ANALYSIS OF ARCHAEAL COMMUNITY DYNAMICS IN FULL-SCALE ANAEROBIC REACTORS USING FLUORESCENT IN SITU HYBRIDIZATION

by
Sair Sinan Kestelli
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ABSTRACT

In order to benefit from great potential of anaerobic reactors in terms of achieving high Chemical Oxygen Demand (COD) removal efficiencies (> %90), careful operation and control monitoring the conventional parameters such as pH, alkalinity, temperature, VFA concentration, etc. are required. These parameters are actually monitored to maintain optimum environmental conditions for a stable and a highly active microbial community in the reactor. However, little attention has been paid to determination of the composition and activity of the microbial community. The maintenance of active methanogenic populations in an anaerobic reactor is especially critical for stable performance. In this study, composition of methanogenic archaeal populations in three full-scale upflow anaerobic sludge blanket (UASB) reactors, namely IUASB (Istanbul), TUASB (Tekirdag) and CUASB (Canakkale) treating alcohol distillery effluents were analyzed using fluorescence in situ hybridization (FISH). The reactors were also investigated in terms of acetoclastic methanogenic capacity using specific methanogenic activity (SMA) test. The results of this study were then compared with the previous studies considering archaeal community dynamics, operating conditions and performance of the reactors in a long term operating period, years between 2001 and 2005.

The IUASB and CUASB reactors achieved COD removal efficiencies between 60%-80% at organic loading rates (OLR) in a range of 3-7 kg COD/m^3.day and 2-10 kg COD/m^3.day, respectively while the TUASB reactor was operated at OLRs 3-5 kg COD/m^3.day achieving COD removal efficiencies between 50%-65% in year 2005. Throughout year 2005, the three UASB reactors had been operated under a food to microorganisms (F:M) ratio of 0.02-0.09 which is much lower than the typical values (0.4-0.6) reported for similar reactors. Also, temperature of CUASB reactor was maintained in a range of 15-25^{o}C while that of IUASB reactor was 30^{o}C±1^{o}C in 2005, which are lower than the optimum temperature levels for mesophilic reactors (35-37^{o}C). All other operational parameters were maintained within their desired ranges.

According SMA tests' results, the PMP rates of the IUASB, TUASB and CUASB reactors' sludges were 192 mL CH_4/gVSS.day, 132 mL CH_4/gVSS.day and 167 mL

CH_4/gVSS.day, respectively, in year 2005. These values are lower than the PMP rates of anaerobic reactors successfully treating similar wastewaters reported in literature (>300 mL CH_4/gVSS.day).

According to FISH results, the relative abundance of archaeal cells within the IUASB, TUASB and CUASB reactors' sludges increased from 14.2%±0.3% to 20.7%±0.9%, 15.0%±0.7% to 23.5%±0.2% and 14.6%±0.7% to 22.3%±0.8%, respectively between years 2004 and 2005. *Methanosaeta* spp. was the most abundant methanogen in the three UASB reactors' sludges. The relative abundance of acetoclastic genus *Methanosaeta* in IUASB and CUASB reactor sludges increased from 58.0%±2.1% to 71.8%±5.5% and 53.0%±0.7% to 55.0%±1.4% of the archaeal subpopulation, respectively. Meanwhile, the relative abundance of acetoclastic genus *Methanosaeta* in TUASB reactor sludge decreased from 79.0%±1.4% to 60.4%±0.6%. In the same period, the relative abundance of hydrogenotrophic methanogens, *Methanobacteriales* within the archaeal subpopulation in IUASB, TUASB and CUASB reactor sludges increased from 10.0%±0.7% to 32.7%±3.8%, 24.0%±0.7% to 43.7%±0.3% and 39.0%±0.7% to 43.5%±0.3%, respectively. Other important archaeal groups such as *Methanosarcina*, *Methanococcales* and *Methanogenium* relatives were not observed in the three UASB reactors' sludges in November 2005.

ÖZET

Anaerobik reaktörlerin sahip olduğu yüksek kimyasal oksijen ihtiyacı (KOİ) giderimi gerçekleştirme potansiyelinden faydalanmak için, pH, alkalinite, sıcaklık, Uçucu Yağ Asitleri (UYA) gibi geleneksel parametrelerin düzenli olarak ölçülerek işletme şartlarının kontrol edilmesi gerekmektedir. Bu parametrelerin kontrol altında tutulması, reaktör içinde amaçlanan stabil ve aktif bir mikrobiyal komünite için optimum koşulların sağlanması için gereklidir. Ancak, mikrobiyal komünitenin aktivitesinin ve kompozisyonunun belirlenmesi konularına yeterli önem gösterilmemiştir. Ayrıca komünite içinde yeterli sayıda ve aktif metanojenik popülasyonun tutulması da reaktörün uzun vadede stabilitesinin sağlanması açısından önem arzetmektedir. Bu çalışmada, alkollü içki endüstrisi atıksuyu arıtımında kullanılan ve IUASB (İstanbul), TUASB (Tekirdağ) ve CUASB (Çanakkale) olarak isimlendirilen, üç farklı yukarı akışlı anaerobik çamur yatağı (UASB) reaktörün metanojenik arkeyal kompozisyonu floresanlı yerinde hibritleşme (FISH) tekniği kullanılarak analiz edilmiştir. Reaktörlerdeki biyolojik çamurun asetoklastik metanojenik aktivitesi ise spesifik metanojenik aktivite (SMA) testi kullanılarak ölçülmüştür. Ayrıca elde edilen sonuçlar, aynı reaktörler üzerinde yapılan önceki çalışmalardan elde edilen verilerle karşılaştırılmış ve arkeyal komünite dinamikleri de gözönüne alınarak, reaktörün uzun vadedeki çalışma şartları ve performansı hakkında değerlendirmeler yapılmıştır.

2005 yılı itibariyle, IUASB ve CUASB reaktörleri, sırasıyla 3-7 kg KOİ/m^3.gün ve 2-10 kg KOİ/m^3.gün lük organik yükleme aralıklarında %60 ile %80 arasında KOİ giderim verimi gerçekleştirirken, TUASB reaktörü 3-5 kg KOİ/m^3.gün lük organik yükleme aralığında çalıştırılmış ve %50 ile %65 arasında KOİ giderim verimi gerçekleştirmiştir. Üç anaerobik reaktör de literatürde benzer reaktörler için belirtilen tipik değerlerin (0.4-0.6) çok altında, 0.02 ile 0.09 arasında değişen substrat/mikroorganizma oranlarında çalıştırılmıştır. Ayrıca CUASB reaktörünün aynı dönemdeki çalışma sıcaklığı 15 ile 25 ^{o}C arasında değişirken, IUASB reaktöründe sıcaklık 30^{o}C±1^{o}C seviyelerinde seyretmiştir. Bu değerler, benzer mezofilik reaktörler için belirtilen optimum sıcaklık seviyesinin (35-37^{o}C) altında kalmıştır. Reaktörlerdeki diğer tüm işletim parametreleri istenen seviyelerde tutulmuştur.

2005 yılında yürütülen SMA testi sonuçlarına göre, IUASB, TUASB ve CUASB reaktör çamurlarının potansiyel metan üretimleri, sırasıyla 192 mL CH_4/gUAKM.gün, 132 mL CH_4/gUAKM.gün ve 167 mL CH_4/gUAKM.gün olarak bulunmuştur. Bu değerler, literatürde benzer atıksuları etkin olarak arıtan anaerobik reaktörler için verilen potansiyel metan üretim hızı değerlerinden (>300 mL CH_4/gUAKM.gün) düşüktür.

Elde edilen FISH sonuçlarına göre, IUASB, TUASB ve CUASB reaktör çamurlarında bulunan arkeyal hücrelerin oranı, 2004-2005 yılları arasında sırasıyla %14.2±%0.3 den %20.7±%0.9 a, %15.0±%0.7 den %23.5±%0.2 ye ve %14.6±%0.7 den %22.3±%0.8 e yükselmiştir. *Methanosaeta* türleri üç anaerobik çamur numunesinde de en yoğun metanojen grubu olarak belirlenmiştir. IUASB ve CUASB reaktör çamurlarında bulunan asetoklastik *Methanosaeta* türüne ait hücrelerin arkeyal altpopülasyonunda oranı 2004-2005 yılları arasında, sırasıyla %58.0±%2.1 den %71.8±%5.5 e ve %53.0±%0.7 den %55.0±%1.4 e yükselmiştir. Aynı dönemde, TUASB reaktör çamurunda bulunan asetoklastik *Methanosaeta* türüne ait hücrelerin oranı ise %79.0±%1.4 den %60.4±%0.6 ya düşmüştür. IUASB, TUASB ve CUASB reaktör çamurlarında arkeyal altpopülasyonunda bulunan *Methanobacteriales* türüne ait hücrelerin oranı ise sırasıyla %10.0±%0.7 den %32.7±%3.8 e, %24.0±%0.7 den %43.7±%0.3 e ve %39.0±%0.7 den %43.5±%0.3 e yükselmiştir. *Methanosarcina*, *Methanococcales* ve *Methanogenium* ve akraba türleri gibi diğer önemli arkeyal gruplarına ise 2005 yılında alınan üç UASB reaktör çamurunda da rastlanmamıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

AMP	Actual Methane Production
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
CUASB	Canakkale Distillery Factory Upflow Anaerobic Sludge Blanket
DGGE	Denaturing Gradient Gel Electrophoresis
FISH	Fluorescent in situ Hybridization
F:M	Food to Microorganisms
HRT	Hydraulic Retention Time
IUASB	Istanbul Distillery Factory Upflow Anaerobic Sludge Blanket
MPN	Most Probable Number
NRB	Nitrate Reducing Bacteria
OLR	Organic Loading Rate
PCR	Polymerase Chain Reactions
PFA	Paraformaldehyde
PMP	Potential Methane Production
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
SMA	Specific Methanogenic Activity
SRB	Sulphate Reducing Bacteria
SRT	Solids Retention Time
SS	Suspended Solids
TOC	Total Organic Carbon
TS	Total Solids
TUASB	Tekirdag Distillery Factory Upflow Anaerobic Sludge Blanket
TVS	Total Volatile Solids
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids

1. INTRODUCTION

Treatment of wastewaters containing high amounts of organic pollutants has been a major field in environmental engineering and sciences. A viable option for this problem has been anaerobic treatment with its advantages such as high efficiency and minimum sludge production. Treatment of industrial wastewater in anaerobic bioreactors has grown in importance especially since the introduction of the Upflow Anaerobic Sludge Blanket (UASB) reactor about 25 years ago (Lettinga et al., 1980). Although the general processes occuring in anaerobic biological wastewater treatment plants, such as hydrolysis, acidogenesis, acetogenesis, methanogenesis, are well understood, the microbial community responsible for these conversions and their qualitative and quantitative contribution to the conversions are far from being clear.

Wastewater characteristics of alcohol distillery industry vary greatly depending on the raw materials used, final product of the factory and production techniques applied. However, commonly wastewaters from this industry are classified as medium to high strength wastewaters. Various wastewater treatment methods are applied to reduce contaminant concentrations to acceptable levels. After some unsuccessful efforts with physco-chemical treatment with various coagulants and aerobic treatment which could not meet desired treatment efficiencies, anaerobic treatment has been realized as an efficient tool to reduce high organic content to acceptable levels. However, huge and simple anaerobic lagoons did not provide enough feasibility and more complex anaerobic systems such as anaerobic filters, expanded/fluidized bed reactors and UASB Reactors were used. UASB Reactors are suitable for highly polluted wastewaters with its granules with high settling velocities, suitability for high flow rates, high degree of contact between wastewater and the bacterial flora (Lester et al., 1986).

Like other anaerobic treatment methods, process control and proper operation of UASB Reactors are crucial for the desired sustainable efficiency to be achieved. Process control in UASB reactors could be performed by various parameters. Most commonly used parameters in anaerobic reactors could be given as efficiency of organic matter removal (BOD, COD, TOC, etc.), levels of Volatile Fatty Acids, VSS/SS ratios, quality or quantity of biogas produced, etc. However, recent studies has shown that these conventional parameters give information just about the current conditions inside the reactor and are far away from determining the current inadequacies and possible problems in near future. (Ince et al., 1994, Monteggia, 1991). Also, proper organic loading rates could not be determined by conventional parameters. Specific Methanogenic Activity (SMA) test is a relatively new and practical test in which potential methane production obtained from the test is used as a parameter to evaluate the performance of the reactor by comparing it with actual methane productions.

Anaerobic treatment systems have their own unique microbial composition like other biological systems. Determination of various species in the reactor will help to understand the microbial population dynamics and provide information about the ecological aspects of the functioning of the system. rRNA based approach using Fluorescent in situ Hybridization (FISH) is an efficient way for determination of microbial population and its applications have led to widen our knowledge on many ecosystems (Amann et al., 1990a; Head et al., 1998; Hugenholtz et al., 1998). This technique has great advantages as it does not need pure cultures, can show three-dimensional spatial distribution and morphology of uncultured cells. Also, target cells can be counted after FISH labeling and probe sensitivity could be controlled even for various communities by using multiple probes with different color labels (Ahring, 2003). It has also been applied to anaerobic treatment systems and provided detailed descriptions of microbial populations in the system (Raskin et al., 1995; Harmsen et al., 1996; Saiki et al., 2002).

In this study, archaeal community structures of three different full-scale UASB Reactors treating alcohol distillery wastewaters were analyzed using FISH; performance of reactors, potential and actual acetoclastic methanogenic activities of the sludges were evaluated considering their microbial compositions. The results of the study were also compared with the previous studies on the same reactors and considering archaeal community dynamics, operating conditions and performance of the reactors were evaluated in a long term.

2. FUNDAMENTALS OF ANAEROBIC DIGESTION

2.1. Process Description

Anaerobic digestion could be defined as a multistage process in which biodegradable organic solids are converted to the end products CH_4, CO_2 and trace amounts of hydrogen in the absence of oxygen. Microbial flora of anaerobic digestion includes many groups of microorganisms which involves in the conversion of complex organic materials to the end products.

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.

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

In the Nine-stage Model by Harper and Pohland (1986), there are 9 biochemical reactions in anaerobic digestion process which are given below and shown diagrammatically in Figure 2.1.

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.

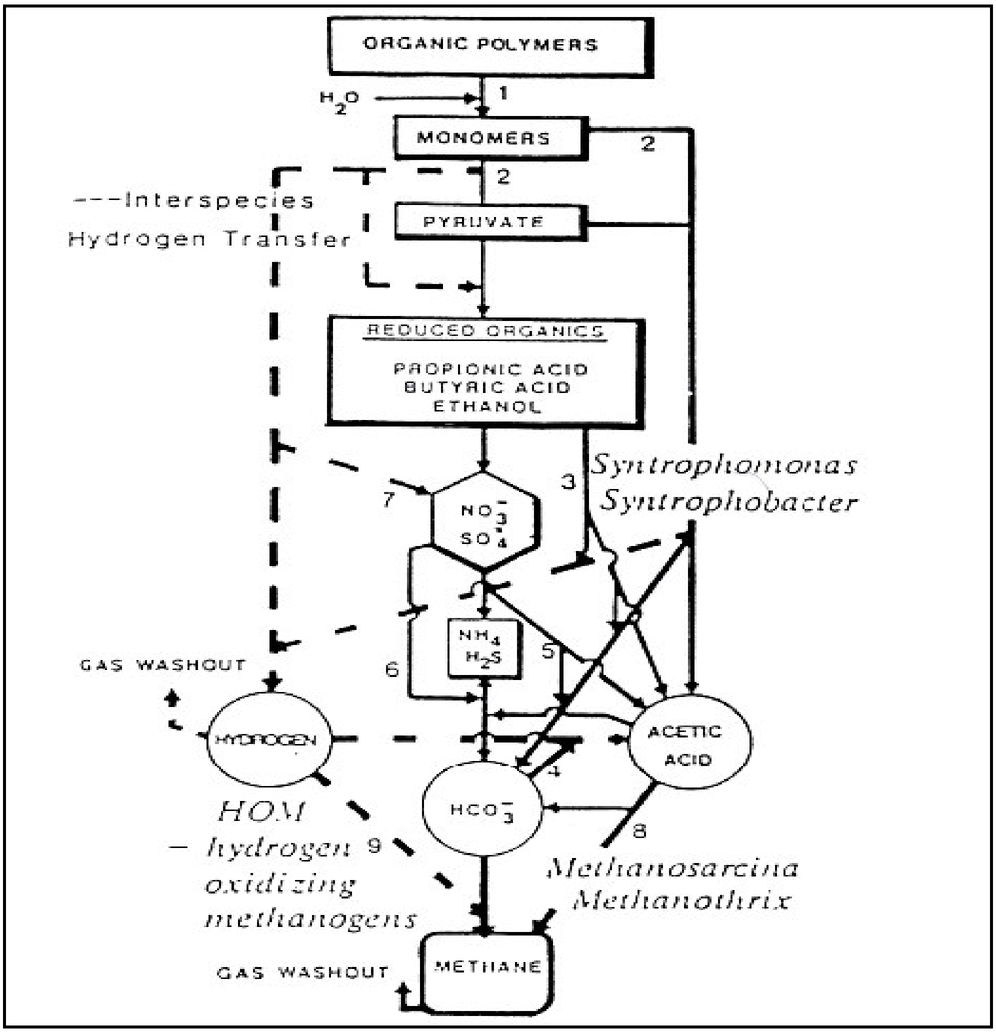


Fıgure 2.1. Substrate Conversion Patterns Associated with the Anaerobic Digestion (Harper and Pohland, 1986).

In the Six-stage Model given by Lester et al. (1986), biochemical reactions are classified into 6 parts which are given below and shown in Figure 2.2.

1. Hydrolysis of proteins, lipids and carbohydrates,
2. Fermentation of amino acids and sugars,
3. Anaerobic (β) Oxidation of higher fatty acids and alcohols
4. Anaerobic Oxidation of intermediary products such as propionate, butyrate,etc.,
5. Decarboxylation of Acetate ($CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$),
6. Hydrogen Oxidation ($CO_2 + 4H^+ \rightarrow CH_4 + 2H_2O$).

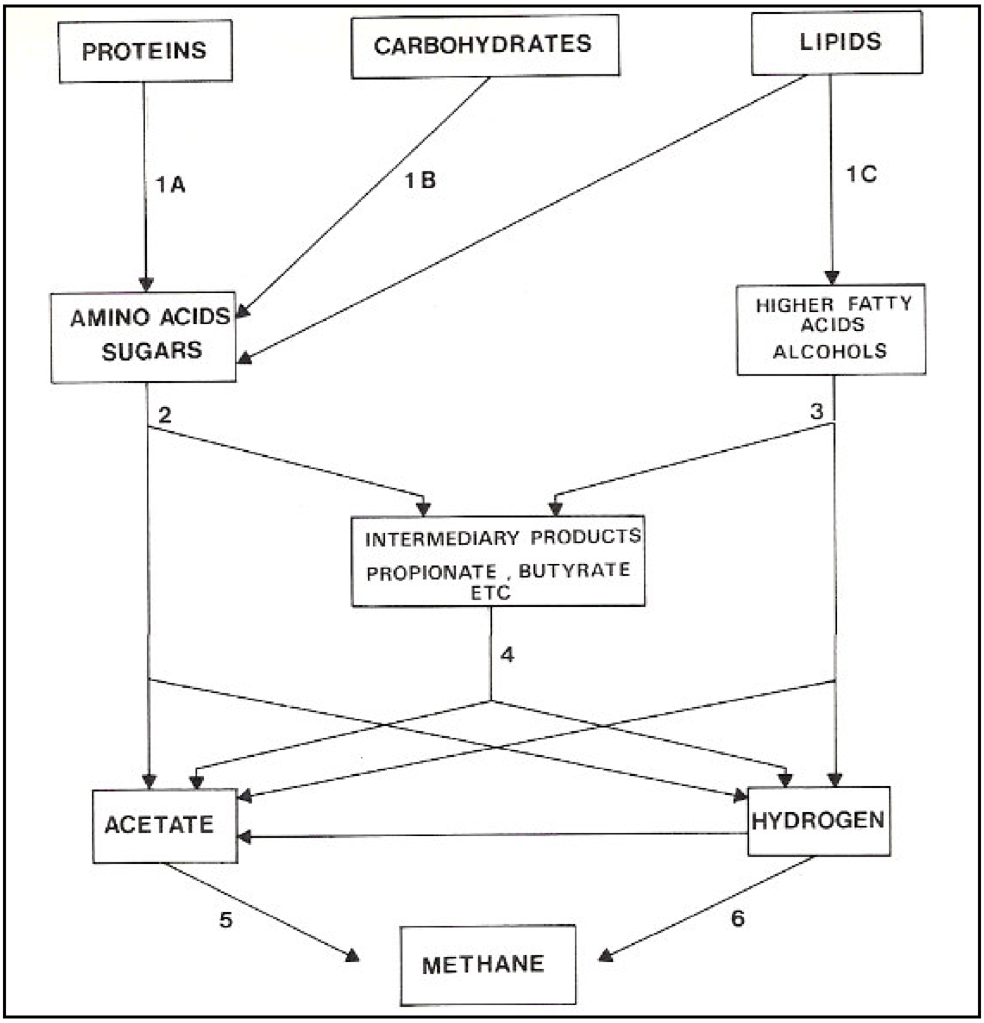


Figure 2.2. Pathway of anaerobic biodegradation
(Lester et al., 1986).

According to Gerardi (2003), anaerobic digestion could be considered as a three-stage process in its simplest form:

1. Hydrolysis,
2. Fermentation (Acidogenesis and Acetogenesis),
3. Methanogenesis.

The first stage of anaerobic digestion, hydrolysis, is the solubilization of particulate organic compounds such as cellulose and colloidal organic compounds such as proteins into soluble compounds that can be absorbed by bacterial cells (Gerardi, 2003). The hydrolysis of these macromolecules under anaerobic conditions is carried out by specific extracellular enzymes of hydrolizing bacteria. These bacteria utilise the organic products of hydrolysis for net biomass synthesis, resulting in a net decay rate for the system that is less than the actual rate of hydrolysis. Hydrolysis rates could therefore be used to define circumstances under which operational instability might be expected, these rates allowing prediction of gas production and degree of stabilization of organic matter (Lester et al., 1986). As particulate organic matter could not pass through the bacterial cell membrane and be utilized for the growth of the bacteria, this step may be rate-limiting for some wastes such as those from pharmaceutical and food industry (Corbitt, 1990).

In the second stage, fermentation, soluble compounds produced through hydrolysis or discharged to the digester are degraded by a large diversity of facultative anaerobes and anaerobes through many fermentative processes (Gerardi, 2003). This stage is mainly divided into two phases, as there are two groups of acid producing bacteria which produce the most important compounds for the following stage, methanogenesis.

The first group of bacteria, called acidogens, are mainly responsible for the fermentation process of hydrolyzed soluble organic compounds such as aminoacids, sugars and long chain fatty acids into carbondioxide, hydrogen gas and volatile fatty acids. Acetic acid, butyric acid and propionic acid are the major products of this step. However, higher fatty acids namely valeric acid, caproic acid, iso-butyric acid, iso-valeric acid and iso-caproic acid can also be produced at lower concentrations.

The second group of acid forming bacteria, acetogens are mainly responsible for the production of acetate which is very crucial in the anaerobic digestion process. Besides acetic acid, they also produce carbondioxide and hydrogen from propionate, butyrate and other higher fatty acids by the β-oxidation process. A molecule is removed from fatty acids having more than two carbons at each reaction until all fatty acids are converted to acetate molecules.

In the final stage, which is generally considered as the rate-limiting step of anaerobic digestion, methanogenesis, acetate, butyrate, and propionate are converted to methane, the most reduced organic molecule. Methanogenesis is a conversion of H_2 and CO_2 by methane-producing bacteria. These bacteria are the most oxygen-sensitive of the bacteria, hence the most strictly anaerobic (Lester et al., 1986). The major part of the carbon flow in a well-operating anaerobic reactor occurs between the fermentative microorganisms and the methanogens. Only between 20 and 30 % of the carbon is transformed into intermediary products before these are metabolized to methane and carbon dioxide as shown in Figure 2.3 and 2.4 (Ahring, 2003).

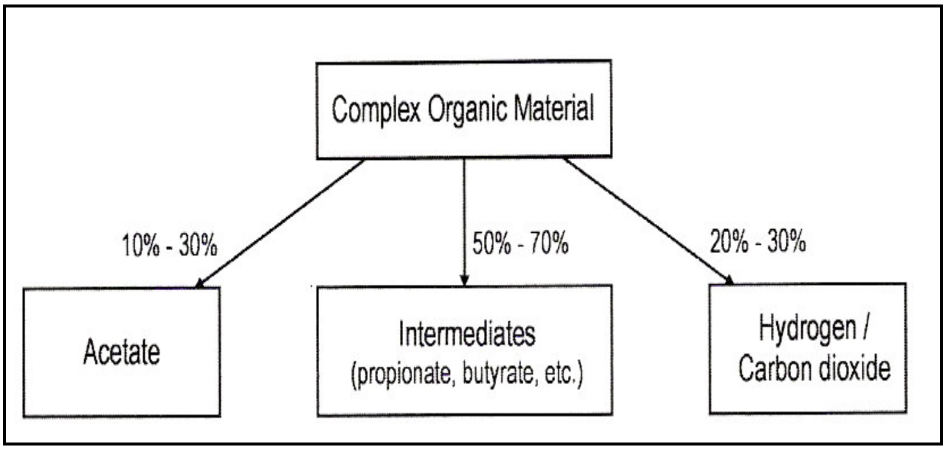


Figure 2.3. Carbon flow in anaerobic environments without active methanogens (Ahring, 2003).

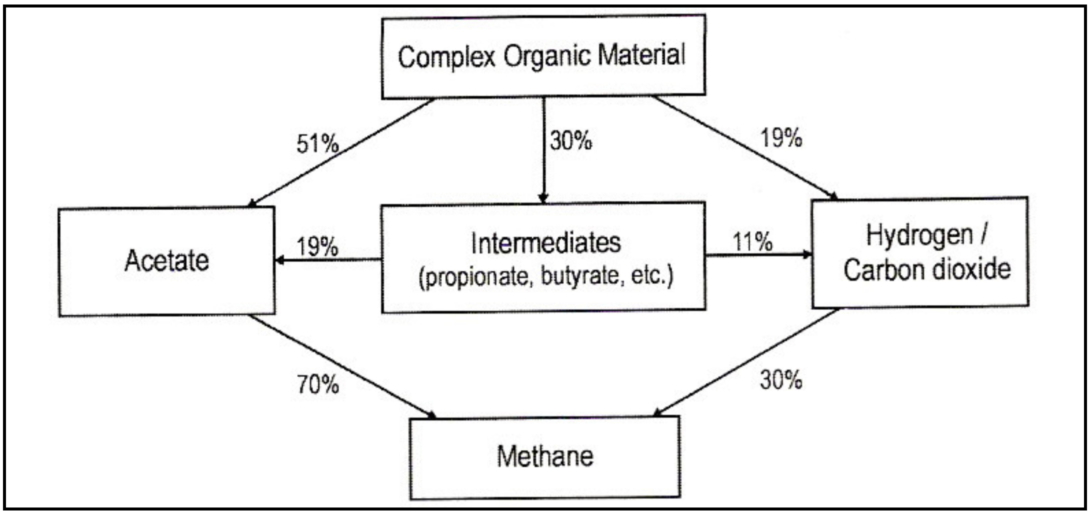


Figure 2.4. Carbon flow in anaerobic environments with active methanogens (Ahring, 2003).

Understanding the microbiology and biochemistry of the stages briefly explained above is crucial in order to achieve cost-effective, stable and highly efficient operation in anaerobic digesters.

2.2. Microbiology and Biochemistry of Anaerobic Digestion

In anaerobic digestion, various microbial groups are involved performing different biochemical reactions in different stages. The major groups of microorganisms and the reactions taking place in anaerobic digestion are as follows:

1. Hydrolytic fermentative bacteria
2. Acidogenic (acid forming) bacteria
3. Hydrogen producing acetogenic bacteria
4. Hydrogen utilizing acetogenic bacteria
5. Carbondioxide reducing methanogens
6. Acetoclastic methanogens

First group of bacteria, as the name implies, are involved in the first stage of anaerobic digestion, hydrolysis. Second, third and fourth group of microorganisms perform acidogenesis and acetogenesis, while the last two groups are responsible for the methanogenesis stage.

2.2.1. Hydrolysis

Hydrolysis is the splitting (lysis) of a compound with water (hydro). In anaerobic digestion, complex insoluble compounds such as particulate and colloidal wastes undergo hydrolysis. These wastes are polymeric substances, which are large insoluble molecules consisting of many small molecules joined together by unique chemical bonds. Hydrolytic bacteria or facultative anaerobes and aerobes that are capable of performing hydrolysis achieve breakage of these bonds.

An example of an insoluble compound that undergoes hydrolysis in an anaerobic digester is cellulose. Cellulose ($C_6H_{12}O_6$) consists of many sugar units or mers of glucose. Although glucose is soluble in water, the joining of the many mers of glucose by unique chemical bonds results in the production of the insoluble polymer cellulose. When cellulose is hydrolyzed in an anaerobic digester, many molecules of soluble glucose are released given in Eq. 2.1.

$$(C_6H_{12}O_6)_n + H_2O \rightarrow nC_6H_{12}O_6 \qquad (2.1)$$

Cellulose is hydrolyzed by the hydrolytic bacterium *Cellulomonas*, which has the special enzyme cellulase (Gerardi, 2003). Another bacterium *Clostridium* is also responsible for degradation of compounds containing cellulose and starch (Lema et al., 1991). Another example of hydrolytic bacteria is *Bacillus*, which degrade proteins and fats (Noike et al., 1985).

Hydrolytic microorganisms could also be classified into cellulytic, proteotic, lipolytic and aminolytic bacteria depending on the substrate used. Examples in each group are *Clostridium thermocellum, Clostiridium bifermentas,* genera of *micrococci* and *Bacillus subtilis*, respectively (Hungate, 1982; Payton and Haddock, 1986).

2.2.2. Acidogenesis and Acetogenesis

The breakdown products such as amino acids, sugars and long chain fatty acids of the hydrolysis phase are converted to the intermediary products acetate, carbondioxide and hydrogen by acid forming bacteria. Obligatory and facultatively anaerobic microorganisms are mainly involved in this stage and single amino acids, pairs of aminoacids and single amino acids with non-nitrogenous compound are used by these microorganisms.

In the acidogenesis stage, single amino acids are used under anaerobic conditions by *clostridia*, *mycoplasmas* and *streptococci*. Butanol, butyric acid, acetone and iso-propanol are generally produced by the genera *Clostiridium* and *Butyribacterium*. For example, *Clostridium butyricum* produces butyrate, *Clostridium acetobutylicum* mainly acetone and butanol and *Clostridium butylicum* produces butanol in addition to hydrogen, carbondioxide and iso-propanol.

In the acetogenesis stage, acetate is produced in several fermentative pathways. A large diversity of bacteria, known as acetogenic or acetate-forming bacteria produce nongaseous acetate. Several biochemical reactions are involved by acetogenic bacteria to produce acetate.

Acetogenic microorganisms utilize mostly H_2 and CO_2 (Eq. 2.2), H_2O and carbonmonoxide (Eq. 2.3), methanol and CO_2 (Eq. 2.4) and six-carbon sugars (Eq. 2.5) to produce acetate (Gerardi, 2003):

$$4H_2 + CO_2 \rightarrow CH_3COOH + 2H_2O \qquad (2.2)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \qquad (2.3)$$

$$4CH_3OH + CO_2 \rightarrow 3CH_3COOH + 2H_2O \qquad (2.4)$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \qquad (2.5)$$

Most homoacetogenic bacteria are gram positive and many are classified in the genus *Clostridium*. Organisms such as *Acetobacterium woodii* and *Clostridium aceticum*can grow through two mechanisms, either chemoorganotrophically by fermentation of sugars given in Eq. 2.5 or chemolithotrophically through the reduction of CO_2 to acetate with H_2 as electron donor given in Eq.2.6.

$$2HCO_3^- + 4H_2 \rightarrow CH_3COOH + 4H_2O \tag{2.6}$$

Acetogenic bacteria grow in a symbiotic relationship with methane-forming bacteria. Acetate serves as a substrate for methane forming bacteria. As an example, when ethanol is converted to acetate, carbondioxide is used and acetate and hydrogen are produced, shown in Equation 2.7.

$$CH_3CH_2OH + CO_2 \rightarrow CH_3COOH + 2H_2 \tag{2.7}$$

Another pathway for acetate production is conversion of glucose to two molecules of pyruvate and two molecules of NADH (the equivalent of $4H^+$) through the glycolytic pathway by homoacetogens given in Eq. 2.8.

$$2\ C_3H_3O_3^- \rightarrow 2\ CH_3COO^- + 2CO_2 + 4H^+ \tag{2.8}$$

Two molecules of CO_2 generated in Eq.2.8 are reduced to acetate by the homoacetate fermentation using the four electrons generated from glucolysis and the four electrons produced from the oxidation of two pyruvates to two acetates. The overall reaction could be written as in Eq.2.9.

$$2\ C_3H_3O_3^- + 4H^+ \rightarrow 3\ CH_3COO^- + H^+ \tag{2.9}$$

2.2.3. Methanogenesis

In this section, methanogens, which are considered as the most important microbial group in anaerobic digestion and biogas production, are explained with a more detailed manner because of the scope and content of this study. Firstly, taxonomy of methanogens is given, then characteristics of methanogens are explained and finally biochemical reactions in the methanogenesis stage are listed.

2.2.3.1. Taxonomy of Methanogens. Prior to the publication of the eighth edition of Bergey's Manual in 1974 (Bryant, 1974), species of methanogenic bacteria were classified together with nonmethanogens, based mainly on morphological criteria. Recognizing the physiological unity of methanogens, Bryant reorganized their taxonomy by bringing them together into a single group (Bryant, 1974). They are also distinguished from true bacteria by a number of characteristics, including the possession of membrane lipids composed of isoprenoids, ether linked to glycerol or other carbohydrates (Langworthy, 1985), a lack of peptidoglican containing muramic acid (Kandler et al., 1977), and distinctive ribosomal RNA sequences (Balch et al., 1979; Woese, 1987).

The validity of this idea was later confirmed by phylogenetic analysis, especially the cataloging and sequencing of 16S rRNA (Balch et al., 1979; Woese, 1987), which documented the unity of methanogens and their evolutionary relationship with some extreme halophiles and extremely thermophilic sulphur dependent organisms. These organisms are members of the kingdom *Archaeobacteria* (Woese et al., 1978). Woese et al. (1990) then proposed a new classification for living organisms, dividing life on earth into three major domains, Bacteria, *Archaea* and Eucarya as shown in Figure 2.5.

The unique phylogenetic status and evolutionary divergence of *Archaea* suggest that they should exhibit wide physiological diversity. However, traditional culture-based studies have shown that opposite was the case. So, within the urkingdom "*Archaea*", two archaeobacterial kingdoms were proposed, Crenarchaeota and Euryarchaeota (Woese et al., 1990). The first kingdom, Crenarchaeota is derived from being phylogenetically close to ancestor or source of *Archaea* (Woese et al., 1990). The second kingdom, Euryarchaeota, is a heterogenous group comprimising a broad spectrum of organisms with varied patterns of metabolism from different habitats. A third archaeal kingdom has been discovered, reporting isolation of several archaeal sequences evoluationary distant from all archaea known to date (Barns et al., 1994). The new group was placed on phylogenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota, as shown in Figure 2.6.

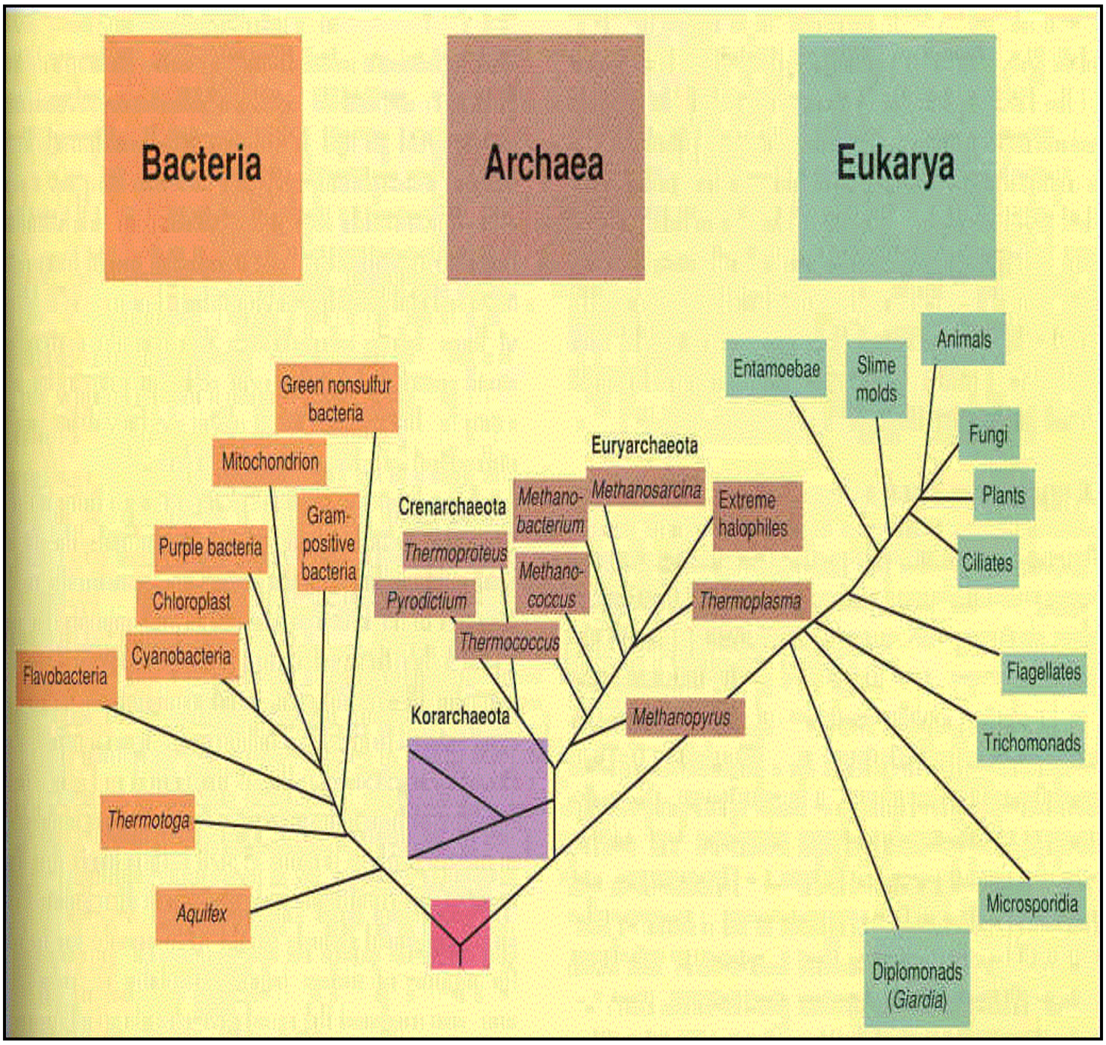


Figure 2.5. Universal phylogenetic tree (Madigan et al., 2002)

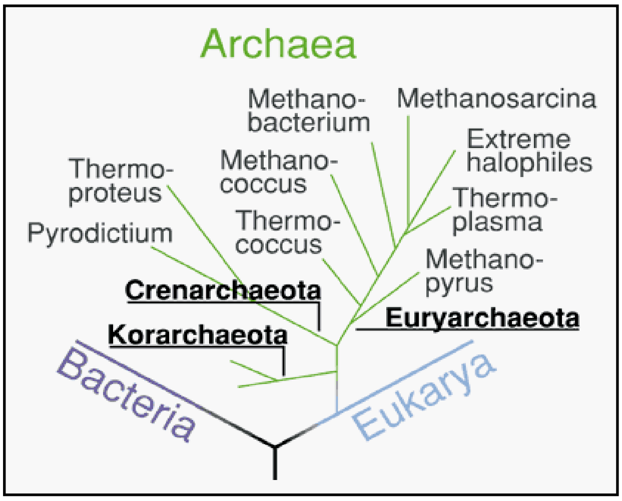


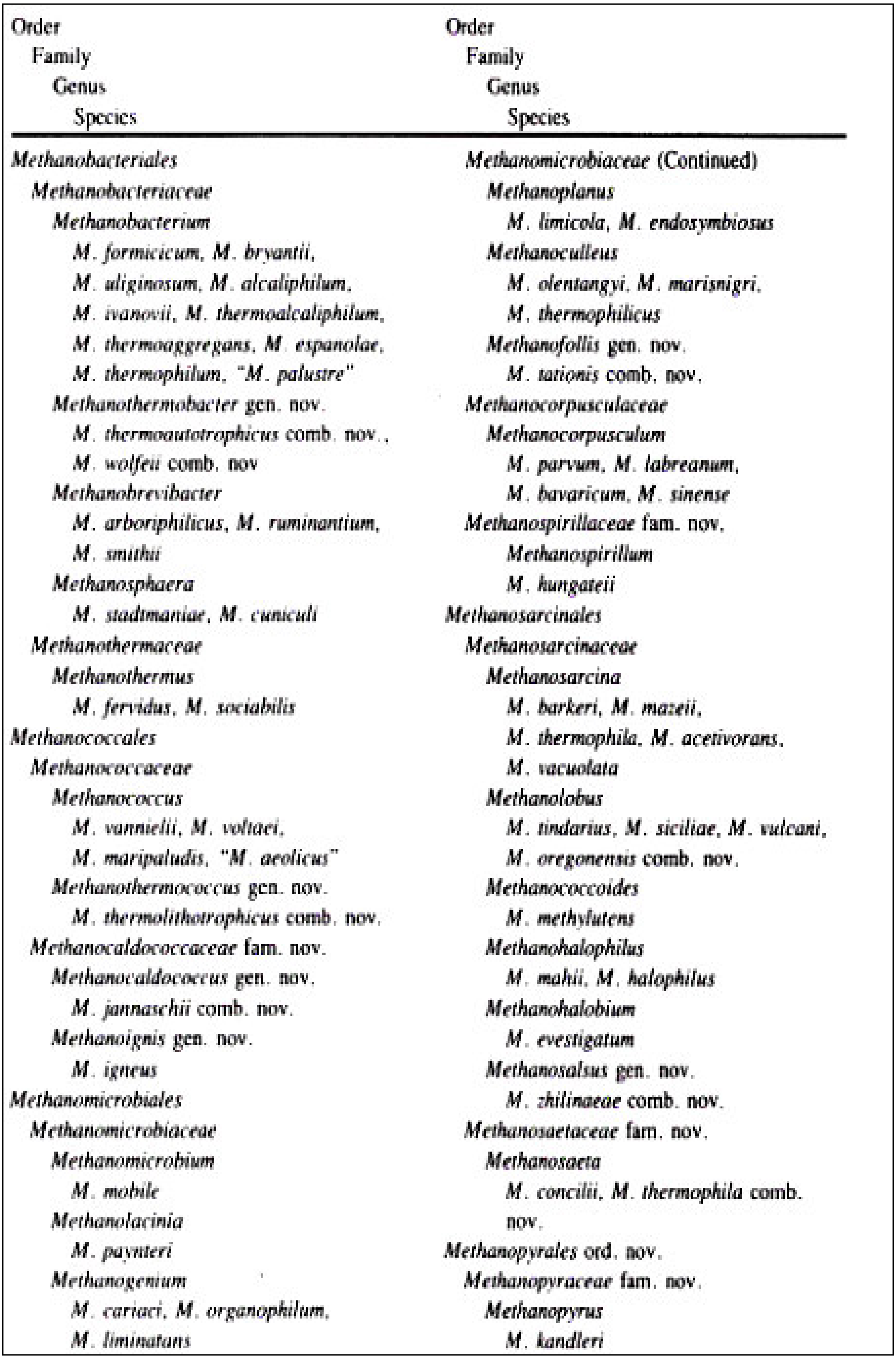
Figure 2.6. Major lineages of *Archaea*: Crenarchaeota, Euryarchaeota and Korarchaeota (Madigan et al., 2002)

Methanogens belong to the kingdom *Archaea* and classified in this kingdom in five orders:

1. *Methanobacteriales*
2. *Methanococcales*
3. *Methanomicrobiales*
4. *Methanosarcinales*
5. *Methanopyrales*

Methanobacteriales, Methanococcales and Methanomicrobiales were described in Bergey's Manual of Systematic Bacteriology (Boone and Mah, 1989). Subsequently, the methylothrophic and acetoclastic methanogens were separated to form Methanosarcinales (Woese et al., 1990). In addition, a novel organism was discovered and placed in a new order, *Methanopyrales* (Burggraff et al., 1991). The resulting taxonomy of methanogens is given in Figure 2.7.

2.2.3.2. Characteristics of Methanogens. As mentioned above, methanogenic *Archaea* have wide diversity and they are divided into five orders with different characteristics. For example, walls of *Methanobacterium* species and relatives include pseudomerin while *Methanococcus* and *Methanoplanus* species include protein or glycoprotein in their cell walls. *Methanosarcina* and relatives contain the metachondroitin (so named because of its structural resemblance to chondroitin sulphate) in their walls. The general characteristics of different methanogenic *Archaea* species are listed in Table 2.1.



- Order
  - Family
    - Genus
      - Species

- *Methanobacteriales*
  - *Methanobacteriaceae*
    - *Methanobacterium*
      - *M. formicicum*, *M. bryantii*,
      - *M. uliginosum*, *M. alcaliphilum*,
      - *M. ivanovii*, *M. thermoalcaliphilum*,
      - *M. thermoaggregans*, *M. espanolae*,
      - *M. thermophilum*, "*M. palustre*"
    - *Methanothermobacter* gen. nov.
      - *M. thermoautotrophicus* comb. nov.,
      - *M. wolfeii* comb. nov
    - *Methanobrevibacter*
      - *M. arboriphilicus*, *M. ruminantium*,
      - *M. smithii*
    - *Methanosphaera*
      - *M. stadtmaniae*, *M. cuniculi*
  - *Methanothermaceae*
    - *Methanothermus*
      - *M. fervidus*, *M. sociabilis*
- *Methanococcales*
  - *Methanococcaceae*
    - *Methanococcus*
      - *M. vannielii*, *M. voltaei*,
      - *M. maripaludis*, "*M. aeolicus*"
    - *Methanothermococcus* gen. nov.
      - *M. thermolithotrophicus* comb. nov.
  - *Methanocaldococcaceae* fam. nov.
    - *Methanocaldococcus* gen. nov.
      - *M. jannaschii* comb. nov.
    - *Methanoignis* gen. nov.
      - *M. igneus*
- *Methanomicrobiales*
  - *Methanomicrobiaceae*
    - *Methanomicrobium*
      - *M. mobile*
    - *Methanolacinia*
      - *M. paynteri*
    - *Methanogenium*
      - *M. cariaci*, *M. organophilum*,
      - *M. liminatans*

- Order
  - Family
    - Genus
      - Species

  - *Methanomicrobiaceae* (Continued)
    - *Methanoplanus*
      - *M. limicola*, *M. endosymbiosus*
    - *Methanoculleus*
      - *M. olentangyi*, *M. marisnigri*,
      - *M. thermophilicus*
    - *Methanofollis* gen. nov.
      - *M. tationis* comb. nov.
  - *Methanocorpusculaceae*
    - *Methanocorpusculum*
      - *M. parvum*, *M. labreanum*,
      - *M. bavaricum*, *M. sinense*
  - *Methanospirillaceae* fam. nov.
    - *Methanospirillum*
      - *M. hungateii*
- *Methanosarcinales*
  - *Methanosarcinaceae*
    - *Methanosarcina*
      - *M. barkeri*, *M. mazeii*,
      - *M. thermophila*, *M. acetivorans*,
      - *M. vacuolata*
    - *Methanolobus*
      - *M. tindarius*, *M. siciliae*, *M. vulcani*,
      - *M. oregonensis* comb. nov.
    - *Methanococcoides*
      - *M. methylutens*
    - *Methanohalophilus*
      - *M. mahii*, *M. halophilus*
    - *Methanohalobium*
      - *M. evestigatum*
    - *Methanosalsus* gen. nov.
      - *M. zhilinaeae* comb. nov.
  - *Methanosaetaceae* fam. nov.
    - *Methanosaeta*
      - *M. concilii*, *M. thermophila* comb. nov.
- *Methanopyrales* ord. nov.
  - *Methanopyraceae* fam. nov.
    - *Methanopyrus*
      - *M. kandleri*

Figure 2.7. Taxonomy of methanogenic bacteria (Boone et al., 1993).

Table 2.1. Characteristics of Methanogenic *Archaea* (Madigan et al., 2000)

Genus	Morphology	Number of Species	Substrate for methanogenesis
Methanobacteriales			
Methanobacterium	Long rods	19	H_2+CO_2, formate
Methanobrevibacter	Short rods	7	H_2+CO_2, formate
Methanosphaera	Cocci	2	Methanol+H_2
Methanothermus	Rods	2	H_2+CO_2, can also reduce S^0; hyperthermophile
Methanococcales			
Methanococcus	Irregular cocci	11	H_2+CO_2, pyruvate+CO_2, formate
Methanomicrobiales			
Methanomicrobium	Short rods	2	H_2+CO_2, formate
Methanogenium	Irregular cocci	11	H_2+CO_2, formate
Methanospirillum	Spirilla	1	H_2+CO_2, formate
Methanoplanus	Plate-shaped cells	3	H_2+CO_2, formate
Methanocorpusculum	Irregular cocci	5	H_2+CO_2, formate,
Methanoculleus	Irregular cocci	6	H_2+CO_2, alcohols, formate
Methanosarcinales			
Methanosarcina	Large irregular cocci in packets	8	H_2+CO_2, methanol, methylamines, acetate
Methanolobus	Irregular cocci in aggregates	5	Methanol, methylamines
Methanohalobium	Irregular cocci	1	Methanol, methylamines;halophilic
Methanococcoides	Irregular cocci	2	Methanol, methylamines
Methanohalophilus	Irregular cocci	3	Methanol,methylamines, methyl sulfides, halophile
Methanosaeta	Long rods to filaments	4	Acetate
Methanopyrales			
Methanopyrus	Rods in chains	1	H_2+CO_2 hyperthermophile, growth at 110 °C

<u>2.2.3.3. Biochemical Reactions in Methanogenesis.</u> Methanogens obtain their energy for reproduction and cellular activity from the degradation of a relatively small number of substrates which are explained in the following sections. The most familiar and frequently acknowledged substrates are acetate and hydrogen. Acetate is commonly split to form methane while hydrogen is combined with carbon dioxide to form methane (Gerardi, 2003).

According to Madigan et al.(2000) at least ten substrates can be converted to methane by pure cultures of methanogens with various biochemical reactions. Three classes of compounds, CO_2-type substrates, methyl substrates and acetate could be given as main groups.

1. CO_2-type substrates
    1.1. Carbon dioxide (with electrons derived from H_2, alcohols, or pyruvate)
    1.2. Formate
    1.3. Carbon monoxide
2. Methyl substrates
    2.1. Methanol
    2.2. Methylamine
    2.3. Dimethylamine
    2.4. Trimethylamine
    2.5. Methylmercaptan
    2.6. Dimethylsulfide
3. Acetotrophic substrate
    3.1. Acetate

In the first class of substrate, CO_2-type substrates including CO_2, formate and carbon monoxide are reduced to methane. Although the reduction of CO_2 to CH_4, shown in Eq. 2.10, is generally H_2 dependent, other substrates in this class can supply the electrons for CO_2 reduction.

$$CO_2+4H_2 \rightarrow CH_4+2H_2O \qquad \Delta G°=-131kJ/mol \qquad (2.10)$$

The second class of methanogenic substrates are methyl group substances which are converted to methane via two conversion mechanisms. First mechanism is the formation of methane by reducing methyl group substances using an external electron donor such as H_2. In the conversion equations methanol (CH_3OH) is used as a model methyl substrate, as given in Eq. 2.11.

$$CH_3OH+H_2 \rightarrow CH_4+H_2O \qquad \Delta G°=-113kJ/mol \qquad (2.11)$$

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, shown in Eq. 2.12.

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \qquad \Delta G° = -319 kJ/mol \qquad (2.12)$$

The final methanogenic substrate is acetate. The conversion mechanism of acetate to methane and carbondioxide is called the acetotrophic reaction. It has been stated that 70% of the methane produced is derived from the acetic acid and the remaining 30% is produced from the reduction of CO_2 (Pavlostathis and Gomez, 1991), given in Eq. 2.13.

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G° = -31 kJ/mol \qquad (2.13)$$

Each of the above reactions are 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.

2.2.4. Microbial Ecology in Anaerobic Digestion

Very few environments exist in which only one population of microorganisms thrives or where populations of microorganisms do not affect each other either positively or negatively. Anaerobic ecosystems such as methanogenic bioreactors are characteristic by their complex food chains and the close symbiotic relationship between the different links in the chain and are often exemplified as classical symbiotic ecosystems in which organisms consume the products of the preceeding link in the chain, rather than consuming each other (Ahring, 2003). Also, symbiotic relationship are commonly found in anaerobic ecosystems. For example, acetogenic bacteria grow in a symbiotic relationship with methane-forming bacteria. Acetate serves as a substrate for methane forming bacteria. As an example, when ethanol is converted to acetate, carbon dioxide is used and acetate and hydrogen are produced, shown in Equation 2.14.

$$CH_3CH_2OH + CO_2 \rightarrow CH_3COOH + 2H_2 \qquad (2.14)$$

When acetate-forming bacteria produce acetate, hydrogen is also produced. If the hydrogen accumulates and significant hydrogen pressure occurs, the pressure results in termination of activity of acetate-forming bacteria and loss of acetate production. However, methane forming bacteria utilize hydrogen in the production of methane and significant hydrogen pressure does not occur (Gerardi, 2003), given in Eq. 2.15.

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad (2.15)$$

Acetate-forming bacteria are obligate hydrogen producers and survive only at very low concentrations of hydrogen in the environment. They can only survive if their metabolic waste –hydrogen- is continously removed. This is achieved in their symbiotic relationship with hydrogen-utilizing bacteria or methane-forming bacteria.

Another microbial group found in anaerobic systems, nitrogen reducers, should theoretically outcompete methanogens due to much higher energy yield of nitrate respiration. This has been verified in a few natural environments (Klüber and Conrad, 1998).

However, in some other studies done on bioreactors, it has been shown that denitrification and methanogenesis can proceed in the same reactor as long as the two processes are spatially separated. For example, Hendriksen and Ahring (1996) found that denitrification took place in the bottom of an upflow anaerobic sludge blanket (UASB) reactor utilizing all available nitrate. Methanogenesis occurred in the uppermost part of the reactor, which was depleted from nitrogen oxides. Also, in a mixed culture system of denitrifying and methanogenic sludge in a digester enriched with methanol, Chen and Lin (1993) observed no competitive interactions between the two communities.

In environments where sulfate is present, sulfate-reducing bacteria will compete with methanogenic consortia for common substrates. Direct competition will occur for substrates like hydrogen, acetate and methanol. Compared to methanogens, sulfate-reducing bacteria are much more versatile than methanogens. Compounds like propionate and butyrate, which require syntrophic consortia in methanogenic environments, are degraded directly by single species of sulfate reducing bacteria (Ahring, 2003).

While little is known about the interspecies competition between methanogens, there is considerable evidence that high acetate concentrations favor the faster growing and more versatile *Methanosarcina*, while the slow growing specialist *Methanosaeta* (formerly *Methanothrix)* is usually favored by low acetate concentrations (Zinder et al., 1984).

Considering the studies done on granular sludge samples, usually *Methanosaeta* species is the predominant species among the *Methanosarcinales* (Zheng and Raskin, 2000). Also, in another study done by Zheng (1999), *Methanosaeta* species were found to serve as backbone for the granulation process and other bacterial and archaeal cells attached them. They also remained dominant in larger cell aggregates and in the mature granules.

Microbial ecology in anaerobic digestion is a relatively new topic in environmental sciences and much needs to be done in order to understand the ecological relationships between different species in the system.

2.2.5. Identification and Classification of Microorganisms

Identification and classification of microorganisms by various methods is crucial in order to understand the microbiology and microbial ecology in a biological system. These methods include determination of the characteristics of microorganisms that lead to the formation of and stability of biomass, and the physiological and ecological properties of the microorganisms in the bioreactor. Insights into the diversity, structure and function of mixed microbial communities in biological reactors are essential for enhancing treatment efficiency and stability against inhibitory compounds. Knowledge about microbial diversity and activity of the seed biomass are also needed for a successful start-up, however, as a general application, seed biomass is taken from another biological reactor unadapted to the new wastewater.

2.2.5.1. Traditional Methods. Classical microbiology techniques used in the identification of microorganisms in bioreactors are often based on cultivation dependent methods on selective growth media and direct microscopic analyses.

Microscopic technique has been used for enumeration of cells for a long time. Although microscopic observations can give an idea about the morphology and size of the microorganisms, for identification of different microorganisms, further investigations should be carried out. Selective plate method is one of these methods. Since it is difficult to distinguish the target microorganism from contaminating colonies and to count them, this method is not generally used. Selective enrichment method can also be used, however, before applying this method, physiological properties of the microorganism should be known. Secondly, growth media which have high concentrations of electron donor or nutrients favor the growth of fast-growing microorganisms. The strain which is aimed to isolate may be oligotroph in its normal environment. Although the disadvantages, these methods are used to understand the function of the community.

For counting microorganisms, Most Probable Number (MPN) technique is commonly used. MPN is not the absolute concentrations of organisms that are present but only a statistical estimate of that concentration (Tchobanoglous and Burton, 1991). The MPN of viable cells is determined by analysis of the number of positive and negative results obtained when testing multiple portions of equal volume and using the Poisson distribution. It is very difficult to estimate the number of the target microorganisms by MPN technique because of media selectivity, particulate matter and long incubation times (Gerhardt et al., 1994).

Cultivation dependent methods are especially difficult to use in anaerobic systems because syntrophic interactions, low growth rates, unknown growth requirements, and obligate anaerobiosis make anaerobic microorganisms difficult to isolate and identify. Particularly, methanogens are among the microorganisms that are most difficult to study by cultivation based techniques.

2.2.5.2. Molecular Methods. The use of macromolecular sequence comparisons to define phylogenetic relationships has revolutionised bacterial taxonomy. Methods based on analyses of nucleic acids allow to study a wide range of microorganisms as they occur in nature without cultivation. The refinement of sequencing techniques and perhaps more notably, the development of the highly versatile Polymerase Chain Reaction (PCR) has greatly facilitated the comparative analysis of large numbers of gene sequences (Saiki et al., 1985). Furthermore, application of PCR to nucleic acids extracted directly from environmental samples enables contemporary researchers to circumvent culture. Thus, culture-independent methods are now widely used for qualitative and quantitative analysis of microbial communities in mixed cultures and environmental samples (Amann et al., 1995).

The analysis of complex ecosystems increasingly utilizes PCR techniques in the selective isolation and subsequent sequencing of genes from heterogeneous templates. The inherent sensitivity of PCR to small quantities of template nucleic acid enables the analysis of discrete easily handled samples. Furthermore, effective protocols have been developed that permit the preparation of suitable template nucleic acids from a range of environmental sources. The speed with which specific genes can be isolated from the environment has rapidly made PCR methods favoured means of molecular analysis. However, for the interpretation of the sequences obtained to be of value, the fidelity of amplicons must be considered with respect to methodological bias and inherent error.

16S rRNA sequence information allows hybridization probes to be designed. The specificity of such probes can be selected to target 16S rRNA sequences in all organisms (universal) or in a specific domain, group, family even species. Labelled oligonucleotides (fluorescent, radioactive, DIG etc.) can be applied to environmental samples or cell enrichment culture, to extracted or amplified nucleic acids or rDNA clones. If there is a match between probes and sample (complementary), the probe anneals to the target region (Amann et al., 1995).

On the other hand, hybridization is widely used in environmental microbiology studies (e.g. De Long, 1992; Raskin et al., 1994a; 1994b; Wagner et al., 2003). One of the disadvantages of this technique is the specificity of probes. Currently, most of the group-specific probes are designed on the basis of sequences of cultured organisms. It is possible to overlook some groups such as recently uncovered wealth of archaeal diversity.

Probing environmental samples with fluorescent labelled oligonucleotide probes (fluorescence in situ hybridization) in combination with conventional epifluorescence microscopy or confocal scanning laser microscopy has many advantages over other techniques: Probing does not introduce the selection biases inherent in culturing techniques; it does not depend on the amplified genes like PCR based methods; metabolically active cells are detected, so descriptions of the physiologically important population members can be obtained; it allows to identify and quantify microorganisms at different levels of phylogenetic depth and localize individual community members in their natural spatial positions, it provides a basis to estimate the in situ growth rates of microorganisms in natural populations.

Biases in PCR and cloning allow only a semi-quantitative assessment of abundance, which, however, form a suitable starting point for more comprehensive quantification with other techniques. Specific PCR approaches allow for more quantitative analysis but the best approach is to count cells specifically visualized by either in situ PCR or fluorescent in situ hybridisation (FISH). The decision on which probes or primers to use can be based on previous phylogenetic studies. Detailed studies of microbial communities with FISH are at present either directed at specific phylogenetic groups or a few broadly defined taxa. Detailed fine scale analysis (e.g. enumeration of the majority of cells present at the species-level) is very labour intensive.

2.2.5.3. Identification and Quantification of Methanogens Morphology and other microbial traits have previously been used for identification and quantification of methanogens. Grotenhuis et al. (1991) microscopically counted cell numbers of methanogens and identified aceticlastic methanogens based on morphology and hydrogenotrophic methanogens by visualizing autoflourescence at 420 nm. Morphology and ultrastructure have also been used extensively in scanning or transmission electron microscopy studies to show the location of certain microorganismsin anaerobic granules by Macleod et al. (1990) and Morgan et al. (1991). Information gained from morphology-based studies are, however, ambiguous and limited since most microorganisms are small in size and simple in morphology and ultrastructure (Ahring, 2003).

When special morphological characteristics or visualization under autoflourescence are not available, physiological and biochemical traits have been used for identification. Also, enrichments on defined substrates have been helpful to identify prevalent species in anaerobic granules by Wu et al. (1992) and MPN estimates have been used for quantification of different trophic groups of anaerobic microorganisms (Grotenhuis et al., 1991; Wu et al., 1992). However, these methods are cultivation dependent and therefore limited with the ability of microorganisms to grow under laboratory conditions. A very small fraction of the microorganisms in nature is culturable by present cultivation techniques, because of unrecognized nutrient and growth conditions and interruptions due to syntrophic interaction between different microbial groups. Also culturing may be especially difficult for anaerobes due to their low growth rates and instantenous nutritional and environmental requirements (Raskin et al., 1995).

More direct methods have been recently developed for identification, quantification and localization of microorganisms in environmental samples. Immunology techniques use monoclonal or polyclonal species-specific antibodies to detect and even quantify the abundance of cultivable microorganisms in environmental samples (Macario et al., 1991; Ovreas et al., 1997). In combination with electron microscopy, antibodies have been used to localize microorganisms in sections of anaerobic granules (Grotenhuis et al., 1991). The major disadvantages of immunotechnology are the need for axenic cultures or defined co-cultures to produce the specific anti-bodies and the high specifity that limits the detection to the species or subspecies level (Amann et al., 1995; De Long, 1992).

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). Nucleic acid-based methods allow microbial community characterization without cultivation. So far, ribosomal RNA (rRNA) and ribosomal DNA (rDNA) have been the most commonly used target nucleic acids in microbial ecology studies. In this study, 16s rRNA oligonucleotide probes will be used to identify and quantify anaerobic microorganisms in the UASB reactor sludges.

2.3. Applied Configurations of Anaerobic Digestion

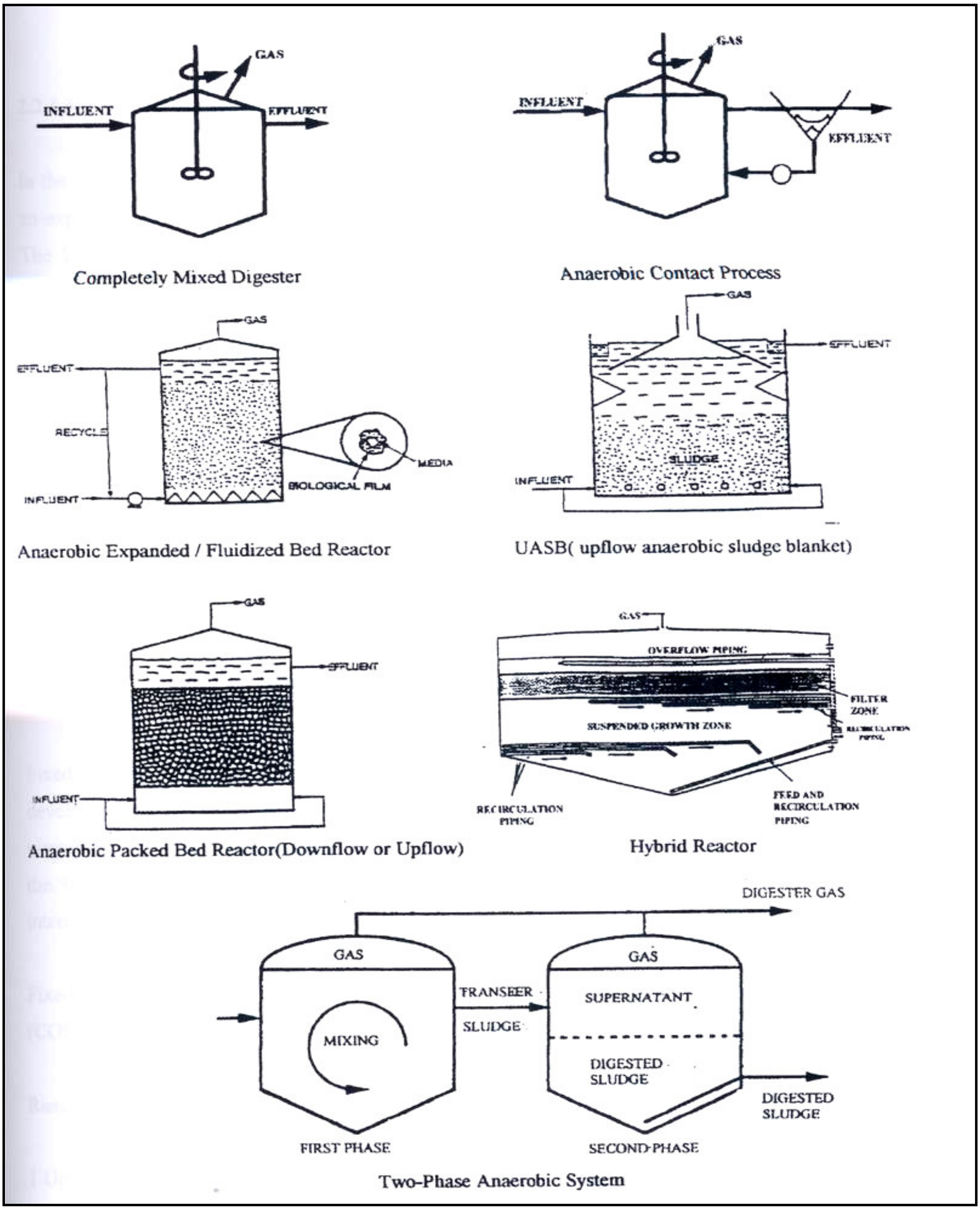
Despite the fact that anaerobic treatment technology is a relatively recently-developed and applied tool for waste treatment, many configurations has been designed and applied. The more common processes used are given in Figure 2.8.

The configurations used in anaerobic digestion could be classified into four groups:

1. Suspended Growth Systems
2. Attached Growth Systems
3. Hybrid Systems
4. Two-phase Systems

2.3.1. Suspended Growth Systems

2.3.1.1. Completely Mixed Digester. Completely mixed digesters are among the earliest configurations of anaerobic digestion and it involves the application of the conventional flow-through tank without any biomass recycle. Waste streams with high concentrations of particulates and very high concentrations of soluble biodegradable organic materials could be applied to these systems. With the chracateristics of the design, the average retention time of anaerobic microorganisms in the reactor (SRT) is almost equal to hydraulic retention time (HRT). Because of the slow growth of methanogens, process stability can be limited by the short SRTs and large reactor volumes are required to maintain necessary SRTs. With the relatively low biomass concentrations and short operating SRTs, loading rates are typically low (1-10 kg COD/m^3.day). Proper mixing conditions provide uniform conditions such as substrate concentrations, temperature and pH throughout the reactor and minimize dead volume accumulation and flow channeling.



Completely Mixed Digester

Anaerobic Contact Process

Anaerobic Expanded / Fluidized Bed Reactor

UASB(upflow anaerobic sludge blanket)

Anaerobic Packed Bed Reactor(Downflow or Upflow)

Hybrid Reactor

Two-Phase Anaerobic System

Figure 2.8. Typical Reactor Configurations used in Anaerobic Treatment (Tchobanoglous and Burton, 1991).

2.3.1.2. Anaerobic Contact Processes. Very long hydraulic retention times (HRT) of completely mixed digesters, as it is equal to the solid retention time (SRT) in the system, cause more difficult operation, higher reactor volumes. This disadvantage is overcomed in anaerobic contact process by separating and recycling biomass back to the anaerobic reactor, which is still completely mixed, but includes an additional settling tank and a sludge-recycling unit. So SRT can be controlled independently from the HRT by changing the amount of sludge recycle. Therefore, high treatment efficiency can be achieved by using short HRTs and smaller digesters due to the longer SRTs obtained with sludge recycle. Organic loading rates of 0.5 to 10 kgCOD/m^3.day can be applied to the reactor with HRTs of range between 0.5 and 5 days.

2.3.1.3. Upflow Anaerobic Sludge Blanket (UASB) Reactor. The upflow anaerobic sludge blanket (UASB) system has found wide application in the treatment of industrial wastewaters, particularly those produced in agriculturally based industries (Britz et al., 1999). For example, UASB systems have been successfully applied to treat wastewaters from sugar, potato processing, slaughterhouse, meat packing, paper mill, food and yeast industries (Lin and Yang, 1991). Also the UASB system has been shown to be a feasible method for treatment of alcohol distillery effluents, COD removal efficiencies in the range of 65%-95% can be achieved depending largely on the kind of raw material used and on the process conditions applied (Driessen et al., 1994).

In the UASB process, the waste to be treated is introduced in the bottom of the reactor. The wastewater flows upward through a sludge blanket composed of biologically formed granules. This granular sludge consists of anaerobic microorganisms, which are still visible as granules after settling and is considered as a major form for immobilization of microorganisms. Similar to biofilms, granular sludge provides minimized mass transfer limits, optimal micro-environment and protection for microorganisms such as methanogens and synthrophic bacteria. Treatment occurs as the wastewater comes in contact with the granules. The gases produced under anaerobic conditions (mainly CO_2 and CH_4) cause internal circulation, which helps in the formation and maintanance of the biological granules. Some of the gas produced within the sludge blanket becomes attached to the biological granules. The free gas and the particles with the attached gas rise to the top of the reactor. The particles that rise to the surface strike the bottom of the degassing baffles, which cause the attached gas bubblesto be released. The degassed granules typically drop back to the surface of the sludge blanket. The free gas and gas released from the granules is captured in the gas collection domes located in the top of the reactor. Liquid containing some residual solids and biological granules passes into a settling chamber, where the residual solids are separated from the liquid. The separated solids fall back through the baffle system to the top of the sludge blanket. To keep the sludge blanket in suspension, upflow velocities in the range of 0.6-0.9 m/h have been used (Tchobanoglous and Burton, 1991).

2.3.2. Attached Growth Systems

2.3.2.1. Fixed Bed Processes. Fixed bed processes contain a flooded bed of inert filter medium which is used for the development of high biomass concentrations required for efficient anaerobic treatment of wastewaters. While wastewater is passing through the medium, soluble organic compounds in the feed diffuse in surfaces of the attached biomass where the organics are converted to intermediate and final products namely methane and carbondioxide. Fixed bed processes can be used for almost all types of industrial wastewaters with low (COD<1000 mg/L) to intermediate (COD>20000 mg/L) concentrations.

2.3.2.2. Anaerobic Expanded/Fluidized Bed Processes. In the expanded/fluidized bed systems, the biomass is attached to the surface of small particles having low specific gravity particles that are kept in suspension by the upward velocity of the flow of the feed and recycle. The particles which are generally in 0.45-0.7 mm diameter and made of materials such as porous alumina, high-density plastic beads and quartzite sand provide a very large specific surface for biological growth as a thin film. Therefore, high biomass concentrations that are not subject to diffusional limitations can be developed on the surface of the particles. Biomass retains longer in the reactor because particles increase the settling velocity of the attached biofilm.

2.3.3. Hybrid Systems

Examples of hybrid systems shown in Figure 2.3.1. have simple design and require no special gas or sludge separation device. While UASB reactors are limited by the settling properties of the granular sludge, anaerobic filters are restricted with channeling and plugging due to the accumulation of suspended biomass in the bottom. So in a hybrid system as a combination of a UASB and an anaerobic filter in the top part of the UASB reactor can overcome the disadvantages of both of the configurations. There are numerous examples of such hybrid systems applied in the industry which make use of both of the systems hybridized.

2.3.4. Two-phase Systems

Different groups of bacteria which can show variations with respect to physiology, nutritional requirements, growth, metabolic characteristics and sensitivity to environmental conditions play role in the anaerobic biodegradation of organic matter (Ghosh et al., 1975; Ince et al., 1995a; Ince et al., 1995b; Ince et al., 1997). Environmental conditions can be optimized for the acid and methane-forming bacteria by using two completely mixed biochemical reactors in series in two-phase systems. Although there are numerous chemical and physical separation techniques, it is generally accepted that the most appropriate method to achieve this is by means of kinetic control which provides the required growth rates of each in separate reactors (Pohland and Ghosh, 1971; Ghosh et al., 1975; Ince et al., 1995a). Although these systems may establish proper conditions for the phased microorganisms, it also requires more extensive care and proper operating conditions in each reactor in order provide the contunity in the preceding reactions.

2.4. Environmental Factors and Process Control in Anaerobic Digestion

2.4.1. pH

pH is an important parameter which affects the solubility of substances and the reaction behavior of microorganisms thereby influencing performance of anaerobic digestion. In a properly operating anaerobic digester a pH of between 6.8 and 7.2 occurs as volatile acids are converted to methane and carbon dioxide (Gerardi, 2003). It is reported that pH below 6.0 are inhibitory to methanogens whereas acid forming microorganisms can live at this pH and continue to produce volatile fatty acids despite low pH, therefore aggravating the environmental conditions further (Lester et al., 1986; Malina et al., 1992). Methanogenesis continue at lower pH values at reduced rates but instability is observed due to the destruction of the bicarbonate buffer system under the excess production and accumulation of organic fatty acids. Optimum growth pH for some methanogenic *Archaea* are given in Table 2.2.

Table 2.2. Optimum pH for some methanogenic *Archaea* (Gerardi, 2003)

Genus	**Optimum pH Range**
Methanosphaera	6.8
Methanothermus	6.5
Methanogenium	7.0
Methanolacinia	6.6-7.2
Methanomicrobium	6.1-6.9
Methanospirillium	7.0-75
Methanococcoides	7.0-7.5
Methanohalobium	6.5-7.5
Methanolobus	6.5-6.8
Methanothrix	7.1-7.8

2.4.2. Temperature

The temperature is a significant parameter affecting microbial systems 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 have been shown 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). A sudden alteration in reactor temperature of even a few degrees may result in a corresponding increase in VFA and COD in the effluent because of a marked upset in microbial metabolism. Temperature fluctuations become more important in high loading rates.

Most methanogenic *Archaea* are active in two temperature ranges. These ranges are the mesophilic range (35-37^{o}C) and thermophilic range (50-60^{o}C). At temperatures between 40^{o}C and 50^{o}C, methane-forming organisms are inhibited. Digester performance decreases significantly somewhere near 42^{o}C, as this represents the transition from mesophilic and thermophilic range (Gerardi, 2003).

2.4.3. Nutrients

Methanogens need trace amounts of elements called as micronutrients besides nitrogen and phosphorus for their fundamental requirements of bacterial metabolism (Speece et al., 1983). The most significant micronutrients considered as necessary for various conditions of active methanogenesis are iron, nickel, magnesium, calcium, sodium, barium, tungstate, molybdate, selenium and cobalt (Henze et al., 1983). Some of the elements such as selenium, tungsten and nickel are important in the enzyme systems of acetogenic and methanogenic *Archaea* (Lester et al., 1986).

2.4.4. Toxic Substances

Anaerobic digestion is known as a sensitive process to inhibitory or toxic substances which affect the activities of anaerobic bacteria. These substances may result from either influent waste stream or the metabolic activities of the digester bacteria themselves. Toxic compounds influence anaerobic digestion either by slowing down the rate of metabolism at low concentrations or killing the organism.

Toxicity in anaerobic systems may be mainly pH related and concentration related. Ammonia, VFA and sulphides are mainly considered as pH related as they dissociate in water depending on pH and dissociation constants. In dissociated form, concentrations of ammonia above 1000 mg/L, VFA above 1000 mg/L and dissolved sulphides above 50 mg/L (for dispersed sludge) to 250 mg/L (for granular sludge) are considered toxic to anaerobic microorganisms (Arceivala, 1998).

Methanogenesis is generally the most sensitive step to these materials although all groups involved in process can be affected. Common toxic substances in anaerobic digestion causing severe operational failures are volatile fatty acids especially propionate, sulfide, ammonia, heavy metals, cyanide, organic solvents and etc.

2.4.5. Process Monitoring and Control

Much research has been carried out on the microbial, biochemical and physical characterization of the various process configurations and their operational sensitivities. However, a universally accepted menu of indicator parameters, sufficient to allow feedback control for the variety of process types and configurations used has not been established. As a result, monitoring and control is largely reactionary, without a sufficient linkage between fundamental principles and applications of the technology in practice. The issues have been widely discussed by Hickey et al., (1991) and Weiland and Rozzi (1991).

Monitoring could be implemented in either the liquid/slurry phase or in the gas phase, with the former more frequently involving measurements of pH, total and individual volatile acids, alkalinity and COD, TOC, VSS or other indicator parameter changes and the latter more frequently involving gas production and quality, mainly including analyses for methane and carbondioxide. Depending upon process circumstances, these analyses may be augmented by the measurement of specific inorganic and organic compounds, microbial/biomass characterization and gas constituents such as hydrogen, carbon monoxide, hydrogen sulphide, ammonia and trace volatile compounds.

pH, alkalinity and volatile fatty aicds are an integral expression of the acid-base conditions of anaerobic microbial treatment processes. Monitoring of pH, either internal or external to the anaerobic microbial treatment process can be used as a control technique, and methods are available to provide appropriate pH adjustments or buffer capacity as required (Loewenthal et al., 1991). Alkalinity or buffer requirements for pH adjustmentcan generally be estimated on the basis of neutralization of excess volatile acids and dissolved carbon dioxide (Li and Sutton, 1984). The results obtained from several studies (Anderson and Yang, 1992; Rozzi et al., 1985) have shown that the bicarbonate concentration is a more sensitive state parameter than both alkalinity and pH for anaerobic digester control. The bicarbonate concentration in an anaerobic digester can be calculated according to the different methods developed by Anderson and Yang (1992), Rozzi and Bunetti (1981). Monitoring individual volatile acids, particularly propionic acid, can also be used to direct loading adjustments and to prevent substrate overloads. Alkali consumption for pH control has been used as a process variable and indirect measure of total volatile acids.

Gas phase monitoring is a frequently applied technique for assessing the efficiency and state of anaerobic microbial stabilization processes. A decrease in the methane content of the gas phase normally signals a concomitant decrease in treatment efficiency of continous-flow systems. Unfortunately, such gas quality changes usually occur after a stress is imposed, and thereby reflect an effect rather than a warning of impending problems.

Several studies (Monteggia, 1991; James et al., 1990) showed that monitoring any changes in the numbers or activities of the methanogenic bacteria in the reactor using such available techniques as Microscopic Count, Most Probable Number (MPN), Coenzyme F_{420}, ATP, Dehydrogenic Activity and Specific Methanogenic Activity (SMA) can be used as a control parameter. The latter technique is more reliable and makes the determination of the most appropriate organic loading rate (OLR) possible rather than the use of conventional parameters such as pH, alkalinity, COD, VFA and gas yield which only provide concerning the current conditions inside the reactor. In addition, the SMA test provides a safe guideline for potential further increases in OLR (Ince et al., 1995a). Various methods have been developed by Van den Berg et al. (1974), Owen et al. (1979), Valcke and Verstraete (1983), de Zeeuv (1984), Dolfing and Bloemen (1985), James et al. (1990), Monteggia (1991) and Soto et al. (1993) to determine the methanogenic activity of anaerobic sludges.

The technique described by Monteggia (1991) determines the acetoclastic methanogenic activity of the sludge. The reason for using this technique is that approximately 70 % of the methane formed during the anaerobic digestion of a complex substrate results from acetic acid (Zinder, 1993).

The SMA test can also be used for the determination of the optimum operating conditions of anaerobic reactors. Three fundamental operating conditions were defined by Monteggia (1991) in a study of laboratory-scale upflow anaerobic sludge blanket reactors. Operating condition one corresponded to an actual methane production (AMP) in the digester of 60% of the potential methane production (PMP) of the sludge using the SMA test, thus resulting in high operating stability and an excellent COD removal. Operating condition two was identified as being from approximately 60% to 100% of the PMP, resulting in a lower COD removal and a stability dependent on the available alkalinity. Operating condition three took place at excessive organic loading rates (i.e. where the AMP in the digester is greater than the PMP) resulting in an irreversible imbalance in the sequential stages of anaerobic biodegradation.

The identification of methanogenic species using recently developed molecular techniques such as FISH and DGGE as a control parameter of a digester has become increasingly attractive. For instance, any deterioration in the performance of a digester may have been due to the change in the dominant species or to the species composition.

2.5. Alcohol Distillery Industry

The distilled spirits industry includes the production of whisky, gin, vodka, rum, cognac, raki, etc. Distilled spirits production may also the production of secondary products such as distillers dried grains used for livestock feeds and other feed food components. In this section, production processes of alcohol distillery industry and treatment of effluents of this industry are given.

2.5.1. Production Processes

Distilled spirits can be produced by a variety of processes. Changing on the final product and different production techniques, usually grains are mashed and fermented to produce an alcohol/water solution, that is distilled to concentrate the alcohol. Then, if necessary, distilled product is aged to provide color, flavor and aroma.

2.5.2. Treatment of Alcohol Industry Wastewater Streams

Distilleries are one of the highest consumers of raw water with consumption in the range of 25-175 L/L of alcohol in India (Uppal, 2004). The raw water requirement includes both process and non-process applications. Water consumption in process application (e.g. yeast propagation, molasses preparation, steam generation etc.) is in the range of 14.5-21.4 L/L of alcohol production. Water consumption in non-process applications (e.g. cooling water, steam generation, for making potable liquor etc.) is much higher, between 102.65 aand 240 L/L of alcohol production (Ansari, 2004). Most units depend on natural sources of water supply such as ground water and surface water (rivers, canals, etc.) for their raw water requirement. Distilleries are also one of the 17 most polluting industries listed by the Central Pollution Control Board (CPCB) of India. For every litre of alcohol produced, molasses-based distilleries generate 8-15 L of wastewater characterized by high BOD (45-60 000 mg/L), high COD (80-160 000 mg/L) and dark colour.

Treatment of distillery effluents could be performed with different methods, including aerobic treatment, anaerobic treatment, physico-chemical treatment methods and combinations of these. Treatment systems consisting of only aerobic reactors are rarely used as they have high energy requirements, high amount sludge production and the need to perform post-treatment reducing the levels to acceptable levels. Application of only physico-chemical treatment methods such as ozonation, ultrafiltration,etc. are also not feasible, as they also require high energy and are not able to remove high organic content of distillery effluents cost-effectively. Therefore, generally a main treatment stage is applied and following, a post treatment method is used.

As the main treatment stage, biological treatment systems are usually used to reduce high organic content of distillery effluents significantly as physico-chemical treatment of distillery effluents has met with little success (Sheehan and Greenfield, 1980) and aerobic reactors could not provide such a high removal efficiency for high strength distillery effluents. Anaerobic biological treatment of high-strength distillery effluent is a proven technology that has been widely applied (Rajeshwari et al., 2000). Among anaerobic treatment systems, high flow rate systems are more preferred as high organic removal rate per unit reactor volume could be achieved. These high rate systems could be classified into three, fixed film, suspended growth and hybrid systems as given in the previous sections.

Following, usually aerobic treatment is applied for further organic matter removal and if necessary, advanced treatment techniques including membrane filtration activated carbon, ion exchange, etc. are applied to obtain high quality water stream from the effluents.

3. AIM OF THE STUDY

Maintenance of active methanogenic populations in an anaerobic treatment system is critical for stable performance. Any imposed stress may lead to a change in the microbial population dynamics of the system which may ultimately be reflected in reactor performance. However, little attempt has been made to assess performance of full-scale anaerobic reactor in relation to the make up of reactor biomass in terms of qualitative and quantitative measures of methanogenic species and their activities. Malfunctions may be detected at a very early stage by monitoring changes in numbers and activities of methanogenic species in anaerobic reactors so that process conditions can be changed and a complete collapse of the reactor can be avoided.

In this study, it was therefore aimed to:

- analyze archaeal community structures of the three full-scale UASB reactors treating alcohol distillery wastewaters,
- determine potential acetoclastic methanogenic activities of the UASB reactors' sludges,
- evaluate operational conditions and performances of the reactors considering microbial composition and potential methane productions of the reactors,
- compare the results of this study with those of the previous studies on the same reactors considering archaeal community dynamics, reactors' performances and operating conditions in a long term operation period.

4. MATERIALS AND METHODS

4.1. Description of the UASB Reactors

In this study, anaerobic reactors in the wastewater treatment systems of Istanbul, Canakkale and Tekirdag alcohol distilleries were investigated. In all of the three treatment systems, anaerobic treatment is used as the main treatment option and aerobic treatment is applied for the removal of the remaining organic matter in the wastewater in order to achieve desired concentrations in the effluent of the treatment system.

In the systems, firstly all anise seeds are separated from the wastewater by pumping the raw wastewater through a screen having a pore size of 1 mm. Filtered wastewater passes to a sedimentation tank for primary settling purposes and pH, temperature, COD and nutrients etc of the wastewater are then adjusted to desired values for anaerobic treatment. UASB systems are applied in the anaerobic treatment stage. Volumes of the UASB reactors are 143 m^3, 476 m^3 and 190 m^3 in Istanbul, Tekirdag and Canakkale alcohol distillery facilities, respectively. Istanbul UASB reactor (IUASB), which is the oldest one among three, has been built in 1996 and operated since then, where Canakkale UASB reactor (CUASB) and Tekirdag UASB reactor (TUASB) has been in operation since 1997 and 1998, respectively. CUASB and TUASB reactors were both seeded with the anaerobic sludge taken from IUASB reactor. Total Volatile Solids (TVS) concentrations of the seed sludge were 70 000-80 000 mg/L. Height of all the granular sludge blankets at the bottom of the each UASB reactor is 1.5 m. In the reactor, the separators are used to hold the granular sludge and collect the biogas produced. Collected biogas is then sent to the gas buffer to be balanced and used or burned in the gas flear. Recirculation is applied in each reactor with different proportions depending on the operating conditions. The reactors are designed to be operated at mesophilic range (35-37^{o}C) and in a pH range of 6.4 - 7.2 although some deviations occur during operation.

Flow diagrams of wastewater treatment systems of Istanbul, Tekirdag and Canakkale alcohol distilleries are given in Figures 4.1-4.3.

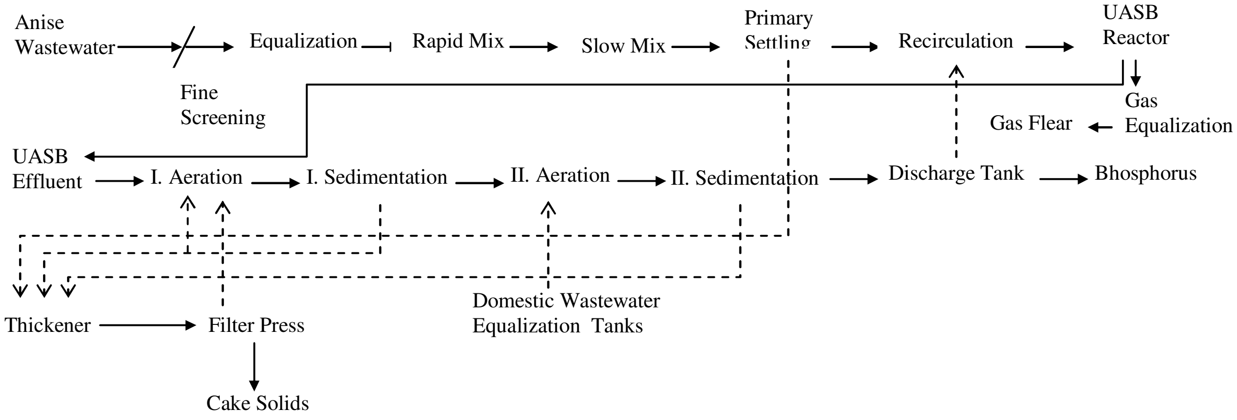


Figure 4.1. Flow diagram of wastewater treatment process of Istanbul alcohol distillery.

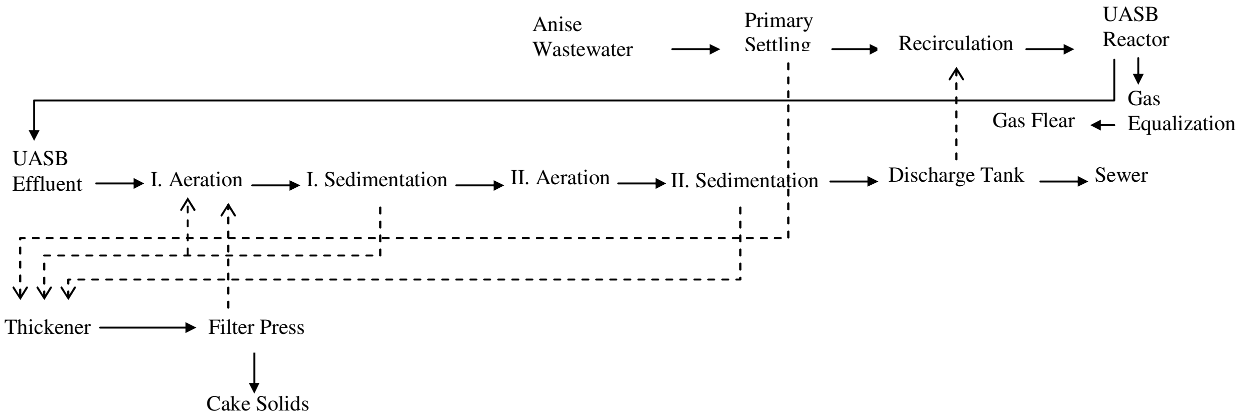


Figure 4.2. Flow diagram of wastewater treatment process of Tekirdag alcohol distillery.

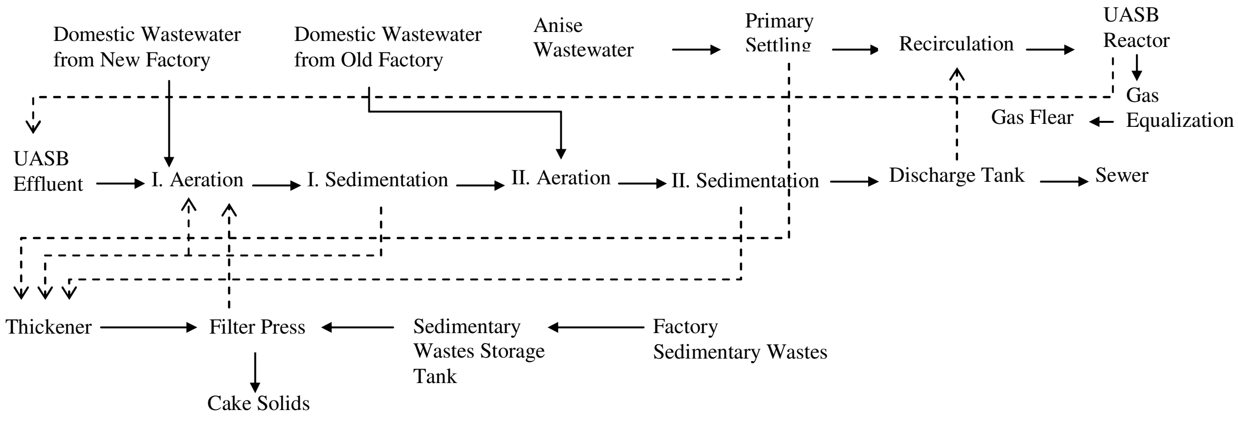


Figure 4.3. Flow diagram of wastewater treatment process of Canakkale alcohol distillery.

4.1. Wastewater Characteristics

Distilleries mainly produce raki, cognac, wine, beer, liquor, etc. as their final products. Raki is produced at Istanbul and Tekirdag distilleries while in Canakkale, cognac is produced. As the production stages significantly affect wastewater characteristics, a brief description of raki and cognac production is given below.

Raki is produced from dry grapes and anise in two steps, suma production and raki production. In suma production, firstly dry grapes are grinded into small pieces and mixed with water in order to be prepared for fermentation. The mixture is fermented for 45 to 56 hours and the fermented sugar is pumped into a distillation column where water is separated from less volatile substances. Following fermentation, the degree of alcohol is increased to 93.5-95.0% in the rectification unit. Finally, the degree of alcohol is decreased to 45-50 % in the cooling and resting unit, sugar is added concurrently and the liquid called “suma” is produced. In the second step, raki production, produced suma is maturated, softened and aged in a tank for one to four months where also anise seeds are added for aromatic taste.

Cognac is produced from dry and young white wines. Double distillation method is used in cognac production. Firstly, wines are passed to the first distillation unit, where the low strength of the wines are increased to about 28% alcoholic volume and more aroma is given. In the second distillation step, the raw cognac spirit is distilled for 12 hours, where the strength is increased to 69-72% alcoholic volume and is then matured in oak tuns of 350 L during 3-25 years or more. During maturation, cognac evaporates 2-4% per year and its strength reduces to 40 % alcoholic volume.

Wastewater characteristics of the three distilleries are given in Table 4.1.

Table 4.1. Characteristics of the three alcohol distillery wastewaters.

Parameter (mg/L)	Alcohol Distilleries		
	Tekirdag (Raki)	Canakkale (Cognac)	Istanbul (Raki)
COD	27 000-32 000	11 000-23 000	25 000-33 000
BOD_5	13 000-15 000	6 000-12 500	12 000-16 000
TKN	500-700	300-350	350-450
SO_4	-	-	50-100
Total-P	120-150	150-200	150-250
Ca^{++}	-	-	170-240
pH^{*}	4.0-6.0	6.5-7.0	5.5-6.0

* unitless

4.2. Analytical Methods

During the operation of the full-scale UASB reactor temperature, pH, COD, BOD and biogas production rates were monitored. Gas compositions for SMA tests were determined using a Hewlet Packard 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).

4.4. Specific Methanogenic Activity (SMA) Test

4.4.1. Description of the SMA Test Equipment

In this study, a fully computerized specific methanogenic activity (SMA) test unit originally developed by Monteggia (1991) and modified by Ince et al. (1995a) was used to determine acetoclastic methanogenic activity. The schematic diagram of the unit is shown in Figure 4.4.

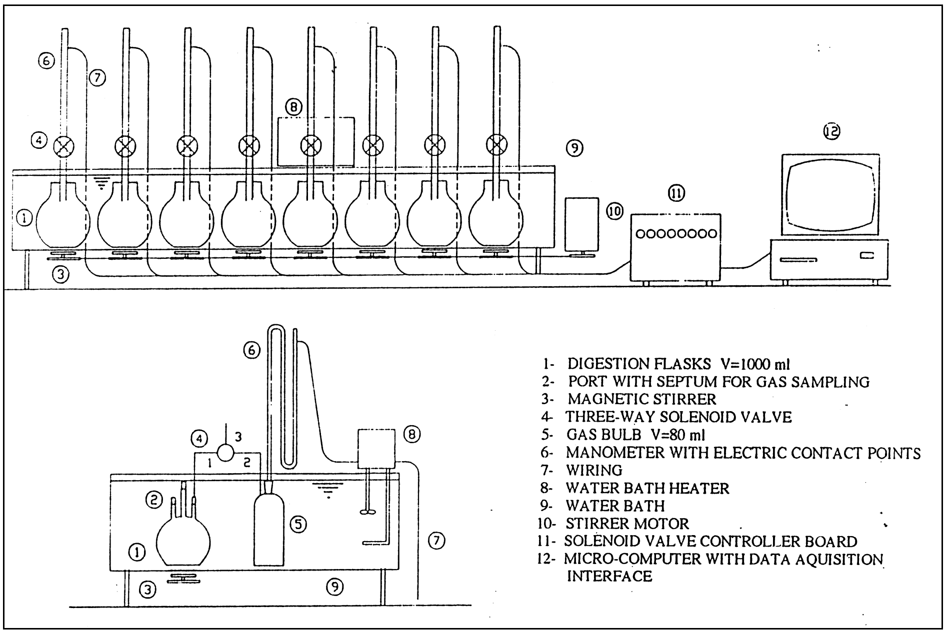


Figure 4.4. Experimental set-up for the SMA test unit.

As shown, the SMA unit consists of eight 1 L digestion flasks placed into a water bath which provides constant temperature levels in the flasks. Mixing is provided by magnetic stirrers running at a speed of 60 rpm. pH is adjusted before the operation of the system by using 1 N NaOH and 2 N HCl.

Gas measurement system contains a manometer and tubing for interconnection between the anaerobic reactor and the other units, shown in Figure 4.5.

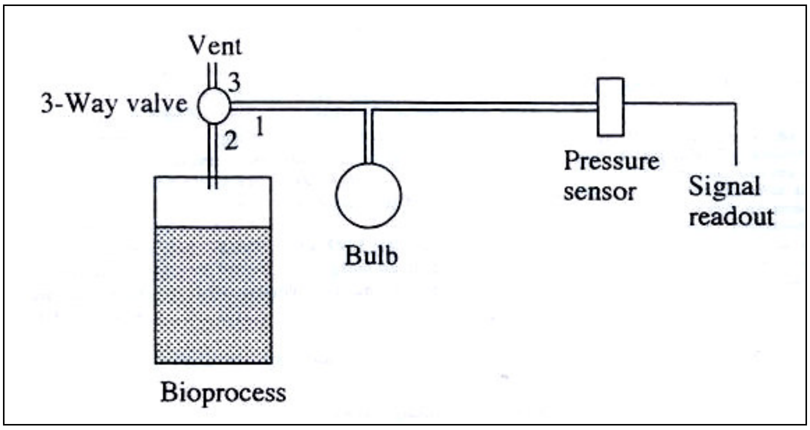


Figure 4.5. Schematic description of the gas flow metering system.

A computer supported with an 8 channel analog input board model DAS 800 (supplied by Metrabyte Corporation) is connected to the gas flow metering system which is used to simultaneously monitor the gas production of the eight independent digesters.

The device used for calibration of the eight digesters with their respective gas flow meters is shown in Figure 4.6 and was described by Monteggia (1991). The eight digesters and the respective gas flow meters were individually calibrated by injecting a known volume of gas.

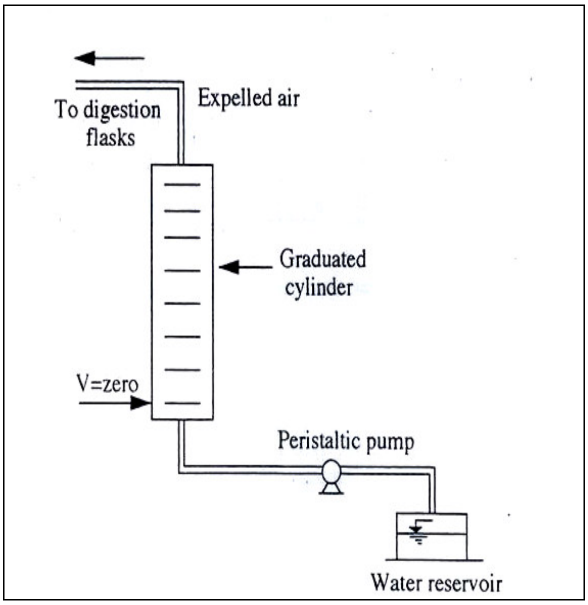


Figure 4.6. Calibration device.

Working principle of the unit is as follows: The gas metering system in the unit uses a solenoid valve set before. The valve is controlled with a pressure measurement device (manometer or pressure transducer). There is a gas bulb for temporary storage of the gases and a line for interconnection between anaerobic reactor and the units of the system. Normally, two ports of the valve are open (1 and 2) and communicate with the pressure measurement device and the gas bulb. During operation, the pressure in the reactor increases gradually because of the biological activity and the resulting biogas production. When the pressure inside the system reaches a set value, the control system sends an electrical signal to a control interface that activates the three-way solenoid valve, simultaneously closing the second port and opening the third port to the atmosphere. So, excess gas in the gas valve is also released. The complete release of the gas lasts 3 seconds and a cycle is completed while another one starts. The number of cycles completed in every hour is recorded in the microcomputer and givenas the test result.

4.4.2. Experimental Procedure of Specific Methanogenic Activity Test

The procedure for the application of the SMA Test and operation of the SMA unit was as follows:

1. Before starting the experiment (preferably 12 hour in advance), total volatile solids content (TVS) of the sludge sample to be analyzed was determined.
2. The concentration of total volatile solids (TVS) in the reactors was brought about 2000 mg/L by diluting sludge sample with a mineral stock solution given in Table 4.2..

Table 4.2. Mineral stock solution for SMA test (Valcke and Verstraete, 1983).

Chemical	Final Concentration (mg/L)
KH_2PO_4	2500
K_2HPO_4	1000
NH_4CI	1000
$MgCI_2$	100
$Na_2S.7H_2O$	100
Yeast extract	200

3. Sample sludge and mineral stock solution were taken to the digester flask.
4. The pH of the reactors was adjusted to 6.8.
5. Reactors were flushed with nitrogen gas with a pressure about 5-10 PSI for about 10 minutes to maintain anaerobic conditions in the reactor. The taps of the reactors were closed immediately after flushing and all connections of the SMA test unit were greased in order to prevent air leakage.
6. Water level in manometer was adjusted by using respirometer.
7. Temperature of the reactors content were maintained at 35±0.5 °C by heating water bath with the heater in the unit.

8. The test sample was acclimated for 12-16 hours. Gas production during the acclimation was neglected.
9. Acetate solution with a pre-determined concentration was added to the reactor.
10. Mixing system was opened and data collection system was started. In the data collection system, biogas production was saved automatically for every hour.
11. Methane concentration was determined at regular intervals by taking 1 mL gas sample.
12. When the biogas production levels decreased to very low amounts, meaning that acetoclastic methanogenic activity was about to end, the system was stopped and collected data were used to calculate specific methanogenic activity.

4.4.3. Calculation of Specific Methanogenic Activity

With the data collected, the potential methane production of the sample was calculated by the formula expressed below in Eq 4.1.

$$\text{SMA (mL CH}_4\text{/gVSS.d)} = (A \times B \times C \text{ x } 24) / (D \times E) \qquad (4.1)$$

A: Biogas production per hour, (mL/h)
B: Methane content of biogas produced, (%)
C: Valve factor,
D: Active volume of the SMA test reactor, L
E: Concentration of biomass in SMA test reactor, g/L

4.4.4. Feed and Seed Sludge for SMA tests

Acetate was used as feed substrate during SMA tests, since approximately 72% of the methane formed during anaerobic digestion of complex substrate results from acetic acid (Zinder, 1993). Acetate concentrations in a range of 2000-5000 mg/L were initially tested in order to reach maximum potential methane production (PMP) rate during the SMA tests.

Among those, 3000 mg/L acetate concentration was found to be optimum. TS and TVS concentrations of the three UASB reactors sludges are given in Table 4.3.

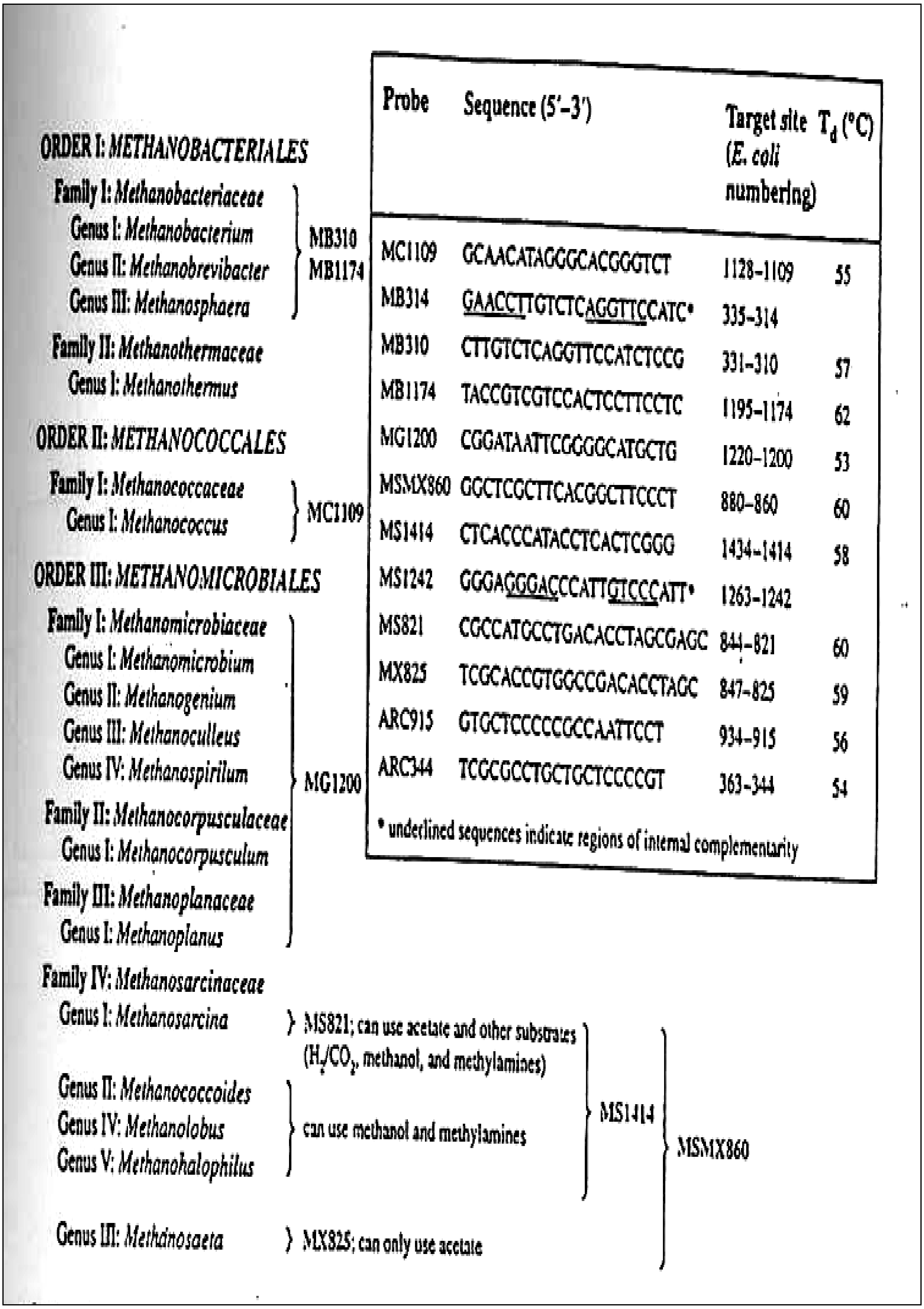
Table 4.3. TS and TVS concentrations of the three UASB reactors' sludges.

Parameter (mg/L)	TUASB reactor	CUASB reactor	IUASB reactor
TS	60 000	125 000	110 000
TVS	55 000	115 000	100 000

4.3. Fluorescent in situ Hybridization (FISH)

In this study, fluorescent in situ hybridization (FISH) is used for the determination of archaeal composition in the sludge samples. As explained in the previous chapter, fluorescent probes give superb spatial reslotion and can be detected instantenously by epiflourescence microscopy. Fluorescently mono-labelled, rRNA-targeted oligonucleotide probes have allowed the detection of individual cells (Hansen et al., 1999). The target group of microorganisms in this study were *Archaea* and methanogens. Oligonucleotide probes designed for methanogens and all *Archaea* are given in Figure 4.7.

The oligonucleotide probes, their specificities, and conditions of stringency used for FISH in this study are given in Table 4.4. Two negative controls were prepared in the hybridization experiments; one of these controls was used to assess nonspecific binding (probe Non338), and the other (lacking a probe) was used to monitor autofluorescence. All probes were obtained commercially (Qiagen, Germany). The manufacturer provided the oligonucleotide probes HPLC grade and freeze-dried state. They were resuspended in TE buffer (10 mM Tris, pH 7.5-8.0, 1 mM EDTA) to obtain a 500 ng/mL stock concentration and were then stored at -20^{o}C. Small aliquots were prepared in order to prevent freeze/thaw of high volumes of diluted probes. Flourescent dyes Tamra, Cy3 and flourescein, which are commonly found in FISH studies, were used in the study.



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

* underlined sequences indicate regions of internal complementarity

Figure 4.7. Oligonucleotide probes designed for methanogens and all *Archaea* (Raskin et al., 1994b).

Table 4.4. 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	*Methanogenium* relatives	CGGATAATTCGGGGCATGCTG	CY3	Raskin et al., 1994a
MSMX860	*Methanosarcinaceae*	GGCTCGCTTCACGGCTTCCCT	CY3	Raskin et al., 1994a
MS1414	*Methanosarcina* + relatives	CTCACCCATACCTCACTCGGG	CY3	Raskin et al., 1994a
MS821	*Methanosarcina*	CGCCATGCCTGACACCTAGGCCAGC	CY3	Raskin et al., 1994a
MX825	*Methanosaeta*	TCGCACCGTGGCCGACACCTAGC	TAMRA	Raskin et al., 1994a
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	CY3	Stahl et al, 1988
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	Fluorescein	Amann et al. 1990
UNIV1392	Virtualy all known organisms	ACGGGCGGTGTGTAC	TAMRA	Pace et al., 1986
NON338	Non sense probe	ACTCCTACGGCAGGCAGC	TAMRA	Manz et al., 1992

4.5.1. Collection and Short Term Fixation of Sludge Samples

Triplicate sludge samples were taken into sterile falcon tubes and diluted with absolute ethanol (1:1, v/v) on-site and tubes were transferred to the laboratory in cool boxes maintained at 4°C or less. Upon arrival, samples were stored at -20°C and fixed within a week.

4.5.2. Standard Paraformaldehyde (PFA) Fixation

For PFA fixation, an appropriate amount of granular sludge sample was taken, 5 g of granules were weighed and the size of the granules were decreased significantly by using a morter. The crushed granular sludge was then diluted in 20 ml of sterile distilled water. 500 µL of mixture was washed once with phosphate-buffered saline (PBS) [130 mM NaCl, 10 mM sodium phosphate, pH 7.2] and resuspended in 0.25 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°C. After fixation, cells were washed once with PBS, resuspended in 1.5 mL of PBS-absolute ethanol (1:1, v/v). Fixed sludge samples could be stored at -20°C for a long time.

4.5.3. Sybr Green I Staining

In this study, Sybr Green I staining was applied for two purposes. Firstly, intact cells in the samples were visualized. Secondly, the number of total microorganisms in the samples was estimated by the quantification of microorganisms stained with Sybr Green I.

The fixed samples were washed with PBS and then resuspended in 1 mL of PBS. Serial dilutions were prepared with PBS containing SYBR Green I (5×10^{-4} dilution of the stock) and were incubated for 15 min. The cells were washed with PBS and resuspended in 100 μL of PBS. 10 μL aliquots were spotted onto a gelatin-coated slide and air dried. One drop of Citifluor antifadent (Citifluor Ltd., U.K.) was added to the sample. Counts were obtained by using an Olympus BX 50 Epifluorescence Microscope equipped with a 100 W high-pressure mercury lamp, U-MWIB and U-MWG filter cubes. Dilutions that resulted in between about 50 and 300 cells per field of view were used.

4.5.4. Hybridization

Appropriate amount of the fixed samples which resulted in between about 50 and 300 cells per field of view (based on the total cell counts) were dehydrated at room temperature in increasing concentrations of ethanol (50%, 80% and 98%). Dehydrated samples were resuspended in 40μL of hybridization buffer (0.9 mol/L NaCl, 2 mg/L Ficoll, 2 mg/L Bovine Serum Albumin, 2 mg/mL polyvinyl pyrrolidone, 5 mmol/L EDTA, pH 8.0, 25 mmol/L NaH_2PO_4, pH 7.0, 0.1% SDS, 5-35% deionised formamide) and prehybridized at the intended hybridisation temperature for 20 min (Amann et al., 1990; Manz et al., 1992). After prehybridisation, 2 μL of probe (50 ng/μL) was added and incubated at the optimal hybridisation temperature for the given probe for at least 4 hours or overnight. Following hybridization, the cells were washed twice in wash buffer containing 20 mmol/L Tris-HCl (pH 7.2), 0.01% SDS, 5 mmol/L EDTA and between 0.9 mol/L and 56 mmol/L NaCl according to the formula of Lathe (1985) for 15 min at the optimal washing temperature before a final wash with deionized water. The cells were resuspended in 100 μL of deionized water, and a 10 μL aliquot was placed on a gelatin-coated slide and air dried. One drop of Citifluor antifadent (Citifluor Ltd., U.K.) was added to the sample.

Table 4.5. Optimum hybridization conditions for oligonucleotide probes

Probe	**Formamide concentration**	**Hybridization temperature**	**Washing temperature**	**NaCl Concentration**
MC1109	0%	46 °C	48 °C	450 mM
MB310	0%	46 °C	48 °C	450 mM
MG1200	0%	46 °C	48 °C	450 mM
MSMX860	0%	46 °C	48 °C	450 mM
MS1414	0%	46 °C	48 °C	450 mM
MS821	0%	46 °C	48 °C	450 mM
MX825	0%	46 °C	48 °C	450 mM
ARC915	0%	46 °C	48 °C	450 mM
EUB338	0%	46 °C	48 °C	450 mM
UNIV1392	0%	46 °C	48 °C	450 mM

For each sample hybridization, two negative controls were prepared; 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 prepared to assess success of cell permeabilization and rRNA content of the cells with universal probe UNIV1392.

4.5.5. Microscopy

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 a special software supplied by the camera manufacturer (Diagnostic Instruments Ltd., UK). The images were processed and analyzed using Image-Pro Plus version 5.1 image analysis software (Media Cybernetics, U.S.A.).

Different flourochromes are excited and emitted at different wavelengths. Optimum emission and excitation wavelengths and corresponding filter cubes for the flourochromes used in this study are given in Table 4.6.

Table 4.6. Optimum emission and excitation wavelengths and corresponding filter cubes for the flourochromes used in this study

Flourochrome	**Colour of Flourescence**	**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	550	565	U-MWG
SYBR GREEN I	Green	494	521	U-MWIB

4.5.6. Quantification of Microorganisms

Quantification of microorganisms in the three sludge samples were conducted by using Image-Pro Plus version 5.1. image analysis software. Quantification involves counts of total microorganisms with Sybr Green staining and counts of specific methanogenic groups with other oligonucleotide probes by using FISH.

Quantification of Total Microorganisms. For each sample, firstly Sybr Green I DNA stain was used to determine the average number of total microorganisms. For all times, triplicate samples were collected from the three UASB reactors and 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.

Quantification of Archaea and Methanogens. Quantification of methanogens involves application of FISH technique with oligonucletiode probes specific for different methanogenic groups given in Table 4.4. For all times, triplicate samples were collected from the UASB reactor and counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated. So a representative number of microorganisms in each group was found.

5. RESULTS AND DISCUSSION

5.1. Performances of the UASB reactors

Performances of the UASB reactors were evaluated in terms of COD removal efficiency and organic loading rate. The source data for the year of 2005, which were used to estimate COD removal efficiency and organic loading rate, were taken from Mey İçki Co. General Directorship. These information were then compared with the results of a study completed in 2004 by Kolukırık investigating the performances of the same reactors.

Changes in COD removal efficiency and organic loading rate of the IUASB reactor throughout the year 2005 are given in Figure 5.1.

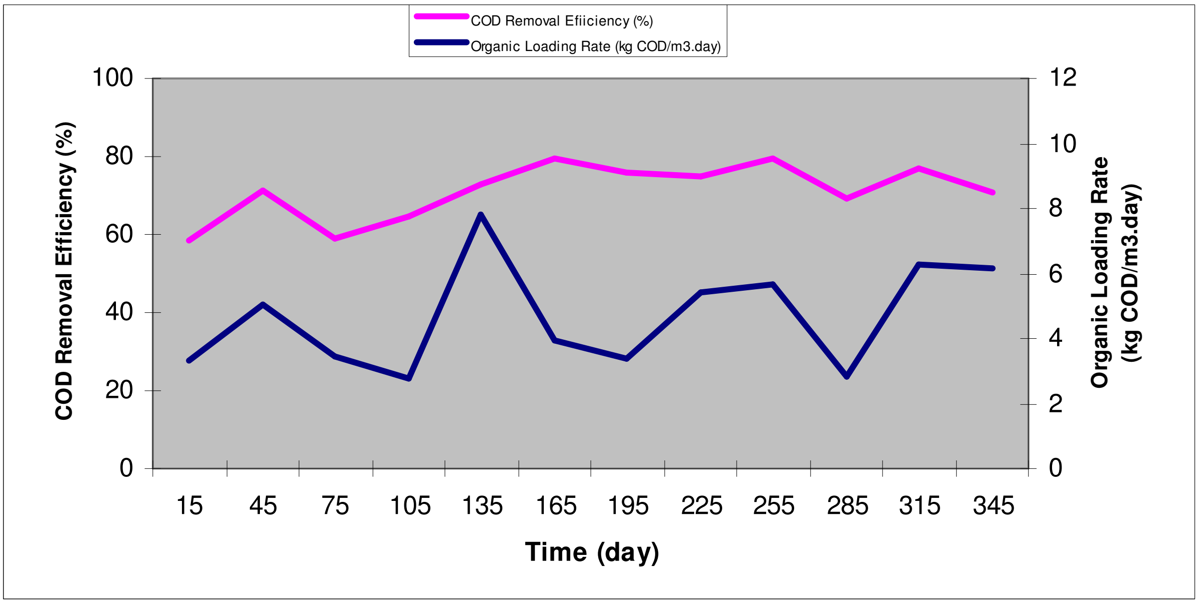


Figure 5.1. Changes in COD removal efficiency and OLR of the IUASB reactor throughout the year 2005.

The IUASB reactor achieved COD removal efficiencies between 60-70% at OLRs in a range of 3-5 kg COD/m^3.day in the first 4 months of the year 2005. After this period, the reactor achieved better COD removal efficiencies which were no lower than 70 %, at OLRs applied in a range of 3-8 kg COD/m^3.day. The UASB reactor had been operated under a F:M (food to microorganisms) ratio between 0.03 and 0.08 which was much lower than the typical values reported for similar reactors (Ince et al., 2005; Baier and Delavy 2005; Driessen et al., 1994; Ince et al., 1994). Also the temperature within the IUASB reactor was maintained at 30°C, which was lower than the optimum operating temperature of mesophilic reactors (35-37 °C). Operating the reactor at temperature levels closer to the mesophilic range could have increased COD removal efficiencies of the reactor.

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Changes in COD removal efficiency and organic loading rate of the IUASB reactor between 2001 and 2004 are given in Figure 5.2.

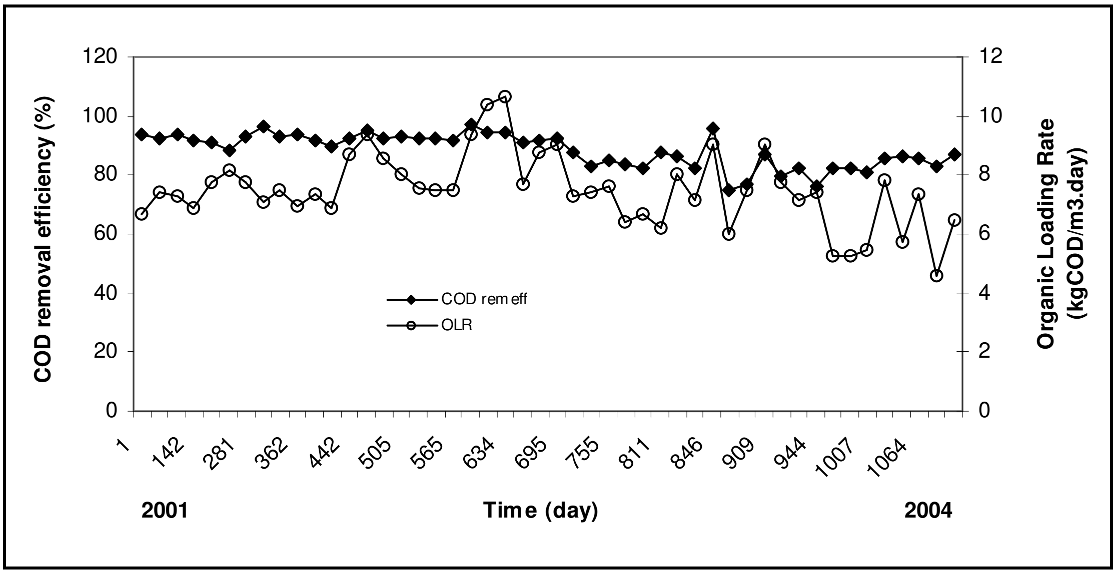


Figure 5.2. Changes in COD removal efficiency and OLR of the IUASB reactor between years 2001 and 2004 (Kolukırık, 2004).

The IUASB reactor performed well achieving COD removal efficiencies of no lower than 80% at OLRs in a range of 6-11 kg COD/m3.day over a period of 1000 days between 2001 and 2004. However, COD removal efficiency decreased slightly in the last year of operation.

Comparing the performances between 2001-2004 and the year 2005, the decrease in the COD removal efficiencies continued, as also OLRs applied were still far from the typical OLRs applied for such UASB reactors and have a overall decreasing projection between 2001 and 2005.

Changes in COD removal efficiency and organic loading rate of the TUASB reactor throughout year 2005 are given in Figure 5.3.

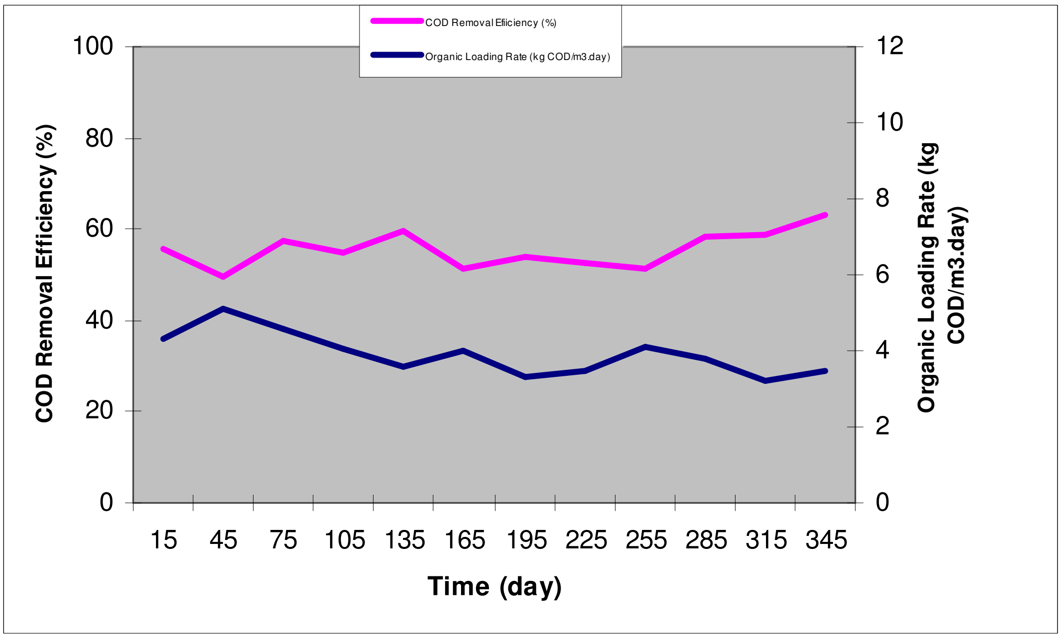


Figure 5.3. Changes in COD removal efficiency and organic loading rate of the TUASB reactor throughout the year 2005.

The TUASB reactor achieved COD removal efficiencies between 50-65% at OLRs in a range of 3-5 kg COD/m^3.day throughout year 2005. The UASB reactor had been operated under a F:M (food to microorganisms) ratio between 0.06 and 0.09 which is also very low compared to the typical values reported for similar reactors. Although the reactor was operated in the optimum range of pH and temperature throughout the year, the OLRs applied were lower than the typical values for UASB reactors. This could be also the reason for the COD removal efficiencies being lower than the desired values.

Changes in COD removal efficiency and OLR of the TUASB reactor between 2002 and 2004 are given in Figure 5.4.

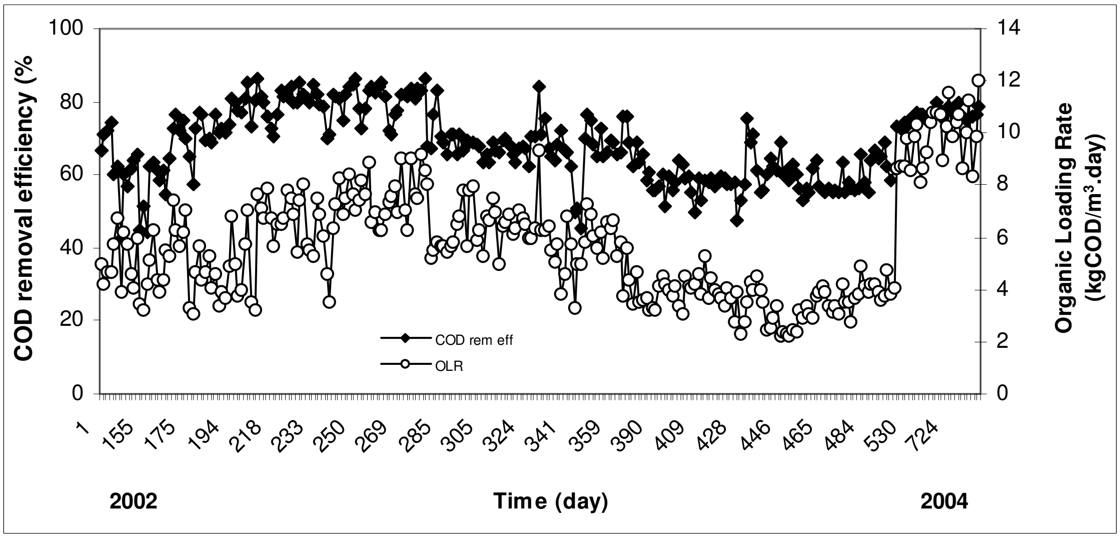


Figure 5.4. Changes in COD removal efficiency and organic loading rate of the TUASB reactor between years 2002 and 2004 (Kolukırık, 2004).

Between years 2002 and 2004, performance of the TUASB reactor in terms of COD removal efficiency was in a range of 60-80% at OLRs in a range of 2.5 and 12 kg COD/m3.day.

Comparing the performances between years 2002-2004 and year 2005, depending on the OLRs applied, the COD removal efficiency remained lower than the typical range in such UASB reactors in both periods. Although there was an increase in the COD removal efficiency at the end of the year 2003 and beginning of 2004, which seemed to be due to the increasing OLRs applied, desired performance (COD removal efficiency > 80%) could still have not been achieved throughout year 2005.

Changes in COD removal efficiency and organic loading rate of the CUASB reactor throughout year 2005 are given in Figure 5.5.

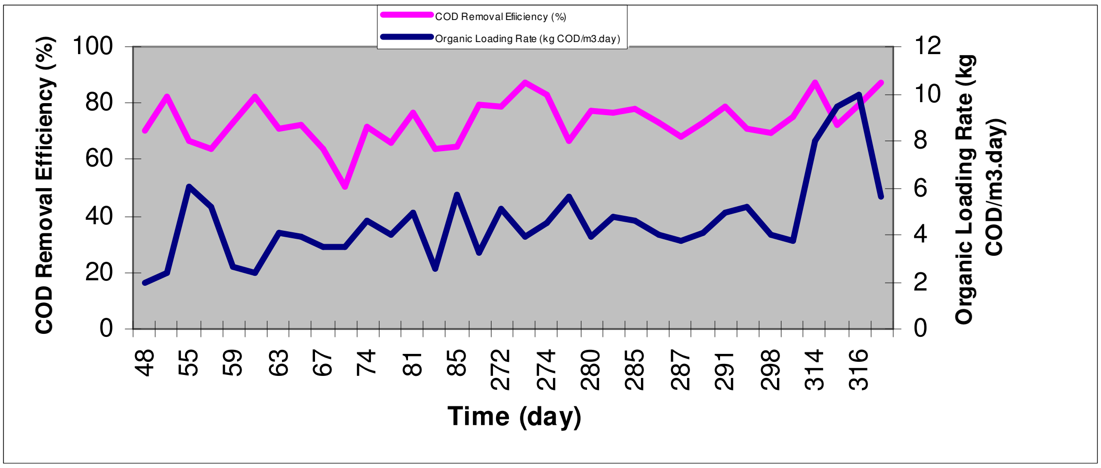


Figure 5.5. Changes in COD removal efficiency and OLR of the CUASB reactor throughout the year 2005.

The CUASB reactor achieved COD removal efficiencies between 60-80% at OLRs in a range of 2-10 kg COD/m^3.day in 2005. The UASB reactor had been operated under a F:M (food to microorganisms) ratio between 0.02 and 0.09 which is also much lower than the typical values reported for similar reactors. The direct relation between the OLRs applied and COD removal efficiencies achieved could be observed in the similar behaviour of the OLR and COD removal efficiency curves. At the end of the year, at higher OLRs applied, increasing from 4 to 10 kg COD/m^3.day, the reactor performed better achieving COD removal efficiencies usually above 80 %. The temperature in the reactor was usually in a range of 20-25 ^{o}C, which was lower than the optimum temperature range for mesophilic reactors (35-37^{o}C). Operating the reactor at temperature levels close to this range could have given an increase in COD removal efficiency of the reactor.

Changes in COD removal efficiency and organic loading rate of the CUASB reactor between 2001 and 2004 are given in Figure 5.6.

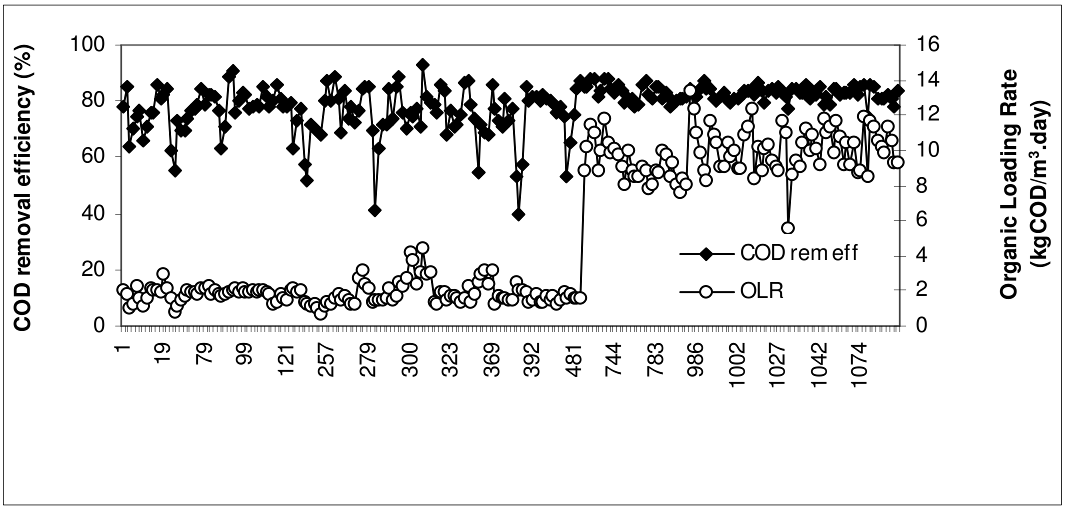


Figure 5.6. Changes in COD removal efficiency and organic loading rate of the CUASB reactor between years 2001 and 2004 (Kolukırık, 2004).

Between 2001 and 2004, performance of the CUASB reactor in terms of COD removal efficiency varied between 70% and 85% at OLRs in a range of 1 to 12 kg COD/m^3.day. Higher OLRs resulted in higher COD removal efficiencies and also better reactor stability was achieved. Comparing the performances between 2001-2004 and 2005, there was a decrease in the COD removal efficiencies of the reactor between 2004 and 2005. However, in the last quarter of year 2005, COD removal efficiencies achieved were very close to those at the end of 2003 which could be mainly due to higher organic loadings applied.

5.2. SMA Test Results

SMA tests were carried out to determine the potential loading capacity and optimum operating conditions of the UASB reactor. The results of the test were then compared with those obtained from a previous study using the same UASB reactors (Kolukırık, 2004).

The results of the SMA test applied to the IUASB sludge in November 2005 are given in Figure 5.7. Potential methane production (PMP) of the sludge sample was found to be 192 mL CH_4 /gVSS.day. The corresponding actual methane production (AMP) rate data of the IUASB reactor were not available in 2005. When the PMP rate is compared to AMP the average PMP rates between 2001 and 2004, 40 mL CH_4/ g VSS.day, AMP/PMP ratio was found to be 0.21 showing that the anaerobic sludge was operating at only 21% of its potential acetoclastic methanogenic capacity. These results showed that the UASB reactor was under loaded compared to its maximum loading capacity.

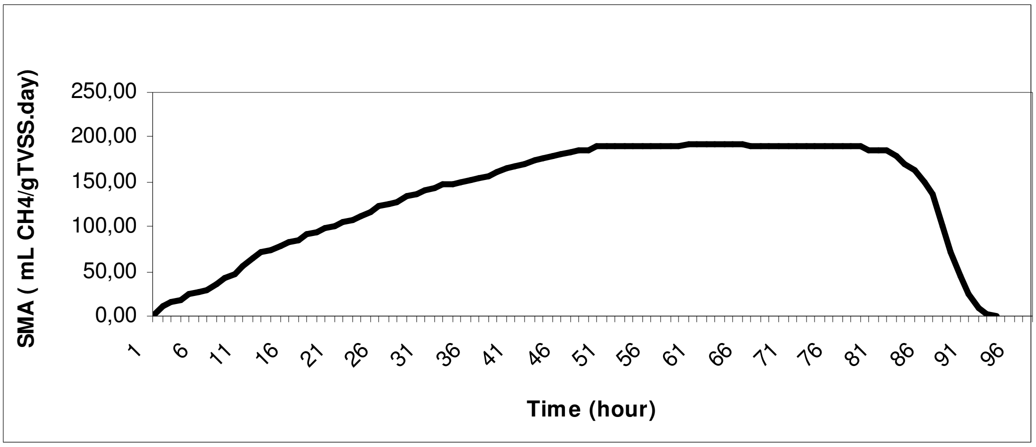


Figure 5.7. SMA test results for the IUASB reactor sludge (November 2005).

SMA test results for the IUASB reactor sludge between 2001 and 2004 are given in Figure 5.8.

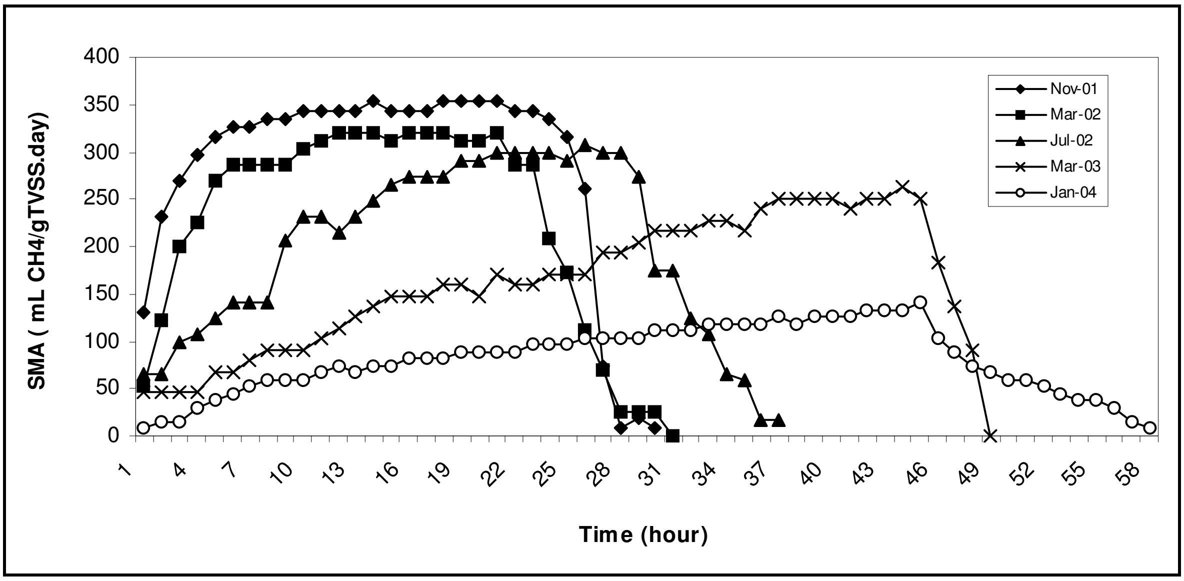


Figure 5.8. SMA test results for the IUASB reactor sludge (2001 - 2004) (Kolukırık, 2004).

When the results of SMA test carried out in November 2005 were compared with those in 2004, a 62% decrease in PMP rate of the anaerobic granular sludge was observed.

Comparing the results of the SMA test carried out in 2005 with those in 2004, an increase from 133 mL CH_4 /gVSS.day to 192 mL CH_4 /gVSS.day in potential methane production of the IUASB reactor sludge was observed. However, the loss in potential methane production, started from 2002 until 2004, was not significantly recovered in 2005. This low potential acetoclastic methanogenic activity could have been due to retaining a high amount of granular sludge within the UASB reactor resulting in a low F:M ratio of 0.03-0.08.

The results of the SMA test applied to the TUASB reactor sludge in November 2005 are given in Figure 5.7. PMP of the sludge sample was found to be 132 mL CH_4 /gVSS.day. The corresponding AMP rate data of the TUASB reactor in 2005 were not available. When the PMP rate was compared to the average AMP rate between 2002 and 2004, 41 mL CH_4/gVSS.day, AMP/PMP ratio was found to be 0.31 showing that the anaerobic sludge was operating at only 31% of its potential acetoclastic methanogenic capacity. These results revealed that the TUASB reactor was also under loaded compared to its maximum loading capacity.

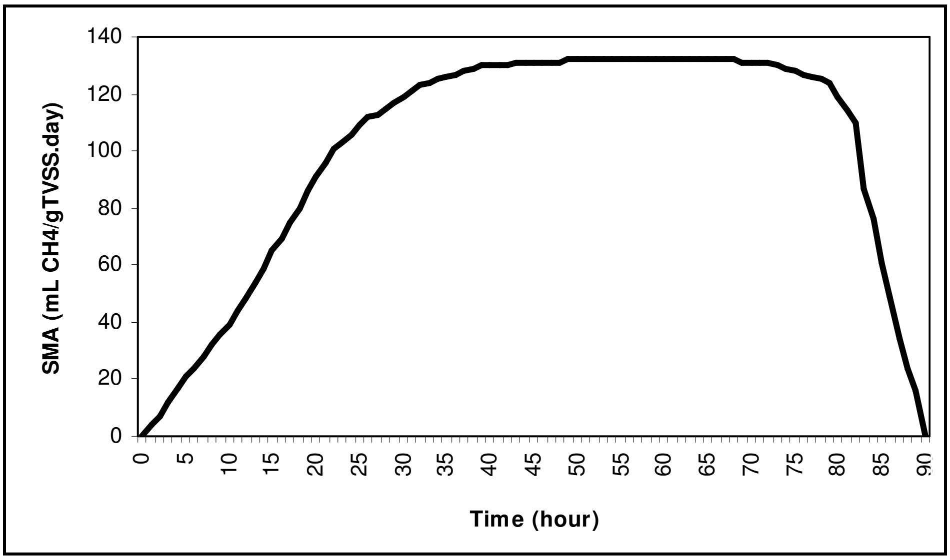


Figure 5.9. SMA test results for the TUASB reactor sludge (November 2005).

SMA test results of the TUASB reactor sludge between 2002 and 2004 are given in Figure 5.10.

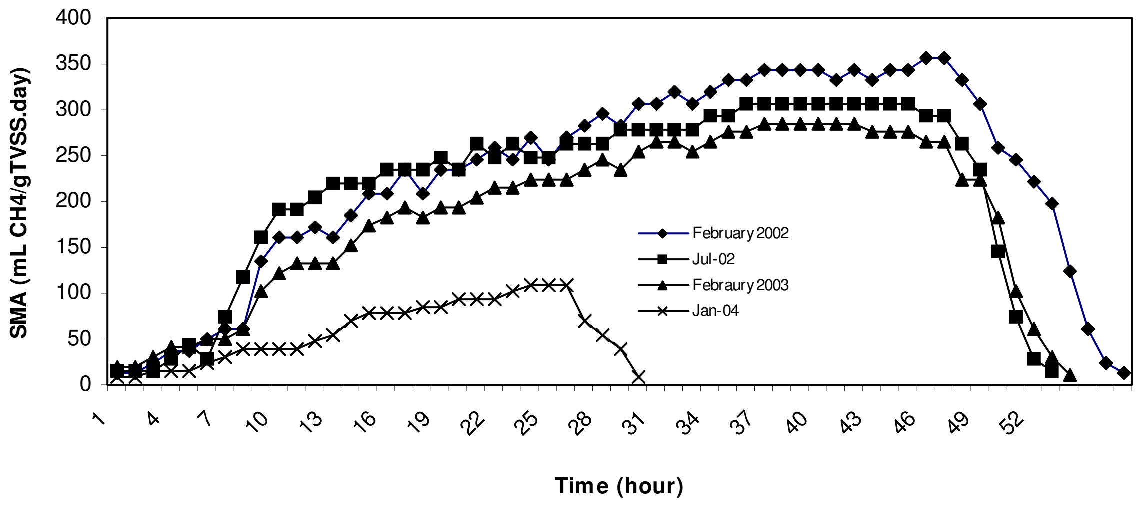


Figure 5.10. SMA test results for the TUASB reactor sludge (2002 – 2004) (Kolukırık, 2004).

Comparing the results of the SMA test carried out in 2005, with those in 2004, an increase from 109 mL CH_4/gVSS.day to 132 mL CH_4/gVSS.day in potential methane production of the TUASB reactor sludge was observed. However, the loss in potential methane production, started from 2002 until 2004, was not significantly recovered in 2005. As in the case of IUASB reactor, this low value in potential acetoclastic methanogenic activity could have been due to retaining a high amount of granular sludge within the UASB reactor resulting in a F:M ratio of 0.06-0.09.

The results of the SMA test applied to the CUASB sludge in November 2005 are given in Figure 5.7. PMP of the sludge sample was found to be 167 mL CH_4 /gVSS.day. The corresponding AMP rate data of the CUASB reactor in 2005 were not available. When the PMP rate was compared to the average AMP rate between 2002 and 2004, 46.5 mL CH_4/gVSS.day, AMP/PMP ratio was found to be 0.28 showing that the anaerobic sludge was using 28% of its potential acetoclastic methanogenic capacity in 2005. These results also showed that the CUASB reactor was under loaded compared to its maximum loading capacity as it was the case for IUASB and TUASB reactors.

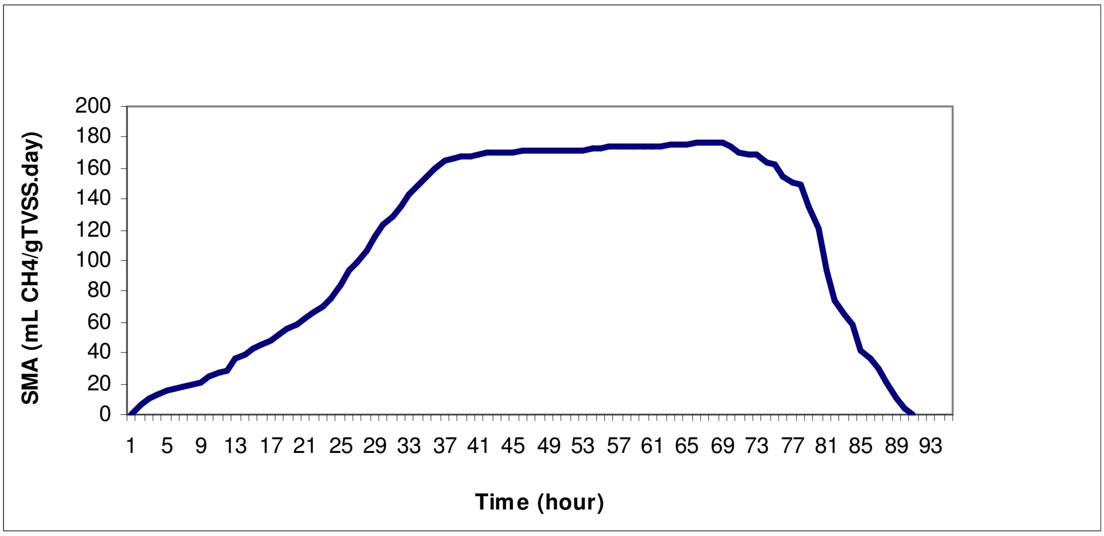


Figure 5.11. SMA test results for the CUASB reactor sludge (November 2005).

SMA test results of the CUASB reactor sludge between 2002 and 2004 are given in Figure 5.12.

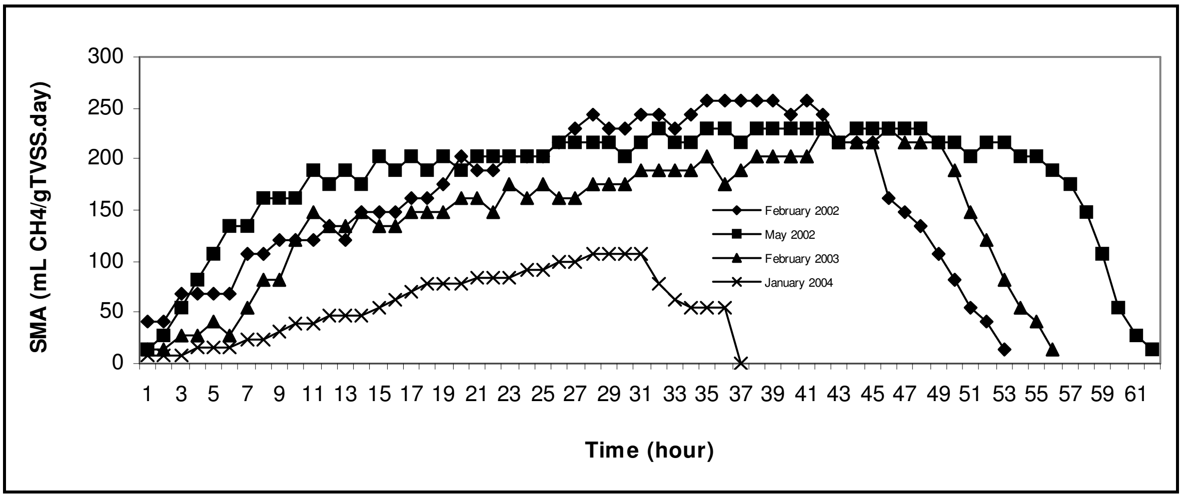


Figure 5.12. SMA test results for the CUASB reactor sludge (2002 – 2004) (Kolukırık, 2004).

Comparing the results of the test carried out in 2005, with those in 2004, an increase from 108 mL CH_4/gVSS.day to 167 mL CH_4/gVSS.day in potential methane production of the CUASB reactor sludge was observed. The loss in potential methane production, started from 2002 until 2004, was significantly recovered in 2005. However, PMP rate of the CUASB sludge in 2005 was still lower than that of good quality UASB granular sludges reported previously. The low value in potential acetoclastic methanogenic activity could have been due to retaining a high amount of granular sludge within the UASB reactor resulting in a F:M ratio of 0.02-0.09 and maintaining temperature levels (15-25^{o}C) lower than the typical range (35^{o}C) for mesophilic UASB reactors.

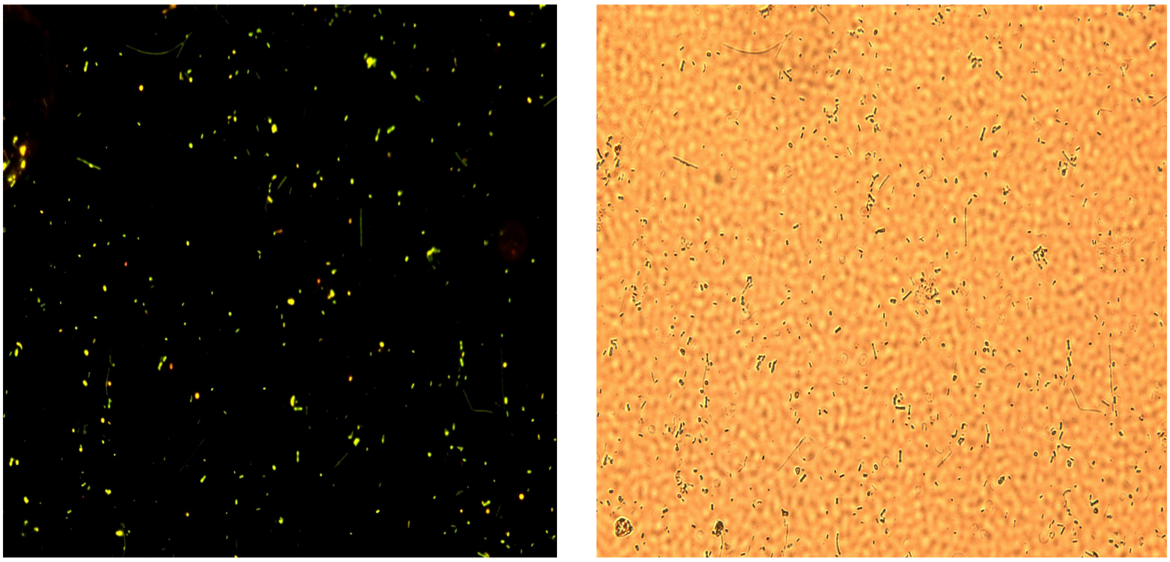
5.3. FISH Results

Samples were stained with SYBR Green before hybridization to observe intact cell concentration in the UASB sludges. Staining with Sybr Green was also used in quantification of total microorganisms.

The microbial community structures of the three UASB sludges were characterized using fluorescent rRNA targeted oligonucleotide probes specific for *Archaea* and phylogenetically defined groups of methanogens. For each sample hybridization, two negative controls were prepared; 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 prepared to assess success of cell permeabilization and rRNA content of the cells with universal probe UNIV1392.

Microscopic analyses of the granular sludge samples from the three UASB reactors were also examined in terms of granule size and rod length.

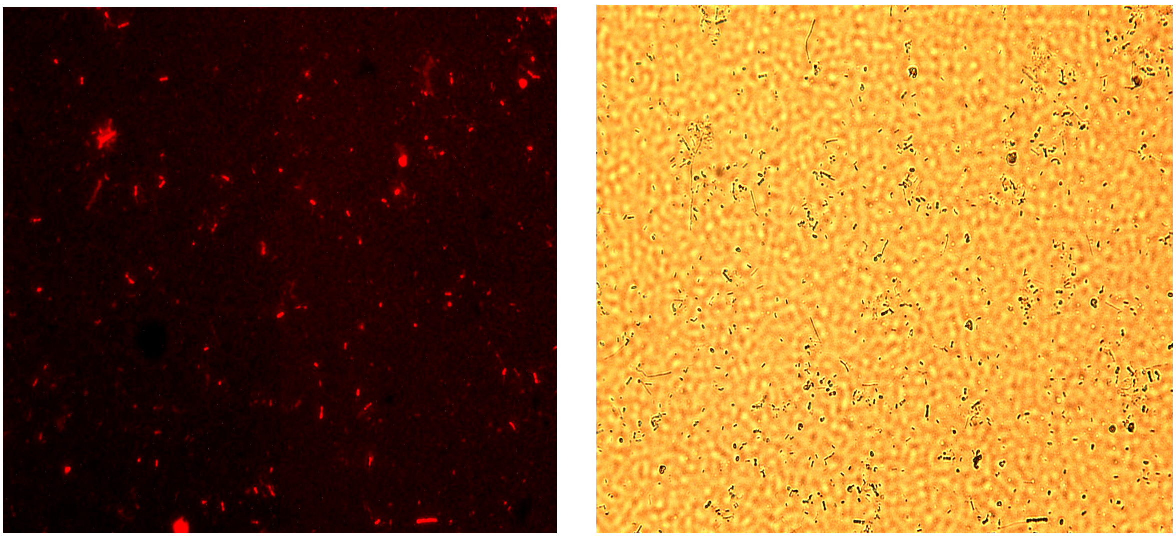
Epifluorescence micrograph examples of the Sybr Green I stained and hybridized IUASB sludge samples are shown in Figure 5.13-5.16.



a. b.

Figure 5.13. Epifluorescence micrograph example of the IUASB reactor's sludge sample stained with Sybr Green I.

a) Cells stained with Sybr Green I stain. b) Light microscope view of the image.

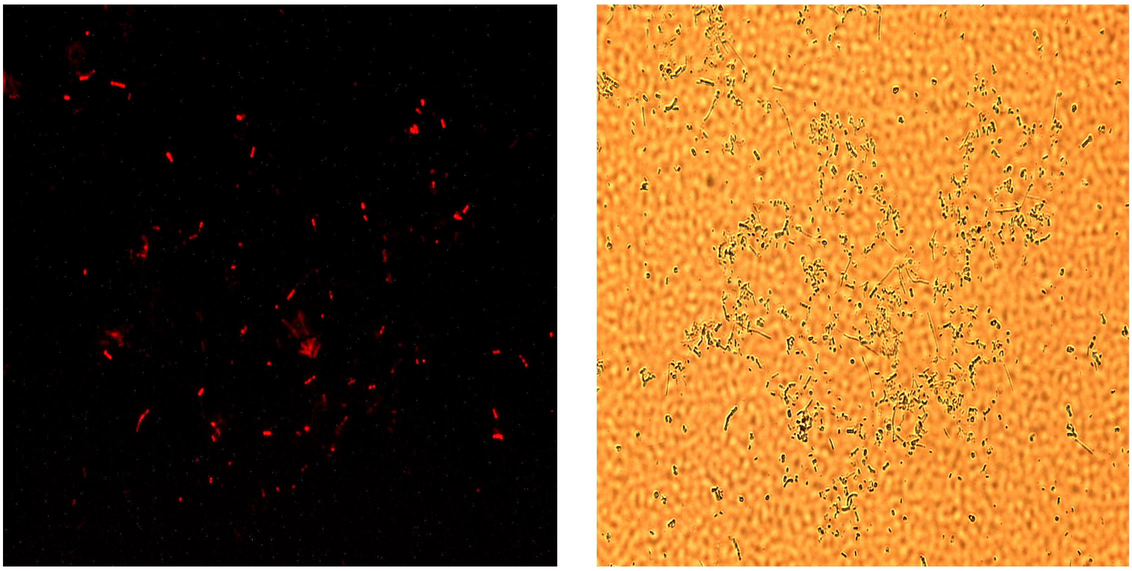


a. b.

Figure 5.14. Epifluorescence micrograph example of the IUASB reactor's sludge sample hybridized with ARC 915 probe (*Archaea*).

a) Cells hybridized with ARC 915 probe (*Archaea*).

b) Light microscope view of the image.

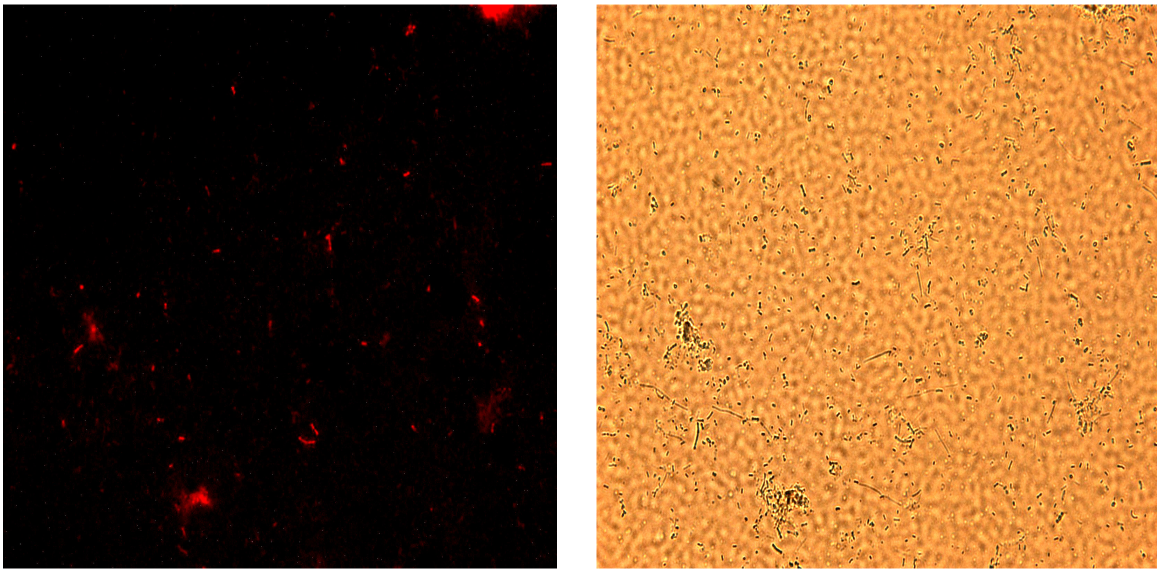


a. b.

Figure 5.15. Epifluorescence micrograph example of the IUASB reactor's sludge sample hybridized with MX 825 probe (*Methanosaeta*).

a) Cells hybridized with MX 825 probe (*Methanosaeta*).

b) Light microscope view of the image.



a. b.

Figure 5.16. Epifluorescence micrograph example of the IUASB reactor's sludge sample hybridized with MB 310 probe (*Methanobacteriales*).

a) Cells hybridized with MB310 probe (*Methanobacteriales*).

b) Light microscope view of the image.

Figure 5.17 shows the quantification results of the IUASB sludge sample in November 2005.

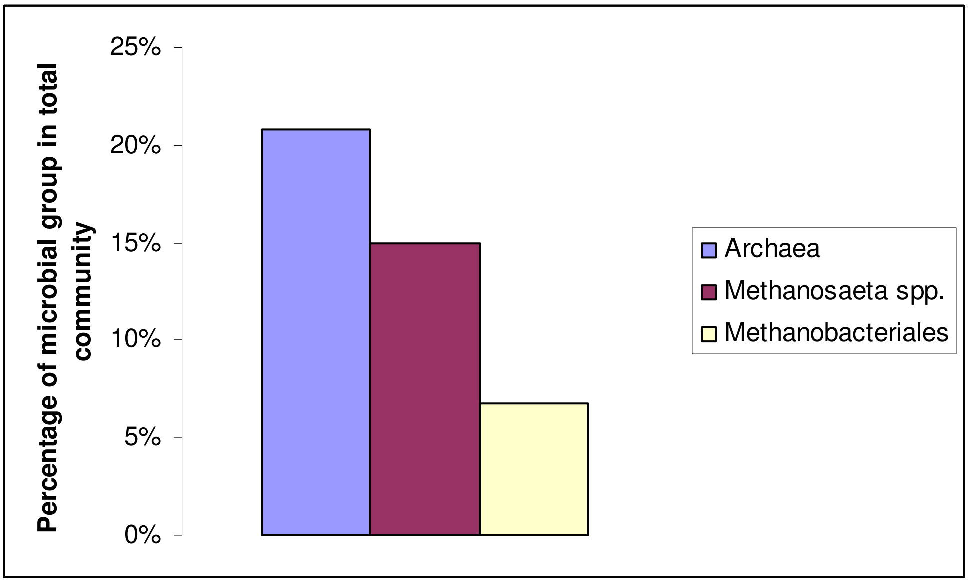


Figure 5.17. FISH Quantification results for IUASB Sludge in November 2005.

Results in November 2005 showed that 20.7%±0.9% of the cells in IUASB reactor sludge belonged to the archaeal domain. Archaeal population in anaerobic reactors has been shown previously to range from 10 to 90% of the total prokaryotic cells (Raskin et al. 1994b, 1995, 1996; Harmsen et al., 1996; Ficker et al., 1999; Angenent et al., 2000; Gonzalez Gil et al., 2001; Hansen et al., 1999; Tay et al., 2001; Saiki et al., 2002; Montenegro et al., 2003; Angenent et al., 2002 and 2004). The relative abundance of *Archaea* in the current study were similar to archaeal abundance in anaerobic sewage sludge digesters (Raskin et al. 1994b, 1995, 1996; Hansen et al., 1999). In this study, the archaeal subpopulation of the IUASB reactor sludge consisted of 71.8%±5.5% of members of the genus *Methanosaeta*, and 32.7%±3.8% of *Methanobacteriales*. Other important archaeal microbial groups such as *Methanosarcina*, *Methanococcales*, *Methanogenium* relatives were not observed in the IUASB sludge in November 2005.

Figure 5.18. shows the quantification results obtained with fluorescent rRNA targeted oligonucleotide probes and light microscope views between 2002 – 2004.

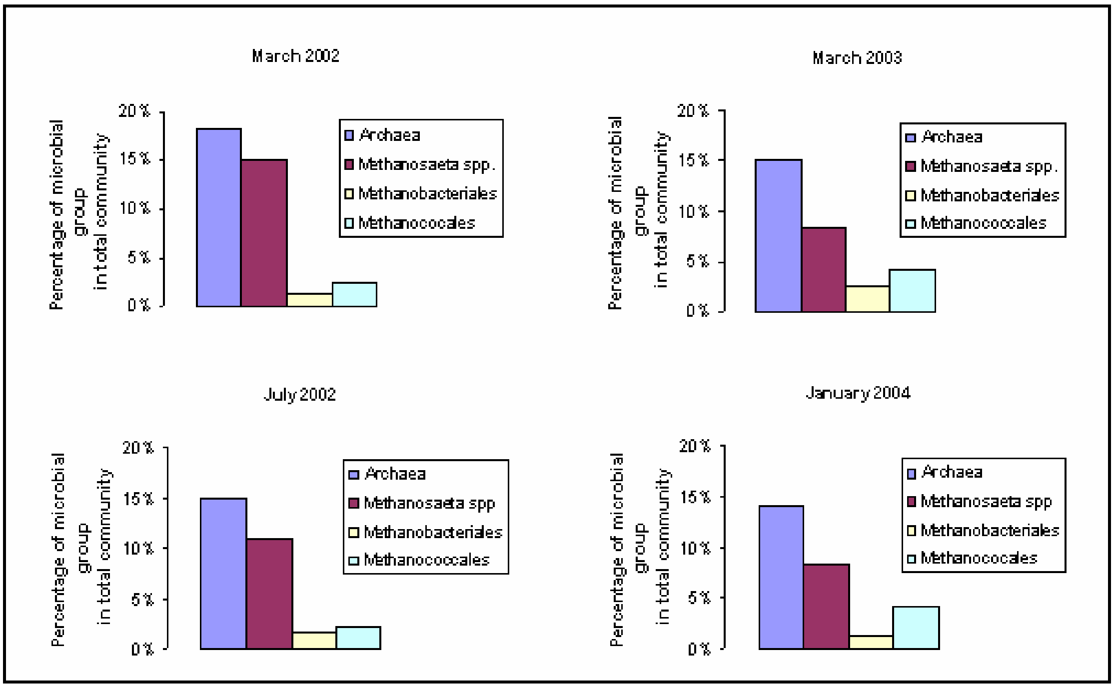
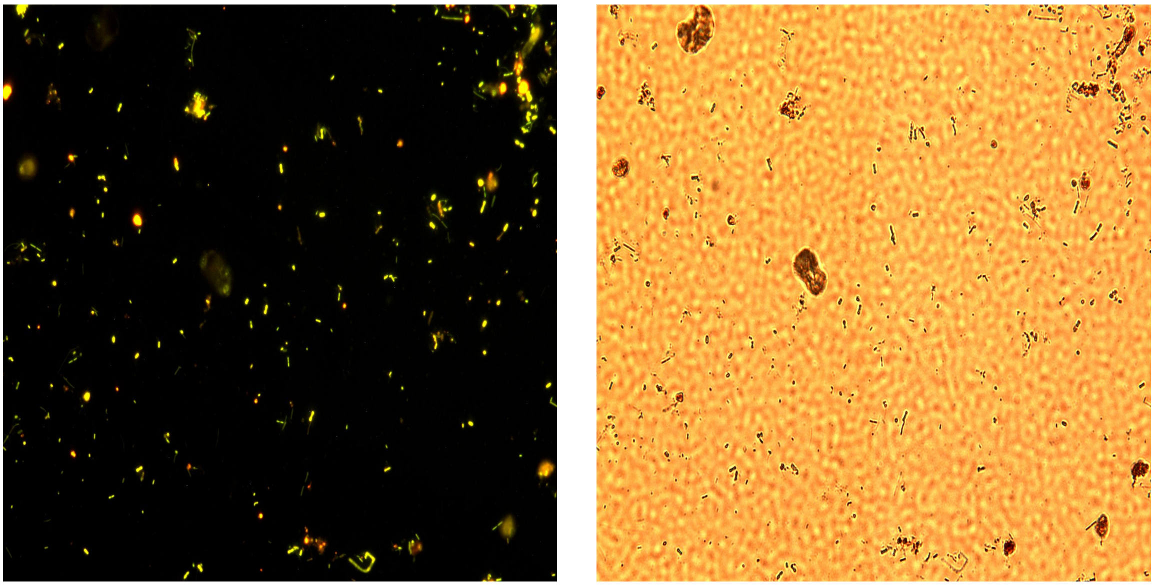


Figure 5.18. FISH Quantification results for IUASB sludge between 2002-2004 (Kolukırık, 2004).

Between 2002-2004, the archaeal subpopulation consisted of members of the genus *Methanosaeta*, *Methanobacteriales* and *Methanococcales* while the relative abundance of *Methanosaeta* spp. in the archaeal subpopulation decreased from 83.0%±1.6% to 58.0%±2.1% in this period. Comparing these results with those taken in November 2005, the relative abundance of the archaeal cells increased from 14.2%±0.3% to 20.7%±0.9% between January 2004 and November 2005. An increase in the relative abundance of members of the acetoclastic genus *Methanosaeta* from 58.0%±2.1% to 71.8%±5.5% of the archaeal population was accompanied by an increase in the relative abundance of hydrogen-oxidizing *Methanobacteriales* from 10.0%±0.7% to 32.7%±3.8% and the resulting dissappearence of the genus *Methanococcales* in the archaeal subpopulation.

Microscopic examination of the IUASB reactor sludge sample showed that while the the diameters of the granules varied between 1.0-2.4 mm, the rodlengths of the *Methanosaeta* spp. and *Methanobacteriales* spp. measured from epifluorescence micrographs by the help of image analysis software were in a range of 1.6-4.1 µm and 2.2-6.3 µm, respectively.

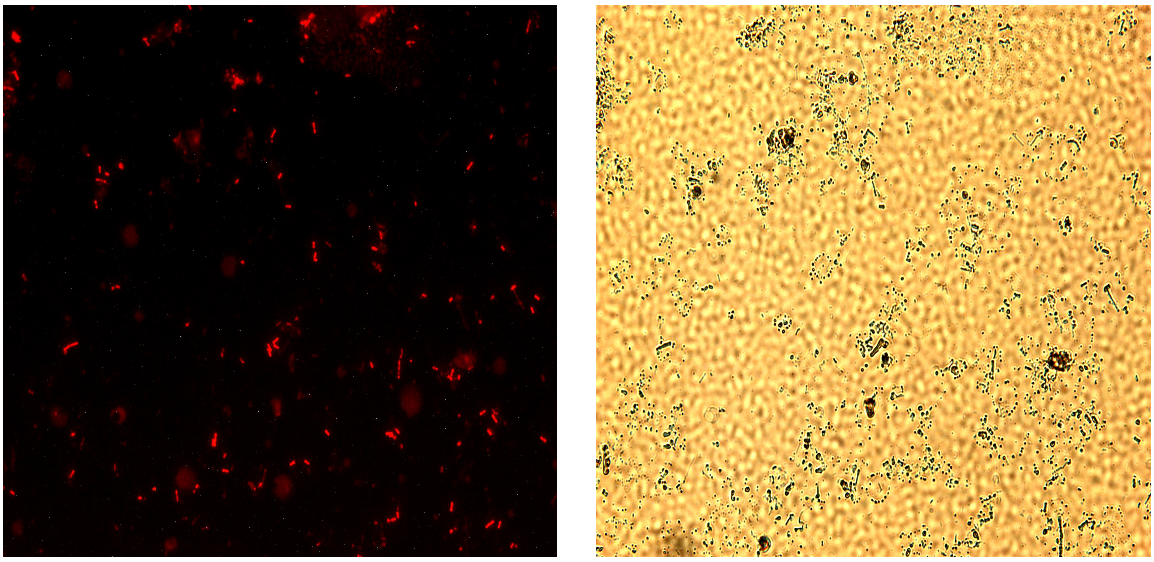
Epifluorescence micrograph examples of the Sybr Green I stained and hybridized TUASB sludge samples are shown in Figure 5.19-5.22.



a. b.

Figure 5.19. Epifluorescence micrograph example of the TUASB reactor’s sludge sample stained with Sybr Green I.

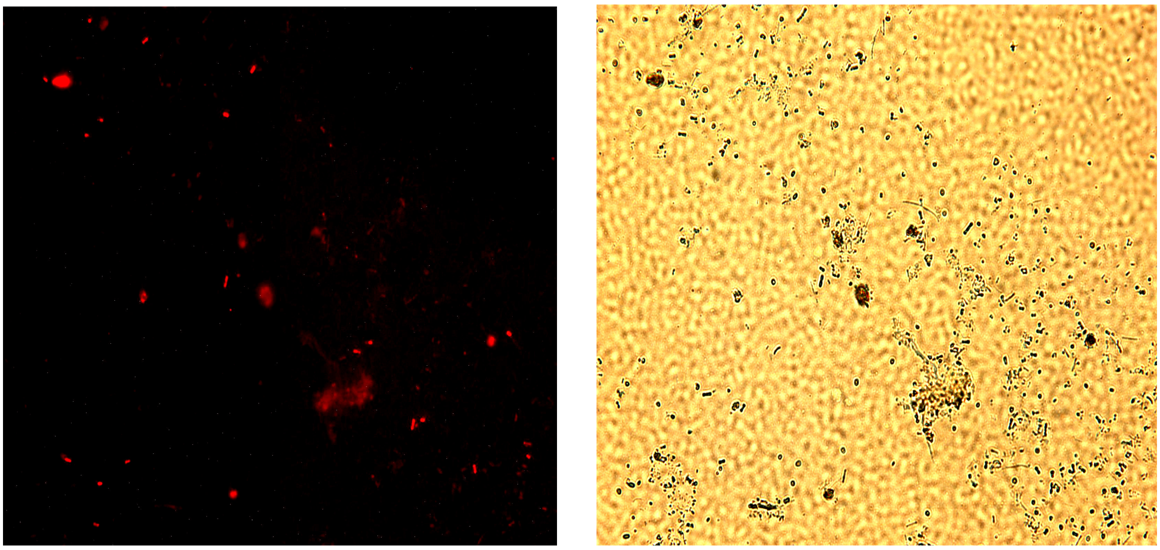
a) Cells stained with Sybr Green I stain. b) Light microscope view of the image.



a. b.

Figure 5.20. Epifluorescence micrograph example of the TUASB reactor’s sludge sample hybridized with ARC 915 probe (*Archaea*).

a) Cells hybridized with ARC 915 probe (*Archaea*) b) Light microscope view of the image.

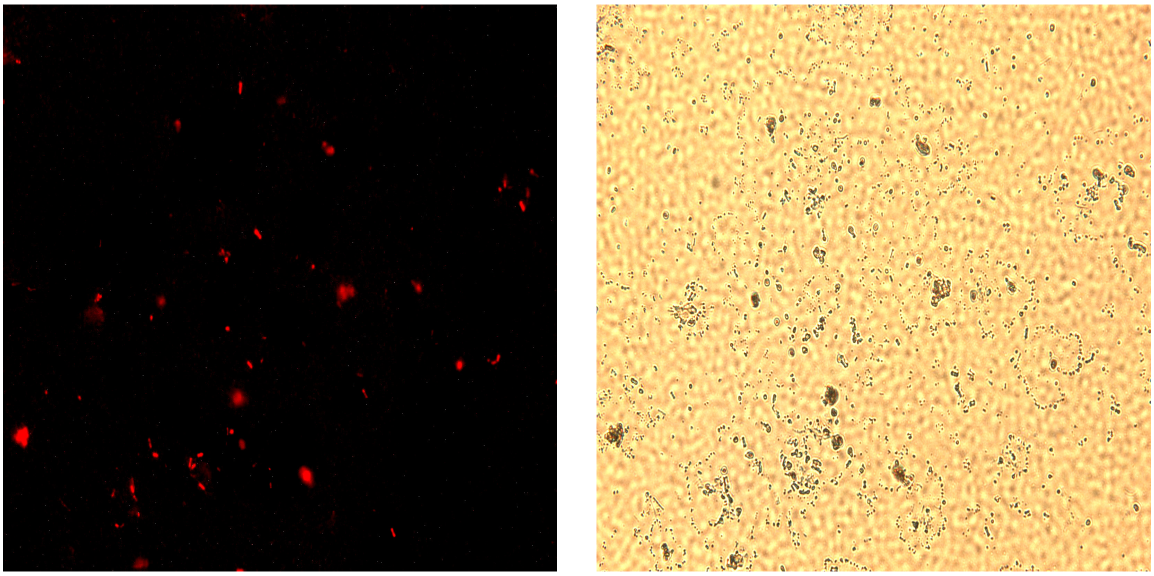


a. b.

Figure 5.21. Epifluorescence micrograph example of the TUASB reactor’s sludge sample hybridized with MX 825 probe (*Methanosaeta*).

a) Cells hybridized with MX 825 probe (*Methanosaeta*).

b) Light microscope view of the image.



a. b.

Figure 5.22. Epifluorescence micrograph example of the TUASB reactor’s sludge sample hybridized with MB 310 probe (*Methanobacteriales*).

a) Cells hybridized with MB310 probe (*Methanobacteriales*).

b) Light microscope view of the image.

Figure 5.23 shows the quantification results of the TUASB sludge sample in November 2005.

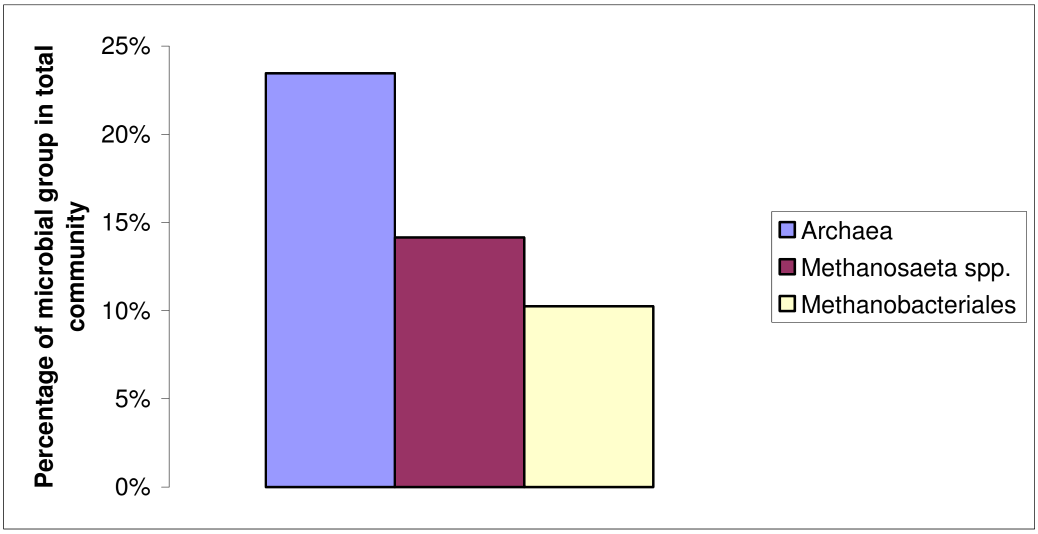


Figure 5.23. FISH Quantification results for TUASB Sludge in November 2005.

Results in November 2005 showed that 23.5%±0.2% of the cells in TUASB reactor sludge belonged to archeal domain. The archaeal subpopulation consisted of 60.4%±0.6% of members of the genus *Methanosaeta*, and 43.7%±0.3% of *Methanobacteriales*. Other important archaeal microbial groups such as *Methanosarcina*, *Methanococcales*, *Methanogenium* relatives were not observed in the TUASB sludge in November 2005.

Figure 5.24. shows the quantification results obtained with fluorescent rRNA targeted oligonucleotide probes and light microscope views in TUASB reactor between 2002 – 2004.

Between 2002-2004, the archaeal subpopulation of the TUASB reactor sludge consisted of members of the genus *Methanosaeta* and *Methanobacteriales* while the relative abundance of *Methanosaeta* spp. in the archaeal subpopulation decreased from 90.0%±1.2% to 79.0%±1.4% in this period. Comparing these results with those taken in November 2005, the relative abundance of the archaeal cells in TUASB reactor sludge increased from 15.0%±0.7% to 23.5%±0.2% between January 2004 and November 2005. A decrease from 79.0%±1.4% to 60.4%±0.6% was observed in the relative abundance of members of the acetoclastic genus *Methanosaeta* in the archaeal subpopulation while a corresponding increase occurred in the relative abundance of hydrogen-oxidizing *Methanobacteriales* from 24.0%±0.7% to 43.7%±0.3%.

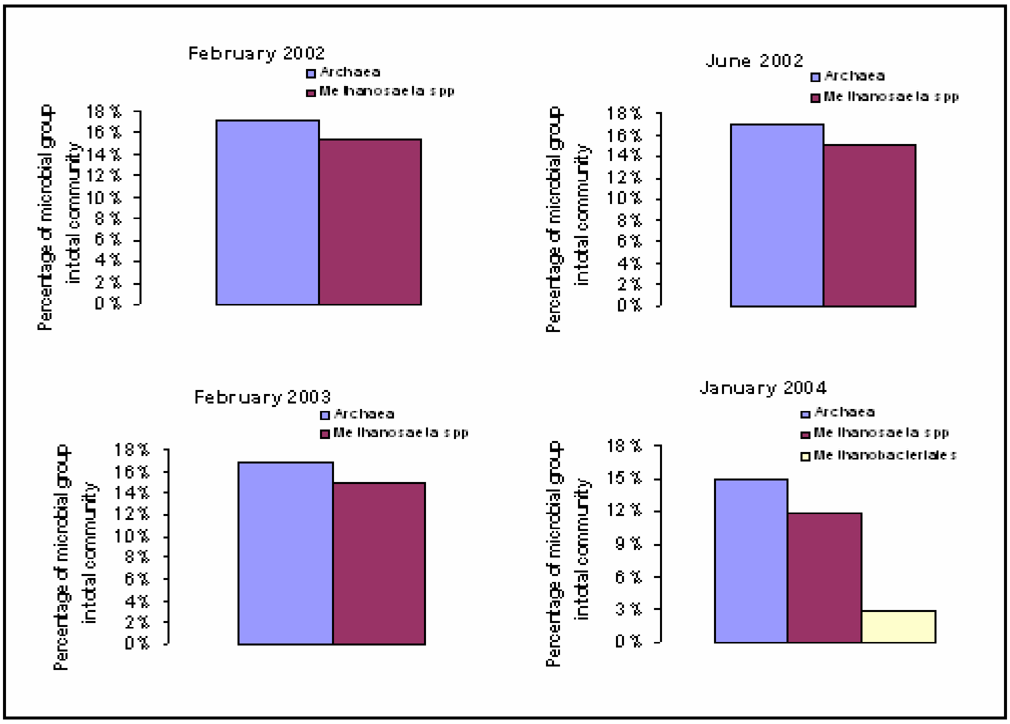
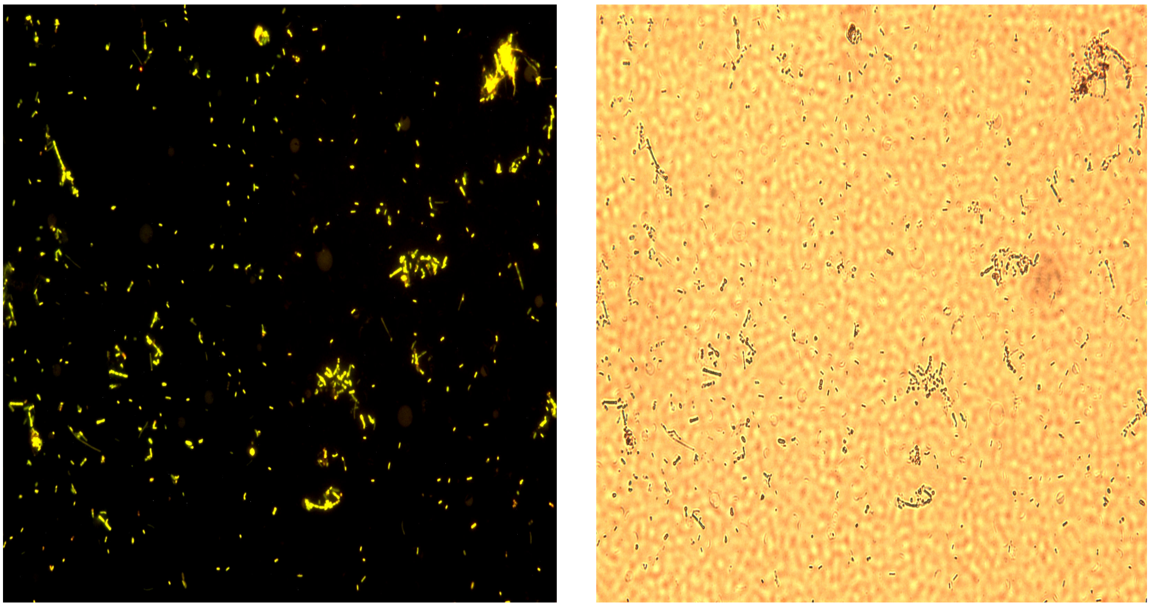


Figure 5.24. FISH Quantification results for TUASB Sludge between 2002 – 2004. (Kolukırık, 2004).

Microscopic examination of the TUASB reactor sludge sample showed that while the diameters of the granules were in a range of 0.7-1.8 mm, the rodlengths of the *Methanosaeta* spp. and *Methanobacteriales* spp. measured from epifluorescence micrographs by the help of image analysis software varied between 1.8-3.9 µm and 1.6-5.3 µm, respectively.

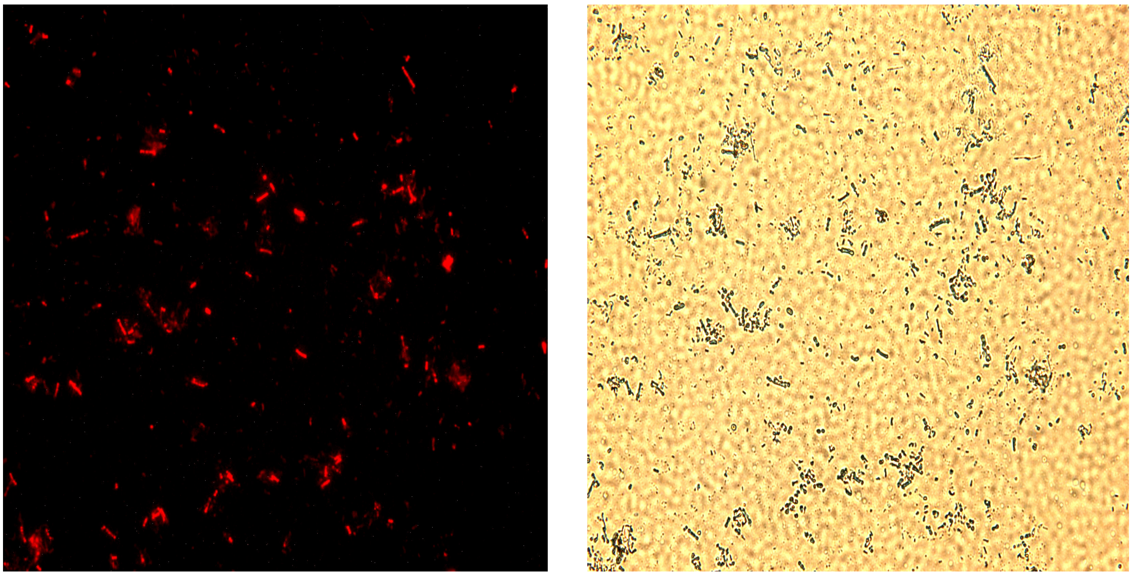
Epifluorescence micrograph examples of the Sybr Green I stained and hybridized CUASB sludge samples are shown in Figure 5.25-5.28.



a. b.

Figure 5.25. Epifluorescence micrograph example of the CUASB reactor’s sludge sample stained with Sybr Green I.

a) Cells stained with Sybr Green I. b) Light microscope view of the image.

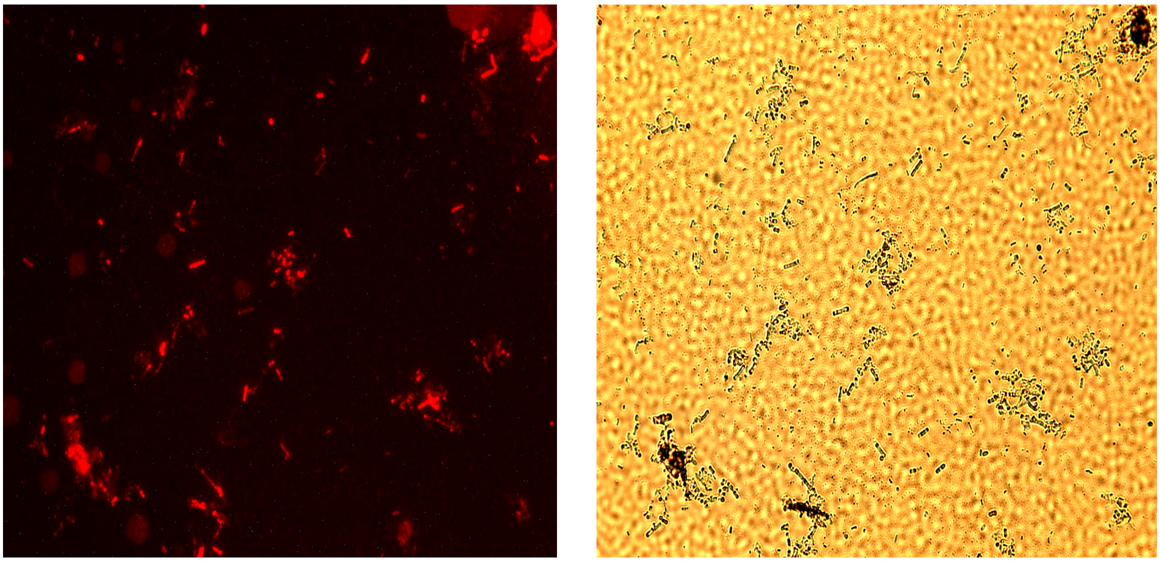


a. b.

Figure 5.26. Epifluorescence micrograph example of the CUASB reactor’s sludge sample hybridized with ARC 915 probe (*Archaea*).

a) Cells hybridized with ARC 915 probe (*Archaea*).

b) Light microscope view of the image.

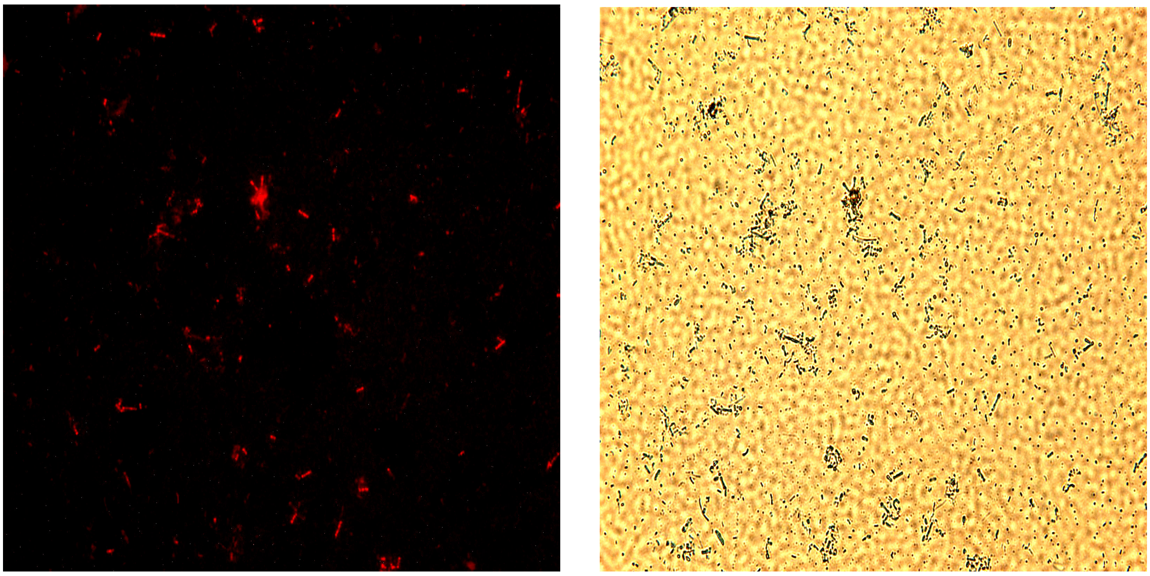


a. b.

Figure 5.27. Epifluorescence micrograph example of the CUASB reactor's sludge sample hybridized with MX 825 probe (*Methanosaeta*).

a) Cells hybridized with MX 825 probe (*Methanosaeta*).

b) Light microscope view of the image.



a. b.

Figure 5.28. Epifluorescence micrograph example of the CUASB reactor's sludge sample hybridized with MB 310 probe (*Methanobacteriales*).

a) Cells hybridized with MB310 probe (*Methanobacteriales*).

b) Light microscope view of the image.

Figure 5.29 shows the quantification results of the CUASB sludge sample in November 2005.

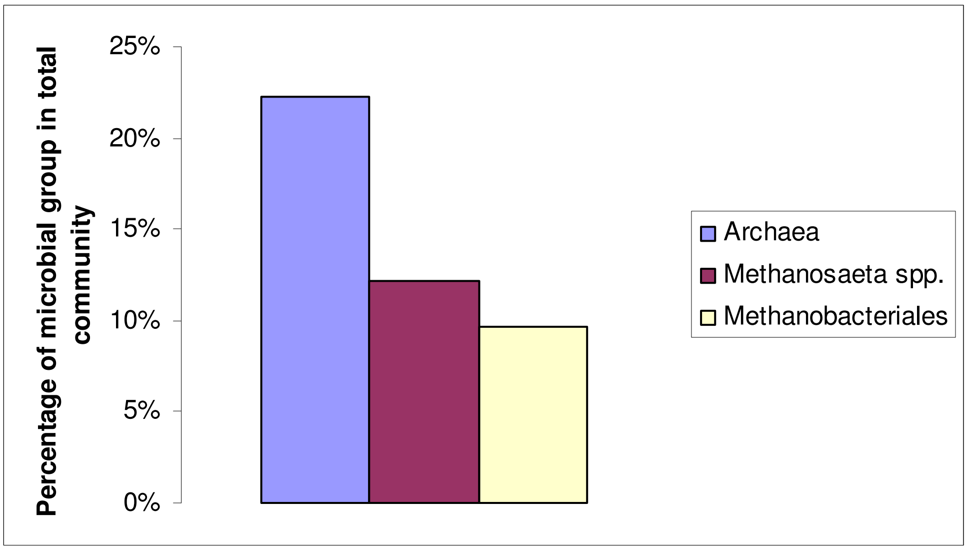


Figure 5.29. FISH Quantification results for CUASB Reactor Sludge in November 2005.

Results in November 2005 showed that 22.3%±0.8% of the cells in CUASB reactor sludge belonged to archaeal domain and the archaeal subpopulation consisted of 55.0%±1.4% of members of the genus *Methanosaeta*, and 43.5%±0.3% of *Methanobacteriales*. Other important archaeal microbial groups such as *Methanosarcina*, *Methanococcales* and *Methanogenium* relatives were not observed in the CUASB sludge in November 2005.

Figure 5.30. shows the quantification results obtained with fluorescent rRNA targeted oligonucleotide probes and Sybr Green I stain in CUASB reactor between 2002 – 2004. Between 2002-2004, the archaeal subpopulation consisted of members of the genus *Methanosaeta* and *Methanobacteriales* while the relative abundance of *Methanosaeta* spp. in the archaeal subpopulation decreased from 59.0%±2.6% to 53.0%±0.7%.

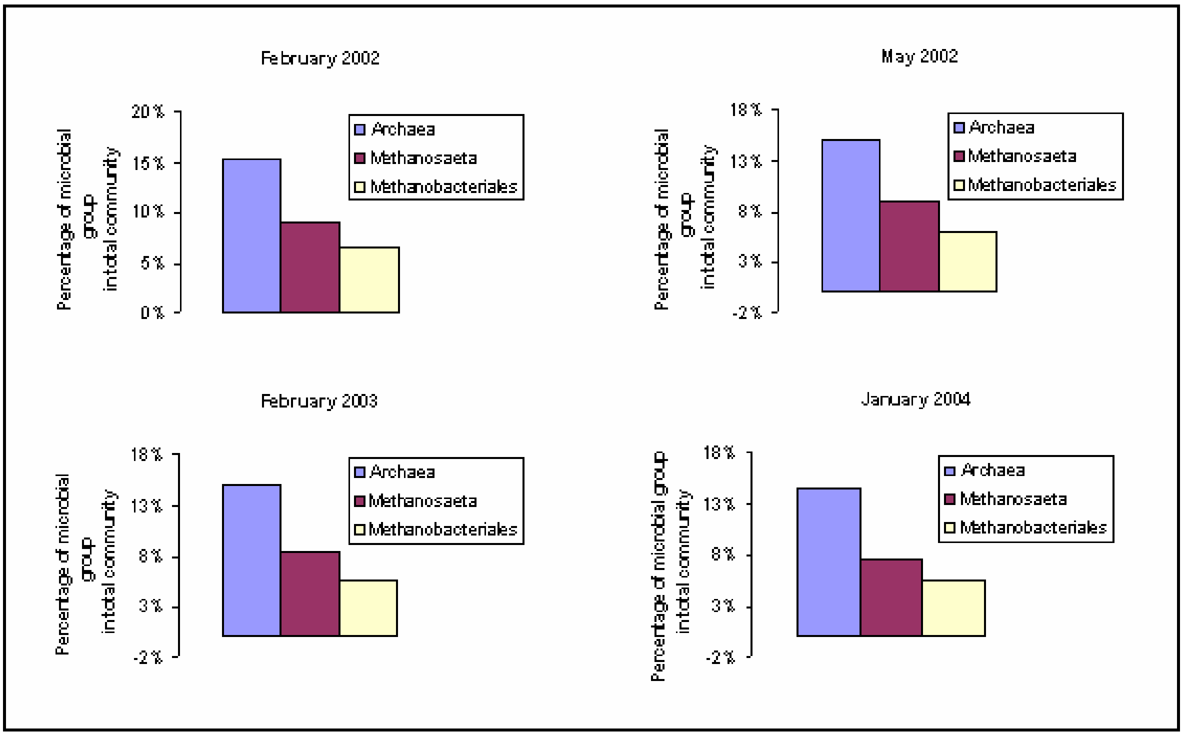


Figure 5.30. FISH Quantification results for CUASB Reactor Sludge between 2002-2004 (Kolukırık, 2004).

Comparing these results with those taken in November 2005, the relative abundance of the archaeal cells increased from 14.6%±0.7% to 22.3%±0.8% between January 2004 and November 2005. The relative abundance of members of the acetoclastic genus *Methanosaeta* increased from 53.0%±0.7% to 55.0%±1.4% of the archaeal subpopulation and an increase in the relative abundance of hydrogen-oxidizing *Methanobacteriales* from 39.0%±0.7% to 43.5%±0.3% was observed in the same period.

Microscopic examination of the CUASB reactor sludge sample showed that while the the diameters of the granules varied between 0.3-1.5 mm, the rodlengths of the *Methanosaeta* spp. and *Methanobacteriales* spp. measured from epifluorescence micrographs by the help of image analysis software were in a range of 1.3-4.4 µm and 1.6-6.0 µm, respectively.

5.4. Overall Discussion

Reactor performances, potential acetoclastic methanogenic activities and archaeal population dynamics of the three full-scale UASB reactors were given in the previous sections. An overall discussion considering these results is made in this section.

The full-scale UASB reactors showed similar patterns in terms of COD removal efficiencies and the OLRs applied. Applied OLRs were usually lower in 2005 compared to those between 2001 and 2004. Also, overall COD removal efficiencies decreased in 2005. The decrease in COD removal efficiency in 2005 could be due to many reasons some of which may be specific for each reactor (e.g., operation of the IUASB and CUASB reactors not at optimum temperature levels optimum for mesophilic reactors). However, the main reason behind this seemed to be OLRs applied to the reactors. The UASBs studied here were operated towards the lower end of typical organic loadings for UASB reactors and UASB reactors treating alcohol distillery effluents have been shown to be effective at OLRs in the range of 10-20 kg COD m^3/day achieving COD removal efficiencies between 65 and 95% (Driessen et al., 1994). However, OLRs applied were usually lower than this range between 2001 and 2005. Also, in the same period, differences in the OLRs applied affected corresponding COD removal efficiencies respectively. In other words, the UASB reactors were underloaded compared to the optimum range of OLRs applied and increases in OLRs which usually resulted in higher COD removal efficiencies could verify it.

Another important parameter about the operation of the biological systems is the F:M ratio. The F:M ratios of the anaerobic reactors were under 0.1 during the operation period which is much lower than the typical values reported for similar reactors in literature. (Ince et al., 2005; Baier and Delavy 2005; Driessen et al., 1994; Ince et al., 1994). Under low F:M ratios, there is little substrate available per unit of biomass which can cause degradation of organics for cell maintenance and will decrease the conversion of organics to new cell matter eventually resulting in cell yield decreases. Therefore, prolonged operation under very low F:M ratios might have lead to a decrease in the amount of active cells maintained within the UASB reactor.

Potential acetoclastic methanogenic activities of the UASB sludges were determined by SMA tests. The results in three UASB reactors ranged from 132 to 192 mL CH_4/g VSS.day, which were also lower than the PMP rates of anaerobic reactors treating similar wastewaters reported in literature (>300 mL CH_4/gVSS.day) (Ince et al., 2001 and 2002; Driessen et al., 1994). Although there were some increases in PMP of the sludges in year 2005 compared to year 2004, the loss in potential acetoclastic methanogenic activity between years 2002 and 2004 were not significantly recovered in year 2005. This could have been mainly due to low F:M ratios applied in the anaerobic system. Sponza et al. (2002) studied relationships between SMA and F:M ratio during granulation process in a laboratory-scale UASB reactor and reported that increasing the F:M ratio from 0.2 to 0.64 had a pronounced effect on the increase in SMA. Baier and Delavy (2005) also reported that operational conditions with high F:M ratios would lead to a significantly better solids removal efficiency of a full scale UASB reactor.

As the AMP data were not available in 2005, the average data of AMP rates between years 2002 and 2004 were taken as a reference and AMP/PMP ratios were found to be in a range of 0.21-0.31, lower than values reported for successfully operating similar reactors. Monteggia (1991) studied the effect of AMP/PMP ratio on the performance of a laboratory-scale UASB reactor. It was reported that operating conditions corresponded to an AMP/PMP ratio of 0.6 resulted in high operating stability and an excellent COD removal. Ince et al. (1995) used this approach to determine a suitable organic loading rate during the start-up phase of a laboratory-scale anaerobic reactor. It was also reported that the anaerobic reactor achieved excellent COD removal rates at an AMP/PMP ratio of 0.45. AMP/PMP ratios in a range of 0.14-0.28 reveal that the UASB reactors should be loaded at higher organic loading rates or sludge should be withdrawed in order to maintain AMP/PMP ratio of 0.6-0.7 which can ensure desired reactor performance with safer operation (Ince et al., 1995, 2001; Monteggia, 1991).

Microscopic analysis of the granular sludges from the three UASB reactors were also being investigated in terms of rodlength and granule size. The results showed that the granules of the IUASB reactor were bigger than the other two UASB reactors. The IUASB reactor also showed the highest PMP rate and highest relative abundance of *Methanosaeta* spp. in the archaeal subpopulation among the three UASB reactors. These results are consistent as it is generally assumed that predominance of *Methanosaeta* spp. in the archaeal subpopulation improves acetoclastic methanogenic activity and granulation (MacLeod et al., 1990; Wiegant, 1988; Rocheleau et al., 1999). It was also found out that the rods of the *Methanobacteriales* spp. were longer than those of the *Methanosaeta* spp. in the three UASB reactors' sludge samples.

The archaeal community structures of the UASB reactors were characterized using fluorescent rRNA targeted oligonucleotide probes specific for *Archaea* and phylogenetically defined groups of methanogens. The relative amount of archaeal cells in all of the reactors increased significantly in 2005. This could be due to two main reasons, the technique applied to perform total cell count and the composition of the sludge blanket of the reactor. In this study, total cell count was performed using Sybr Green I staining while in the previous study (Kolukırık, 2004), total cell counts were obtained from light microscope views. Although light microscope views give more visual details about the composition and morphology of the sludge, Sybr Green I staining is more specific for the determination of the cells, because organic/inorganic components in the sludge other than microbial cells are being ignored. Besides, light microscope views of the images could be used as a control tool, which was also adopted in this study. Another reason for the increase in the archaeal cells could be the composition of the sludge blanket. On the basis of published data it appears that the relative abundance of *Archaea* is high when medium contain monomers and that it is decreased when the medium contains biopolymers as the carbon source. However, it was also reported that variations in process type, operating conditions and reactor performance could have resulted in changes in the composition rather than the size of the archaeal communities present in anaerobic reactors (Ahring, 2003; Leclerc et al., 2004).

Three UASB reactors have shown similar archaeal composition, consisting of two microbial families, *Methanosaeta* and *Methanobacteriales*. While *Methanococcales spp.* have disappeared with the accompanying increase in *Methanosaeta* and *Methanobacteriales spp.* in the IUASB reactor, the relative abundance of *Methanosaeta* spp. in archaeal subpopulation decreased and that of *Methanobacteriales* spp. increased in TUASB reactor. The archaeal composition of CUASB reactor did not change significantly during the monitoring period.

The archaeal compositions of the full-scale UASB reactors analyzed in this study were shown to be similar to other studies previously carried out on the same reactors. In the study of Akarsubasi et al. (2005), seed sludge used in a lab-scale CSTR reactor was taken from the IUASB reactor in year 2001. The potential acetoclastic methanogenic activity of the sludge was found to be 466 mL CH_4/gVSS.day, then. Archaeal Denaturing Gradient Gel Electrophoresis (DGGE) profiles of the IUASB reactor sludge, shown in Figure 5.31., exhibited predominance of *Methanosaeta concilii*-like sequences (10N, Lane 9) and *Methanobacteriales*-like sequences (9N, Lane 10), which was also found in IUASB reactor in this study by FISH. In another study by Akarsubasi et al. (2006), archaeal composition of the IUASB and TUASB sludges taken in 2001 were determined by DGGE technique. The PMP rates of the IUASB and TUASB sludges were 350 and 376 mL CH_4/gVSS.day, respectively, then. The DGGE profiles, shown in Figure 5.32., revealed that the overall composition within the IUASB and TUASB reactors was almost the same. Fasta 3 analysis showed that most closely related sequences to methanogenic *Archaea* were *Methanobacterium formicicum* (T1) and *Methanosaeta soehngenii* (T3) with 100% similarity to sequences in public databases. The relative band intensities of the profiles showed that while *Methanosaeta*-like species have been found in almost same band intensities in both reactors, *Methanobacteriales*-like species have shown higher band intensities in the TUASB reactor compared to the IUASB reactor. It was also shown by this study that the relative abundance of *Methanobacteriales spp.* in archaeal subpopulation in TUASB was higher than that in IUASB reactor in 2005.

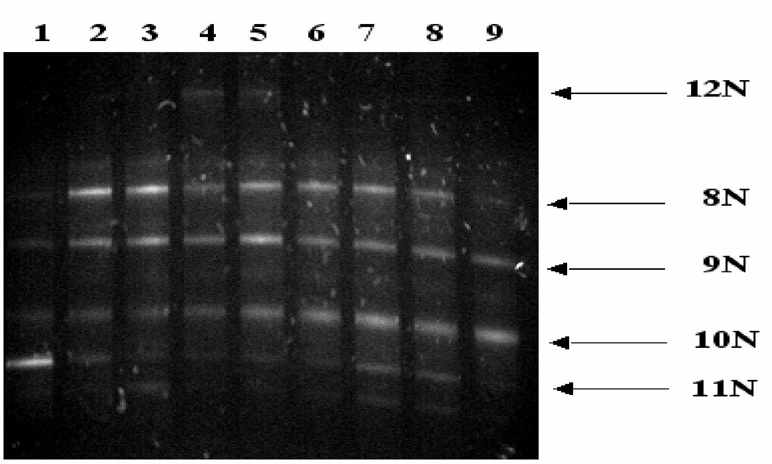


Figure 5.31. Archaeal Denaturing Gradient Gel Electrophoresis (DGGE) profiles (lanes 1-8 corresponds to feeding regime and lane 9 seed sludge) (Akarsubasi et al., 2005).

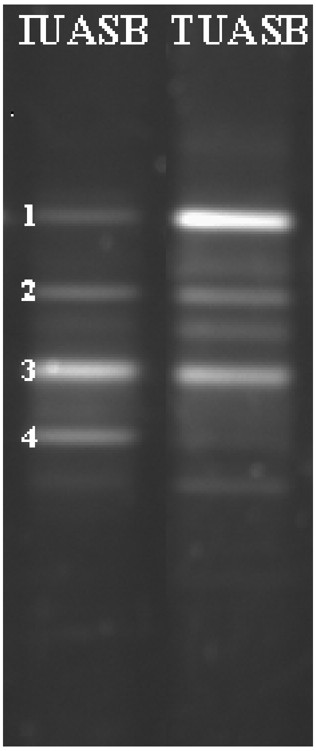


Figure 5.32. Denaturing Gradient Gel Electrophoresis (DGGE) profiles of Archaeal amplicons from IUASB and TUASB reactors in 2001
(Position 1 belongs to *Methanobacterium formicicum*, while position 3 belongs *Methanosaeta soehngenii* spp.) (Akarsubasi et al., 2005).

Among acetoclastic methanogens, *Methanosaeta spp.* were the dominant species while *Methanosarcina spp.* were not observed in the UASB reactors. *Methanosarcina* spp. and *Methanosaeta* spp. have been identified as important methanogens in granular sludge from upflow anaerobic sludge blanket (UASB) reactors (Hofman-Bang et al., 2003). *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). These kinetic data suggest that low effluent concentration of acetate from a UASB reactor would be expected if the granules were dominated by *Methanosaeta* spp. Therefore, low effluent concentrations of acetate would be a reason for the dominance of *Methanosaeta* spp. in the reactors. Also, numerical dominance of the genus *Methanosaeta* compared to other methanogens in anaerobic reactors has been reported previously (Ficker et al. 1999; Merkel et al., 1999; Sekiguchi et al., 1999; Plumb et al., 2001, Angenent et al., 2004). *Methanosaeta* spp. are regarded as being important for the formation and maintenance of granular sludge (Rocheleau et al., 1999), therefore it is generally assumed that numerical dominance of *Methanosaeta* over other methanogens should be favored in granular sludge systems.

Microbial compositions of the different UASB sludges were also investigated by FISH in other studies. A study about the granule composition in a full-scale UASB reactor treating wastewater from a corn wet milling plant (Zheng and Raskin, 2000) showed that the archaeal domain constituted between 30% and 50% of total 16S rRNA. *Methanosarcinales* and *Methanobacteriales* were the two dominant methanogenic groups. *Methanomicrobiales* constituted less than 5% while *Methanococcales* were almost absent. The results of another study on the microbial diversity of a full-scale UASB reactor treating wastewater from a cheese factory (Casserly and Erijman, 2002) reveal that *Methanosarcinales*, *Methanobacteriales*, *Methanococcales* and *Methanogenium* species were present in the UASB sludge samples. Among the family *Methanosarcinaea, Methanosarcina* and relatives (specific for MS1414 probe) dominated. *Methanosarcina* and *Methanosaeta* species were present in the UASB sludge in close amounts.

The relative abundance of *Methanosaeta* spp. in archaeal subpopulation increased in IUASB reactor sludge between years 2004 and 2005 while the archaeal composition of CUASB reactor did not change significantly in the same period. On the other hand, predominance of *Methanosaeta* spp. within the TUASB reactor decreased over time in this study with a change to greater abundance of hydrogenotrophic methanogens. Other studies have also reported that the general tendency of hydrogenotrophic methanogens to dominate and loss of Methanosaeta spp. in anaerobic reactors (Petersen and Ahring, 1992; Schnurer et al., 1994 and 1999; Griffen et al., 1998; Delbes et al., 2001; Angenent et al., 2002; Pender et al., 2004; Akarsubasi et al., 2005; Collins et al., 2005). This has led to the suggestion that acetate is being converted to H_2/CO_2 in these ecosystems by a reverse reaction of acetate-oxidizing organisms (possibly by homoacetogenic bacteria) and further to methane by hydrogenotrophic methanogens (Delbes et al., 2001, Angenent et. al., 2002) in TUASB reactor. Among the hydrogenotrophic methanogens, *Methanobacteriales* was found to be dominant within the reactors. It was also previously reported that among the hydrogenotrophic methanogens, *Methanobacteriales* followed by *Methanomicrobiales* were dominant and *Methanococcales* were almost absent within both full-scale and lab-scale UASB reactors (Hofman-Bang et al., 2003; Raskin et al., 1995). The production of methane from acetate through this syntrophic consortium appears to be more common in stressed systems (Schnurer et al., 1994 and 1999; Petersen and Ahring, 1992; Angenent et al., 2002). The stress on the anaerobic systems in this study might have been originated from operation of the three full-scale UASB reactors under F:M ratios much lower than the typical values reported for similar reactors and operation of CUASB and IUASB reactors at temperature levels lower than typical values for mesophilic anaerobic reactors.

6. CONCLUSION

The results of the study showed that operation of UASB reactors at low organic loading rates and F:M ratios resulted in low COD removal efficiencies and reduced granular sludge quality. These consequences were also verified by low PMP values and AMP/PMP ratios. The archaeal composition of the three UASB reactors consisted of same species, *Methanosaeta* spp. and *Methanobacteriales* spp. but the archaeal population dynamics were different in the three reactors. While there was an increase in the relative abundance of *Methanosaeta* spp. in the archaeal subpopulation in IUASB reactor sludge between years 2004 and 2005, the predominance of Methanosaeta spp. in the TUASB reactor have tend to change to hydrogenotrophic methanogens in year 2005. Compared to other two UASB reactors, CUASB reactor showed a more stable archaeal community structure in a long term., the archaeal subpopulation almost equally dominated by Methanosaeta spp. and Methanobacteriales between years 2002 and 2005.

The significant decreases in the activities of methanogens in the UASB reactors studied here could not have been determined by measurement of parameters conventionally used to monitor anaerobic treatment reactors such as pH, temperature, alkalinity, gas production and gas composition, removal of organic matter, etc. These can only provide information about the current conditions inside the reactor. In this study, the results of SMA test and FISH were correlated with each other and combined with the performance and operation data available to analyze archaeal community dynamics in the three full scale UASB reactors and the overall evaluation produced valuable results about the quantity of active methanogenic populations in the reactors. There are few published studies available that present results for both techniques for full-scale anaerobic reactors. Future studies using these tools to analyze anaerobic reactors in terms of microbial population dynamics will definitely provide a clearer picture about the microbiological and ecological aspects of anaerobic treatment. Verification of the microbial composition by other molecular methods such as DGGE will also help to achieve a better understanding in the microbial population dynamics of anaerobic reactors.

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APPENDIX A: OPERATIONAL DATA OF THE UASB REACTORS IN YEAR 2005

Table A.1. Operational Data of the IUASB Reactor in 2005.

Time (Month)	COD_{in} (mg/L)	COD_{out} (mg/L)	Flow (m^3/day)	Temp. (^{o}C)	pH	COD Removal Efficiency (%)	Organic Loading Rate (kg COD/m^3.day)
1	1525	630	312	30	7.1	58.69	3.33
2	2305	665	312	29	7.2	71.15	5.03
3	1580	645	312	32	7.2	59.18	3.45
4	1265	450	312	31	7.4	64.43	2.76
5	3585	970	312	30	7.0	72.94	7.82
6	1805	370	312	30	7.0	79.50	3.94
7	1545	375	312	31	7.0	75.73	3.37
8	2475	620	312	30	7.1	74.95	5.40
9	2590	525	312	30	7.0	79.73	5.65
10	1305	400	312	30	7.1	69.35	2.85
11	2890	670	312	29	7.3	76.82	6.31
12	2815	830	312	30	7.3	70.52	6.14

Table A.2. Operational Data of the TUASB Reactor in 2005.

Time (Month)	COD_{in} (mg/L)	COD_{out} (mg/L)	Flow (m^3/day)	Temp. (^{o}C)	pH	COD Removal Efficiency (%)	Organic Loading Rate (kg COD/m^3.day)
1	3417	1510	600	33.5	7.1	55.81	4.31
2	4058	2048	600	33.8	6.9	49.53	5.12
3	3616	1539	600	34.2	7.0	57.44	4.56
4	3228	1454	600	34.5	7.1	54.96	4.07
5	2840	1152	600	35	7.0	59.44	3.58
6	3160	1536	600	35	6.9	51.39	3.98
7	2644	1212	600	35	7.1	54.16	3.33
8	2755	1308	600	35	6.9	52.52	3.47
9	3255	1588	600	35	7.0	51.21	4.10
10	3006	1252	600	35	7.1	58.35	3.79
11	2549	1055	600	35	7.0	58.61	3.21
12	2761	1012	600	35	7.0	63.35	3.48

Table A.3. Operational Data of the CUASB Reactor in 2005.

Time (day)	COD_{in} (mg/L)	COD_{out} (mg/L)	Flow (m^3/day)	Temp. (^oC)	pH	COD Removal Efficiency (%)	Organic Loading Rate (kg COD/m^3.day)
48	2560	768	144	16.9	7.29	70.00	1.94
52	3200	576	144	16.0	7.01	82.00	2.43
55	8000	2688	144	16.0	6.58	66.40	6.06
56	6880	2496	144	18.0	6.95	63.72	5.21
59	3520	960	144	18.0	6.97	72.73	2.67
61	3200	576	144	19.0	6.96	82.00	2.43
63	5440	1600	144	20.0	7.11	70.59	4.12
64	5180	1440	144	19.0	7.02	72.20	3.93
67	4640	1664	144	17.0	6.97	64.14	3.52
70	4640	2304	144	18.0	6.96	50.34	3.52
74	6080	1728	144	19.0	7.11	71.58	4.61
75	5280	1792	144	20.0	7.02	66.06	4.00
81	6560	1536	144	23.8	7.28	76.59	4.97
82	3360	1216	144	22.6	7.28	63.81	2.55
85	7520	2688	144	22.9	7.19	64.26	5.70
235	4320	896	144	26.4	7.27	79.26	3.27
272	6720	1408	144	29.0	7.53	79.05	5.09
273	5120	640	144	28.1	7.44	87.50	3.88
274	5920	1024	144	28.8	7.41	82.70	4.49
277	7360	2432	144	29.0	7.43	66.96	5.58
280	5120	1152	144	25.6	7.36	77.50	3.88
284	6240	1472	144	23.7	7.33	76.41	4.73
285	6080	1344	144	22.5	7.39	77.89	4.61
286	5280	1408	144	23.5	7.40	73.33	4.00
287	4960	1600	144	24.1	7.38	67.74	3.76
288	5440	1472	144	25.7	7.40	72.94	4.12
291	6560	1408	144	26.6	7.45	78.54	4.97
295	6880	1984	144	23.9	7.37	71.16	5.21
298	5280	1600	144	26.4	7.43	69.70	4.00
301	4960	1216	144	27.1	7.47	75.48	3.76
314	10560	1344	144	23.5	7.25	87.27	8.00
315	12460	3456	144	23.6	7.31	72.26	9.44
316	13120	2720	144	23.9	7.42	79.27	9.94
320	7360	960	144	21.6	7.45	86.96	5.58

APPENDIX B: SMA TEST RESULTS FOR THE UASB REACTOR SLUDGE SAMPLES

Table B.1. SMA Test Results for the IUASB Reactor Sludge in November 2005.

Time (hour)	SMA (mL CH_4/gVSS.day)	Time (hour)	SMA (mL CH_4/gVSS.day)
1	**0**	55	189
2	11	56	189
3	16	57	189
4	19	58	189
5	24	59	189
6	27	60	192
7	29	61	192
8	35	62	192
9	43	63	192
10	48	64	192
11	56	65	192
12	64	66	192
13	72	67	189
14	75	68	189
15	77	69	189
16	83	70	189
17	85	71	189
18	90	72	189
19	93	73	189
20	98	74	189
21	101	75	189
22	104	76	189
23	106	77	189
24	112	78	189
25	117	79	189
26	122	80	186
27	125	81	186
28	128	82	186
29	133	83	178
30	136	84	170
31	141	85	162
32	144	86	149
33	146	87	136
34	146	88	101
35	149	89	72
36	152	90	45
37	154	91	24
38	157	92	8
39	160	93	3
40	165	94	0
41	168	95	0
42	170	96	0
43	173		
44	176		
45	178		
46	181		
47	184		
48	186		
49	186		
50	189		
51	189		
53	189		

Table B.2. SMA Test Results for the TUASB Reactor Sludge in November 2005.

Time (hour)	SMA (mL CH_4/gVSS.day)	Time (hour)	SMA (mL CH_4/gVSS.day)
1	0	57	132
2	4	58	132
3	7	59	132
4	12	60	132
5	17	61	132
6	21	62	132
7	24	63	132
8	28	64	132
9	32	65	132
10	36	66	132
11	39	67	132
12	44	68	132
13	48	69	132
14	54	70	131
15	59	71	131
16	65	72	131
17	69	73	131
18	75	74	130
19	80	75	129
20	86	76	128
21	91	77	127
22	96	78	126
23	101	79	125
24	103	80	124
25	106	81	119
26	109	82	114
27	112	83	110
28	113	84	87
29	115	85	76
30	117	86	61
31	119	87	49
32	121	88	34
33	123	89	24
34	124	90	16
35	125	91	0
36	126	92	0
42	130		
43	130		
44	131		
45	131		
46	131		
47	131		
48	131		
49	131		
50	132		
51	132		
52	132		
53	132		
54	132		
55	132		
56	132		

Table B.3. SMA Test Results for the CUASB Reactor Sludge in November 2005.

Time (hour)	SMA (mL CH_4/gVSS.day)	Time (hour)	SMA (mL CH_4/gVSS.day)
1	0	57	167
2	6	58	167
3	11	59	167
4	13	60	167
5	15	61	167
6	17	62	167
7	18	63	167
8	19	64	167
9	21	65	167
10	25	66	167
11	27	67	167
12	29	68	167
13	36	69	167
14	39	70	167
15	43	71	167
16	46	72	167
17	48	73	167
18	52	74	161
19	56	75	161
20	58	76	161
21	62	77	158
22	66	78	155
23	70	79	155
24	75	80	155
25	85	81	154
26	94	82	151
27	99	83	150
28	106	84	135
29	115	85	121
30	124	86	93
31	129	87	74
32	135	88	65
33	143	89	58
34	149	90	42
35	155	91	37
36	160	92	30
42	165	93	21
43	166	94	10
44	167	95	4
45	167	96	0
46	167		
47	167		
48	167		
49	167		
50	167		
51	167		
52	167		
53	167		
54	167		
55	167		
56	167		

APPENDIX C: MORE EPIFLUORESCENCE MICROGRAPH EXAMPLES OF THE SYBR GREEN I STAINED AND HYBRIDIZED UASB REACTOR SLUDGE SAMPLES

IUASB Reactor

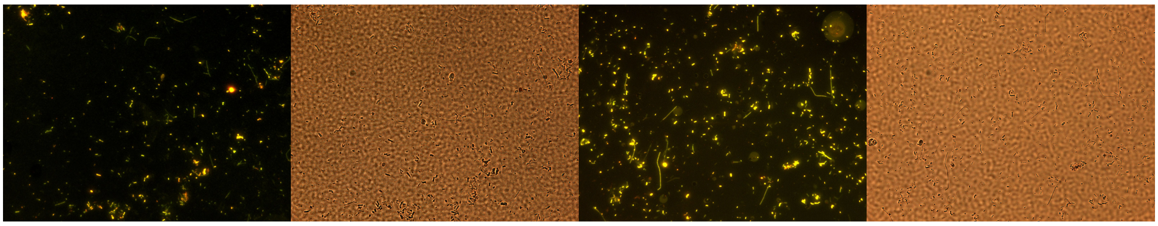

Figure C.1-4. More epifluorescence micrograph examples of Sybr Green I stained IUASB sludge samples and their corresponding light microscope views.

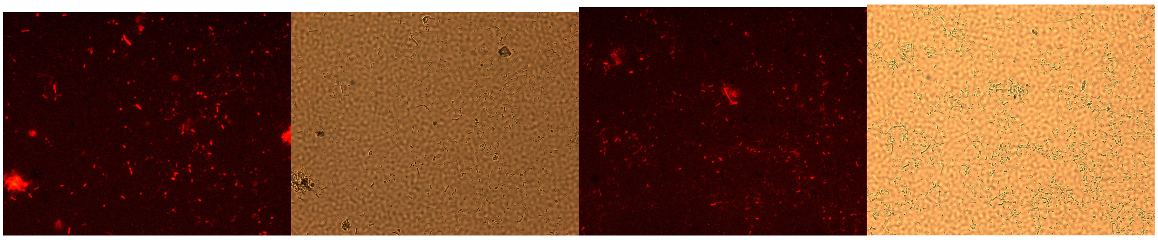

Figure C.5-8. More epifluorescence micrograph examples of IUASB sludge samples hybridized with ARC 915 probe (*Archaea)* and their corresponding light microscope views

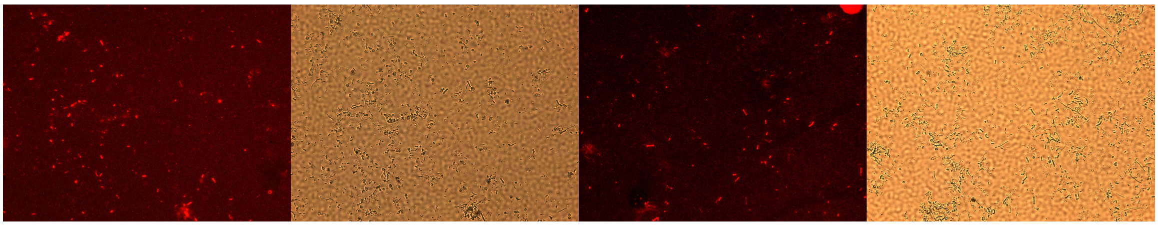

Figure C.9-13. More epifluorescence micrograph examples of IUASB sludge samples hybridized with MX 825 (*Methanosaeta*) probe and their corresponding light microscope views.

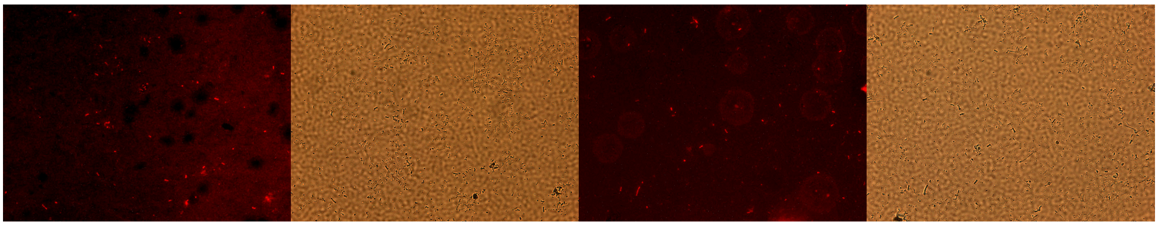

Figure C.14-17. More epifluorescence micrograph examples of IUASB sludge samples hybridized with MB 310 probe (*Methanobacteriales*) and their corresponding light microscope views.

TUASB Reactor

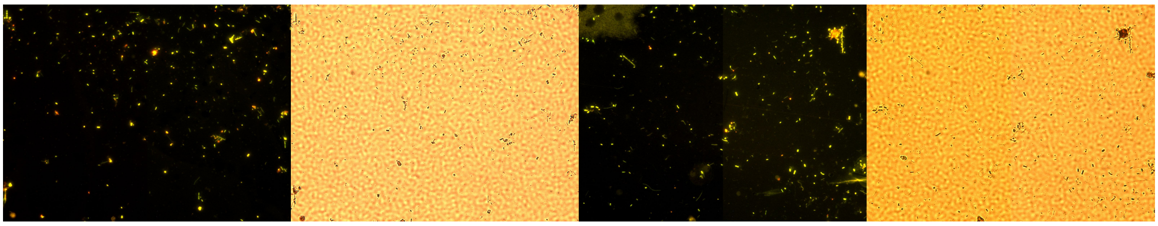

Figure C.18-21. More epifluorescence micrograph examples of Sybr Green I stained TUASB sludge samples and their corresponding light microscope views.

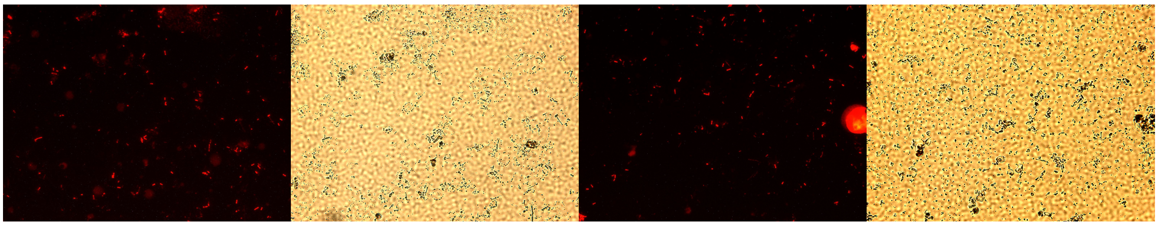

Figure C.22-25. More epifluorescence micrograph examples of TUASB sludge samples hybridized with ARC 915 probe (*Archaea)* and their corresponding light microscope views

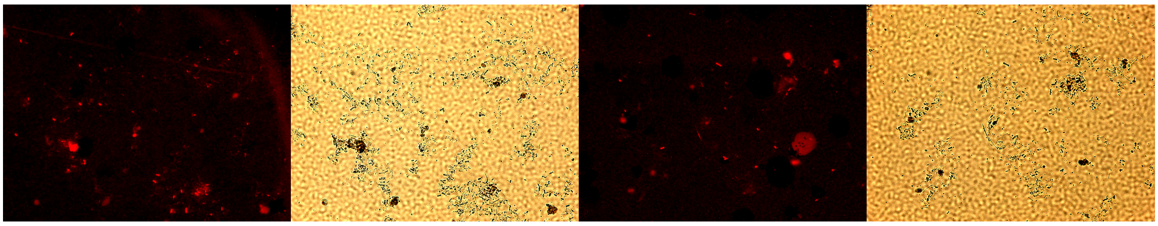

Figure C.26-29. More epifluorescence micrograph examples of TUASB sludge samples hybridized with MX 825 probe (*Methanosaeta*) probe and their corresponding light microscope views.

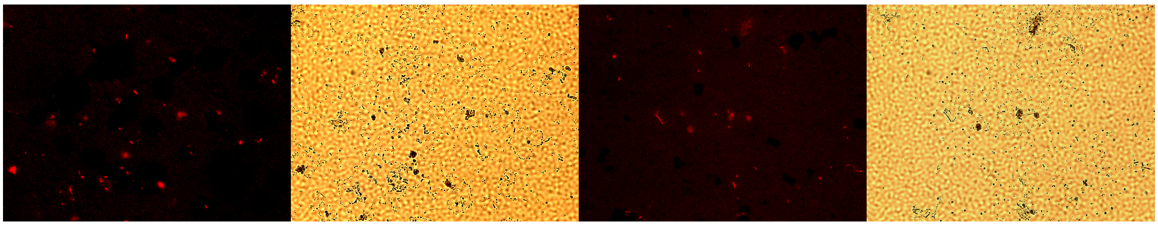

Figure C.30-33. More epifluorescence micrograph examples of TUASB sludge samples hybridized with MB 310 probe (*Methanobacteriales*) and their corresponding light microscope views.

CUASB Reactor

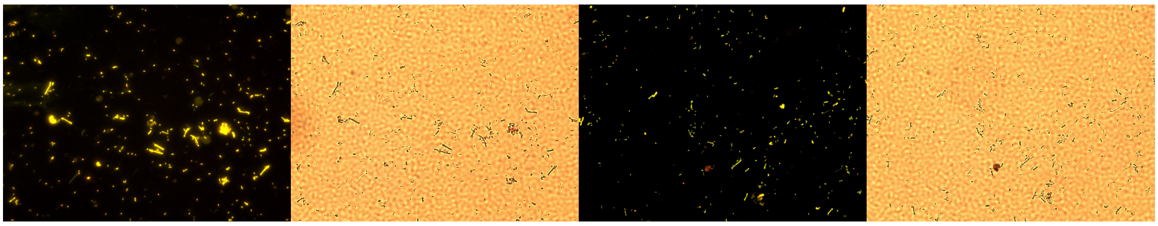

Figure C.34-37. More epifluorescence micrograph examples of Sybr Green I stained CUASB sludge samples and their corresponding light microscope views.

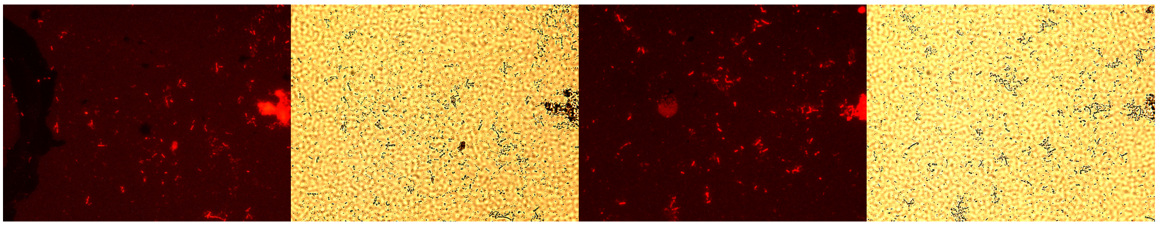

Figure C.38-41. More epifluorescence micrograph examples of CUASB sludge samples hybridized with ARC 915 probe (*Archaea)* and their corresponding light microscope views

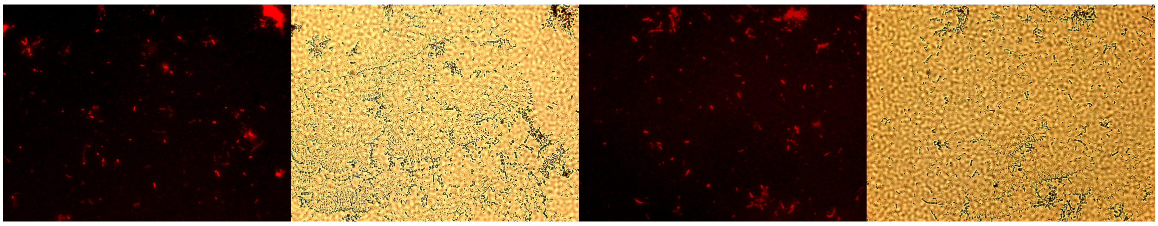

Figure C.42-45. More epifluorescence micrograph examples of CUASB sludge samples hybridized with MX 825 probe (*Methanosaeta*) probe and their corresponding light microscope views.

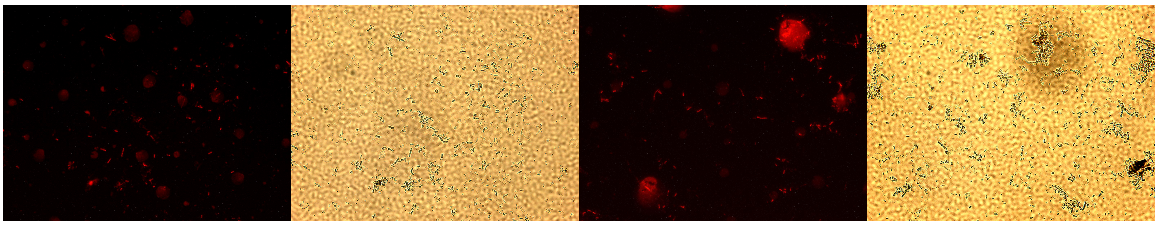

Figure C.46-49. More epifluorescence micrograph examples of CUASB sludge samples hybridized with MB 310 probe (*Methanobacteriales*) and their corresponding light microscope views.

APPENDIX D: QUANTIFICATION DATA OF THE UASB REACTOR SLUDGE SAMPLES

Table D.1. Quantification Data of IUASB Reactor Sludge in November 2005.

	SYBR GR			ARC 915			MX 825			MB 310		
	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3
Image 1	391	482	388	74	83	71	73	44	50	28	26	48
Image 2	274	505	354	78	104	56	69	52	58	22	17	23
Image 3	294	401	321	84	59	71	55	47	59	18	24	26
Image 4	424	314	420	62	99	64	53	43	47	20	24	40
Image 5	417	276	363	80	63	87	66	45	55	15	25	26
Image 6	274	402	348	76	61	97	76	63	71	26	16	41
Image 7	298	289	352	88	79	78	52	68	58	14	26	19
Image 8	319	411	435	50	85	90	46	44	40	25	21	25
Image 9	376	387	401	68	75	78	70	41	58	17	28	21
Image10	247	341	396	66	59	79	43	38	41	30	31	18
Total	3314	3808	3778	726	767	771	603	485	537	215	238	287
Average	331.4	380.8	377.8	72.6	76.7	77.1	60.3	48.5	53.7	21.5	23.8	28.7

Table D.2. Quantification Data of TUASB Reactor Sludge in November 2005.

	SYBR GR			ARC 915			MX 825			MB 310		
	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3
Image 1	316	318	326	92	84	68	42	33	48	45	33	26
Image 2	266	273	320	56	72	46	28	37	42	22	24	22
Image 3	305	329	291	66	81	53	26	47	49	20	26	20
Image 4	229	306	333	62	62	81	31	29	30	47	21	25
Image 5	313	314	255	68	64	61	60	35	70	31	36	23
Image 6	362	280	274	86	77	83	46	66	35	35	39	35
Image 7	288	221	256	64	65	66	70	49	44	16	34	38
Image 8	337	279	298	91	79	58	34	30	49	46	28	30
Image 9	326	219	293	61	86	60	33	33	36	38	21	41
Image10	376	281	260	55	58	71	40	34	47	21	31	34
Total	3118	2820	2906	701	728	647	410	393	450	321	293	294
Average	311.8	282	290.6	70.1	72.8	64.7	41	39.3	45	32.1	29.3	29.4

Table D.3. Quantification Data of CUASB Reactor Sludge in November 2005.

	SYBR GR			ARC 915			MX 825			MB 310		
	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3
Image 1	282	339	483	55	86	98	44	31	53	38	58	49
Image 2	277	414	361	86	81	99	40	27	24	32	30	54
Image 3	335	294	315	73	65	75	32	26	57	28	26	43
Image 4	365	287	323	81	54	71	42	51	50	21	18	37
Image 5	348	303	302	79	74	69	51	37	65	31	49	24
Image 6	398	297	341	87	66	80	36	50	45	23	18	22
Image 7	371	306	391	82	54	74	53	43	34	21	31	30
Image 8	359	301	361	68	66	69	61	47	27	40	22	22
Image 9	411	312	375	66	79	83	28	23	32	38	30	31
Image10	251	301	379	81	104	66	40	52	47	47	48	26
Total	3397	3154	3631	758	729	784	427	387	434	319	330	338
Average	339.7	315.4	363.1	75.8	72.9	78.4	42.7	38.7	43.4	31.9	33	33.8