E+06	3.55 E+06
1.5 + 1	2.70 E+06	0.53 E+06	5.54 E+06

5.3. FISH Results of Control and Solvent Added Reactors

Before viewing active populations, pre-existing populations in terms of microbial diversity in seed sludge was investigated by DGGE. Bionumerics 5.0 was used to analyze the data obtained by DGGE. To explore the microbial structure in the seed sludge archaeal and bacterial populations were investigated separately. In Figure 5.1, 1, 2 and 3 stand for DGGE marker, *Archaea* and bacteria respectively. According to the results, the population diversity in the bacterial domain was found to be considerably higher than the archaeal domain in the unfed seed sludge as it is previously reported (Collins et al., 2003). This can be attributed to availability of much more diverse substrate for the bacterial populations compared to archaeal populations represented mostly by the methanogens in anaerobic bioreactors. 23 bacterial and 10 archaeal bands have been observed each corresponded to a different specie.

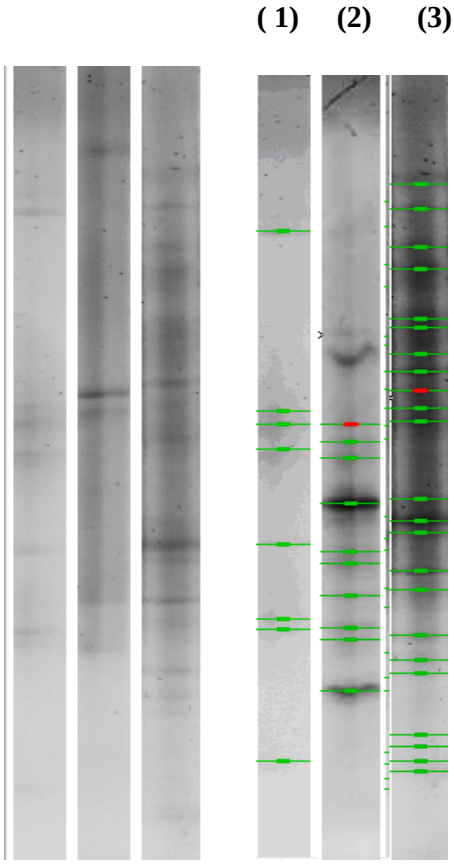
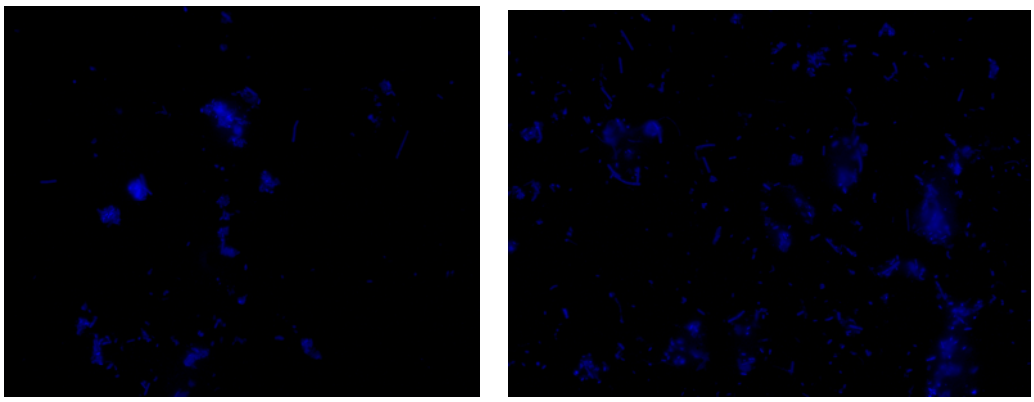


Figure 5.10. Unprocessed and processed DGGE picture.

The microbial community structure of the sludges taken from lab-scale anaerobic batch reactor were characterized using fluorescence rRNA targeted oligonucleotide probes specific for bacteria, *Archaea* and phylogenetically defined groups of methanogens. Sludge samples were initially stained by DAPI before hybridization to observe intact cell concentration. For each sample hybridization, two negative controls were used; one of these controls was used to assess nonspecific binding (with Non338 probe), and the other (lacking a probe) was used to monitor autofluorescence. In addition to negative controls, one positive control was used 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 stain to visualize intact cells in the samples. 10 random fields of views were used for each quantification study.

FISH Results of Control Reactor

As mentioned above, before hybridization, DAPI staining was applied to the sludge samples to indicate intact cell concentration. Eight different dilutions were used to find the optimum dilution to apply. 1/2 dilution factor and 15 µl sample volume were decided to be optimum and applied to the FISH analyses that are done during the study. DAPI results of the control sludge are shown in Figure 5.11.



(a) 1/2 dilution factor, 10 µL sample (b) 1/2 dilution factor, 20 µL sample

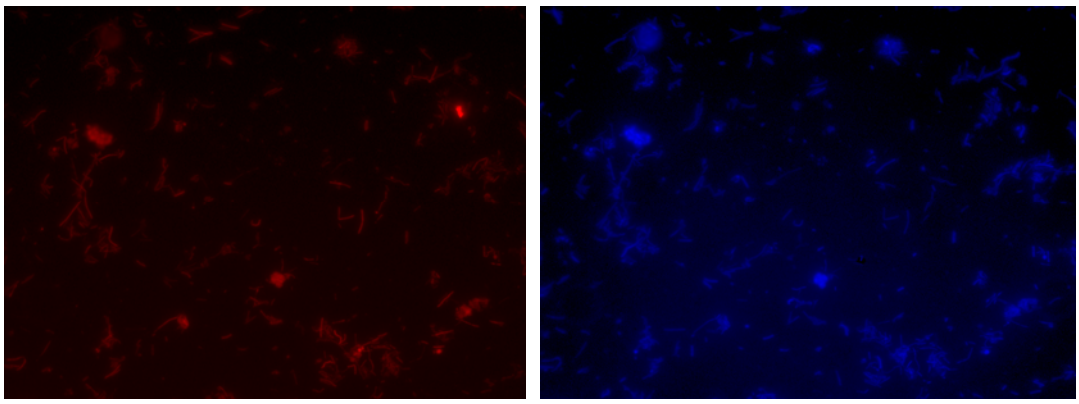
Figure 5.11. DAPI results of control sludge.

As can be seen from Table 5.5., total active microorganisms of the control reactor were found to be 27.2 ± 4.0% (mean ± standard deviation) (with Univ1392 probe). 18.2 ± 2.0% of the seed sludge consisted of eubacteria (with Eubmix probe) and archaeal population was detected as 10.5 ± 1.2% (with Arc915 probe) Similarly, Archaeal population in anaerobic reactors has been shown previously to range from 10% to 90% of the total prokaryotic cells in the literature (Raskin et al., 1994b and 1996; Harmsen et al., 1996; Ficker et al., 1999; Gonzalez-Gil et al., 2001; Tay et al., 2001; Saiki et al., 2002; Angenent et al., 2004). The archaeal subpopulation was composed of 29.1 ± 0.9% of members of the genus *Methanosaeta* (With Mx825 probe), 28.0 ± 1.5% *Methanosarcina* and relatives (with Ms1414 probe), 24.0 ± 1.3% *Methanobacteriales* (with Mb310 probe), 12.0 ± 0.4% *Methanococcales* (with Mc1109 probe), 2.1 ± 0.3% *Methanomicrobiales* (with Mg1200 probe). Epifluorescence micrographs of the control sludge are shown in Figure 5.12.

Dominance of *Methanosaeta* which is assumed to improve granulation and result in more stable reactor performance, (Hulshoff, 1988) under typical loadings and reactor configurations especially in UASB and EGSB reactors was previously shown in many other studies (Ficker et al., 1999; Merkel et al., Sekiguchi et al., 1999). In addition, Kolukirik (2004) investigated a full-scale UASB reactor treating an alcohol distillery effluent in terms of performance, acetoclastic methanogenic capacity and archaeal composition. The findings indicated that the archaeal subpopulation consists of 59 ± 2.6% of members of the genus *Methanosaeta* and 40 ± 1.3% *Methanobacteriales*.

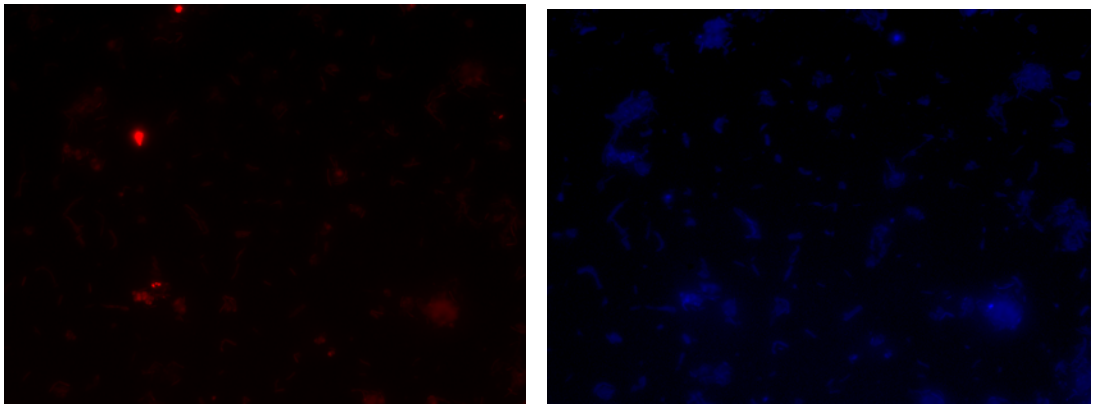
Some sources of the literature stated that in addition to *Methanosaeta,* other acetate-utilizing methanogenic genera *Methanosarcina* has been identified as an important methanogen in granular sludge from anaerobic reactors (De Zeeuw, 1984; Grotenhuis, 1988; Hulshoff, 1989; Schmidth, 1996). *Methanosaeta spp.* is known to grow only on acetate (Jetten et al., 1992). Besides acetate, *Methanosarcina spp.* is also capable of growing on substrates such as methanol, methylamines, and sometimes hydrogen and carbon dioxide. *Methanosaeta* spp. have a lower growth rate at high acetate concentrations than do *Methanosarcina spp*., but their affinity for acetate is 5 to 10 times higher (Zinder, 1990; Jetten et al., 1992).

Although acetate is the only substrate in the control reactor hdyrogenotropic methanogens were present in the system as previously cited in the literature (Schnuner et al., 1999). In the control sludge, the percentage of the hydrogenotrophic methanogens *Methanobacteriales* relatives (8.4% of the active microbial community) was higher than *Methanomicrobiales* and *Methanococcales* relatives. Similarly, some studies in the literature reported *Methanobacteriales* relatives as dominant in hydrogenotrophic methanogens. It was previously reported that among the hydrogenotrophic methanogens, *Methanobacteriales* followed by *Methanomicrobiales* were dominant methanogens and *Methanococcales* were almost absent within both full-scale and lab-scale UASB reactors (Hofman-Bang et al., 2003). Also *Methanobacteriales* such as *Methanobacterium* and *Methanobrevibacter* have been reported to be the dominant hydrogen and formate-consuming methanogens (Raskin et al., 1994) whereas in some other studies *Methanomicrobiales* were reported as dominant. For the other hydrogenotrophic methanogen *Methanococcales,* it was stated in the literature that they were almost absent within granular sludge (Hofman-Bang et al., 2003) or has been reported to play relatively small role in sludge (Raskin et al., 1995). The predominance of *Methanobacteriales* and less abundance of *Methanomicrobiales* and *Methanococcales* in our study is difficult to explain, since ecological significance of different hydrogen and formate utilizing methanogens and the competition for common substrates among these populations have been studied less extensively than the competition for acetate among acetoclastic methanogens (Raskin et al., 1996; McMahon et al., 2001; Stams et al., 2003).



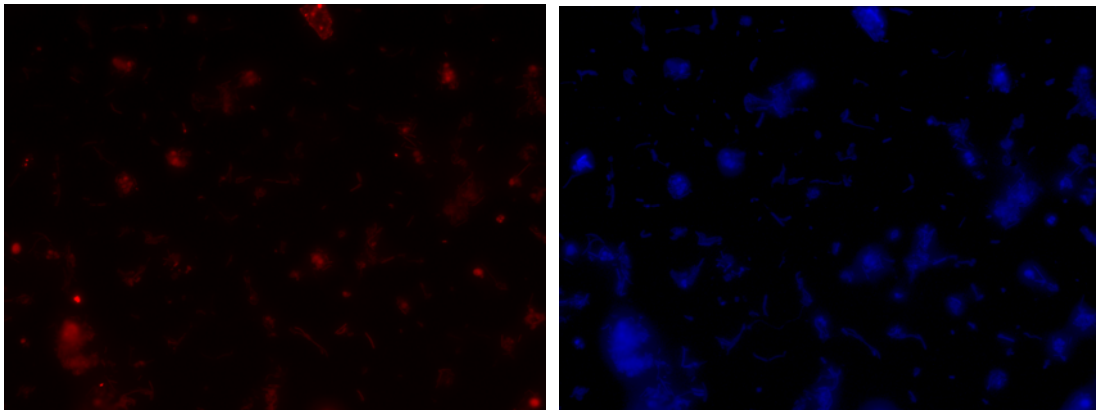
(a) (b)

Active cells hybridized with UNIV1392 probe.



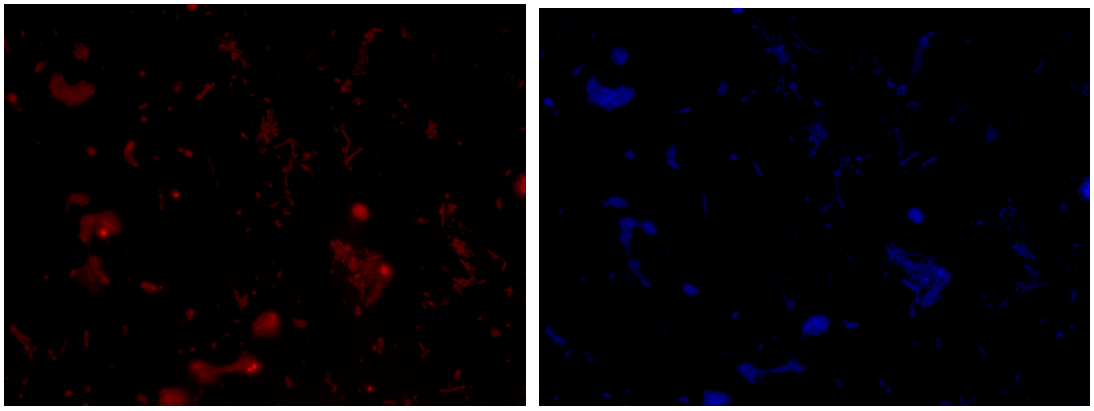
(a) (b)

Eubacteria hybridized with EUBMIX probe.



(a) (b)

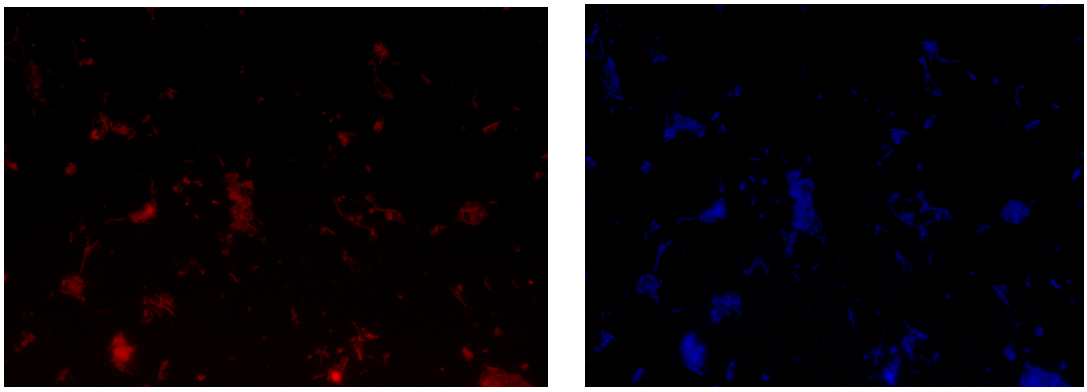
Archaea hybridized with ARC195 probe .



(a) (b)

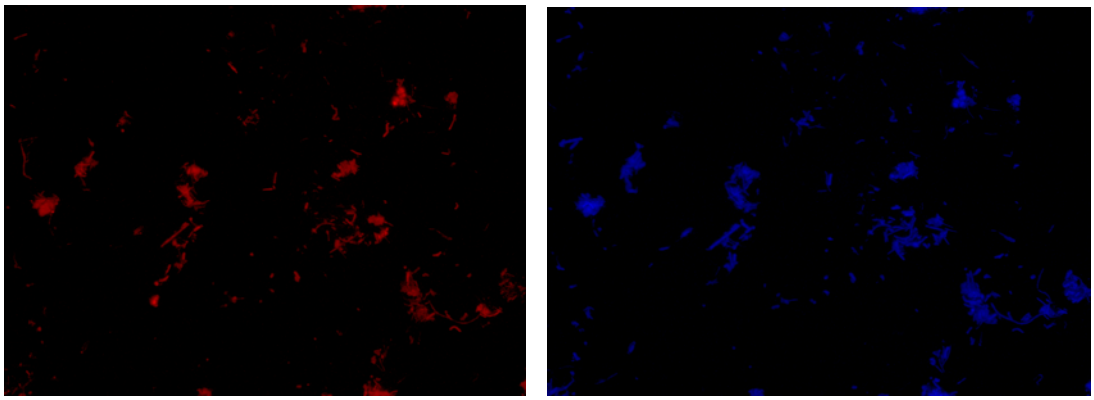
Methanosaeta hybridized with MX825 probe.

Figure 5.12. Epifluorescence micrographs of the hybridized sludge samples.
(a) Fluorescent and (b) DAPI images



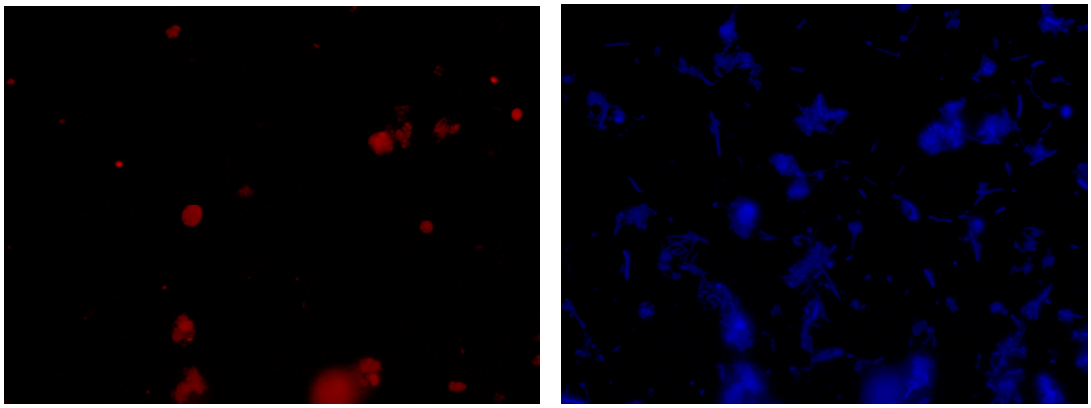
(a) (b)

Methanosarcina and relatives hybridized with MS1414 probe.



(a) (b)

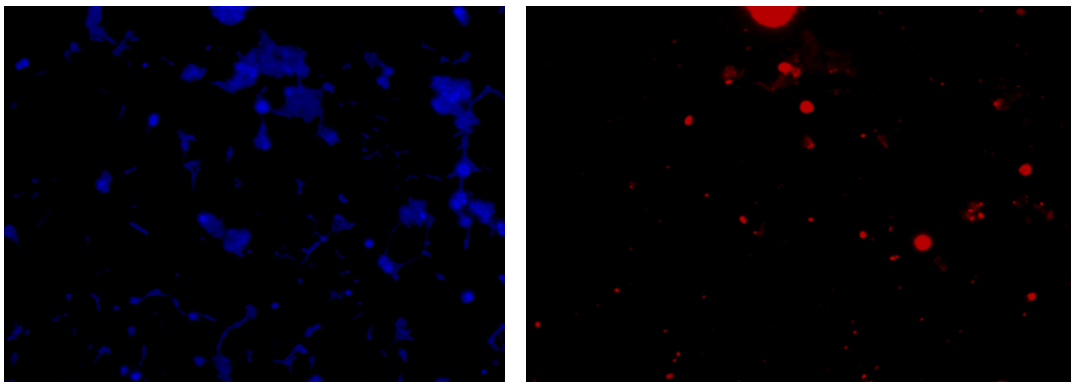
Methanobacteriales hybridized with MB310 probe.



(a) (b)

Methanococcales hybridized with MC1109 probe.

Figure 5.12. (continued) Epifluorescence micrographs of the hybridized sludge samples.
(a) Fluorescent and (b) DAPI images



(a) (b)

Methanomicrobiales hybridized with MG1200 probe.

Figure 5.12.(continued) Epifluorescence micrographs of the hybridized sludge samples. (a) Fluorescent and (b) DAPI images.

FISH Results of Methanol Added Reactors

In 0.3 M methanol added reactor 42.2 ± 1.2% of the cells in the lab-scale anaerobic batch reactor sludge gave positive signal with UNIV1392 probe, that is, 42.2% of the microorganisms were metobolically active. Bacterial and archaeal population were detected 30.4 ± 1.1% and 17.3 ± 2.0% respectively. The Archaeal subpopulation composed of 33.4 ± 0.8% of members of the genus *Methanosarcina* and relatives, 29.7 ± 0.4% *Methanosaeta*, 25.6 ± 1.2% *Methanobacteriales*, 14.4 ± 0.3% *Methanococcales*, 2.0 ± 1.2% *Methanomicrobiales* (Table 5.5.).

In the 1.0 M methanol added reactor 69.6 ± 0.2% of the cells were metobolically active which was an evident of the increase in percentage of active cells with the increase in methanol concentration. Bacterial and archaeal population were detected as 29.2 ± 0.4% and 40.3 ± 0.1% respectively. The archaeal subpopulation composed of members of the genus 37.5 ± 0.2% *Methanosaeta*, 25.1 ± 0.2% *Methanobacteriales*, 17.2 ± 0.4% of *Methanosarcina* and relatives, 14.9 ± 0.3% *Methanococcales*, 1.7 ± 0.3% *Methanomicrobiales*.

Methanosarcina and relatives were the dominant population and an increase in the total number of active cells was observed compared to control reactor in 0.3 M methanol added reactor. Jones (1991) stated that *Methanosarcina spp*. is the most versatile methanogen can use H_2/CO_2, methanol, methylated amines and pyruvate besides acetate supporting the increase in the number of *Methanosarcina ssp*. and relatives due to their ability to use methanol. Therefore, methanol became an additional substrate for microorganisms in the methanol added reactors whereas acetate was the only substrate in the control reactor.

Table 5.5. FISH results of methanol added reactors.

	Control sludge	0.3 M methanol added reactor	1.0 M methanol added reactor
Active Cells (%)	27.2 ± 4.0	42.2 ± 1.2	69.6 ± 0.2
Eubacteria (%)	18.2 ± 2.0	30.4 ± 1.1	29.2 ± 0.4
Achaea (%)	10.5 ± 1.2	17.3 ± 2.0	40.3 ± 0.1
Methanosaeta (%)	29.1 ± 0.9	29.7 ± 0.4	37.5 ± 0.2
Methanosarcina and relatives (%)	28.0 ± 1.5	33.4 ± 0.8	17.2 ± 0.4
Methanobacteriales (%)	24.0 ± 1.3	25.6 ± 1.2	25.1 ± 0.2
Methanococcales (%)	12.0 ± 0.4	14.4 ± 0.3	14.9 ± 0.3
Methanomicrobiales (%)	2.1 ± 0.3	2.0 ± 1.2	1.7± 0.3

Table 5.6. Standardized FISH results of methanol added reactor (% in active cell).

	Control Sludge	0.3 M methanol added sludge	1.0 M methanol added sludge
Methanosaeta	3.04	5.13	15.11
Methanosarcina and relatives	2.84	5.77	6.93
Methanobacteriales	2.42	4.42	10.11
Methanococcales	1.26	2.49	6.0
Methanomicrobiales	0.2	0.3	0.6

FISH Results of Toluene Added Reactors

FISH was also applied to the sludge samples taken from toluene added reactors in order to indicate the microbial community change in the sludge samples. In 0.5 mM toluene added reactor, active cells, eubacteria and archaeal population were detected as 74.6 ± 1.2%, 25.1 ± 0.9%, 49.0 ± 1.3% respectively. FISH results showed that the percentage of the genuses of *Methanobacteriales, Methanosaeta* and *Methanosarcina* were found as 50.0 ± 1.3%, 30 ± 0.3% and 10.1 ± 0.4%, respectively. In addition, archaeal subpopulation was composed of 8.1 ± 1.0% *Methanococcales*, 5.0 ± 1.1% *Methanomicrobiales*.

In 1.5 mM toluene added reactor, 72.1 ± 0.4% of the cells were metabolically active. 17.0 ± 1.6% and 54.3 ± 2.1% of these active cells were belonged to domain eubacteria and *Archaea* respectively. Archaeal subpopulation were consisted of 46.5 ± 1.3%, 27.6 ± 1.9%, 9.2 ± 1.3%, 6.0 ± 1.1% and 3.8 ± 0.2% of the genuses of *Methanobacteriales*, *Methanosaeta*, *Methanosarcina*, *Methanococcales* and *Methanomicrobiales* respectively.

In 4.0 mM toluene added reactor, 75.0 ± 0.4% of the cells were metabolically active. 20.0 ± 1.6% and 50.3 ± 2.1% of these active cells were eubacteria and *Archaea* respectively. Archaeal subpopulation were consisted of 46.1 ± 1.8%, 31.1± 1.2%, 9.3 ± 1.3%, 5.1 ± 1.1% and 2.2 ± 0.2% of the genuses of *Methanobacteriales*, *Methanosaeta*, *Methanosarcina*, *Methanococcales* and *Methanomicrobiales* respectively (Table 5.7.).

The increase in the toluene concentration didn't have an adverse affect on the number of active cells. Toluene added reactors had the highest active microbial population in number compared to other reactors including control reactor. Archaeal population was found to be higher than bacterial population which can be supported by the literature stating that toluene biodegredation starts in the methanogenesis stage with a pH 7.0-7.2 of anaerobic digestion, especially faster in the beginning of the metanogenesis. The degredation rate decreases after the stabilization of methanogenesis stage (Mrowiec et al., 2005). The results were inaccordance with previous studies. Oz (2008) investigated the effects of solvents and solvent mixtures on methanogenic activity and microbial composition of anaerobic sludge taken from single phase anaerobic reactor. FISH results

were given for IC_{50} concentrations of solvents. Methane production did not show a positive correlation with active microbial community in the case of toluene added reactor. Although decrease in the methane production was observed decrease in active cells could not be observed.

The dominance of *Methanobacteriales* was observed in the toluene added reactors. The population of *Methanosarcina* and relatives decreased significantly. *Methanosaeta* was found to be the second dominant genus in the reactor. Supporting the results, in a study of Oz (2008), an ASBR reactor was operated with toluene-containing synthetic wastewater. According to the FISH results, *Methanobacteriales* species was found to be the most abundant species following *Methanosaeta* and resistant to IC_{50} concentration of toluene (1.2 mM) toluene added anaerobic reactor during the study. In another study, the anaerobic sludge was dominated by acetoclastic genus *Methanosaeta* of which were slightly effected by increasing toluene concentrations. Toluene didn't have any effect on relative abundance of *Methanosaeta spp*. which was between 73% and 68% of the archaeal population (Ince et al., 2007). In addition, a full-scale upflow anaerobic sludge blanket (UASB) reactor was investigated in terms of archaeal composition, acetoclastic methanogenic capacity and performance over a 2-year period to observe the effect of toluene. An increase in the relative abundance of hydrogenotrophic *Methanobacteriales* was reported from non-detectable levels to 24% (Kolukirik et al., 2007). Enright et al. (2005) supports the results indicating the dominance of *Methanobacteriales* with the study in which two expanded granular sludge bed-anaerobic filter (EGSB-AF) bioreactors were operated at 15^0C for the treatment of toluene-contaminated volatile fatty acid-based wastewater. Metabolic assays suggested that a psychrotolerant H2/CO2-utilizing methanogenic community developed in the toluene degrading biomass (Enright et al., 2007a).

Table 5.7. FISH results of toluene added reactors.

	Control sludge	0.5 mM toluene added reactor	1.5 mM toluene added reactor	4.0 mM toluene added reactor
Active Cells (%)	27.2 ± 4.0	74.6 ± 1.2	72.1 ± 0.4	75.0 ± 0.4
Eubacteria (%)	18.2 ± 2.0	25.1 ± 0.9	17.0 ± 1.6	20.0 ± 1.6
Archaea (%)	10.5 ± 1.2	49.0 ± 1.3	54.3 ± 2.1	50.3 ± 2.1
Methanosaeta (%)	29.1 ± 0.9	30.0 ± 0.3	27.6 ± 1.9	31.1 ± 1.2
Methanosarcina and relatives (%)	28.0 ± 1.5	10.1 ± 1.4	9.2 ± 1.3	9.3 ± 1.3
Methanobacteriales(%)	24.0 ±1.3	50.0 ± 1.3	46.1 ± 1.8	46.5 ± 1.3
Methanococcales (%)	12.0 ± 0.4	8.1 ± 1.0	6.0 ± 1.1	5.1 ± 1.1
Methanomicrobiales (%)	2.1 ± 0.3	5.0 ± 1.1	3.8 ± 0.2	2.2 ± 0.2

Table 5.8. Standardized FISH results of toluene added reactors. (% in active cell).

	0.5 mM toluene added reactor	1.5 mM toluene added reactor	4.0 mM toluene added reactor
Methanosaeta	14.7	14.9	15.5
Methanosarcina	14.7	4.9	4.7
Methanobacteriales	24.5	24.5	23.4
Methanococcales	3.9	3.2	2.6
Methanomicrobiales	2.4	2.3	1.1

FISH Results of Iso- propanol Added Reactors

In 0.1 M iso-propanol added reactor, active cells, eubacteria and archaeal population were detected as 37.0 ± 1.1%, 16.1 ± 0.8%, 20.0 ± 1.3% respectively. FISH results indicated that the percentage of the genuses of *Methanosaeta, Methanobacteriales,* and *Methanosarcina* were found as 50.0 ± 0.2%, 25.0 ± 1.3%, and 12.5 ± 0.4%, respectively. In addition, archaeal subpopulation was composed of 7.5 ± 0.2% *Methanococcales*, 5.0 ± 1.1% *Methanomicrobiales.*

An increase in the percentage of active cells was observed with the increase in the concentration of iso-propanol as in the case of methanol added reactors. In 1.0 M iso-propanol added reactor active cells, eubacteria and archaeal population were detected as 49.3 ± 1.1%, 12.1 ± 0.8%, 34.2 ± 1.3% respectively. FISH results showed that the percentage of the genuses of *Methanosaeta, Methanobacteriales,* and *Methanosarcina* were found as 43.8 ± 0.2%, 16.1 ± 1.2%, and 26.9 ± 0.5% respectively. In addition, archaeal subpopulation was composed of 11.7 ± 1.2% *Methanococcales*, 3.1 ± 0.2% *Methanomicrobiales* as shown in Table 5.9.

Both of the iso-propanol added reactors showed a higher percentage of active population compared to control reactor which is an evident for degredation of iso-propanol to some extent. The findings were inaccordance with the previous studies which have shown that iso-propanol can be oxidised by hydrogenophilic methanogens to acetone during growth on H_2/CO_2 (Widdel, 1986; Widdel et al., 1988) and homoacetogenic bacteria capable of metabolising iso-propanol to acetate and higher fatty acids have also been reported (Eichler and Schink, 1984). Similarly, methanogenic activity tests had been carried out to determine the effect of solvents; methanol, toluene and iso-propanol on anaerobic sludge which was taken from brewery wastewater treating anarobic reactor operated in single phase lab-scale anaerobic batch reactor. IC_{50} concentrations of methanol, iso-propanol and toluene were applied to SMA test reactors. The smallest active population was observed for the iso-propanol added reactor among toluene and methanol added reactors (Oz, 2007). The predominance of *Methanosaeta* could be observed in the reactor followed by *Methanobacteriales*. The predominance order changes with the increased molarity of iso-propanol and *Methanosarcina* became the dominant genus in the reactor.

Table 5.9. FISH results of iso- propanol added reactors.

	Control sludge	0.1 M iso-propanol added reactor	1.0 M iso-propanol added reactor
Active Cells (%)	27.2 ± 4.0	37.0 ± 1.1	49.3 ± 1.1
Eubacteria (%)	18.2 ± 2.0	16.1 ± 0.8	12.1 ± 0.8
Archaea (%)	10.5 ± 1.2	20.0 ± 1.3	34.2 ± 1.3
Methanosaeta (%)	29.1 ± 0.9	50.0 ± 0.2	43.8 ± 0.2
Methanosarcina and relatives (%)	28.0 ± 1.5	12.5 ± 0.4	26.9 ± 0.5
Methanobacteriales(%)	24.0 ±1.3	25.0 ± 1.3	16.1 ± 1.2
Methanococcales(%)	12.0 ± 0.4	7.5 ± 0.2	11.7 ± 1.2
Methanomicrobiales(%)	2.1 ± 0.3	5.0 ± 1.1	3.1 ± 0.2

Table 5.10. Standardized FISH results of iso-propanol added reactors (% in active cell).

	0.1 M iso-propanol added reactor	1.0 M iso-propanol added reactor
Methanosaeta	10.0	15.0
Methanosarcina	2.5	9.2
Methanobacteriales	4.0	5.5
Methanococcales	1.5	4.0
Methanomicrobiales	1.0	1.1

FISH Results of Toluene + Methanol Added Reactors

In 0.5 mM toluene + 1.0 M methanol added reactor, active cells, eubacteria and archaeal population were detected as 38.0 ± 2.1%, 24.0 ±1.8% , 12.0 ± 2.3% respectively. FISH results showed that the percentage of the genuses of *Methanobacteriales,*

Methanosaeta, Methanosarcina, Methanococcales and *Methanomicrobiales* were found as 16.0 ± 1.2%, 50.4 ± 0.7%, 15.7 ± 0.4%, 15.4 ± 0.2% and 4.0 ± 1.1% respectively as shown in Table 5.11.

In 1.5 mM toluene + 1.0 M methanol added reactor active cells, eubacteria and archaeal population were detected as 31.3 ± 1.1%, 20.4 ± 0.8%, 10.1 ± 1.3% respectively. FISH results showed that the percentage of the genuses of *Methanobacteriales, Methanosaeta, Methanosarcina, Methanococcales* and *Methanomicrobiales* were found as 30.0 ± 1.1%, 30.2 ± 0.2%, 20.7 ± 0.3%, 19.0 ± 1.2% and 2.0 ± 0.8% respectively.

The multiple effect of solvents on the percentage of active population was much more pronounced than single effects of toluene and methanol. In contrast to the effects of single solvents, a decrease in the percentage of active cells was observed with the increase in concentration. The genuses *Methanobacteriales* and *Methanosaeta* dominated the microbial community as in the cases of methanol and toluene added reactors. By the increase in toluene concentration, an increase in the percentage of *Methanobacteriales* was observed in accordance with the previous findings indicating the high resistivity of *Methanobacteriales* especially in toluene added reactor.

Table 5.11. FISH results of toluene + methanol added reactors.

	Control sludge	0.5 mM toluene +1.0 M methanol added reactor	1.5 mM toluene +1.0 M methanol added reactor
Active Cells (%)	27.2 ± 4.0	38.0 ± 2.1	31.3 ± 1.1
Eubacteria (%)	18.2 ± 2.0	24.0 ± 1.8	20.4 ± 0.8
Archaea (%)	10.5 ± 1.2	12.0 ± 2..3	10.1 ± 1.3
Methanosaeta (%)	29.1 ± 0.9	50.4 ± 0.7	30.2 ± 0.2
Methanosarcina and relatives (%)	28.0 ± 1.5	15.7 ± 0.4	20.7 ± 0.3
Methanobacteriales(%)	24.0 ±1.3	16.0 ± 1.2	30.0 ± 1.1
Methanococcales(%)	12.0 ± 0.4	15.4 ± 0.2	19.0 ± 1.2
Methanomicrobiales(%)	2.1 ± 0.3	4.0 ± 1.1	2.0 ± 0.8

Table 5.12. Standardized FISH results of methanol + toluene added reactors (% in active cell).

	0.5 mM toluene +1.0 M methanol added reactor	1.5 mM toluene + 1.0 M methanol added reactor
Methanosaeta	6.0	3.1
Methanosarcina	1.9	2.1
Methanobacteriales	2.0	3.0
Methanococcales	1.8	1.9
Methanomicrobiales	0.5	0.2

The results of the study can be summarized as:

- The number of acetyl-CoA synthetase gene of *Methanosaeta concilii* did not change significantly for the methanol and toluene + methanol added reactors. However, the gene could not be detected for the toluene and iso-propanol added reactors.
- Inhibition in methane production for selected concentrations of solvents was observed for iso-propanol, methanol + toluene, methanol and toluene in decreasing order.
- According to FISH results, percentage of active cells observed for toluene, methanol, iso-propanol and toluene + methanol reactors was in decreasing order. Control reactor had the lowest percentage of active cells. This could be due to single type of substrate (acetate) feeding.

According to the seed sludge used and reactor type, the results obtained may vary a lot in the literature. Table 5.13. shows IC_{50} concentrations of solvents used in this study and other studies gathered from literature.

There are many studies about degradation of organic solvents in anaerobic processes in literature, however number of recent studies regarding inhibition are limited. According to Table 5.13. IC_{50} concentration of methanol for different seed sludges and reactor types is in the range of 0.4 – 1.35 M. Initial inhibition concentration and IC_{50} concentrations for toluene also vary in literature. Although Edwards and Galic (1994) could not observe any inhibition up to 1.8 mM, Ghosh et al. (1996) found 0.5 mM to be initial inhibition

concentration. IC_{50} concentration found for toluene was 4.0 mM in this study and 1.2 mM reported by Oz in 2008. Regarding IC_{50} concentration for iso-propanol, the results of this study and the study of Oz were close to each other which might be due to the similar seed sludges used.

Table 5.13.Comparative studies for inhibition.

Solvent	Author	Type	Seed sludge	IC_{50}
Methanol	Enright et al. (2005)	EGSB lab scale	granular sludge from a citric acid production plant	0.95 M
	Enright et al. (2005)	EGSB lab scale	sludge treating alcohol distillery wastewater	1.35 M
	Ayman Oz (2005)	lab-scale anaerobic batch reactor	from the EGSB reactor used at a brewery	0.4 M
	This study	lab-scale anaerobic batch reactor	from the UASB reactor treating raki wastewater	0.7 M
Toluene	Edwards and Galic (1994)	Lab-scale 250 ml microcosm	a creosote-contaminated sediment	Up to 1.8 mM No inhibition
	Ghosh et al. (1996)	continuous-flow,mesophilic plug-flow digester	Anaerobic inoculum from digesters in the waste water treatment plant.	0.5 mM no inhibition
	Oz (2008)	lab-scale anaerobic batch reactor	from the EGSB reactor used at a brewery	1.2 mM
	This study	lab-scale anaerobic batch reactor	from the UASB reactor treating raki wastewater	4 mM
Iso-propanol	Oz (2008)	lab-scale anaerobic batch reactor	from the EGSB reactor used at a brewery	0.4 M
	This study	lab-scale anaerobic batch reactor	from the UASB reactor treating raki wastewater	0.5 M (After second exposure)

Table 5.14. Comparative studies for FISH method.

Solvent	Study	Type	Seed sludge	Dominant genus
Methanol	Ayman Oz (2008)	lab-scale anaerobic batch reactor	from the EGSB reactor used at a brewery	*Methanosaeta*
	Gözdereliler (2008)	lab-scale anaerobic batch reactor	from the EGSB reactor used at a brewery	*Methanosaeta* and *Methanosarcina*
	This study	lab-scale anaerobic batch reactor	from the UASB reactor treating raki wastewater	*Methanosaeta*
Toluene	Kolukirik et al. (2007)	A full-scale upflow anaerobic sludge blanket (UASB) reactor	from the EGSB reactor used at a brewery	Significant increase in *Methanobacteriales*
	Ayman Oz (2008)	lab-scale anaerobic batch reactor	from the EGSB reactor used at a brewery	Significant increase in *Methanobacteriales*
	This study	lab-scale anaerobic batch reactor	from the UASB reactor treating raki wastewater	*Methanobacteriales*
Iso-propanol	Ayman Oz (2008)	lab-scale anaerobic batch reactor	from the EGSB reactor used at a brewery	*Methanosaeta*
	This study	lab-scale anaerobic batch reactor	from the UASB reactor treating raki wastewater	*Methanosaeta*

There are very few studies regarding effects of solvents on microbial community structure. Therefore, FISH results were only compared with very limited number of studies (Kolukirik, 2007; Gözdereliler, 2008, Oz, 2008). When methanol was used as an organic solvent, *Methanosaeta* was found to be the dominant genus in studies given in Table 5.14. Dominance or increase of *Methanobacteriales* observed in toluene added reactors in studies of Kolukirik et al. (2007) and Oz (2008) was in accordance with the findings of this study. For iso-propanol added reactors, dominance of *Methanosaeta* was reported in this study and by Oz (2008). Parallelity in dominant genuses that were found in these studies can be due to similar type of seed sludges used.

6. CONCLUSION

In this study, single and multiple effects of methanol, toluene, iso-propanol and methanol + toluene on methane production, expression level of acetyl-CoA synthetase gene of *Methanosaeta* and microbial community structure were investigated.

According to the findings of the study for methanol added reactor, succesful degredation was observed for 0.1 M and 0.3 M methanol. Methane production was higher than the control reactor due to the degradation of methanol. Beyond 0.7 M methanol, degradation tended to decrease as a result of inhibition to bacterial growth due to toxicity caused by high concentration of substrate and decrease in methane production was observed. In toluene added reactor methane production was decreased with increasing concentrations and 50% inhibition was observed for 4.0 mM toluene added reactor. Iso-propanol had the most pronounced effect on biogas production. The results obtained for exploring the multiple effects of methanol + toluene indicated that multiple efffect of solvents was harsher than single effects of solvents and has the second most severe effect on biogas production.

In the methanol and methanol + toluene added reactors the number of acetyl-CoA synthetase gene increased following the second exposure. However, after the third exposure the number of acetyl-CoA synthetase genes didn't change significantly. In the toluene and propanol added reactors an increase in the number of acetyl-CoA synthetase genes was observed in accordance with the methanol added reactor. However acetyl-CoA synthetase genes could not be detected after the final exposure.

According to the FISH results, toluene added reactor had the highest active population and the percentage of the active population did not changed with increasing concentrations of toluene. *Methanosaeta* was found to be the second dominant population following *Methanobacteriales* in toluene added reactor. *Methanosaeta* was generally the dominating genus in other solvent added reactors. FISH results showed that the dominating genuses in the reactor were *Methanosaeta, Methanobacteriales* and *Methanosarcina* respectively. In addition, due to the FISH results, *Methanomicrobiales* had the lowest

population in all reactors and *Methanococcales* usually had a bigger population than *Methanomicrobiales* but smaller than other genuses. The results showed that there was a distinct shift from acetoclastic methanogens to hydrogenotrophic methanogens in response to stress conditions. *Methanobacteriales* were found to be more resistant to higher concentrations as in other reactors. The increase in the addition of toluene to methanol gave rise to a decrease in microbial community conflicting with single effects of solvents. Multiple effects of solvents had more adverse effect on active microbial cells.

FISH results obtained didn't show a positive correlation between active population and methane production. It is interesting to conclude that high percentage of active microbial community was not observed to be necessarily an evidence for high methane production. According to the Q-PCR results it was found that toluene and iso-propanol inhibited the acetyl- CoA synthetase enzyme which is the one of the reasons of decrease in methane production. Although the inhibition of acetyl- CoA synthetase enzyme, the active percentage of *Methanosaeta concilii* which is known to grow only on acetate increased. It might be an evidence for *Methanosaeta concilii* might be much more metabolicly diverse than previously thought.

7. RECOMMENDATIONS

This study is a part of a completed project (106Y241, 'Determination of Interaction between Anaerobic Treatment of Organic Solvent Containing Industrial Wastewater with Its System Dynamics Using Molecular Tools'). In this project, effects of chosen solvents on an anaerobic sludge taken from a full scale UASB reactor treating alcohol (raki) distillery wastewater. In this study, the effect of solvents on methanogenic activity, acetyl-CoA pathway and microbial community of the sludge was evaluated. However, it should be mentioned that the study has been carried out with non-acclimated sludges in order to determine the acute effect of methanol on microbial diversity. For further study, the study should be carried out by acclimated sludge to selected solvents.

The main focus in this study was the identification of methanogens. Proteobacterial population should be also identified and quantified to clearly understand non-methanogenic steps together with methanogenic archaeal composition.

This study might be enlenghtened in time and done with a bigger scale lab reactors to better understand and interpret the effects of solvents on methanogenic activity, acetyl-CoA pathway and microbial community in the long run.

In this study the effect of solvents on the acetyl-CoA pathway was investigated by quantification of mRNAs by Q-PCR technique and microbial community were investigated by FISH. Additionally MAR-FISH method might be used to evaluate substrate uptakes by different phylogenetic groups by using radio-labeled subtrates. The technique can help to achieve a better understanding of metabolism of microorganisms.

REFERENCES

Aceti, D.J, Ferry, J.G. 1988. Purification and characterization of acetate kinase from acetate-grown Methanosarcina thermophila. Evidence for regulation of synthesis. Journal of Biological Chemistry, 263 (30), 15444-15448.

Andrade, I.M., Buitron, G., 2004. Influence of the origin of the inoculum on the anaerobic biodegradability test. Water Science and Technology, 49, 11, 53-59.

Alagappan, G., Cowan, R., 2001. Biokinetic models for representing the complete inhibition of microbial activity at high substrate concentrations. Biotechnology and Bioengineering, 75, 4, 393-405.

Alagappan, G., Cowan, R., 2003. Substrate inhibition kinetics for toluene and benzene degrading pure cultures and a method for collection and analysis of respirometric data for strongly inhibited cultures. Biotechnology and Bioengineering, 83, 7, 798-809.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D. A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology, 56, 1919-1925.

Amann, R.I., Ludwig, W., Schleifer K., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiological Reviews, 59, 143-169.

Amann, R.I., 1995. Fluorescently-labelled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. Molecular Ecology, 4, 543–554.

Amann, R.I., Fuchs, B.M., Behrens, S., 2001. The identification of microorganisms by fluorescence *in situ* Hybridization. Current Opinion in Biotechnology, 12, 231–236.

Anderson, G.K., Kasapgil, B., Ince, O., 1994. A microbial study of two-stage anaerobic digestion during start up. Water Research, 28, 11, 2382-2392.

Angelidaki, I., Ellegaard, L., Ahring, B.K., 1993. A mathematical model for dynamic simulation of anaerobic digestion of complex substrates: focusing on ammonia inhibition. Biotechnology and Bioengineering, 42, 159–166.

Angenent, L.T., Sung, S., Raskin, L., 2004. Formation of granules and *Methanosaeta* fibres in an anaerobic migrating blanket reactor (AMBR). Environmental Microbiology, 6, 315-322.

APHA-AWWA-WPCF., 1997. Standard methods for the examination of water and wastewater. 18th edition, Washington, D.C. American Public Health Association.

Araujo, J.C., Brucha, G., Campos, J.R.,Vazoller R.F., 2000. Monitoring the development of anaerobic biofilms using fluorescent *in situ* hybridization and confocal laser scanning microscopy. Water Science and Technology, 41, 69.

Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R., Wolfe, R.S., 1979. Methanogens: Reevaluation of a unique biological group. Microbiological Reviews, 40, 260-296.

Beller, H.R., Edwards, E.A., 2000. Anaerobic toluene activation by benzylsuccinate synthetase in a highly enriched methanogenic culture. Applied and Environmental Microbiology, 66, 12, 5503-5505.

Bhattacharya, S.K., Safferman, A.G., 1989. Determination of bioavailable nickel concentrations in inhibited methanogenic systems. Environmental Technology Letters, 10 (8), 725–730.

Blum, D.J.W., Speece, R.E., 1991. A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. Journal Water Pollution Control Federation, 63, 198–207.

Bordel, S., Muñoz, R., Díaz, L.F., Villaverde, S., 2007. New insights on toluene biodegradation by *Pseudomonas putida* F1: Influence of pollutant concentration and excreted metabolites. Applied Microbiology and Biotechnology, 74, 857-866.

Borja, R., Sanche´z, E., Duran, M.M., 1996. Effect of the clay mineral zeolite on ammonia inhibition of anaerobic thermophilic reactors treating cattle manure. Journal of Environmental Science and Health, 31 (2), 479–500.

Braun, M., Stolp, H., 1985. Degradation of methanol by a sulfate reducing bacterium. Archeal Microbiology, 142, 77–80.

Cattony, E.B., Chinalia, F.A, Ribeiro, R., Zaiat, M., Foresti, E., Varesche, M.B., 2005. Ethanol and toluene removal in a horizontal-flow anaerobic immobilized biomass reactor in the presence of sulfate. Biotechnology and Bioengineering, 91, 244-53.

Chen, Y., Chen, J.J., Creamer, K.S., 2007. Inhibiton of anaerobic digestion process: A review. Bioresource Technology, 99, 4044-4064.

Cicerone, R. J., Oremland, R. S., 1988. Biogeochemical aspects of atmospheric methane. Global Biogeochemistry Cycles, 2 (4), 299–327.

Collins, G., Woods, A., McHugh, S., Carton, M.W., O'Flaherty, V., 2003. Microbial community structure and methanogenic activity during start-up of psychrophilic anaerobic digesters treating synthetic industrial wastewaters. FEMS Microbiology Ecology, 46, 159-170.

Dahllöf, I., 2002. Molecular community analysis of microbial diversity. Current Opinion in Biotechnology, 13, 213–217.

De Bok, F.A.M., Plugge, C.M., Stams, A.J.M., 2004. Interspecies electron transfer in methanogenic propionate degrading consortia. Water Research, 38, 1368-1375.

Delbe´s, C., Moletta, R., Godon, J.J., 2001. Bacterial and archaeal 16S rDNA and 16S rRNA dynamics during an acetate crisis in an anaerobic digester ecosystem. FEMS Microbiology Ecology, 35, 19–26.

De Long, E.F., Wickham, G.S., Pace N.R., 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single microbial cells. Science, 243, 1360-1363.

De Witt., 1999. Toluene and xylenes annual, De Witt & Company Inc., Houston, Texas, 143–181.

De Zeeuw, W., 1984. Acclimatization of anaerobic sludge for UASB-reactor start-up. PhD thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Dinopolou, G., Sterritt, R.M., Lester, J.N., 1988. Anaerobic acidogenesis of complex wastewater. 2. Kinetics of growth, inhibition, and product formation. Biotechnology and Bioengineering, 31, 969–978.

Driessen, W., Tielbaard, M., Habets, L. and Yspeert, P., 2000. Anaerobic treatment of evaporator condensates from the chemical pulp industry. In “VI Latin-American Workshop and Seminar on Anaerobic Digestion” 127–135, Pernambuco, Brazil.

Eastmen, J.A., Ferguson, P., 1981. Solubilization of particulate carbon during the acid phase of anaerobic digestion. Journal Water Pollution Control Federation, 53,352-366.

Edlund, A., Jansson, J.K., 2006. Changes in active bacterial communities before and after dredging of highly polluted Baltic Sea sediments. Applied and Environmental Microbiology, 72, 6800–6807.

Edwards, E.A., Edwards, A.M., Grbic-Galic, D., 1994. A method for the detection of metabolites at very low concentration: application to the detection of metabolites of anaerobic toluene degradation. Applied and Environmental Microbiology, 60, 323–327.

Edwards, E.A., Grbic-Galic, D., 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Applied and Environmental Microbiology, 60, 313-322.

Edwards, E.A., Wills, L.E., Reinhard, M., Grbic-Galic, D., 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Applied and Environmental Microbiology, 58, 794-800.

Eggen, R., Geerling A., Boshoven, A., De Vos, W., 1991. Cloning, sequence analysis, and functional expression of the acetyl coenzyme a synthetase gene from methanothrix soehngenii in escherichia coli. Journal of Bacteriology, 173, 6383-6389.

Eichler, B., Schink, B., 1984. Oxidation of primary aliphatic alcohols by *Acetobacterium carbinolicum sp*. nov., a homoacetogenic anaerobic. Archives of Microbiology, 140, 147-52.

Enright, A., McHugh, S., Collins, G., O'Flaherty, V., 2005. Low-temperature anaerobic biological treatment of solvent containing pharmaceutical wastewater. Water Research, 39, 4587–4596.

Enright, A., Collins, G. O'Flaherty, V., 2007a. Low-temperature anaerobic biological treatment of toluene-containing wastewater. Water Research, 41, 7, 1465-1472.

Enright, A., Collins, G. O'Flaherty, V., 2007b. Temporal microbial diversity changes in solvent-degrading anaerobic granular sludge from low-temperature (15 °C) wastewater treatment bioreactors. Systematic and Applied Microbiology, 30, 471–482.

Evans, P.J., Ling, W., Goldschmidt, B., Ritter, E.R., Young, L. Y., 1992. Metabolites formed during anaerobic transformation of toluene and o-xylene and their proposed relationship to the initial steps of toluene mineralization. Applied and Environmental Microbiology, 58, 496-501.

Fernandez, A., Huang, S., Seston, S., Xing, J., Hickey, R., Criddle, C., Tiedje, J., 1999. How stable is stable? function versus community composition. Applied and Environmental Microbiology, 65, 3697–3704.

Ficker, M., Krastel, K., Orlicky, S., Edwards, E., 1999. Molecular characterization of a toluene-degrading methanogenic consortium. Applied and Environmental Microbiology, 65, 5576–5585.

Fielding, E.R., Archer, D.B., De Macario, E.C., Macario, A.J.L., 1988. Isolation and characterization of methanogenic bacteria from landfills. Applied and Environmental Microbiology, 54(3), 835-836.

Florencio, L., Field, J.A., Lettinga, G., 1994. Importance of cobalt for individual trophic groups in an anaerobic methanol-degrading consortium. Applied and Environmental Microbiology, 60, 227-234.

Florencio, L., 1994. The fate of methanol in anaerobic bioreactors. Ph.D thesis, Wageningen University.

Fox, P., Ketha, S., 1996. Anaerobic treatment of high-sulphate wastewater and substrate interactions with isopropanol. Journal of Environmental Engineering, 122, 989–994.

Fricke, W.F., Seedorf, H., Henne, A., Krüer, M., Liesegang, H., Hedderich, R., Gottschalk, G., Thauer, R.K., 2006. The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H2 for methane formation and ATP synthesis. Journal of Bacteriology, 188(2), 642-58.

Fries, M.R., Zhou, J., Chee-Sandford, J., Tiedje, J.M., 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Applied and Environmental Microbiology, 60, 2802-2810.

Garcia, J. L., Patel, B.K.C., Ollivier, B., 2000. Taxonomic phylogenetic and ecological diversity of methanogenic *Archaea*. Anaerobe, 6(4), 205-226.

Gerardi, M.H., 2003. The Microbiology of anaerobic digesters: John Wiley & Sons Inc. ISBN 0-471-20693-8, 51–57, New Jersey.

Ghosh, S., Liu T., Fukushi, K. 1996. Anaerobic biodegredation of toluene in a plug-flow reactor. Proceedings of the HSRC/WERC Joint Conference on the Environment.

Giovannoni, S.J., DeLong., E.F, Olsen, G.J., Pace, N.R., 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of whole microbial cells. Journal of Bacteriology, 170, 720-726.

Gonzalez-Gil, G., Kleerebezem, R., Lettinga, G., 1999. Effects of nickel and cobalt on kinetics of methanol conversion by methanogenic sludge as assessed by on-line CH4 monitoring. Applied and Environmental Microbiology, 65, 1789–1793.

Gözdereliler, E., 2008. Evaluation of methanol effect on an anaerobic sludge using methanogenic activity measurements and fluorescent *in situ* hybridization, M.S. Thesis, Bogazici University.

Grbić-Galić, D., Vogel, T.M., 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Applied and Environmental Microbiology, 53, 2, 254–260.

Grotenhuis, J.T.C., Koornneef, E., Plugge, C.N., 1988. Immobilization of anaerobic bacteria in methanogenic aggregates. In Lettinga, G., Zehnder, A. J. B., Grotenhuis,T. C. L. Hulshoff, W.(ed.), Granular anaerobic sludge: microbiology and technology. Pudoc Wageningen, Wageningen, The Netherlands.

Gujer, W., Zehnder, A.J.B., 1983. Conversion processes in anaerobic digestion. Water Science and Technology, 15, 49-77.

Harmsen, H.M, Kengen, H.M.P., Akkermans, A.D.L., Stams, A.J.M., De Vos, W.M., 1996. Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by *in situ* hybridization using 16S rRNA-based oligonucleotide probes. Applied and Environmental Microbiology, 62, 1656–63.

Harper, S. R., Pohland, F.G., 1986. Recent developments in hydrogen management during anaerobic wastewater treatment. Biotechnology and Bioengineering, 27, 585-602.

Heipieper, H.J., Weber, F.J., Sikkema, J., Kewelch, H., De Bont, J.A.M., 1994. Mechanisms of resistance of whole cells to toxic organic solvents. Trends in Biotechnology, 12, 409–415.

Henry, P.M., Donlon, B.A., Lens P.N., Colleran, E.M., 1996. Use of anaerobic hybrid reactors for treatment of synthetic pharmaceutical wastewaters containing organic solvents. Journal of Chemical Technology and Biotechnology, 66, 251-264.

Henze, M., Harremoes, P., 1983. Anaerobic treatment of wastewater in fixed film rectors: A literature review. Water Science and Technology, 15, 1-101.

Hilton, B.L., Oleszkiewicz, J.A., 1988. Sulphide-induced inhibition of anaerobic digestion. Journal of Enviromental . Engineering, 114, 1377–1391.

Hofman-Bang, J., Zheng, D., Westermann, P., Ahring, B.K., Raskin, L., 2003. Molecular ecology of anaerobic reactor systems. Advances in Biochemical Engineering/Biotechnology, 81, 151-203.

Hugenholtz, P., Pitulle, C., Hershberger, K.L., Pace, N.R., 1998. Novel division level bacterial diversity in a Yellowstone hot spring. Journal of Bacteriology, 180, 366–376.

Hulshoff, L.W., 1989. The phenomenon of granulation of anaerobic sludge. Ph.D thesis, Wageningen University.

Hutnan, M., Mrafková, L., Drtil, M., Derco J., 1999. Methanogenic and nonmethanogenic activity of granulated sludge in anaerobic baffled reactor. Chemistry Papers, 53 (6), 374-378.

Hwang, S.C.J., Lee, C.M., Lee, H.C., Pua, H.F., 2003. Biofiltration of waste gases containing both ethyl acetate and toluene using different combinations of bacterial cultures. Journal of Biotechnology, 105, 83-94.

Imachi, H., Sekiguchi, Y., Kamagata, Y., Ohashi, A., Harada, H., 2000. Cultivation *and in situ* detection of a thermophilic bacterium capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens in a thermophilic methanogenic granular sludge. Applied Environmental Microbiology, 66, 3608-3615.

Ince, B., Selcuk, A., Ince, O., 2002. Effect of a chemical synthesis-based pharmaceutical wastewater on performance, acetoclastic methanogenic activity and microbial population in an upflow anaerobic filter. Journal of Chemical Technology and Biotechnology, 77, 711-719.

Jetten, M., Stams, A., Zehnder, A.,1989. Purification and characterization of an oxygen-stable carbon monoxide dehydrogenase of *Methanothrix soehngenii*. European Journal of Biochemistry, 181, 437- 441.

Jetten, M.S.M., Stams, A.J.M., Zehnder, A.J.B., 1992. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina spp.* FEMS Microbiological Reviews, 88, 181–198.

Jia, Z., 2009. Real time quantative PCR. http://structure.biochem.queensu.ca/labjournalclub/Labmeeting_QPCR_vin.pdf

Jin, P., Bhattacharya, S.K., Williama, C.J., Zhang, H., 1998. Effects of sulfide addition on copper inhibition in methanogenic systems. Water Research, 32, 977–988.

Jones, W.J., 1991. Diversity and physiology of methanogens. In: Rogers JE & Whitman WB (Eds) Microbial production and consumption of greenhouse gases: methane, nitrogen oxides, and halomethanes, 39-55. American Society for Microbiology, Washington.

Joulian, C., Ollivier, B., Patel, B.K.C., Roger, P.A., 1998. Phenotypic and phylogenetic characterization of dominant culturable methanogens isolated from ricefield soils. FEMS Microbiology Ecology, 25(2), 135-145.

Kandler, O., Hensel, R., Mayr, U., Stetter, K.O., 1977. Comparative studies of lactic acid dehydrogenases in lactic acid bacteria. I. Purification and kinetics of the allosteric L-lactic acid dehydrogenase from *Lactobacillus casei ssp*. and *Lactobacillus curvatus*. Archaeal Microbiology, 112, 81–93.

Karakashev, D., Batstone, D.J., Angelidaki, I., 2005. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Applied and Environmental Microbiology, 71, 331-338.

Kayhanian, M., 1994. Performance of a high-solids anaerobic digestion process under various ammonia concentrations. Journal of Chemical Technology and Biotechnology, 59 (4), 349.

Kolukirik, M., 2004. Determination of archaeal population dynamics in full-scale UASB reactors using fluorescent *in situ* Hybridization Technique, M.S. Thesis, Istanbul Technical University.

Kolukirik, M., Ince, O., Kasapgil Ince, B., 2007. Methanogenic community change in a full-scale UASB reactor treating alcohol distillery wastewaters in terms of performance and methanogenic activity. Journal of Chemical Technology and Biotechnology, 80, 138-144.

Koster, I.W., Rinzema, A., De Vegt, A.L., Lettinga, G., 1986. Sulfide inhibition of the methanogenic activity of granular sludge at various pH levels. Water Research, 20, 1561–1567.

Koster, I.W., Lettinga, G., 1984. The influence of ammonium-nitrogen on the specific activity of pelletized methanogenic sludge. Agricultural Wastes, 9, 2.5–216.

Koster, I.W., Lettinga, G., 1988. Anaerobic digestion at extreme ammonia concentrations. Biological Wastes, 25, 51–59.

Kroeker, E.J., Schulte, D.D., Sparling, A.B., Lapp, H.M., 1979. Anaerobic treatment process stability. Journal Water Pollution Control Federation, 51, 718– 727.

Langworthy, T. A., 1985. Lipids of archaebacteria, In Woese, C. R., Wolfe, R. S., (Eds.) The bacteria, Academic Press Orlando, Florida, 8, 459-497.

Lema, J.M., Iza, R., Garcia, P., Fernandez, F., 1991. Chemical reactor engineering concepts in design and operation of anaerobic treatment processes, Water Science and Technology, 24(8), 79-86.

Lester, J.N., Stronach, S.M., Rudd, T., 1986. Anaerobic digestion process in industrial wastewater treatment, Springer Verlag, Berlin.

Lettinga, G., Van Geest, A.T., Hobma, S., Van der Laan, J., 1979. Anaerobic treatment of methanolic wastes. Water Research, 13, 725-737.

Lettinga, G., 1995. Anaerobic digestion and wastewater treatment systems. Antonie van Leeuwenhoek, 67, 3-28.

Liu, W.T, Chan, O.C., Fang, H.H.P., 2002. Microbial community dynamics during start-up of acidogenic anaerobic reactor. Water Research, 36 , 3203–3210.

Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic *Archaea*. Annual of New York Academy of Sciences, 1125, 171-189.

Llobet-Brossa, E., Rosselló-Mora, R., Amann, R.E., Microbial community composition of Wadden Sea sediments as revealed by fluorescence *in situ* hybridization. Applied and Environmental Microbiology, 64 (7), 2691-2696.

Lovley, D.R., Lonergan, D.J., 1990. Anaerobic oxidation of toluene, phenol and p-cresol by the dissimilatory iron-reducing organism GS-15. Applied and Environmental Microbiology, 56, 1858-1864.

Loy, A., Horn, M., Wagner, M., 2003. probeBase—an online resource for rRNA targeted oligonuleotide probes. Nucleic Acids Research, 31 (1), 514–516.

Lueders, T., Manefield, M., Friedrich, M.W., 2004. Enhanced sensitivity of DNA and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. Environmental Microbiology, 6, 73–78.

Macarie, H., 2000. Overview of the application of anaerobic treatment to chemical and petrochemical wastewaters. Water. Science and Technology, 42, 201–214.

Madigan, M.T., Martinko, J..M., Parker, J., 2002. Brock biology of microorganisms, (10^{th} edition), Prentice Hall, Inc., New Jersey.

Madigan, M.T., Martinko, J.M., Parker J., 2000 Brock biology of microorganisms, (9^{th} edition) Prentice Hall, Inc., New Jersey .

Malina, J.F., Pohland, F.G., (Eds), 1992. Design of anaerobic process for the treatment of industrial and municipal wastes, Technomic Publishing Co., U.S.A.

Malinen, E., Kassinen, A., Rinttilä, T., Palva, A., 2003. Comparison of real-time PCR with SYBR green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted

oligonucleotide probes in quantification of selected faecal bacteria. Microbiology, 149, 269-277.

Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.H., 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Sytematic and Applied Microbiology, 15, 593-600.

McCarty, P.L., 1971. Energetics and kinetics of anaerobic treatment. Advances in Chemical Services, 105, 91-107.

McCarty, P.L., 2001. The development of anaerobic treatment and its future. Water Science and Technology, 44(8), 149–156.

McHugh, S., Carton, M., Collins, G., O'Flaherty, V., 2004. Reactor performance and microbial community dynamics during anaerobic biological treatment of wastewaters at 16–37 °C. FEMS Microbiology Letters, 48, 369–378.

Meckenstock, R.U., Morasch, B., Griebler, C., Richnow, H.H., 2004. Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. Journal of Contaminant Hydrology, 75, 215–255.

Merkel, W., Manz, W., Szewzyk, U., Krauth, K., 1999. Population dynamics in anaerobic wastewater reactors: modelling and *in situ* characterization. Water Research, 33, 2392–2402.

Miller, T.L., Wolin, M.J., Kusel, E., 1986. Isolation and characterization of methanogens from animal feces. Systematic Applied Microbiology, 8, 234-238.

Minami, K., Okamura, K., Ogawa, S., Naritomi, T., 1991. Continuous anaerobic treatment of wastwater from kraft pulp mill. Journal of Fermentation and Bioengineering, 71, 270–274.

Mohan, S.V., Prakasham, R.S., Satyavathi, B., Annapurna, J., Ramakrishna, S.V., 2001. Biotreatability studies of pharmaceutical wastewater using an anaerobic suspended film contact reactor. Water Science and Technology, 43, 2, 271-276.

Morris, C.E., Bardin, M., Berge, O., Frey-Klett, P., Fromin, N., Girardin, H., Guinebretiere, M..H., Lebaron, P., Thiery, J.M., Troussellier, M., 2002. Microbial biodiversity: approaches to experimental design and hypothesis testing in primary scientific literature from 1975 to 1999. Microbiology and Molecular Biology Reviews, 66, 592-616.

Moyer, C.L., Dobbs, F.C., Karl, D.M., 1994. Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi seamount, Hawaii. Applied and Environmental Microbiology 60, 871-879.

Mrowiec, B., Suschka, J., Keener, T. C., 2005. Formation and biodegradation of toluene in the anaerobic sludge digestion process. Water Environment Research, 77(3), 274.

Muyzer, G., De Waal, E.C., Utterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology, 59, 695–700.

Muyzer, G., Smalla, K., 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek, 73, 127–141.

Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinion in Microbiology, 2, 317–322.

Myabhate, S.P., Gupta S.K., Joshi S.G., 1988. Biological treatment of pharmaceutical wastewater. Water Air and Soil Pollution, 38, 189–197

Nanninga, H.J., Gottschal, J.C., 1986. Isolation of a sulfate reducing bacterium growing with methanol. FEMS Microbiology, 38, 125–130.

Noike, T.G., Endo, J.E., Chang., I., Yaguehi, I., Matsumoto, J.I., 1985. Characteristics of carbohydrate degradation and the rate-limiting step in anaerobic digestion, Biotechnology and Bioengineering, 27, 1482-1489.

OECD, 1993. Organization for economic co-operation and development. chemicals testing draft OECD guidelines for the testing of chemicals - Sections 1-5.

O'Flaherty, V., Lens, P., Leahy, B., Colleran, E., 1998. Long-term competition between sulphate-reducing and methane-producing bacteria during full-scale anaerobic treatment of citric acid production wastewater. Water Research, 32, 815–825.

Ince, O., Kolukirik, M., Cetecioglu, Z., Eyice, O., Inceoglu, O., Ince, B., 2009. Toluene inhibition of an anaerobic reactor sludge in terms of activity and composition of acetoclastic methanogens. Journal of Environmental Science and Health-Part A: Toxic/Hazardous Substances & Environmental Engineering, 44, 14.

Oktem, Y.A., Ince, O., Donnelly, T., Sallis, P., Ince, B., 2008. Anaerobic treatment of a chemical synthesis-based pharmaceutical wastewater in a hybrid upflow anaerobic sludge blanket reactor. Bioresource Technology, 99(5), 1089-96.

Ollivier, B., Cayol, J.L., Patel, B.K.C., Magot, M., Fardeau, M.L., Garcia, J.L., 1997. *Methanoplanus petrolearius spp.* a novel methanogenic bacterium from an oil-producing well. FEMS Microbiology Letters, 147(1), 51-56.

Olsen, G.J., Woese, C.R., 1993. Ribosomal RNA: a key to phylogeny. Journal of Federation of American Societies for Experimental Biology (FASEB), 7, 113-23.

Oz, N., Ince, O., Ince, B., 2004. Effect of wastewater composition on methanogenic activity in an anaerobic reactor. Journal of Environmental Science and Health, Part A., 39,

(11), 2941-53.

Oz, N., Ince, O., Ince, B., Akarsubasi, A.T., Eyice, O., 2003. Microbial population dynamics in an anaerobic CSTR treating a chemical synthesis-based pharmaceutical wastewater. Journal of Environmental Science and Health, 38, 2029–2042.

Oz, N., 2008. Analysis of microbıal communities associated with anaerobic solvent degradation in sequencing batch reactors by traditıonal and molecular tools. Ph.D Thesis, Bogazici University.

Pace, N.R., Stahl, D.A., Lane, D.J., Olsen, G.J., 1986. The analysis of natural microbial populations by ribosomal RNA sequences. Advanced Microbial Ecology, 9, 1-55.

Paulo, P.L., Jiang, B., Rebac, S., Hulshoff-Pol, L., Lettinga, G., 2001. Thermophilic anaerobic digestion of methanol in UASB reactor. Water Science and Technology, 44, 129–136.

Paulo, P.L, Stams, J.M., Field, J.A., Dijkema, C., Van Lier, J.B., Lettinga, G., 2003. Pathways of methanol conversion in a thermophilic anaerobic (55^{o}C) sludge consortium. Applied Journal of Microbiology and Biotechnology, 63, 307-314.

Pavlostathis, S.G., Giraldo-Gommez, E., 1991. Kinetics of anaerobic treatment. Water Science Technology, 24 (8), 35-39.

Payton, M.A., Haddock, B.A., 1986. Principles of biotechnology: scientific fundamentals. Comprehensive Biotechnology, 1. Ed. M. M. Young, Pergamon.

Pernthaler, J., Alfreider, A., Posch, T., Andreatta, S., Psenner, R., 1997. *In situ* classification and image cytometry of pelagic bacteria from a high mountain lake. Applied and Environmental Microbiology, 63, 4778-4783.

Pohland, F.G., Suidan, M.T., 1987. Prediction of pH stability in biological treatment systems. Chemistry of Wastewater Technology, 37, 441-463.

Poulsen, L.K., Ballard, G. Stahl, D.A., 1993. Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. Applied and Environmental Microbiology, 59,1354–1360.

Raskin, L., Stromley, M.J., Rittmann, E., B, Stahl, A. D., 1994. Group-Specific 16S rRNA hybridization probes to describe natural communities of methanogens. Applied and Environmental Microbiology, 4, 1232-1240.

Raskin, L., Poulsen, L.K., Noguera, D.R., Rittmann, B.E., 1994a. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. Applied and Environmental Microbiology, 60(4), 1241-1248.

Raskin, L., 1994b. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Applied and Environmental. Microbiology, 60, 1232-1240.

Raskin, L., Stromley, J.M., Rittmann, B.E., Stahl D.A., 1994b. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Applied and Environmental Microbiology, 60, 1232-1240.

Raskin, L., Rittman, B.E., Stahl, D.A., 1996. Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. Applied and Environmental Microbiology, 62, 3847-3857.

Ravenschlag, K., Sahm, K., Amann, R., 2001. Quantitative molecular analysis of the microbial community in marine Arctic sediments (Svalbard). Applied and Environmental Microbiology, 67, 387–395.

Robbins, J.E., Gerhard, S.A., Kappel, T.J., 1989. Effects of ammonia in anaerobic digestion and an example of digestor performance from cattle manure protein mixtures. Biological Wastes, 27, 1–14.

Roest, K., 2007. Microbial community analysis in sludge of anaerobic wastewater treatment systems: Integrated culture dependent and culture-independent approaches. Ph.D.thesis, Wageningen University.

Rogers, J.B., Du Teau, N.M., Reardon, K.F., 2000. Use of 16S-rRNA to investigate microbial population dynamics during biodegradation of toluene and phenol by a binary culture. Biotechnolgy and Bioengineering, 70, 4, 436-445.

Sanz, J.L., Kochling, T., 2006. Molecular biology techniques used in wastewater treatment: An overview, Applied and Environmental Microbiology, 22, 237–248.

Saiki, Y., Iwabuchi, C., Katami, A., Kitagawa, Y., 2002. Microbial analyses by fluorescence *in situ* hybridization of well-settled granular sludge in brewery wastewater treatment plants. Journal of Bioscience and Bioengineering, 93, 601-606.

Schink, B., 1997. Energetics of syntrophic coorperation in methanogenic degradation. Microbiological Molecular Biology Reviews, 61, 262-280.

Schmidt, J. E., Ahring., B.K., 1996. Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. Biotechnology and Bioengineering, 49, 229–246.

Schnurer, A.G., Zellner, G., Svensson, B., 1999. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. FEMS Microbiology Ecology, 29, 249–261.

Sekiguchi, Y., Kamagata, Y., Nakamura, K., Ohashi, A., Harada, H., 1999. Fluorescence *in situ* hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Applied and Environmental Microbiology, 65, 1280–1288.

Sikkema, J., De Bont, J.A.M., Poolman, B., 1994. Interactions of cyclic hydrocarbons with biological membranes. Jounal of Biological Chemistry. 26, 8022– 8028.

Smith K.S., Ingram–Smith C., 2007. *Methanosaeta,* the forgetten methanogen. Trends in Microbiology, 15 (4), 150-155.

Sorensen, J., Christiensen, D., Jorgensen, B.B., 1981. Applied and Environmental Microbiology, 5, 42.

Soto, M., Mende´z, R., Lema, J.M., 1993. Sodium inhibition and sulphate reduction in the anaerobic treatment of mussel processing wastewaters. Journal of Chemical Technology and Biotechnology, 58, 1–7.

Speece, R.E., 1983. Anaerobic biotechnology for industrial wastewater treatment. Environmental Science and Technology, 17, 416-427.

Speece, R.E., Parkin, G.F., 1983. The response of methane bacteria to toxicity. In: Proceedings of the 3rd International Symposium on Anaerobic Digestion, Boston, MA.

Sprott, G.D., Patel, G.B., 1986. Ammonia toxicity in pure cultures of methanogenic bacteria. Systematic Applied Microbiology, 7, 358–363.

Stahl, D. A., Flesher, B., Mansfield, H.R., Montgomery, L., 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Applied and Environmental Microbiology, 54, 1079-1084.

Stams, A.J.M., 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie van Leeuwenhoek, 66, 271-294.

Stams, A.J.M., De Bok, F.A.M., Plugge, C.M., Van Eekert, M.H.A., Dolfing, J., Schraa, G., 2006. Exocellular electron transfer in anaerobic microbial communities. Environmental Microbiology, 8, 371-382.

Sterritt, R.M., Lester, J.N., 1980. Interaction of heavy metals with bacteria. Science of the Total Environment, 14 (1), 5–17.

Stover, E. L., Brooks, S., Munirathinam, K., 1994. Control of biogas H_2S concentrations during anaerobic treatment. American Institute for Chemical Engineers Symposium series, 300, 90.

Stronach, S.M., Rudd, T., Lester, J.N., 1986. Anaerobic Digestion Process in Industrial Wastewater Treatment, Springer Verlag, Berlin.

Suzuki, M.T., Giovannoni, S.J., 1996. Bias caused by template annealing in the ampli®cation of mixtures of 16S rRNA genes by PCR. Applied and Environment Microbiology, 62, 625-630.

Tagawa, T., Syutsubo, K., Sekiguchi, Y., Ohashi, A., Harada, H., 2000. Quantification of methanogen cell density in anaerobic granular sludge consortia by fluorescence *in situ* hybridization. Water Science and Technology, 42 (3–4), 77–82.

Terzis, E., 1994. Anaerobic treatment of industrial wastewater containing organic solvents. Water Science and Technology, 29, 9, 321-329.

Teske, A., Wawer, C., Muyzer, G., Ramsing, N.B., 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments.Applied and Environmental Microbiology, 62, 1405.

Thauer, R.K., Jubgermann, K., Decker, K., 1977. Energy conservation in chemothrophic anaerobic bacteria. Bacteriology Reviews, 41, 100-180.

Thauer, R. K., Shima, S., 2006. Biogeochemistry: methane and microbes, Nature, 440, 878-879.

Torsvik, V., Goksoyr, J., Daae, F.L., 1990. High diversity in DNA of soil bacteria. Applied and Environmental Microbiology, 56, 782–787.

Torsvik, V., Sorheim, R., Goksoyr, J., 1996. Total bacterial diversity in soil and sediment communities—a review. Journal of Industrial Microbiology, 17, 170-178.

Torsvik, V., Ovreas, L., 2002. Microbial diversity and function in soil: from genes to ecosystems. Current Opinions in Microbiology, 5, 240-245.

Torsvik, V., Ovreas, L., Thingstad, T.F., 2002. Prokaryotic diversity – magnitude, dynamics, and controlling factors. Science, 296, 1064-1066.

Upton, M., Hill, B., Edwards, C., Saunders, J.R., Ritchie, D.A., Lloyd, D., 2000. Combined molecular ecological and confocal laser scanning microscopic analysis of peat bog methanogen populations. FEMS Microbiology. Letters, 193 (2), 275–281.

Urakawa, H., Yoshida, T., Nishimura, M., Ohwada, K., 2000. Characterization of depth-related population variation in microbial communities of a coastal marine sediment using 16S rDNA-based approaches and quinone profiling. Environmental Microbiology, 2, 542-554.

Vance D., 1997. What is toxic and what is not? Environmental Technology, 7 (3), 28-29.

Van Lier, J.B., Van Der Zee, F.P., Rebac, S., Kleerebezem, R., 2001. Advances in high rate anaerobic treatment: Staging of reactor systems. Anaerobic digestion for sustainable development, 1, 17-26.

Vogels, G.D., Keltjens, J.T., 1988. Biochemistry of methane production,. In A.J.B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, Inc., New York. 707-770.

Weijma, J., 2000. Methanol as electron donor for thermophilic biological sulphate and sulphite reduction. Ph.D thesis, Wageningen University.

Whitman, W.B., Ankwanda, E., Wolfe, R.S., 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. Journal of Bacteriology, 149, 852-863.

Widdel, F., 1986. Growth of methanogenic bacteria in pure culture with 2-propanol and other alcohols as hydrogen donors. Applied and Environmental Microbiology, 51, 1056-1062.

Widdel, F., Rouviere, P.E., Wolfe, R.S., 1988. Classification of secondary alcohol-utilizing methanogens including a new thermophilic isolate. Archives of Microbiology, 150, 477-481.

Wiegant, W.M., Zeeman, G., 1986. The mechanism of ammonia inhibition in the thermophilic digestion of livestock wastes. Agricultural Wastes, 16, 243–253.

Woese, C., Fox, G.E., 1977. Molecular ecology of anaerobic reactor. Proceedings of the National Academy of Science,74(11), 5088-5090.

Woese, C.R., 1987. Bacterial evolution. Microbiology Reviews, 51, 221–271.

Woese, C.R., Kandler, O., Whelis, M.L., 1990. Towards a natural system of organisms; proposal for the domains *Archaea*, bacteria and eukarya. Proceedings of National Academy of Sciences, 87, 4576-4579.

Wu, J.H., Liu, W.T., Tseng, I.C., Cheng, S.S., 2001. Characterization of microbial consortia in a terephthalate-degrading anaerobic granular sludge system. Microbiology, 147, 373–38

Yamaguchi, T., Yamazaki, S., Uemura, S., Tseng, I.-C., Ohasi, A., Harada. H., 2001. Microbial-ecological significance of sulfide precipitation within anaerobic granular sludge revealed by micro-electrodes study. Water Research, 35, 3411–3417.

Yang, J., Speece, R.E., 1986. The effects of chloroform toxicity on methane fermentation. Water Research, 20, 1273–1279.

Youngseob, Y., Jaai, K., Seokhwan, H., 2005. Use of real-time pcr for group-specific quantification of aceticlastic methanogenic anaerobic processes: Population dynamics and community structures. Wiley Inter Science, 93, 3, 423-433.

Zhang, T., Noike, T., 1994. Influence of retention time on reactor performance and bacterial trophic populations in anaerobic digestion processes. Water Research, 28, 27–36.

Zeeman, G., Wiegant, W.M., Koster-Treffers, M.E., Lettinga, G., 1985. The influence of the total ammonia concentration on the thermophilic digestion of cow manure. Agricultural Wastes, 14, 19–35.

Zinder S. H., Cardwell, S.C., Anguish, T., Lee, M., Koch M., 1984. Methanogenesis in a thermophilic (58^0C) anaerobic digester: Methanothrix sp. as an important acetoclastic methanogen. Applied and Environmental Microbiology, 47, 796-807.

Zinder, S.H., 1990. Conversion of acetic acid to methane by thermophiles. FEMS Microbiological Reviews, 75, 125–138.